

1 *Article for special issue of Harmful Algae on toxic cyanobacteria*

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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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## 23 **Abstract**

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

43

## 44 **Introduction**

45 Blooms of toxic cyanobacteria have become a common occurrence in water bodies  
46 worldwide. One of the most pervasive bloom-forming cyanobacteria in freshwater ecosystems is  
47 *Microcystis*. In temperate systems, this organism overwinters in the benthos and during the  
48 summer rises to the epilimnion where it can accumulate to form blooms and scums on the water  
49 surface (Reynolds and Rogers, 1976; Ibelings et al., 1991). Blooms of *Microcystis* generally  
50 occur when water temperatures exceed 15°C (Okino, 1974; Reynolds et al., 1981; Jacoby et al.,  
51 2000) and the occurrence of blooms has been linked to anthropogenic nutrient loading (Perovich  
52 et al., 2008; Dolman et al., 2012). Many *Microcystis* strains can produce the potent hepatotoxin  
53 microcystin, and thus persistent blooms pose a risk to those who use impaired water resources  
54 for drinking water supplies, recreational activities, and fisheries. Microcystins are the only  
55 cyanotoxins for which the World Health Organization has set drinking and recreational water  
56 standards and are typically the only cyanotoxins screened for by municipal management agencies  
57 (Chorus and Bartram, 1999; Hudnell et al., 2008). As global climate changes, the occurrence and  
58 intensity of *Microcystis* blooms is expected to increase (Paerl and Huisman, 2008; Michalak et  
59 al., 2013; Paerl and Otten, 2013).

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

## 64 **Geographic distribution**

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

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

## 91 **Toxins**

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

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

110 the majority of cyanobacteria, including *Microcystis*, may produce BMAA (Cox et al., 2005),  
111 whereas many other investigators have failed to identify this compound (Faassen, 2014). BMAA  
112 is a non-protein amino acid which that has been linked to neurodegenerative diseases, including  
113 amyotrophic lateral sclerosis, Parkinson's, and Alzheimer's Disease (Cox and Sacks, 2002;  
114 Bradley and Mash, 2009; Banack et al., 2010; Holtcamp, 2012). The exact mode of BMAA  
115 toxicity is still under investigation, with both acute and chronic mechanisms indicated (Lobner et  
116 al., 2007). Perhaps the most methodical investigation to date was conducted by Réveillon et al.  
117 (2014), who reanalyzed a number of cyanobacterial isolates, including *M. aeruginosa* PCC 7806,  
118 that were reported as BMAA producers. Notably, using highly sensitive and specific hydrophilic  
119 interaction chromatography coupled to tandem mass spectrometry (HILIC-MS/MS), they failed  
120 to detect BMAA in any cultures. Both free and bound forms of the closely related isomer, 2,4-  
121 diaminobutyric acid (DAB) were detected in all cultures, and may be the compound identified as  
122 BMAA in previous studies using other analytical approaches. Beyond direct toxins, *Microcystis*  
123 has also been shown to produce compounds that act as endocrine disruptors; while not lethal to  
124 fish, these compounds regulate genetic elements associated with sexual maturity and  
125 differentiation (Rogers et al., 2011).

126         Microcystin was originally identified as Fast-Death Factor (Bishop et al., 1959), but was  
127 renamed microcystin a few years later (Konst et al., 1965). Microcystins are cyclic heptapeptides  
128 which contain a unique  $\beta$ -amino acid, Adda (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-  
129 4,6-dienoic acid; Rinehart et al., 1988; Tillett et al., 2000). Microcystins generally contain two  
130 conventional D-amino acids in positions one and six, a D-erythro- $\beta$ -methylaspartic acid in  
131 position three, and position seven is often *N*-methyldehydroalanine (Tillett et al., 2000). To date,  
132 over 100 different microcystin congeners have been characterized (Puddick et al., 2014), mostly

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

138 Different microcystin congeners vary in toxicity from essentially non-toxic (e.g.,  
139 [(6Z)-Adda<sup>5</sup>] microcystin-LR, LD<sub>50</sub> > 1,200 µg kg<sup>-1</sup>) to highly toxic (e.g., microcystin-LR, LD<sub>50</sub>  
140 = 50 µg kg<sup>-1</sup>; Rinehart et al., 1994). Toxicity is manifested as an irreversible covalent bond  
141 formed between the toxin and protein phosphatases, especially in hepatocytes, which leads to  
142 subsequent cell structure damage (Goldberg et al., 1995; Maynes et al., 2006; Feurstein et al.,  
143 2009; Feurstein et al., 2010) and can result in liver disease as well as nephrotoxicity (Milutinović  
144 et al., 2003). This toxicity has led the World Health Organization (WHO) to propose a drinking  
145 water guideline of 1 µg l<sup>-1</sup> for the common hydrophilic variant microcystin-LR (WHO, 2003).

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

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

158         Microcystin concentrations have been determined in cultures and environmental samples  
159 using a variety of methods including protein phosphatase inhibition assays, enzyme-linked  
160 immunosorbent assay (ELISA), chemical derivatization with gas chromatography-mass  
161 spectrometry analysis, and high performance liquid chromatography (HPLC) coupled to either  
162 ultra-violet, photodiode array detector or mass spectrometry detection (Spooof, 2005; Sangolkar et  
163 al., 2006). Choice of the most appropriate analytical method requires consideration regarding  
164 sensitivity, specificity, and associated consumables and equipment costs. For instance, ELISA  
165 broadly detects all microcystin congeners, but provides no information on which specific  
166 congeners are present, however the equipment required to perform the assay is minimal.  
167 Variations in detection methodology in concert with different starting materials (i.e., dried,  
168 filtered, wet samples) and methods of microcystin extraction make comparisons of toxin  
169 concentrations among waterbodies and between studies challenging. Nevertheless, it is clear that  
170 microcystin concentrations can reach extremely high levels during *Microcystis* blooms  
171 worldwide. For example, levels reached 7,300  $\mu\text{g g}^{-1}$  dry weight (dw) in China (Zhang et al.,  
172 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  
173 (Jones et al., 1995), 19.5  $\text{mg l}^{-1}$  in Japan (Nagata et al., 1997) and 36  $\text{mg l}^{-1}$  in New Zealand  
174 (Wood et al., 2006).

