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The genetic and ecophysiological diversity of *Microcystis*

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Originality Significance Statement

Toxic cyanobacterial blooms are proliferating globally due to cultural eutrophication, climate change, and ecosystems made vulnerable by invasive species. Among the most prevalent concerns is *Microcystis*, which threatens freshwater ecosystems and drinking water supplies by formation of dense blooms and production of microcystins, a diverse class of liver toxins. Diverse *Microcystis* strains exhibit remarkable variation in their physiology, habitat, and ability to produce toxins. This strain-level variation is recognized as a key determinant of the ecological success of *Microcystis* and the toxicity of its blooms. However, the links between genotype and phenotype and how they underpin the environmental distribution of strains and production of toxins remain unclear. This review synthesizes current knowledge on the environmental distribution and genetic and phenotypic diversity of *Microcystis* strains and identifies knowledge gaps and promising paths for future research.

Summary

Microcystis is a cyanobacterium that forms toxic blooms in freshwater ecosystems around the world. Biological variation among taxa within the genus is apparent through genetic and phenotypic differences between strains and via the spatial and temporal distribution of strains in the environment, and this fine-scale diversity exerts strong influence over bloom toxicity. Yet we do not know how varying traits of *Microcystis* strains govern their environmental distribution, the tradeoffs and links between these traits, or how they are encoded at the genomic level. Here we synthesize current knowledge on the importance of diversity within *Microcystis* and on the genes and traits that likely underpin ecological differentiation of taxa. We briefly review spatial and environmental patterns of *Microcystis* diversity in the field and genetic evidence for cohesive groups within *Microcystis*. We then compile data on strain-level diversity regarding growth responses to environmental conditions and explore evidence for variation of community interactions across *Microcystis* strains. Potential links and tradeoffs between traits are identified and discussed. The resulting picture, while incomplete, highlights key knowledge gaps that need to be filled to enable new models for predicting strain-level dynamics, which influence the development, toxicity, and cosmopolitan nature of *Microcystis* blooms.

Introduction

Toxic cyanobacterial blooms degrade drinking water supplies and freshwater ecosystems around the world (Huisman *et al.*, 2018). Driven mainly by cultural eutrophication, these blooms are expected to become even more intense, frequent, and widespread with climate change as increasing temperature and water stratification favor bloom-forming cyanobacteria (Paerl and Huisman, 2008, 2009; O'Neil *et al.*, 2012; Michalak *et al.*, 2013; Paerl and Otten, 2013b; Visser *et al.*, 2016; Ho *et al.*, 2019). Among the most common and harmful of all bloom-forming cyanobacteria is *Microcystis*, which is widespread in temperate and subtropical lakes, reservoirs, and rivers in at least 108 countries on six continents (Harke, Steffen, *et al.*, 2016). *Microcystis* produces a variety of natural products, with the main focus on microcystins due to their environmental abundance and known toxic effects (Welker *et al.*, 2006; Kehr *et al.*, 2011; Dittmann *et al.*, 2015; Lezcano *et al.*, 2019; Pérez-Carrascal *et al.*, 2019; Pearson *et al.*, 2020). Recent years have seen *Microcystis* blooms in large lakes that were among the largest in recorded history (Michalak *et al.*, 2013), that shut down drinking water supplies in large cities (Qin *et al.*, 2010; Steffen *et al.*, 2017), and that expanded into lakes, rivers, and estuaries that were previously unaffected (Kramer *et al.*, 2018).

What factors have contributed to the rise of *Microcystis* blooms in recent decades? Phosphorus loading is a key determinant of bloom size, *i.e.*, *Microcystis* biomass (Stumpf *et al.*, 2012; Michalak *et al.*, 2013), thus management strategies are often focused on P (Ohio EPA, 2013). However, in some cases blooms have become more intense and frequent without apparent increases in phosphorus loading, highlighting the complexity of the issue and the important role of factors such as top-down and bottom-up ecological controls (Vanderploeg *et al.*, 2001; Sarnelle *et al.*, 2005), the form of phosphorus (Kane *et al.*, 2014), nitrogen availability (Paerl *et al.*, 2016), and competitive interactions that may be shifting along with climate change (Paerl and Huisman, 2008; Sandrini *et al.*, 2016). Furthermore, the factors that drive bloom formation and those that drive toxicity may be uncoupled owing to intracellular regulation of toxin

production as well as the relative proportion of toxic and non-toxic *Microcystis* cells within blooms (Rinta-Kanto *et al.*, 2009; Wilhelm and Boyer, 2011).

Remarkably, *Microcystis* dominates phytoplankton communities across a wide range of physicochemical conditions that vary both between lakes around the world and within lakes over time and seasonal changes. Several key traits appear to underpin the success of *Microcystis* over such wide-ranging conditions. First, *Microcystis* can sustain high biomass despite low concentrations of inorganic phosphorus, in part because of its ability to access benthic nutrients by vertical migration (Gobler *et al.*, 2016). Second, increased availability of nitrogen from anthropogenic sources likely helps *Microcystis*, which does not fix its own nitrogen, compete with other phytoplankton (Gobler *et al.*, 2016; Paerl *et al.*, 2016; Nolan and Cardinale, 2019). Third, *Microcystis* produces a variety of natural products that can deter grazers and inhibit competitors (Van Wichelen *et al.*, 2016). Fourth, *Microcystis* forms gas vesicles that allow it to effectively migrate vertically in the water column in order to find optimal light intensity and quality, and to form surface scums that reduce light availability to other phototrophs (Komárek, 2003). Fifth, by forming large colonies of cells, *Microcystis* can evade predation by key zooplankton taxa (Ger *et al.*, 2016). Finally, *Microcystis* effectively overwinters on the sediments, allowing rapid initiation of blooms during favorable conditions (Humbert *et al.*, 2005; Welker *et al.*, 2007; Kitchens *et al.*, 2018).

Another explanation for the widespread ecological success of *Microcystis* is its phenotypic diversity across taxa within the genus. The question of whether there are truly different species of *Microcystis* is considered in the section on genetic diversity below. There are also various definitions of “strain”; here we use the term in a broad sense to refer to natural intra-species variants with distinct genotypes and/or phenotypes, including both cultured isolates as well as uncultured entities observed through molecular methods, recognizing that thresholds to delineate strains are undefined (Van Rossum *et al.*, 2020). The diversity of *Microcystis* strains is apparent both through observations of spatial and temporal patterns in the field and the widely varying traits of strains studied in the laboratory. Such intraspecific diversity

enables rapid shifts in strains optimized to varying environmental conditions and biotic interactions while maintaining dominance at a coarser phylogenetic level (Muraille, 2018; Willis and Woodhouse, 2020), and likely plays a key role in the widespread occurrence and persistence of *Microcystis* blooms.

