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

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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., [(6*Z*)-Adda^5] microcystin-LR, LD_{50} > 1,200 µg kg^{-1}) to highly toxic (e.g., microcystin-LR, LD_{50} = 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

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 (Spoof, 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 μg g^{-1} dry weight (dw) in China (Zhang et al.,
172 1991), 7,100 μg g^{-1} dw in Portugal (Vasconcelos et al., 1996), 4,100 μg g^{-1} dw in Australia
173 (Jones et al., 1995), 19.5 mg l^{-1} in Japan (Nagata et al., 1997) and 36 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

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 (*mcyABCDEF*) 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 *mcy* 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 *mcy* genes in *Microcystis* is thought to be regulated via a bidirectional promoter that is located between the *mcyA* and *mcyD* 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).

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 μmol of photons m^{-2} s^{-1}) and between medium and high light (31 and 68 μmol of photons
232 m^{-2} s^{-1}; respectively). Phelan and Downing (2011) found a strong correlation between
233 microcystin concentration and growth rate under high light (37 μmol photons $m^{-2}s^{-1}$)
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

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

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}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.

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 verses 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_2) 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_2, 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

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

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

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 N_2 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* N_2 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_2 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,

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 CO_2 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 it 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-N_2 fixing cyanobacteria such as *Microcystis* with N_2 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 PO_4^{3-}) 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 N_2 fixers over *Microcystis* and no significant increases in N_2 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 N_2-fixing taxa under conditions highly favorable for N_2 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

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_2-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_2 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_2 levels, *Microcystis* is known to
614 have both high- and low-affinity bicarbonate uptake systems as well as two CO_2 uptake systems
615 (Sandrini et al., 2014). At high pCO_2, *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_2 limitation, further promote cyanobacterial dominance. *Microcystis* appears to
622 be well adapted to high or low CO_2 concentrations (Sandrini et al., 2015), characteristics that
623 likely permit it to continue to dominate blooms, even as CO_2 concentrations are drawn-down to
624 low levels. Transitions of CO_2 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_2. Further, the response of other freshwater phytoplankton to
627 changing CO_2 levels has been poorly studied. As such, there remain significant unknowns
628 regarding how rising levels of atmospheric CO_2 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

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, CO_2 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

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

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 metatrascriptomic 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 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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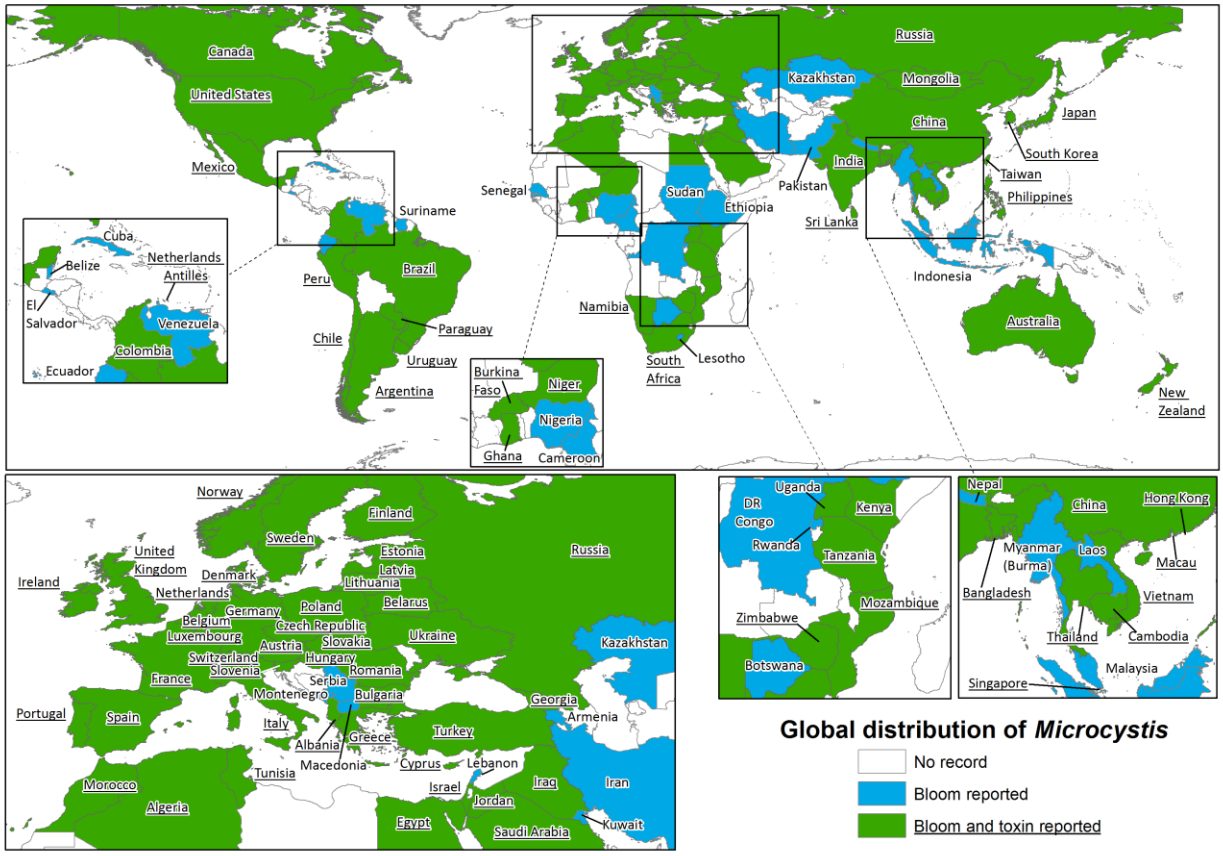
Tables and Figures