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



179 and only observed relatively minor (3- to 4-fold) shifts in microcystin production. Another  
180 popular hypothesis as discussed below (*Grazing* section) is that microcystins act as feeding  
181 deterrents for predators such as zooplankton and fish (Jang et al., 2003; Jang et al., 2004).  
182 However, phylogenetic analysis suggests that the genes responsible for microcystin synthesis  
183 pre-date the eukaryotic lineage (Rantala et al., 2004). More recently, factors such as chelation of  
184 metals (Humble et al., 1997; Sevilla et al., 2008), intraspecies communication (Schatz et al.,  
185 2007), colony formation (Gan et al., 2012), and protein-modulation (Zilliges et al., 2011) have  
186 been implicated as potential functions for microcystin. A brief review of studies in these areas is  
187 given below.

188         Culture-based studies have shown that microcystin concentrations are generally highest  
189 between 20 and 25°C (van der Westhuizen and Eloff, 1985; Watanabe and Oishi, 1985; van der  
190 Westhuizen et al., 1986; Codd and Poon, 1998; Amé and Wunderlin, 2005). Dziallas et al.  
191 (2011) provided further evidence regarding the influence of temperature on microcystin  
192 production by incorporating gene expression assays. These authors found that the fraction of  
193 microcystin-producing *M. aeruginosa* were significantly lower at 32°C than at 20 and 26°C,  
194 although microcystin concentrations increased at these higher temperature (26 and 32°C).  
195 Temperature has also been shown to alter ratios of microcystin congeners. Using batch cultures  
196 and a natural population of *M. aeruginosa* kept at 20°C, microcystin-LR was predominately  
197 produced, whereas at 28°C the ratio of microcystin-LR and microcystin-RR remained constant  
198 (Amé and Wunderlin, 2005).

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

202 batch cultures of axenic *Microcystis*, microcystin production decreased in proportion to cell  
203 division when the culture became nitrogen (N) limited, suggesting that microcystin production is  
204 controlled by environmental effects related to the rate of cell division (Orr and Jones, 1998).  
205 Similarly, using continuous cultures under either N (Long et al., 2001) or phosphorus (P)  
206 limitation (Oh et al., 2000), a linear relationship was also observed between microcystin  
207 production and growth rate. Downing et al. (2005) suggested that considering a single nutrient in  
208 isolation was an oversimplified approach and found microcystin quotas to be positively  
209 correlated with nitrate uptake and cellular N content, and negatively correlated with carbon  
210 fixation rate, phosphate uptake, and cellular P. They concluded that microcystin quotas were  
211 controlled by variables other than growth rate, with N having the most significant effect. In  
212 support of this, Harke and Gobler (2013) observed that under conditions of low inorganic N,  
213 many of the peptide synthesis genes in the microcystin synthetase cassette (*mcyABCDEF*) were  
214 downregulated and microcystin content per cell decreased when cells were N limited.  
215 Furthermore, increases in exogenous N concentrations have been associated with increases in  
216 microcystin (Van de Waal et al., 2009; Horst et al., 2014; Scott et al., 2014; Van de Waal et al.,  
217 2014). Increases in microcystin concentrations or expression of individual *mcy* genes during N  
218 limitation have also been observed (Ginn et al., 2010; Pimentel and Giani, 2014) suggesting a  
219 more complicated relationship or perhaps strain to strain variability.

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

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

228 Light has also been investigated as a factor controlling microcystin synthesis. For  
229 instance, Kaebernick et al. (2000) demonstrated that light intensity affects microcystin  
230 synthase expression, whereby increases in transcription occurred between dark and low light  
231 ( $16 \mu\text{mol of photons m}^{-2} \text{ s}^{-1}$ ) and between medium and high light (31 and  $68 \mu\text{mol of photons}$   
232  $\text{m}^{-2} \text{ s}^{-1}$ ; respectively). Phelan and Downing (2011) found a strong correlation between  
233 microcystin concentration and growth rate under high light ( $37 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ )  
234 conditions for *Microcystis aeruginosa* PCC 7806 and suggested a possible role for  
235 microcystin in protection against photo-oxidation. There is also some evidence to suggest  
236 microcystins may allow *Microcystis* to acclimate to high light and oxidative stress (Zilliges et  
237 al., 2011). Alexova et al. (2011b) showed that microcystins bind to proteins under high light and  
238 during periods of oxidative stress. Interestingly, oxidative stress is often brought about by  
239 supersaturated oxygen conditions produced by vigorously photosynthesizing surface blooms  
240 themselves. Paerl and Otten (2013) suggested that under these conditions, microcystins act as  
241 protectants against cellular damage during active surface bloom formation. Indeed, microcystin  
242 production is often highest during early nutrient-replete phases of the bloom (Davis et al., 2010),  
243 when photosynthetic oxygen production is maximal (Otten et al., 2012). Using a DNA  
244 microarray based on the genome of *M. aeruginosa* PCC 7806, Straub et al. (2011) demonstrated  
245 that the biosynthesis of microcystins occurred primarily during the light period, although this has  
246 been disputed by Penn et al. (2014) who found microcystins were produced throughout the  
247 day/night cycle in natural populations of *Microcystis*. One possible explanation for the often