Much of the prior work on strain variability within *Microcystis* has focused on the fact that some strains have genetic capability to produce microcystins ("toxigenic") whereas others do not ("non-toxigenic"). This trait receives attention because microcystin is a primary concern for human and animal health and the relative abundance of toxigenic and non-toxigenic cells within *Microcystis* blooms is a key determinant of microcystins concentrations in blooms (Carmichael and Gorham, 1981; Ohtake *et al.*, 1989; Vezie *et al.*, 1998; Davis *et al.*, 2009; Gobler *et al.*, 2016) together with the amount of microcystins produced per cell (Chorus, 2001; Jahnichen *et al.*, 2001). Indeed, *Microcystis* strains are often referred to as "toxic" and "non-toxic" based on ability to produce microcystins. In fact, this simplified classification scheme hides the fact that collectively, *Microcystis* produces a large array of secondary metabolites with possible toxic properties including over 200 (Puddick *et al.*, 2014; Taranu *et al.*, 2019) different microcystin variants (congeners), which range in their toxicity (Rinehart *et al.*, 1994; Chernoff *et al.*, 2020). These toxin congener profiles differ between strains (Puddick *et al.*, 2014), and environmental conditions (Amé and Wunderlin, 2005; Taranu *et al.*, 2019). Understanding and modeling the ecology and physiology of these strains will be critical to inform forecasts and management of toxic blooms (Hellweger *et al.*, 2019). However, despite the important role of strain diversity in shaping the ecological success of *Microcystis* and toxicity of blooms, and the prevalence of genotypic and phenotypic variability within *Microcystis* (Harke, Steffen, *et al.*, 2016; Pérez-Carrascal *et al.*, 2019) (and bacterial species in general (Van Rossum *et al.*, 2020), a complete understanding of how genetic and phenotypic traits are distributed across the *Microcystis* genus, and what trade-offs may exist between toxin production and other traits key to their ecological success, remains elusive.

With the recent acceleration of genomics-enabled research, this review aims to provide a timely synthesis of our current knowledge and to identify critical knowledge gaps in the genetic and phenotypic diversity of *Microcystis* strains. We focus on traits likely to underpin a strain's fitness in response to environmental parameters or community interactions (Litchman *et al.*, 2007; Zwart *et al.*, 2015). In the interest of exposing links between genotype and phenotype, we draw on data from studies of pure cultures. However, inherent limitations of cultures should be recognized. Strains in culture are biased toward those collected from eutrophic conditions because of difficulty in isolating and culturing strains that are not adapted to the usually high concentration of nutrients found in typical culture media (Wilson *et al.*, 2006). Investigators tend to work with axenic unicellular cultures available from culture collections, whereas in nature *Microcystis* usually grows in multicellular colonies. Although the colonial form likely influences several key traits, it is usually lost over time and is rarely maintained for more than a year or two in culture. Toxin profiles can change over time because of spontaneous mutations (Kaebernick *et al.*, 2001). These factors, along with differences in culture conditions between studies, can result in different trait values even for the same isolate. Finally, laboratory strains are often characterized from a small subset of perspectives, thus holding incomplete information on physiological properties, genome sequence, and toxin content (microcystin congeners or other secondary compounds), and thus concealing potential strain-dependent links between these traits.

Spatial and temporal patterns of strain diversity suggest ecologically distinct strains

Various methods have been used to study the diversity of *Microcystis* taxa in the environment, ranging from microscopy to characterization of isolated cultures to cultivation-independent molecular analyses. Cultivation methods impart major biases on which strains of *Microcystis* are retrieved (Wilson *et al.*, 2006) and morphology does not accurately reflect phylogeny or function (Kondo *et al.*, 2000; Otsuka *et al.*, 2001; Harke, Steffen, *et al.*, 2016), thus much work has focused on cultivation-independent genetic methods that can quantify the diversity and abundance of *Microcystis* (Baker *et al.*, 2002; Rinta-Kanto *et*

al., 2005; Rinta-Kanto and Wilhelm, 2006). Given the practical importance of understanding controls on bloom toxicity (*i.e.*, toxin concentration relative to biomass), many studies on the diversity of *Microcystis* have focused on the relative abundance of toxigenic and non-toxigenic strains, which often coexist in *Microcystis* blooms. The proportion of toxigenic cells in a *Microcystis* population, which is often estimated by quantifying the number of cells carrying *mcy* genes as a percentage of total *Microcystis* cells (*e.g.*, via 16S rRNA gene copies), can range from essentially 0% to 100% (Yoshida *et al.*, 2007; Davis *et al.*, 2009). This proportion often varies spatially within a water body (Otten *et al.*, 2012) and even within a single water sample according to colony size/morphology, with larger colonies enriched in toxigenic cells in some cases (Kurmayer *et al.*, 2003; Wang *et al.*, 2013). In lakes around the world, a temporal succession of strains is often observed, with toxigenic strains dominating in early/peak blooms and non-toxigenic strains dominating in later stages of blooms (Park *et al.*, 1998; Welker *et al.*, 2007; Briand *et al.*, 2009; Davis *et al.*, 2009, 2010; Bozarth *et al.*, 2010; Singh *et al.*, 2015; Gobler *et al.*, 2016). This succession can explain dynamics of the toxicity of blooms, but in some cases it does not (Kardinaal *et al.*, 2007; Rinta-Kanto *et al.*, 2009). Other water bodies show distinct patterns of strain composition altogether (Yoshida *et al.*, 2007; Joung *et al.*, 2011). Regardless, the oft-observed succession of strains, even in low resolution terms of toxigenic vs non-toxigenic, suggests that there are ecologically distinct strains of *Microcystis*, with specific adaptations to bloom stage in terms of nitrogen requirements, resistance to oxidative stress, and optimal light and temperature conditions for growth (see sections on these topics below). This inference is supported by experiments in which manipulation of environmental conditions results in changes in the composition of toxigenic/non-toxigenic cells in blooms (Davis *et al.*, 2009, 2010).