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

Genus species	Strain	GenBank Assembly or Accession #	16S Identity (%) NIES-843	Genome ANI (%) NIES-843	ANI Fragments	ǂDNA-DNA (%) NIES-843
Microcystis aeruginosa	NIES-843 ●	NC_010296.1	100.00	100.00	29,201	100.0
Microcystis aeruginosa	NIES-2549	CP011304.1	99.79	95.95	10,491	NA
Microcystis aeruginosa	NIES-44	GCA_000787675.1	99.60	96.35	10,695	NA
Microcystis aeruginosa	DIANCHI-905 ●	NZ_AOCI00000000.1	99.59	95.65	10,387	NA
Microcystis aeruginosa	PCC 7005	GCA_000599945.1	99.66	95.90	9,915	NA
Microcystis aeruginosa	PCC 7806 ●	AM778844.1-AM778958.1	99.72	95.64	10,408	NA
Microcystis aeruginosa	PCC 7941 ●	GCA_000312205.1	99.58	95.95	10,912	NA
Microcystis aeruginosa	PCC 9432	GCA_000307995.2	99.73	95.96	10,685	NA
Microcystis aeruginosa	PCC 9443 ●	GCA_000312185.1	99.66	96.16	10,927	NA
Microcystis aeruginosa	PCC 9701	GCA_000312285.1	99.73	96.34	10,539	NA
Microcystis aeruginosa	PCC 9717 ●	GCA_000312165.1	99.80	97.28	12,947	NA
Microcystis aeruginosa	PCC 9806	GCA_000312725.1	99.66	96.18	10,872	NA
Microcystis aeruginosa	PCC 9807 ●	GCA_000312225.1	99.93	95.80	11,503	NA
Microcystis aeruginosa	PCC 9808 ●	GCA_000312245.1	99.73	95.97	10,814	NA
Microcystis aeruginosa	PCC 9809 ●	NZ_CAIO00000000.1	99.73	98.57	15,062	NA
Microcystis aeruginosa	SPC-777 ●	NZ_ASZQ00000000.1	99.66	96.11	10,914	NA
Microcystis aeruginosa	Taihu-98	ANKQ01000001.1	99.46	96.02	11,102	NA
Microcystis sp.	T1-4	NZ_CAIP00000000.1	99.72	95.95	10,594	NA
Microcystis aeruginosa	TAC86 ●	AB012333.1	99.59	NA	NA	75.0
Microcystis flos-aquae	UWOCC C2	AF139328.1	99.65	NA	NA	NA
Microcystis ichthyoblabe	TC24	AB035550.1	99.66	NA	NA	80.7
Microcystis novacekii	BC18	AB012336.1	99.93	NA	NA	74.0
Microcystis panniformis	VN425	AB666076.1	99.58	NA	NA	NA
Microcystis protocystis	VN111	AB666054.1	99.86	NA	NA	NA
Microcystis viridis	CC9	AB035552.1	99.73	NA	NA	91.7
Microcystis wesenbergii	TC7	AB035553.1	99.59	NA	NA	89.7
Microcystis wesenbergii	NIES-107 ●	DQ648028.1	99.72	NA	NA	NA
Aphanocapsa montana	BDHKU210001	NZ_JTJD00000000.1	88.06	80.36	17	NA
Cyanobium gracile	PCC-6307	NC_019675.1	87.62	81.97	19	NA
Gloeocapsa sp.	PCC-7428	GCA_000317555.1	90.18	76.79	52	NA
Gloeocapsa sp.	PCC-73106	GCA_000332035.1	89.74	74.90	95	NA
Synechococcus elongatus	PCC-6301	AP008231.1	89.53	81.33	25	NA