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

251         There is also mounting evidence that microcystins may be involved in cell-to-cell  
252 signaling. For instance, Dittmann et al. (2001) identified a microcystin related protein (*mrpA*)  
253 that shares similarities with proteins thought to be involved in quorum-sensing in *Rhizobium*.  
254 This protein was only present in a wild-type culture but not an inactivated mutant and was  
255 responsive to light, with a rapid decline of transcription under high light conditions. Kehr et al.  
256 (2006) provided further evidence for this process by demonstrating interactions between  
257 microcystin and the lectin microvirin (MVN) isolated from *Microcystis aeruginosa* PCC 7806.  
258 MVN is believed to be involved in the aggregation of single *Microcystis* cells into colonies  
259 (Kehr et al., 2006). Schatz et al. (2007) found that the release of microcystin from lysed cells into  
260 the extracellular environment induced a significant upregulation of *mcyB* and an accrual of  
261 microcystins in remaining *Microcystis* cells. Additionally, Gan et al. (2012) found that high  
262 concentrations of extracellular microcystins significantly enhanced *Microcystis* colony size, and  
263 that microcystins induced the production of extra-cellular polysaccharides. This contributed to  
264 cell colony formation and upregulated genes related to its synthesis. Using field-based studies  
265 (Wood et al., 2011) showed that *Microcystis* sp. can ‘switch’ microcystin production on and off.  
266 Field and experimentally induced 20-fold changes in microcystin quotas within a five-hour  
267 period were observed in concert with up to a 400-fold change in *mcyE* expression (Wood et al.,  
268 2011; Wood et al., 2012). In both studies the changes in microcystin quotas were associated with  
269 increased *Microcystis* densities (i.e., scum formation, in this case defined as a thin (ca. 3 mm)  
270 layer of cells of the lake surface) and were not caused by a shift in the relative abundance of

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

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

## 292 **Genomics and phylogeny**

### 293 ***Genomics of Microcystis spp.***

294           The first *Microcystis* genome was sequenced from the toxic isolate *M. aeruginosa* NIES -  
295 843 (Kaneko et al., 2007), followed shortly by that of *M. aeruginosa* PCC 7806 (Frangeul et al.,  
296 2008). As late as 2015, only two *M. aeruginosa* genomes have been closed, however, the number  
297 of draft genomes has subsequently increased, as strains isolated from diverse locations have been  
298 sequenced. To date, 15 draft genomes are available, sequenced from strains isolated in Japan  
299 (Kaneko et al., 2007; Okano et al., 2015), the Netherlands (Frangeul et al., 2008), China (Yang et  
300 al., 2013; Yang et al., 2015), and Brazil (Fiore et al., 2013) accompany a collection of draft  
301 sequences from Humbert et al. (2013) for isolates from Canada, the Central African Republic,  
302 France, the United States, South Africa, Australia, and Thailand. Genomes range in size from  
303 4.26 Mbp (*M. aeruginosa* PCC 9806) to 5.84 Mbp (*M. aeruginosa* NIES 843). Previous studies  
304 have highlighted genetic diversity between species of *Microcystis*, for example between the  
305 potentially toxic *M. aeruginosa* and nontoxic *M. wesenbergii* (Harke et al., 2012), and  
306 sequencing of such species may reveal important insight into the divergent ecological strategies  
307 that may exist between strains, potentially driven by each strain's unique flexible genes.

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

315 or consecutively throughout the bloom season (Rinta-Kanto et al., 2005; Ha et al., 2009; Baxa et  
316 al., 2010; Wood et al., 2011). Similarly, these tools have bolstered the ability to identify  
317 organism(s) responsible for toxin production, even in mixed phytoplankton communities  
318 (Dittmann and Börner, 2005; Rinta-Kanto and Wilhelm, 2006; Gobler et al., 2007; Steffen et al.,  
319 2014b). Recent efforts have resulted in a better understanding of the factors that drive  
320 *Microcystis* growth and/or toxicity in the environment, including the role of macronutrients such  
321 as phosphorus and nitrogen (Davis et al., 2009; Rinta-Kanto et al., 2009; Sevilla et al., 2010;  
322 Harke et al., 2012), micronutrients such as iron (Sevilla et al., 2008; Alexova et al., 2011a), and  
323 rising global temperatures (Davis et al., 2009) and carbon dioxide (CO<sub>2</sub>) concentrations (Van de  
324 Waal et al., 2011).

325         The combined impact of the availability of *Microcystis* genome information and the  
326 application of high-throughput sequencing and targeted genetic analyses has marked a transition  
327 to global genomic studies of *Microcystis* ecology and physiology, in both laboratory and field  
328 studies. To date, the number of studies employing the genomes of strains NIES-843 and PCC  
329 7806 as type strains far exceeds the usage of the other 13, more recently sequenced genomes. *M.*  
330 *aeruginosa* NIES-843 has been used for transcriptomic (Harke and Gobler, 2013; Penn et al.,  
331 2014; Steffen et al., 2014a; Steffen et al., 2015), proteomic (Alexova et al., 2011b), and  
332 metabolomic studies (Steffen et al., 2014a), as has *M. aeruginosa* PCC 7806 (Straub et al., 2011;  
333 Penn et al., 2014; Makower et al., 2015; Meissner et al., 2015; Sandrini et al., 2015). These  
334 studies have provided insight into the nutritional ecology, responses to changing CO<sub>2</sub>, and toxin  
335 production, among others factors.

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

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

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

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



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

### 365 ***Phylogeny***

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

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

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

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

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

429 strains may be considerably different, even if gene content is shared, owing to the diverse array  
430 (10% or more of the total genome) of transposable elements and insertion and repeat sequences  
431 (Kaneko et al., 2007; Frangeul et al., 2008). As previously mentioned (above), until recently,  
432 only one strain of *Microcystis* had been fully sequenced and its genome closed (NIES-843).  
433 Using Pac Bio RS II long read sequencing a second strain of *Microcystis aeruginosa* (NIES-  
434 2549) has been recently sequenced and its genome closed (Yamaguchi et al., 2015). The  
435 completion of this second genome enables, for the first time, a true assessment of genome  
436 synteny (i.e., gene order/arrangement) in *Microcystis*. Using the bioinformatics program Gepard  
437 (GEnome PAir - Rapid Dotter; Krumsiek et al., 2007) the genome synteny was assessed for these  
438 two strains, and for comparison, the genome synteny analysis originally provided by Novichkov  
439 et al. (2009) was recreated in order to illustrate the five different patterns of genome  
440 rearrangement presently recognized to occur in prokaryotes (Figure 3). This assessment clearly  
441 demonstrates that *Microcystis* retains almost zero genome synteny, a finding in stark contrast to  
442 the synteny values of 68-86% reported elsewhere (Humbert et al., 2013) that relied only upon  
443 relatively short contiguous fragments of draft genomes. Considering that these two *Microcystis*  
444 isolates were both classified as *M. aeruginosa*, exhibited 99.66% 16S rRNA identity, shared  
445 3,342/4,282 coding sequences (CDS), exhibited an ANI of 95.95%, and were both isolated from  
446 the same water body (Lake Kasumigaura, Japan), it is anticipated that all closed *Microcystis*  
447 genomes will exhibit a similar decay pattern in genome synteny. Furthermore, evidence for  
448 active genome rearrangement was recently observed for transposase genes that exhibit  
449 differential transcription patterns in response to nutrient availability in culture (Steffen et al.,  
450 2014a) and in environmental samples (Harke et al., 2015; Steffen et al., 2015). Indeed, these  
451 observations imply that regional heterogeneities in drivers of transposable element activity may