Despite the importance of strain composition in bloom toxicity and dynamics, the genomic and ecophysiological traits that delineate ecologically cohesive groups of strains are unknown. Because *mcy* genes are variably present in *Microcystis* strains and polyphyletic (Tillett *et al.*, 2000, 2001; Rantala *et al.*, 2004), they cannot serve as a phylogenetically accurate or comprehensive marker of the strain

composition of blooms. The 16S rRNA gene is not sufficient to resolve phylogeny of strains; oligotyping of 16S sequences has shown some promise in distinguishing strains, though with significant caveats (Berry *et al.*, 2017). Genes and intergenic regions with higher levels of sequence divergence are required to more accurately profile strain composition in a sample. The intergenic spacer of genes for phycocyanin biosynthesis (*cpcBA* IGS) and the internal transcribed spacer (ITS) of the rRNA operon have been used for this purpose (Janse *et al.*, 2004; Moisander *et al.*, 2009; Bozarth *et al.*, 2010; Wang *et al.*, 2013; Guan *et al.*, 2018; Ho *et al.*, 2019; Chun *et al.*, 2020). Such studies have tracked dynamics of strain composition of *Microcystis* populations across time and space (Bozarth *et al.*, 2010; Otten *et al.*, 2017), identified sources and transport of *Microcystis* cells in watersheds (Otten *et al.*, 2015), and revealed relationships between strain abundance and nutrient levels (Guan *et al.*, 2018) and bacterial and eukaryotic communities (Chun *et al.*, 2020). Finally, the CRISPR-cas loci may provide even greater resolution of strains (Kimura *et al.*, 2018)**,** but does not reflect phylogeny due to frequent horizontal transfer (Varble *et al.*, 2019).

Whole genome sequences provide a complete picture of the gene content of *Microcystis* strains (Humbert *et al.*, 2013; Meyer *et al.*, 2017; Pérez-Carrascal *et al.*, 2019). While *de novo* assembly of genomes from the environment remains challenging due to the highly complex and repetitive nature of the *Microcystis* genome (Humbert *et al.*, 2013), shotgun metagenomics is already providing insights into the environmental distribution and expression of strain-specific genes (Steffen *et al.*, 2012; Meyer *et al.*, 2017), including those encoding toxins and taste and odor compounds (Otten *et al.*, 2016). Even without assembly, shotgun metagenomic approaches offer a range of applications for studying the dynamics of strains in the environment (Van Rossum *et al.*, 2020). Higher resolution reconstruction of strain-specific genomes awaits improvements to assembly and binning software, and especially continued advances in long sequence read technologies. For now, the main value of whole-genome sequencing lies in revealing the genetic diversity of cultured *Microcystis* strains, and in assessing how informative single markers such as the ITS region are relative to whole genome-based phylogenetic relationships.

Genetic diversity of *Microcystis* strains

The rapidly growing number of whole genome sequences provides new views of the genetic diversity of *Microcystis* strains (Humbert *et al.*, 2013; Harke, Davis, *et al.*, 2016; Jackrel et al., 2019; Pérez-Carrascal *et al.*, 2019; Willis and Woodhouse, 2020). To assess how well these cultured strains represent the environmental diversity of *Microcystis,* we constructed a phylogenetic tree of the 16S-23S internal transcribed spacer (ITSc) from all publicly available high-quality draft whole genome sequences of cultured isolates and sequences retrieved by PCR from uncultured *Microcystis* in the field (**Fig. 1**). 128 of the 159 *Microcystis* genomes available on NCBI (accessed on June 1, 2020) had a readily identifiable ITS region in the assembled contigs. Others were likely missing due to known issues with assembly of rRNA operons. The sequences from genomes of cultured *Microcystis* represent clades across the tree of environmental diversity, but they are unevenly distributed, with some narrow clades represented by many genomes (e.g., Mae2 LL/LG) and other clades having no representatives (**Fig. 1**). Thus, the known diversity of *Microcystis* is still sparsely represented by genome sequences, and likely also by cultured isolates. The phylogeny based on ITSc sequences is reasonably consistent with clusters defined by phylogeny of core genes (Pérez-Carrascal *et al.*, 2019). However, in some cases ITSc does not resolve clusters that were resolved by core genes (*e.g*., Mae4, Mae6), and in other cases ITSc sequences show more phylogenetic divergence/dispersion than clusters formed by core genes (*e.g.*, Mae1).

Pairwise comparisons of *Microcystis* genomes showed they all have > 93.6 % average nucleotide identity (ANI) across the shared parts of their genomes with every other *Microcystis* genome, with 99.8 % of comparisons > 94 % ANI and 70 % of comparisons > 95 % ANI (**Fig. 1**, histogram insert). This high ANI relatedness among sequenced *Microcystis* isolates has been consistently observed (Harke, Davis, *et al.*, 2016; Pérez-Carrascal *et al.*, 2019; Willis and Woodhouse, 2020). Based on a commonly used genomic definition of species, *i.e.*, a monophyletic group sharing > 94-95% ANI (Konstantinidis *et al.*, 2006; Chan

et al., 2012)**,** this implies that most sequenced *Microcystis* strains belong to a single species. However, 19 named *Microcystis* species exist, named primarily based on colony morphology (morphospecies). Considering the plasticity observed in morphology within single isolates (Yang *et al.*, 2006), it has long been clear that colony morphology alone does not accurately delineate species within the *Microcystis* genus, and regrouping of multiple morphospecies has been suggested before (Kondo *et al.*, 2000; Otsuka *et al.*, 2001; Harke, Steffen, *et al.*, 2016). Comparison of morphospecies delineations with genomic information shows that distinct clusters of *Microcystis* genomes sharing > 96% ANI only partially correspond to current *Microcystis* morphospecies, and the monophyletic morphospecies often correspond to highly related strains sampled at a single location (Pérez-Carrascal *et al.*, 2019; Willis and Woodhouse, 2020). It has been argued that a strict species definition based on an ANI cutoff of 94-95 % may not match the reality of species delineations across bacterial and archaeal life, and instead that levels of recombination relative to mutation among strains should be evaluated to group strains into species (Bobay and Ochman, 2017; Arevalo *et al.*, 2018; Shapiro, 2018). For *Microcystis*, inferred recombination rates between some of the > 96 % ANI clusters are lower than within some of these genetic clusters, making the case that speciation events have occurred or are ongoing within the *Microcystis* genus (Pérez-Carrascal *et al.*, 2019).