● Denotes microcystin producer ; NA - Not Available ; ǂ 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.



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.

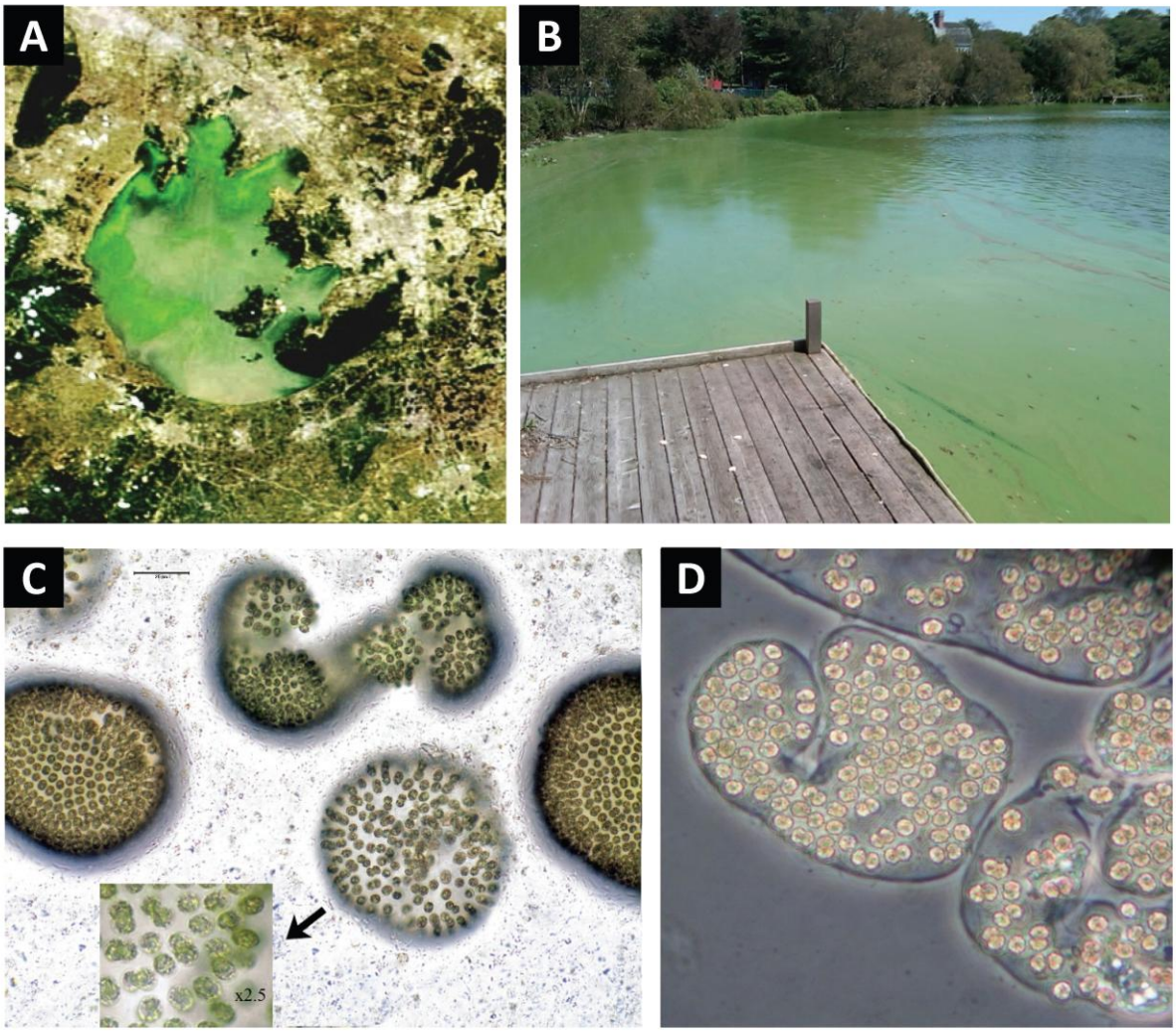


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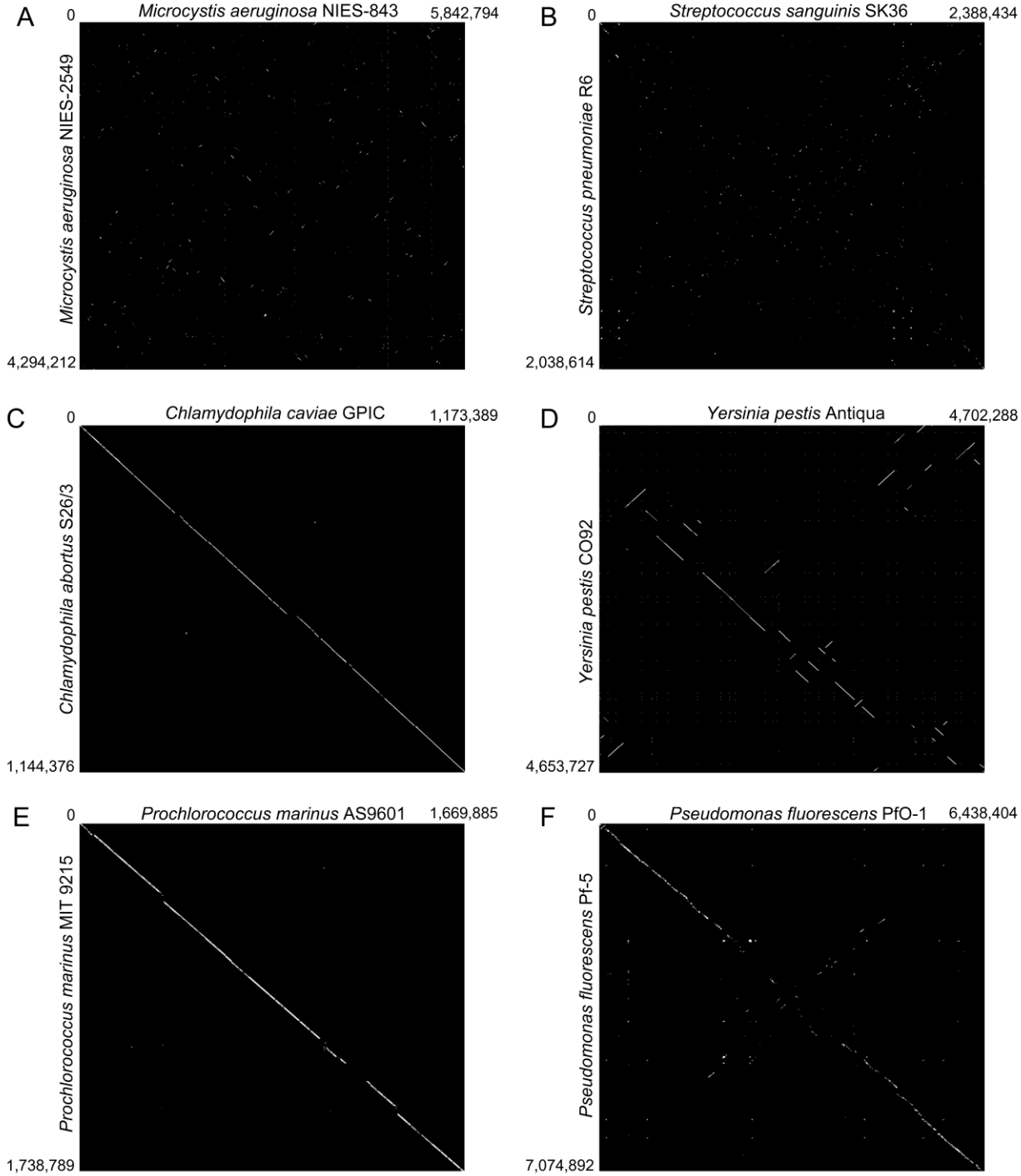
Figure 3 Dot plot matrix comparing whole genome synteny for *Microcystis aeruginosa* (A) relative to five other genera of bacteria known to exhibit different genome rearrangement patterns as described by Novichkov et al., 2009. (B) Complete decay of genome synteny. (C) Absence of rearrangement. (D) Multiple 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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