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

457         Whether or not all *Microcystis* morpho-species should be placed within a single *M.*  
458 *aeruginosa* complex could be construed as a purely esoteric question, but doing so could provide  
459 additional applied benefits for the scientific, research, and managerial communities. For  
460 example, it would simplify the task of microscopic identification and enumeration for public  
461 health purposes and remove much of the subjectivity inherent to each taxonomist. More  
462 importantly, such a unification of *Microcystis* morpho-species would also counter the widespread  
463 belief that certain cyanobacterial species are universally toxic or nontoxic. For example,  
464 *Microcystis wesenbergii* and *Aphanizomenon flos-aquae* are frequently cited by water quality  
465 managers as being nontoxic species despite documented reports to the contrary (Yoshida et al.,  
466 2008).

## 467 **Ecology**

### 468 *Nutrients*

469         Traditionally, P input reductions have been the focus for controlling cyanobacterial  
470 blooms based on the premise that N supplies can be met by N<sub>2</sub> fixation (Schindler et al., 2008).  
471 An important distinction between the genus *Microcystis* and several other major bloom forming  
472 cyanobacterial genera (e.g., *Dolichospermum/Anabaeana*, *Aphanizomenon*, *Cylindrospermopsis*,  
473 *Nodularia*) is that the former is incapable of supplying its N requirements *via* N<sub>2</sub> fixation, while

474 the latter are capable of doing so (Carr and Whitton, 1982; Potts and Whitton, 2000). This  
475 distinction has important ecophysiological and nutrient management ramifications, because  
476 growth and proliferation of *Microcystis* are exclusively reliant on either external N sources  
477 generated by various human activities (whose natural occurrence can be markedly augmented),  
478 including agriculture, urbanization, and industrial pollution or internal regeneration of combined  
479 N forms (largely ammonium). While P input controls are still very much at the center of bloom  
480 management strategies, an increasing number of freshwater ecosystems are now experiencing  
481 expanding blooms of non-N<sub>2</sub> fixers like *Microcystis* and/or *Planktothrix*, despite having such  
482 controls in place. This suggests that anthropogenic N inputs play a role in the global proliferation  
483 of these organisms (Paerl et al., 2014a). Indeed, in numerous eutrophic systems experiencing  
484 both spatial and temporal expansions of *Microcystis* blooms (e.g., Lakes Taihu-China, Erie-  
485 USA/Canada, Okeechobee-Florida/USA, Ponchartrain - Louisiana), it has been shown that N  
486 enrichment plays a key role in bloom proliferation (Paerl and Huisman, 2009; Chaffin and  
487 Bridgeman, 2014; Paerl et al., 2014a; Paerl et al., 2015). Overall, the world-wide proliferation of  
488 *Microcystis* appears closely linked to increases in both P and N loading from expanding human  
489 activities (Paerl, 2014). This conclusion confirms the changing nutrient limitation paradigm,  
490 where N and P co-limitation (and hence the need for N and P nutrient inputs controls) is much  
491 more common than previously thought, especially in eutrophic waters (Dodds et al., 1989; Elser  
492 et al., 2007; Lewis and Wurtsbaugh, 2008; Conley et al., 2009; Lewis et al., 2011; Paerl et al.,  
493 2014b).

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

497 2005). Therefore, eutrophic freshwater ecosystems that contain relatively high concentrations of  
498 reduced N may have a tendency to favor cyanobacterial blooms. This, combined with the fact  
499 that most eutrophic systems are highly turbid and potentially light-limited, will favor  
500 cyanobacterial blooms that can regulate their buoyancy and vertically migrate in order to access  
501 nutrient rich bottom waters (i.e., by sinking) and optimize utilization of radiant energy (by  
502 floating as buoyant surface blooms). *Microcystis* is particularly adept at using such a strategy,  
503 especially during thermally-stratified summer bloom periods, when bottom waters will be  
504 relatively enriched with reduced N, while near-surface irradiance is maximal and reduced N  
505 inventories may be depleted.

506         The ability to migrate vertically also optimizes access to biologically-available P.  
507 *Microcystis* is extremely effective in sequestering sources of P, even at low concentrations  
508 (Jacobson and Halmann, 1982; Kromkamp et al., 1989; Sbiyyaa et al., 1998; Baldia et al., 2007;  
509 Saxton et al., 2012). This strategy is particularly effective in eutrophic, turbid, shallow water  
510 systems in which *Microcystis* can rapidly migrate between P-rich bottom sediments and take  
511 advantage of periodic sediment resuspension due to wind-mixing. By rapidly adjusting its  
512 buoyancy depending on photosynthetic CO<sub>2</sub> fixation versus nutrient acquisition needs,  
513 *Microcystis* can maintain dominance. *Microcystis* is capable of intracellular storage of P  
514 (polyphosphate bodies), enabling it to survive during periods of P deprivation (Carr and Whitton,  
515 1982) and it is also capable of collecting P on its exterior surface (Saxton et al., 2012). Moreover,  
516 *Microcystis* has been shown to upregulate genes to synthesize high-affinity phosphate  
517 transporters and alkaline phosphatases that allow it to persist under low P conditions (Harke et  
518 al., 2012, Harke and Gobler, 2013). In summary, *Microcystis* is exceptionally good at accessing  
519 both N and P via a variety of cellular mechanisms, including buoyancy regulation, cellular