These observations for *Microcystis* stand in contrast to the genus of Earth's most abundant cyanobacterium, *Prochlorococcus*. The ANI among sequenced strains of *Prochlorococcus* can be as low as 65% (Paul *et al.*, 2019), yet genomic subclusters are referred to as 'ecotypes' of a single species, *Prochlorococcus marinus*, rather than different species of *Prochlorococcus* (Biller *et al.*, 2015). Ecotypes, a concept originating from botany, are groups of genetically distinct strains within a species that are adapted to specific environmental conditions (Turesson, 1922). Translating this concept to bacteria poses challenges and the term has been interpreted in various ways. Considering the genome-based species cutoff of 94-95 % ANI, the choice for ecotype rather than species delineations within *Prochlorococcus* seems contrary to genomic evidence. However, a recent analysis inferred substantial homologous

recombination between distantly related strains of *Prochlorococcus*, suggesting the absence of strict species boundaries (Bobay and Ochman, 2017). Going back to the initial definitions of the term ecotype in sexually reproducing organisms, ecotypes are still interfertile with other ecotypes of the same species, but gene flow between ecotypes is limited (though not eliminated) by ecological barriers, i.e., lack of spatial or temporal co-occurrence (Turrill, 1946). Thus, considering the limited evolutionary divergence of core genes among sequenced *Microcystis* genomes, the variable levels of genetic exchange across and within genetic subclusters of *Microcystis* does not necessarily imply that these should be considered distinct species rather than ecotypes that are a product of the ongoing process of speciation. Further sampling of the genomic diversity of *Microcystis* and analyses of the extent to which recombination maintains cohesive subclusters within the genus will help determine whether multiple species of *Microcystis* should be delineated. Alternatively, all genomic subclusters of *Microcystis* may be considered as 'ecotypes' of the most commonly named species, *Microcystis aeruginosa*, which currently already comprises a polyphyletic group of strains distributed across the *Microcystis* phylogeny (Pérez-Carrascal *et al.*, 2019; Willis and Woodhouse, 2020) (**Fig. 2**), as proposed previously (Harke, Davis, *et al.*, 2016).

In the case of *Prochlorococcus*, ecotypes have been linked to various environmental factors, with adaptations to light conditions along vertical layers of the ocean delineating the primary separation between phylogenetic clusters. Adaptations to different levels of iron, temperature, and N and P availability are conserved only at finer phylogenetic scales (Biller *et al.*, 2015; Martiny *et al.*, 2015)**.** *Synechococcus*, another common marine cyanobacterium, has a biogeography that is more evident horizontally, driven by temperature, macronutrients, and iron availability (Sohm *et al.*, 2016). In the case of *Microcystis*, limited mapping of ecological niche to genomic clusters has been carried out, with the exception of the low phosphorus/high phosphorus dichotomy observed in a set of 46 isolates originating from Michigan lakes (Berry *et al.*, 2017; Jackrel *et al.*, 2019). This lack of mapping of environmental niche to genomic clusters highlights an important knowledge gap that should be explored. Conveniently, the presence of a *Microcystis* colony in a water body indicates fitness of the strain in this environment, as

collected colonies are frequently composed of 10^4 to 10^5 cells (Costas *et al.*, 2008). Thus, mapping of environmental conditions of the water body from which a culture originated onto phylogenetic trees may reveal additional environmental optima for genomic subclusters.

In the absence of extensive environmental data to identify ecotypes, we can also resort to a reverse ecology approach, inferring ecology from genomic information. This has been done either by identifying genes specific to each genomic subcluster, as recently done for *Microcystis* (Willis and Woodhouse, 2020), or by determining the levels of recent gene flow to delineate ecologically relevant populations (Arevalo *et al.*, 2018)(Pérez-Carrascal *et al.*, 2019). The latter study found some genomic subclusters to correspond to higher levels of gene flow within than between clusters, but this was not universally the case. The former approach, *i.e.*, inferring functions from genes specific to genotypic clusters, identified genes of potential interest involved in nutrient adaptation, viral defense, membrane biosynthesis, DNA repair, chaperones, and Ca^{2+} transport (Willis and Woodhouse, 2020). We find patterns in the presence of genes involved in key processes between genotypic clusters, suggesting variable adaptations to conditions related to these traits (**Fig. 2**, **Fig. S1**). For example, in the super-clade including Mwe, Mae8, and Mae10 (top of phylogenetic tree) the absence of *mcy* genes correlates with absence of genes for ammonium transporters (*amt*), consistent with a link between microcystin production and uptake of ammonium (Chaffin *et al.*, 2018), but this linkage is not universal across clades. Taken together, this mix-and-match of various genes (and presumably traits) across the sub-species clusters within *Microcystis* suggests a diversity of multi-dimensional niches defined by various combinations of traits. Full implementation of the reverse ecology approach awaits clear delineation of ecologically relevant genotypic clusters and the environments to which they are adapted. Jackrel and colleagues illustrated the promise of this approach by showing that isolates from low-nutrient lakes have genome-wide reductions in nitrogen use and an expansion of phosphorus assimilation genes (Jackrel *et al.*, 2019).

Comparison of genotypic relatedness based on gene content versus phylogeny of core genes shows similarity in some clustering but differences in others (**Fig. 2**). Such discrepancies are expected given that the consensus phylogeny of core genes represents a mixture of many different evolutionary histories of single genes (Bapteste *et al.*, 2007; Thiergart *et al.*, 2014), whereas gene content may be better at differentiating phenotypes of sub-species clusters shaped by horizontal gene transfer (Van Rossum *et al.*, 2020) but does not necessarily reflect the phylogeny of the evolutionary core. Closer examination of geographic and environmental drivers of these evolutionary patterns is needed. In addition, current analyses have not yet taken into account smaller differences in sequence composition of shared genes with functional impact (*e.g.*, variation in microcystin congener biosynthesis) (Mikalsen *et al.*, 2003; Pearson *et al.*, 2004; Tooming-Klunderud *et al.*, 2008) nor evolution of gene expression of shared genes, which has been found to be a driving factor in ecological divergence between closely related organisms (Whitehead and Crawford, 2006; Denef *et al.*, 2010), including *Microcystis* (Srivastava *et al.*, 2019). There are also high levels of genomic variation within genomic clusters, driven by large levels of horizontal gene transfer between *Microcystis* genomes as well as external sources (Humbert *et al.*, 2013; Meyer *et al.*, 2017; Willis and Woodhouse, 2020), further underscoring the extensive genomic diversity within *Microcystis*. Despite these caveats and remaining gaps in knowledge, it is clear that there is a genetic clustering of strains within *Microcystis*, with each cluster having a unique set of flexible genes, suggesting that they are ecologically distinct. Testing this hypothesis will ultimately require phenotypic characterization of isolates from the different clusters; we consider the current state of this endeavor in the next section.

Functional diversity of *Microcystis* strains

Distinct temporal and spatial patterns of strain abundance and genetic clustering of strains suggest ecologically distinct sub-species groups of *Microcystis*, but higher-order and neutral processes also contribute to community assembly and genomic evolution and thus care should be taken not to

automatically assume selection for specific genotypes to explain these observations (Lynch, 2007; Nemergut *et al.*, 2013; Dick, 2018). Direct examination of the distribution of traits across the *Microcystis* phylogeny can further inform the ecological relevance of observed genetic clusters; differentiated traits may represent adaptations to specific environmental conditions. Studies of multiple traits across have shown significant differences between *Microcystis* strains (López-Rodas *et al.*, 2006; Wilson *et al.*, 2006), but to our knowledge these data have not been comprehensively synthesized across studies or been put into phylogenetic context. While we consider each trait independently, in reality they are intertwined. For example, the response to light is temperature-dependent and the response to temperature and oxidative stress depends on nutrient availability (Thomas and Litchman, 2016; Sandrini *et al.*, 2020). Similarly, the response to oxidative stress is also light dependent (Drábková *et al.*, 2007, 2007; Mikula *et al.*, 2012; Piel *et al.*, 2019) and the impact of inorganic carbon availability depends on other co-stressors (Gao *et al.*, 2012; Harvey *et al.*, 2013; Glibert, 2020). The effect of environmental factors, such as stimulation of microcystins production by nitrogen for example, may be linked to growth rate rather than specific metabolic processes (Orr *et al.*, 1998). Further, comparisons across studies may be influenced by conditions that were unreported or difficult to control for, such as subtle differences between light sources or growth media. Finally, growth rates may be influenced by whether strains form colonies or are single celled (Wilson *et al.*, 2006), a property that can change during lab culture. In some cases we can constrain such effects whereas in others there is insufficient information, and these limitations highlight the need for future work, especially studies that specifically assess traits across strains representative of the diversity within the genus.

Colony morphology and buoyancy

Microcystis cells clump together within an exopolysaccharide-rich mucilage matrix to form colonies of various sizes and shapes. Recent evidence from metagenomic sequencing of single colonies or cultures suggests that colonies initiated from clonal expansion from single cells are more prevalent than clumping

of distinct genotypes (Jackrel *et al.*, 2019; Pérez-Carrascal *et al.*, 2020). Although colony morphology was once used for species identification (Otsuka *et al.*, 2000), this trait is now known to change with environmental conditions and season, and genetic data supports the unification of morphospecies into a single species (Harke, Davis, *et al.*, 2016; Xiao *et al.*, 2018; Pérez-Carrascal *et al.*, 2019). In addition, colony morphology varies across strains under the same culturing conditions (Wilson *et al.*, 2006), and has important implications for surface area to volume ratio and thus nutrient uptake and growth rate (Wilson *et al.*, 2010), avoidance of grazing (Webster and Peters, 1978; Fogg, 1991; Yang *et al.*, 2006; see grazing section below), and light competition or availability to individual cells (Reynolds, 1984), suggesting that it could shape the strain composition of *Microcystis* blooms (Wilson *et al.*, 2006). Colony size and morphology also shows a relationship with presence of *mcy* genes for biosynthesis of microcystins (Kurmayer *et al.*, 2003; Via-Ordorika *et al.*, 2004).

Buoyancy regulation is an interplay of vesicle synthesis, protein and carbohydrate production, colony size, and light intensity (Xiao *et al.*, 2012). Like the seasonal succession of morphospecies, gas vesicle formation changes with the season in response to environmental factors (Xiao *et al.*, 2018)**.** Different laboratory strains of *Microcystis* have different gas vesicle production characteristics that are regulated by the *gvp* gene, which varies with light intensity. For example, strains that may not float at typical lab culture light intensities become buoyant at high light intensities and floating strains may sink at high light intensities (Xiao *et al.*, 2012). The extent to which buoyancy varies among strains and how this variation is genetically encoded remains unclear.

Nutrient requirements and biomass stoichiometry

Microcystis blooms are manifestations of excess nutrients, particularly nitrogen (N) and phosphorus (P). Therefore the N and P content of *Microcystis* biomass is a critical trait for understanding how elevated N and P concentrations become blooms (Gerloff and Skoog, 1954), how *Microcystis* competes with other phytoplankton (Olsen, 1989; Olsen *et al.*, 1989), and how the stoichiometry of these elements shapes

toxin production (Van de Waal *et al.*, 2014). Like other phytoplankton, *Microcystis* exhibits substantial plasticity in the quantity of N and P in its biomass (**Fig. 3A-B**). The median strain can vary its P quota (atoms P per cell) by 4-fold, biomass P content by 8-fold, and molar N:P ratio in biomass by 6-fold. While some of the differences among strains in Figure 3A are attributable to differences in culture conditions, the two studies that compared multiple strains indicate that strains differ in both their P content and plasticity (Hesse and Kohl, 2001; Saxton *et al.*, 2012). Some of this intra and inter-strain variability in N and P content is attributable to two determinants of elemental content within phytoplankton: nutrient availability (Rhee, 1973) and growth rate (Droop, 1973), which can act both individually and interactively (Flynn *et al.*, 2010; Hillebrand *et al.*, 2013). Figure 3 (C-D) shows that, within individual strains, biomass N and P content are positively correlated with growth rate, but the differences among strains are substantial. Wide differences in response to N limitation between strains (Brandenburg *et al.*, 2018) further supports strain-dependent quotas. Additional experiments with multiple strains would help to determine the extent of intraspecific variability in these traits. Further, the outcome of competition depends on whether nutrients are supplied constantly or in pulses (Suominen *et al.*, 2017), so the capacity for some *Microcystis* strains to assimilate N and P beyond their immediate needs for growth underscores the need for care when extrapolating from laboratory experiments to dynamics in the field.