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

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

534         It has been proposed that reducing N inputs under elevated P conditions may lead to  
535 replacement of non-N<sub>2</sub> fixing cyanobacteria such as *Microcystis* with N<sub>2</sub> fixing cyanobacterial  
536 bloom species such as *Dolichospermum/Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*,  
537 *Nodularia* (Schindler et al., 2008; Schindler, 2012). This possibility has recently been tested  
538 using *in situ* mesocosms in Lake Taihu (China) by enriching summer cyanobacterial bloom  
539 communities dominated by *Microcystis* with P (as PO<sub>4</sub><sup>3-</sup>) without adding dissolved inorganic  
540 nitrogen (DIN; to enhance N limitation) while ensuring sufficient supplies of iron (Fe) and other  
541 trace metals. Incubations of up to a month under these conditions failed to induce succession of  
542 N<sub>2</sub> fixers over *Microcystis* and no significant increases in N<sub>2</sub> fixation were reported (Paerl et al.,



543 2014b). In fact, net increases in *Microcystis* biomass were observed during the course of the  
544 experiment. This indicated that *Microcystis* was able to effectively compete with N<sub>2</sub>-fixing taxa  
545 under conditions highly favorable for N<sub>2</sub> fixation (Paerl et al., 2014b). In summary, these  
546 findings argue for increased attention to dual nutrient input constraints to deplete the lake of  
547 previously loaded nutrients. Once the overall phytoplankton biomass is reduced by these  
548 measures, it may be possible to shift to a more P-oriented control strategy, although in more  
549 eutrophied ecosystems this may take years to decades to accomplish (Paerl et al., 2014b).

### 550 *Physical factors*

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

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

568         In many instances, buoyancy compensation by *Microcystis* can overcome light to  
569 moderate wind mixing, which enables it to remain in surface waters more readily than other  
570 bloom forming taxa that it may be competing with. For example, in Taihu, China, highly buoyant  
571 *Microcystis* colonies maintain strong dominance in surface waters during N-limited summer  
572 conditions, despite the fact that N<sub>2</sub>-fixing genera (e.g., *Dolichospermum/Anabaena*,  
573 *Aphanizomenon*) are present during this period. This superior buoyancy and the ability to thrive  
574 on regenerated N may contribute to this dominance over radiant energy demanding diazotrophs  
575 during N-limitation (Paerl, 2014).

576         It is well known that vertical stability through stratification and long water replacement  
577 times favor cyanobacteria over eukaryotic phytoplankton (Reynolds et al., 1981; Reynolds,  
578 2006); hence, disruption of these conditions can, under certain circumstances, modulate bloom  
579 dynamics. Vertical mixing devices, bubblers and other means of destratification have proven  
580 effective in controlling *Microcystis* blooms in relatively small lakes and ponds (Visser et al.,  
581 1996). However, these devices have limited applicability in large lake, estuarine and coastal  
582 waters, because they cannot exert their forces over large areas and volumes (Paerl, 2014).

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

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

603         Climatic changes, including rising global temperatures, increasing CO<sub>2</sub> levels, altered  
604 precipitation patterns, and resultant changes in freshwater discharge or flushing rates have  
605 synergistically influenced *Microcystis* bloom dynamics (Paerl and Paul, 2012). Warmer  
606 temperatures favor cyanobacterial blooms over eukaryotic phytoplankton taxa because growth  
607 rates of the former are optimized at relatively high temperatures (Jöhnk et al., 2008; Paerl and  
608 Huisman, 2008, 2009). In addition, warmer global temperatures and changes to precipitation  
609 patterns have led to the earlier onset of and longer lasting conditions favoring cyanobacterial  
610 blooms (Paerl and Huisman, 2008; Paul, 2008; Paerl and Huisman, 2009; Michalak et al., 2013).  
611 Intensification of vertical stratification (Paerl and Huisman, 2009) in combination with

612 eutrophication also appears to be particularly favorable for development and persistence of  
613 *Microcystis* blooms (Jöhnk et al., 2008). With regards to CO<sub>2</sub> levels, *Microcystis* is known to  
614 have both high- and low-affinity bicarbonate uptake systems as well as two CO<sub>2</sub> uptake systems  
615 (Sandrini et al., 2014). At high pCO<sub>2</sub>, *Microcystis* uses its low-affinity bicarbonate uptake  
616 systems and increases biomass as well as increasing cellular chlorophyll *a* and phycocyanin  
617 content, raising PSI/PSII ratios, and decreasing overall dry weight and carbohydrate content  
618 which may improve buoyancy (Sandrini et al., 2015). Steffen et al. (2015) showed that  
619 *Microcystis* transcribed its carbon concentration mechanism genes (ccm) at sites across the  
620 Western Basin of Lake Erie and proposed that conditions of dense algal biomass, with resultant  
621 high-pH and CO<sub>2</sub> limitation, further promote cyanobacterial dominance. *Microcystis* appears to  
622 be well adapted to high or low CO<sub>2</sub> concentrations (Sandrini et al., 2015), characteristics that  
623 likely permit it to continue to dominate blooms, even as CO<sub>2</sub> concentrations are drawn-down to  
624 low levels. Transitions of CO<sub>2</sub> in lakes today due to algal bloom formation and demise (Balmer  
625 and Downing, 2011), however, far exceeds anthropogenic changes that will be produced in the  
626 future from atmospheric CO<sub>2</sub>. Further, the response of other freshwater phytoplankton to  
627 changing CO<sub>2</sub> levels has been poorly studied. As such, there remain significant unknowns  
628 regarding how rising levels of atmospheric CO<sub>2</sub> will affect future *Microcystis* blooms.