Whereas the nutrient content of *Microcystis* influences the amount of biomass that potentially results from excess nutrients, development of a bloom also requires that the concentrations of available nutrients are sufficient for *Microcystis* to maintain a positive net growth rate despite competition from other species (Holm and Armstrong, 1981; Marinho and De Oliveira E Azevedo, 2007). However, no study to date has compared the Monod kinetics (*i.e.*, growth rate as a saturating function of N or P concentration) or Droop kinetics (growth rate as a function of internal quota) for more than a few strains of *Microcystis*. Available data among studies show that the initial slope of the Monod function (d^{-1} μM^{-1}, (Healey, 1980)) ranges from 1.3 to 24 for P (Ahlgren, 1985; Baldia *et al.*, 2007) and 0.014 to 1.3 for N (Kappers, 1985; Tan *et*

al., 2019). While some of this variation in the relationship between resource N and P and growth rate is attributable to differences in experimental design, there is also evidence that strains differ in their affinity for acquiring these elements. Comparison of P uptake kinetics in eight strains of *Microcystis* and showed that the half-saturation constant for phosphate uptake ranged from 9.5-19.5 µM and the apparent maximum uptake rate ranged from 0.27-0.57 µM mg^{-1} h^{-1} (Shen and Song, 2007). Given the variation among strains in both resource use kinetics and biomass elemental content, and the apparent sorting of genotypes based on resource availability (Jackrel *et al.*, 2019), further experiments that systematically characterize this variation would be especially useful.

Nitrogen uptake and metabolism

Nitrogen plays an important role in stimulating and sustaining *Microcystis* blooms, and nitrogen availability appears to strongly influence microcystin cellular quotas (Harke and Gobler, 2013, 2015; Horst *et al.*, 2014). Furthermore, nitrogen addition to natural communities promotes blooms with significantly higher microcystins concentrations to a greater extent than phosphorus additions (Donald *et al.*, 2011; Davis *et al.*, 2015; Jankowiak *et al.*, 2019). The regulation of microcystins production likely involves both C and N metabolism (see below), and the highest concentrations of microcystins correlated with high N:P ratios, in both the lab and the field (Downing *et al.*, 2005; Beversdorf *et al.*, 2015). The rate of microcystins production is tightly coupled to cell division rates, suggesting potential for indirect effects of environmental factors on microcystin production (Orr *et al.*, 1998, Lyck *et al.*, 2004). However, in some cases microcystins production rate is strongly positively correlated with nitrogen uptake rate (and negatively correlated with phosphorus uptake and carbon fixation rates) independent of growth rate (Downing *et al.*, 2005). To our knowledge, no study to date has examined the effect of growth rate and nitrogen availability on microcystins production or cellular content across multiple *Microcystins* strains using a common approach, thus the extent to which these different results can be explained by different strains is unclear.

Both field and lab culture data suggest that there are strain-specific differences in nitrogen metabolism, but the driving mechanisms are still largely unknown. Most strains harbor similar genetic capabilities for nitrogen assimilation and transport (**Fig. 2; Fig. S1**), thus observed phenotypic differences likely indicate differences in regulation of gene expression and/or functional differences (*e.g.*, affinity, kinetics) associated with alleles. This is consistent with differential expression of several proteins involved in nitrogen uptake and metabolism between strains, under both N-replete and N-limited conditions (Alexova *et al.*, 2011, 2016). Ammonium transporters (*amt*, *amtc*) are among the most variably present N-related genes across *Microcystis* strains (**Fig. 2**). Increased availability of inorganic nitrogen sources leads to production of more microcystins by toxigenic strains (Orr and Jones, 1998), which apparently have a higher nitrogen requirement than non-toxigenic strains, and require higher nitrogen concentrations to achieve their maximum growth rate (Vézie *et al.*, 2002). Co-culture competition experiments further suggest that toxigenic strains grow better at high combined (N and P) nutrient concentrations, whereas non-toxigenic strains grow better at low concentrations (Vézie *et al.*, 2002). However, it is unclear whether these conclusions apply broadly to toxigenic and non-toxigenic strains across the diversity of *Microcystis*, or rather are confined to the few strains tested.

Observations of natural communities also suggest that the form and concentration of nitrogen influences the abundance of *Microcystis* strains/species (Monchamp *et al.*, 2014). Nitrogen enrichment, particularly inorganic forms of N like nitrate and ammonia, promotes toxigenic strains over non-toxigenic strains (Davis *et al.*, 2009, 2010). Non-toxigenic strains were stimulated by organic forms of N like urea and glutamic acid (Davis *et al.*, 2010). This could explain in part why community shifts from mostly toxigenic to non-toxigenic strains are often observed midway through the bloom in seasonally stratified systems, as the primary available nitrogen source often changes from inorganic to organic forms (Davis *et al.*, 2010; Beversdorf *et al.*, 2015; Gobler *et al.*, 2016). However, the relative importance of inorganic nitrogen vs organic nitrogen sources in driving *Microcystis* strain composition of blooms remains poorly understood, especially considering that dissolved organic nitrogen concentrations in freshwaters are rarely quantified.

Temperature

Microcystis blooms rarely occur at cooler temperatures (< 20°C). Water temperature affects *Microcystis* in many ways including colony size, metabolic rate, buoyancy, chlorophyll-a concentration, toxin concentration, and growth rate (Wantabe and Oishi, 1985; Staehr and Birkeland, 2006; Bouchard and Purdie, 2011; Li and Reidenbach, 2014; Duan *et al.*, 2018; You *et al.*, 2018). Temperature also influences competition, including between *Microcystis* and other bloom forming algae (Chu *et al.*, 2007; Lürling *et al.*, 2013; Thomas and Litchman, 2016) and between different *Microcystis* strains (Xiao *et al.*, 2017). Cyanobacteria generally have a higher temperature of optimal growth than other freshwater phytoplankton, thus there is an apparent link between increasing temperatures and global proliferation of cyanobacterial blooms (Paerl and Huisman, 2008).