### 629 ***Grazing***

630 The ability of any algal group to form blooms is related to its ability to outgrow  
631 competitors and avoid routes of mortality. In aquatic ecosystems, mortality is generally attributed  
632 to top-down ecological controls such as grazing and viral lysis (Sunda et al., 2006; Smayda,  
633 2008). *Microcystis* has been shown to experience lower rates of mortality than other algae *via*  
634 grazing by zooplankton and bivalves (Vanderploeg et al., 2001; Wilson et al., 2006). Among the

635 zooplankton, larger grazers including daphnids and copepods, are generally less capable of  
636 grazing *Microcystis* than smaller protozoan species (Fulton and Paerl, 1987; Gobler et al., 2007).  
637 While early studies predicted that grazer inhibition may be related to synthesis of microcystin  
638 (Arnold, 1971; Fulton and Paerl, 1987; Rohrlack et al., 1999; DeMott et al., 2001), multiple lines  
639 of evidence demonstrate this is not the case. Rantala et al. (2008) found that the evolution of  
640 microcystin synthesis significantly predated that of metazoans and thus suggested the toxin did  
641 not evolve as a grazing deterrent. Meta-analyses of laboratory studies have concluded that while  
642 *Microcystis* reduces zooplankton population growth rates, the effects are typically not related to  
643 microcystin content of cultures (Wilson et al., 2006; Tillmanns et al., 2008; Chislock et al.,  
644 2013). Within an ecosystem setting, Davis and Gobler (2011) quantified grazing rates by  
645 multiple classes of zooplankton on toxic and non-toxic strains of *Microcystis* in two ecosystems  
646 and found that both microzooplankton and mesozooplankton were capable of grazing both toxic  
647 and nontoxic strains with similar frequencies and rates. Incongruence in culture grazing  
648 experiments may be due to differential production of other, non-microcystin, secondary  
649 metabolites that have not been considered in previous studies.

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

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

660           Outbreaks of *Microcystis* blooms in some lakes in the United States appear to be  
661 stimulated in part by the arrival of recently established zebra mussel (*Dreissena* sp.) populations  
662 (Vanderploeg et al., 2001; Raikow et al., 2004) and this correlation may be linked to the trophic  
663 status of lakes (Sarnelle et al., 2005). While zebra mussel invasions of new ecosystems typically  
664 result in significant reductions in all plankton biomass due to intense filter feeding (Caraco et al.,  
665 1997), *Microcystis* cells consumed by zebra mussels are typically rejected as pseudofeces from  
666 which cells can emerge and regrow (Vanderploeg et al., 2001). Given the ability of zebra  
667 mussels to consume both phytoplankton and zooplankton (Jack and Thorp, 2000; Higgins and  
668 Zanden, 2010; Kissman et al., 2010), *Dreissena* invasions also effectively eliminate competitors  
669 and predators of *Microcystis*. Further, zebra mussels may alter ambient nutrient regimes to favor  
670 *Microcystis*. Zebra mussels can increase concentrations of dissolved organic phosphorus (DOP;  
671 Heath et al., 1995), and under low P loads zebra mussels may promote *Microcystis* blooms  
672 (Sarnelle et al., 2005; Bykova et al., 2006), perhaps via regeneration of organic P given  
673 *Microcystis* has the ability to grow efficiently on DOP using alkaline phosphatase (Harke et al.,  
674 2012).

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

681 suggests a genetic shift occurs in wild zooplankton populations towards populations able to graze  
682 *Microcystis* (Hairston et al., 1999; Sarnelle et al., 2005; Davis and Gobler, 2011; Chislock et al.,  
683 2013). While filter feeding bivalves may ultimately also be capable of such adaptation (Bricelj et  
684 al., 2005), this possibility has yet to be explored.

### 685 ***Microbial Interactions***

686 As a largely colonial bloom forming genus, *Microcystis* has numerous complex  
687 interactions with both bacteria and protists (protozoans, microalgae, fungi; Paerl, 1982; Paerl and  
688 Millie, 1996; Shen et al., 2011; Shao et al., 2014). These interactions can be both intimate, such  
689 as is the case of microbes attached to or existing within *Microcystis* colonies, or more diffuse for  
690 microbes co-occurring in time and space. Regarding bacteria associated with colonies, during the  
691 decline of a *Microcystis* bloom, Parveen et al. (2013) found colonies to be depleted in  
692 *Actinobacteria*, but enriched in *Gammaproteobacteria* and changes in temperature may shape  
693 associated bacterial communities (Dziallas and Grossart, 2012). While many of the functional  
694 roles of *Microcystis*-bacterial associations remain unknown, it is clear that such associations can  
695 be both mutually beneficial as well as antagonistic with regard to their effects on growth  
696 potentials, viability and mortality of *Microcystis* and associated microbes. It has been noted that  
697 photosynthetic performance and growth rates of epiphytized *Microcystis* cells and colonies are  
698 often higher than bacteria-free or axenic cultures (Paerl, 1982; Paerl and Millie, 1996), indicating  
699 a mutualistic, if not symbiotic properties of such associations. Paerl and Millie (1996) speculated  
700 that while heterotrophic bacteria associated with bloom-forming cyanobacteria (e.g.,  
701 *Dolichospermum/Anabaena*, *Microcystis*) clearly benefitted from the organic matter produced by  
702 the cyanobacteria, the cyanobacteria benefitted from organic matter decomposition, CO<sub>2</sub>  
703 production, and nutrient (N, P, Fe and trace metals) regeneration provided by associated

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

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

#### 724 ***Interactions with viruses***

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



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

730         Indeed, viruses that may constrain cyanobacterial blooms have long been a “holy grail”  
731 for microbial ecologists (Safferman and Morris, 1963, 1964). Indeed much of the early work in  
732 virus ecology was dedicated to the idea that bacteriophage might be used to mitigate or even  
733 control harmful cyanobacterial bloom populations in the environment (Safferman and Morris,  
734 1964). Chief amongst these efforts, the study of two virus types (LPP-1 and SM-1) were of  
735 interest, especially as the later was reported to include the bloom-producer *Microcystis*  
736 *aeruginosa* NRC-1 amongst putative hosts (Safferman and Morris, 1967).