As reported previously and compiled here, different *Microcystis* species/strains have distinct growth responses to temperature (Xiao *et al.*, 2017; Bui *et al.*, 2018)(**Fig. 4**). Optimum temperature for growth ranges from 25℃ to 35℃, and this effect appears to be independent of light levels and other experimental conditions, suggesting that optimal growth temperature is a key trait that may differentiate strain niches. Unfortunately, growth rate data at different temperatures are sparse, and thus many of the temperature optima are imprecise. Moreover, there is little data on non-toxigenic strains, and **Fig. 4** does not clarify the relationship between production of microcystins and temperature adaptations. However, taken together with the strong effect of temperature on *Microcystis* physiology, these results suggest that temperature influences bloom occurrence, intensity, and strain composition. Supporting this hypothesis, higher temperature favored toxigenic strains in some studies (Davis *et al.*, 2009; Dziallas and Grossart, 2011). However, in other lab culture experiments, a non-toxigenic strain had higher optimal growth temperature than toxic strains (Thomas and Litchman, 2016). The microcystins quota per cell decreases with increasing temperature (Bui *et al.*, 2018; Peng *et al.*, 2018), counteracting the effect of growth rate and strain selection, which further complicates the effect of temperature on bloom toxicity. However, there is also some evidence that the cell quota of microcystins increases with temperature, thus the

response and threshold of how temperature influences microcystin quota may be strain dependent (van der Westhuizen and Eloff, 1985; Wantabe and Oishi, 1985; Mowe *et al.*, 2015). These inconsistent conclusions from field and lab data highlight persistent gaps in knowledge; we have a highly incomplete picture of how temperature response maps onto genotype and phylogeny.

Light

Light intensity impacts the growth rate (Wiedner *et al.*, 2003), toxin production (Wiedner *et al.*, 2003; Salvador *et al.*, 2016), and buoyancy (Brookes, 2001; Straub *et al.*, 2011) of *Microcystis*, and it is a major factor in determining the growth and cell density of *Microcystis* blooms (Huisman *et al.*, 1999; Tomioka *et al.*, 2011; Verspagen *et al.*, 2014). Responses to light include both short-term cellular acclimation as well as evolutionary adaptation of strains. Studies of gene expression offer insight into the molecular encoding of light acclimation and adaptation (Srivastava *et al.*, 2019). High light intensity causes oxidative stress (Zilliges *et al.*, 2011), presenting a challenge for buoyant scums at the water-air interface (Brookes *et al.*, 2003). The effect of light on *Microcystis* is complex because it depends on several processes and factors, including light quality (*i.e.*, wavelength), turbulence, tolerance of oxidative stress, photoacclimation, and colony formation, which themselves may be dependent on water depth and clarity, temperature, media composition, and adaptation to laboratory culturing conditions. The effect of light is also complex in the context of a community – different cells and colonies (and perhaps strains) in a single habitat can experience vast differences in intensity at a given time, depending on factors such as water depth and microenvironment. Light limitation may increase the phenotypic heterogeneity within *Microcystis* communities, reducing overlap in traits between cells and therefore increasing the probability of individual success (Fontana *et al.*, 2019). These findings suggest that competition, both interspecies and intraspecies, is light-dependent (Fontana *et al.*, 2019).

The diversity of light adaptations across *Microcystis* strains has long been of interest in terms of how it shapes the relative success of toxigenic and non-toxigenic strains of *Microcystis*. In some cases, toxigenic

strains are less competitive under light limitation, perhaps due to the cost of microcystins production (Kardinaal *et al.*, 2007; Renaud *et al.*, 2011; Van De Waal *et al.*, 2011; Briand *et al.*, 2012). Consistent with this conclusion, some toxigenic strains are adapted to high light conditions and non-toxigenic strains to low light conditions (Deblois and Juneau, 2012), and UV-B radiation may inhibit growth of non-toxigenic strains more than toxigenic strains (Yang *et al.*, 2015). Many studies have found that non-toxigenic strains dominate blooms later in the summer, when light and nutrient limitation are highest, indicating that non-toxigenic strains are better at competing for light in the field (Janse *et al.*, 2004; Bozarth *et al.*, 2010). It is also interesting to note that all strains isolated from Michigan lakes with low total phosphorus (which have clear water and thus high light availability) are toxigenic (the Mae2 LL/LG clade in **Fig. 2**), supporting the link between high light and toxicity. However, other studies observed toxigenic strains that dominate under low light, which undermines the link of microcystin production to light adaptation (Schatz *et al.*, 2005; Renaud *et al.*, 2011; Zhai *et al.*, 2013). As indicated in **Fig. 2** and discussed in the conclusion, the ability to produce microcystins is scattered throughout the *Microcystis* phylogeny and it is not directly correlated with presence/absence of many functional genes, raising the question of whether traits are coordinated (co-vary). Production of microcystins is just one trait, which may or may not be linked to other traits. From this viewpoint it is not surprising to see conflicting conclusions for light adaptation of toxigenic and non-toxigenic strains.

A compilation of the growth response of *Microcystis* strains to light intensity shows several interesting trends (**Fig. 5**). First, while *Microcystis* growth occurs across a broad range of light intensities, the optimum for growth for all strains (where apparent) occurs within a relatively narrow range of 25-100 μmol photons m^{-2} s^{-1}. Second, there appear to be differences in optimal light level between strains, independent of temperature and media composition. However, in many cases the optimum is poorly defined due to the limited number of measurements. Third, the shape of the curve for growth response to light varies, indicating that some strains are photoinhibited (*e.g.*, NIES 111, CS 338, CS 558) at high light whereas others are not (*e.g.*, CPCC 299, MIC 08). In part this may be explained by colony-forming traits;

single-cells reach light saturation at lower light levels than colony-forming strains (Chorus, 2001). Unfortunately, colony-forming traits are not widely reported for the strains (**Table S3**). No evidence of a relationship between growth response to light intensity and capability of producing microcystins was identified in our review (**Fig. 5**). While these and other data support the conclusion that light adaptation may be a key trait for differentiating the physiology of *Microcystis* strains, more precise and controlled measurements are needed to directly compare across strains and evaluate links and tradeoffs with other traits.