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

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

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

## 767 **Conclusions**

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

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

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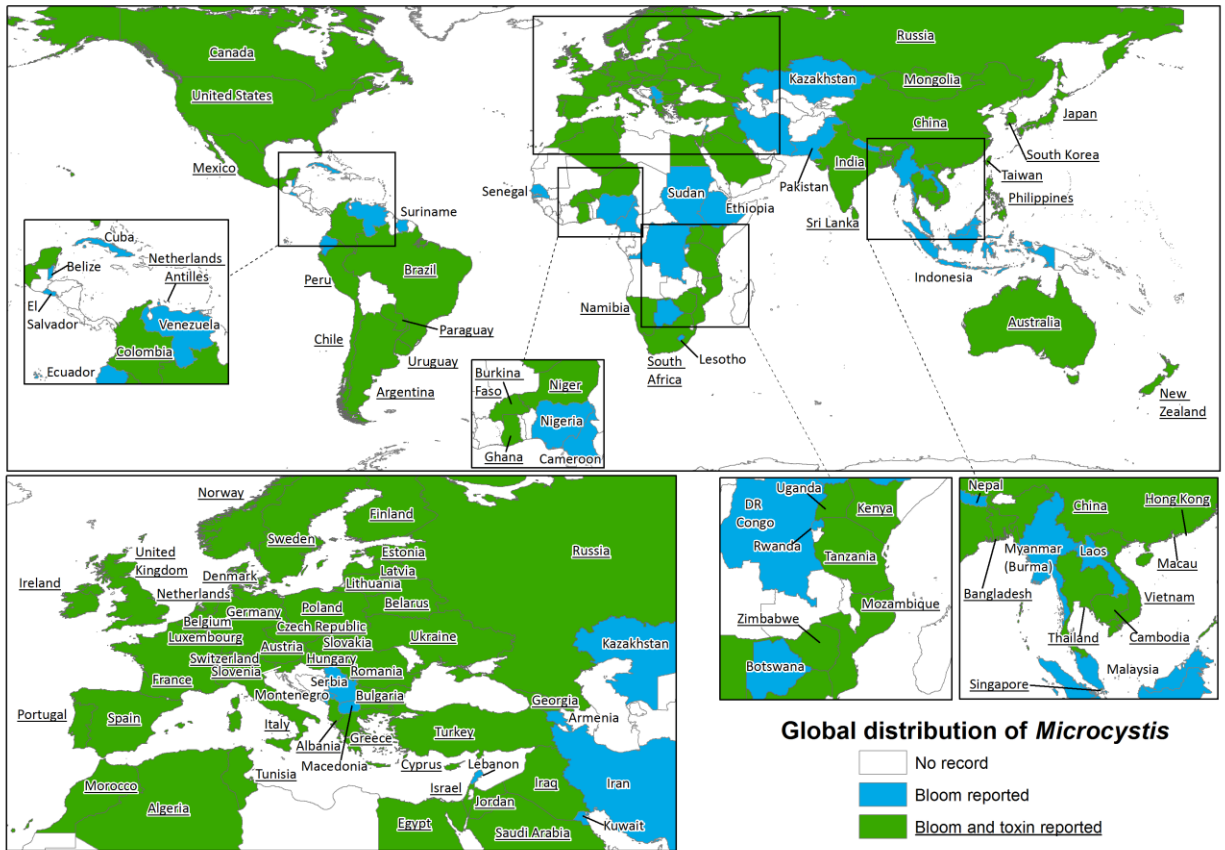
819 **Tables and Figures**

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	<sup>†</sup> 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_AOCI00000000.1	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 ; <sup>†</sup> DNA-DNA hybridization data from Otsuka et al., 2001

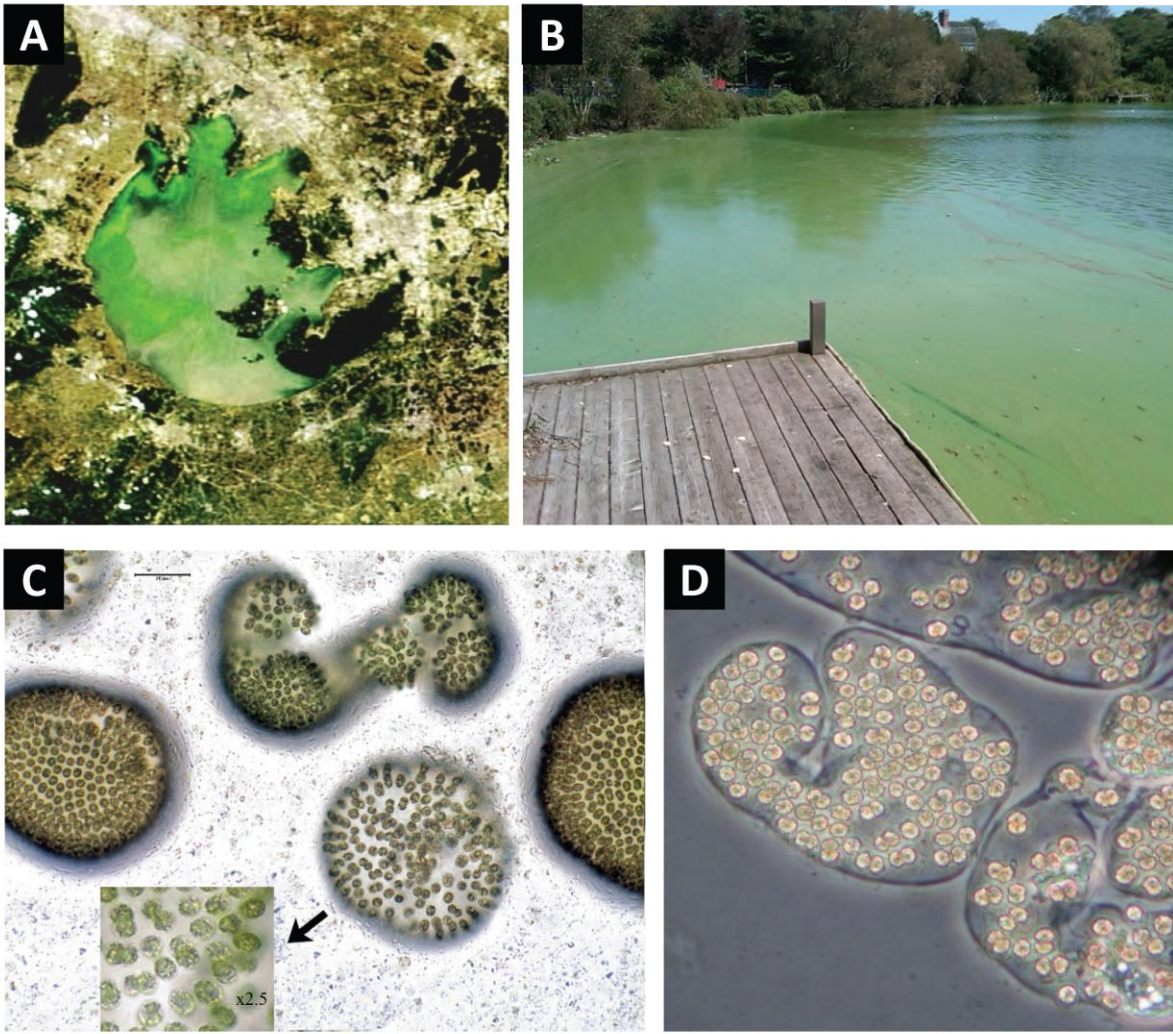
825 **Figure 1** The global occurrence of *Microcystis* blooms and microcystin as determined through literature searches for  
 826 records of *Microcystis* blooms from 257 countries and territories. White indicate no record of bloom or microcystin,  
 827 blue indicates a record was found for the occurrence of a bloom, and green indicates countries or territories where  
 828 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.

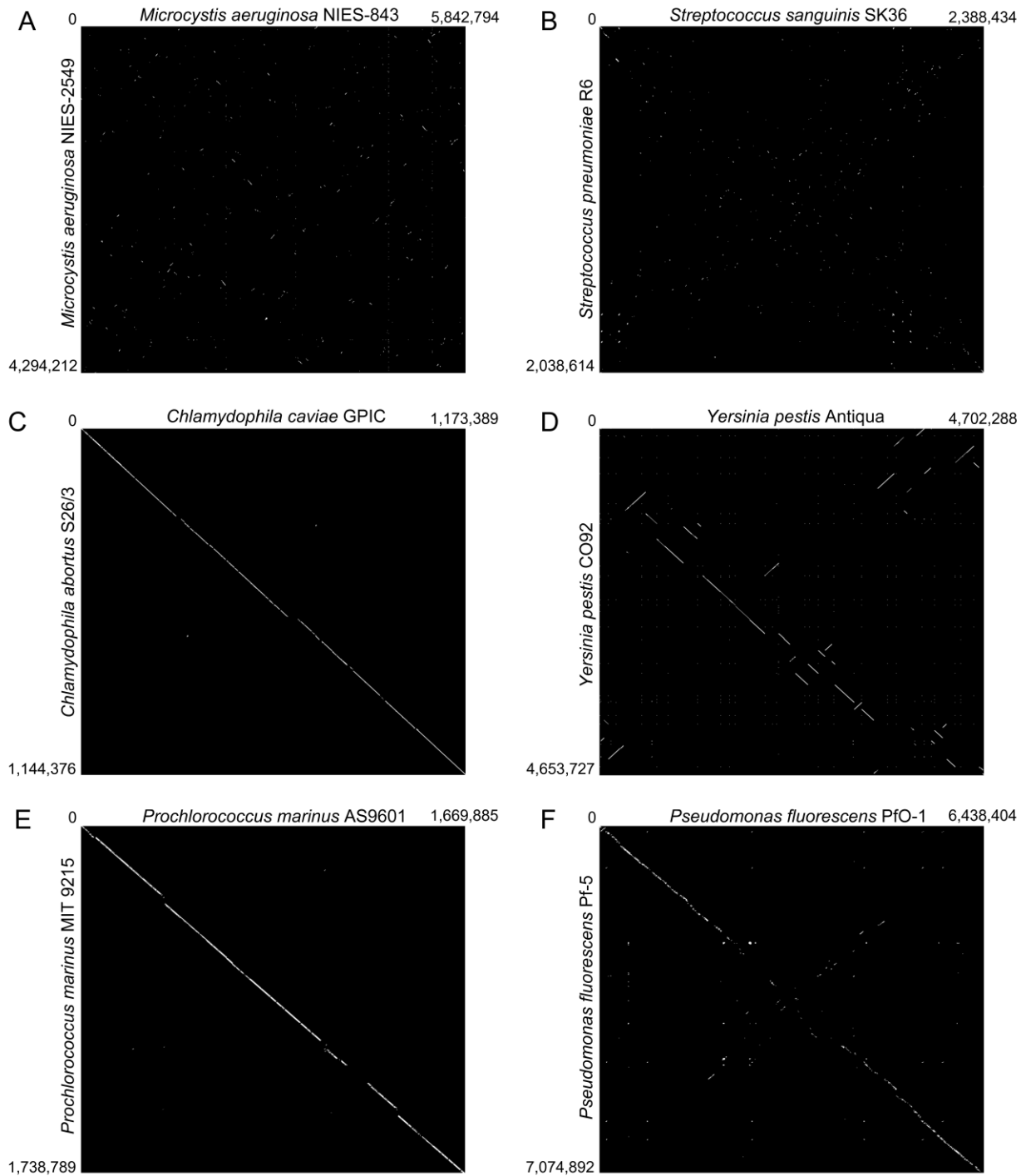


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835 **Figure 3** Dot plot matrix comparing whole genome synteny for *Microcystis aeruginosa* (A) relative to five other  
 836 genera of bacteria known to exhibit different genome rearrangement patterns as described by Novichkov et al., 2009.  
 837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



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