Oxidative stress

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) are ubiquitous in aquatic ecosystems and may influence microbial community composition because of varying sensitivity among taxa due to variable presence of genes for catalase and peroxidase enzymes that decompose H_2O_2 (Perelman *et al.*, 2003; Morris *et al.*, 2008, 2011; Kim *et al.*, 2016). This includes large differences in sensitivity between genera (Ostrowski *et al.*, 2001; Perelman *et al.*, 2003; Morris *et al.*, 2008, 2011; Kim *et al.*, 2016, 2019; Ma *et al.*, 2018; Bayer *et al.*, 2019) as well as subtle differences among different strains (Morris *et al.*, 2011; Bayer *et al.*, 2019), suggesting that ROS may affect community composition at multiple taxonomic levels. Cyanobacteria are particularly sensitive to H_2O_2, enabling treatment of cyanobacterial blooms with H_2O_2, which kills the cyanobacteria, leaves algae and other organisms intact, and decomposes to water and O_2 (Matthijs *et al.*, 2012). H_2O_2 at natural concentrations may also impact the strain composition of *Microcystis* blooms (Paerl and Otten, 2013a; Schuurmans *et al.*, 2018). This hypothesis is supported by findings that microcystins covalently bind to photosynthetic proteins that are sensitive to oxidative damage (Zilliges *et al.*, 2011), thus protecting toxigenic strains and favoring them over non-toxigenic strains in blooms under oxidative stress (Dziallas and Grossart, 2011; Zilliges *et al.*, 2011; Paerl and Otten, 2013a).

To date, the differential impact of H_2O_2 on growth of *Microcystis* strains has only been explored along the toxigenic vs. non-toxigenic dichotomy (Dziallas and Grossart, 2011; Zilliges *et al.*, 2011; Paerl and Otten, 2013a; Schuurmans *et al.*, 2018), yielding conflicting results. In one culturing study, three microcystins producing strains had lower reductions in chlorophyll content than two non-toxigenic strains when treated with H_2O_2 (Dziallas and Grossart, 2011). However, the opposite result was found in a study where the mutant strain unable to produce microcystins degraded H_2O_2 faster than the toxic wild type, and a natural non-toxic strain could recover from large additions of H_2O_2 at low light intensities (Schuurmans *et al.*, 2018). This may indicate that the H_2O_2 sensitivity of *Microcystis* strains varies, albeit not in the previously hypothesized toxigenic vs. nontoxigenic manner. However, the conflicting results could be due to lack of standardized conditions between experiments. The effect of H_2O_2 on cyanobacteria is dependent on light intensity (Drábková *et al.*, 2007; Morris *et al.*, 2011; Piel *et al.*, 2019) and wavelength (Sandrini *et al.*, 2020), deviation from optimal growth temperature (Ma *et al.*, 2018), cell density (Morris *et al.*, 2008, 2011), and associated heterotrophic bacteria (Kim *et al.*, 2019). Therefore, it is important to standardize these parameters in experiments comparing the H_2O_2 sensitivities of cyanobacteria. The aforementioned studies were performed at different light and temperature regimes, which makes interpreting and comparing their results difficult. In addition, Schuurmans et al. largely measured growth inhibition at large H_2O_2 doses, orders of magnitude above concentrations observed in natural waters (Cooper *et al.*, 1989; Cory *et al.*, 2016), and used light levels far below those typical of natural sunlight (Sagert and Schubert, 2000). The Dziallas and Grossart and Zilliges et al. studies did not measure background H_2O_2 concentrations of the growth media during their experiment, so the total H_2O_2 exposure in their experiments is unknown. Therefore, the extent to which naturally occurring H_2O_2 concentrations impact the growth of different *Microcystis* strains is still uncertain.

The distribution of genes encoding catalases and peroxidases among publicly available *Microcystis* genomes is similar to that of *Prochlorococcus* and *Nitrosopumilus* (Morris *et al.*, 2012; Kim *et al.*, 2016, 2019); the majority lack heme catalases and peroxidases and contain multiple peroxiredoxin genes (**Fig.**

2). This suggests that like *Prochlorococcus* and *Nitrosopumilus*, many *Microcystis* strains are sensitive to naturally occurring H_2O_2 concentrations. However, three strains of a nontoxic *Microcystis* clade have the catalase-peroxidase *katG*, the canonical enzyme for degradation of high extracellular H_2O_2 concentrations (Seaver and Imlay, 2001; Perelman *et al.*, 2003; Cosgrove *et al.*, 2007; Morris *et al.*, 2012). In addition, several *Microcystis* genomes have genes annotated as animal haem peroxidases (AHPs), cell surface enzymes that oxidize manganese through superoxide production and H_2O_2 decomposition (Wariishi *et al.*, 1992; Scheeline *et al.*, 1997; Schlosser and Höfer, 2002; Anderson *et al.*, 2009; Andeer *et al.*, 2015). The dual ROS production and decay activity of these enzymes makes it unclear whether they influence oxidative stress in microbes or if the ROS are merely transient intermediates in the oxidation of manganese and other metals (Zinser, 2018). Overall, these genomic insights support the physiological evidence for variation in resistance to ROS across *Microcystis* strains.

Inorganic carbon/pH

Microcystis strains also have distinct adaptations to availability of dissolved inorganic carbon (C_i) (Van De Waal *et al.*, 2011; Sandrini *et al.*, 2016), raising the question of how availability of C_i influences the strain composition, and thus toxicity, of *Microcystis* blooms. Dynamics of C_i play out across several time scales of interest including daily (diel cycles associated with photosynthesis), seasonal (*e.g.*, bloom development), and decadal and longer time scales associated with rising atmospheric CO_2 due to anthropogenic emissions. In all cases, C_i availability is intertwined with pH based on the chemical relation between pH and C_i system; shifts in pH determine the C_i availability for photosynthetic organisms, and photosynthesis alters the pH of the environment. pH can vary by up to 0.5 units on diel cycles (Krausfeldt *et al.*, 2019). On the time scale of bloom development, the initiation of *Microcystis* blooms is typically characterized by neutral or slightly acidic conditions (high pCO_2 concentrations), while dense blooms have pH levels that can exceed 9.5, at which pCO_2 concentrations in the water are essentially negligible, leading to potential carbon limitation (Bañares-España *et al.*, 2006; Gu *et al.*, 2011; Krausfeldt *et al.*, 2019). Lab experiments show that increased C_i availability induces changes in the strain

composition of blooms, suggesting that rising atmospheric CO_2 will shape the strain composition of blooms and evolution of *Microcystis* (Sandrini *et al.*, 2016). Further, both C_i availability and pH have a profound impact on biota at the community and ecosystem levels, likely leading to complex effects that must be considered in addition to the *Microcystis* strain-level effects that we focus on here.

A handful of experiments have explored the impact of C_i availability (pCO_2 of 100 – 1250 ppm) on a variety of *Microcystis* strains, showing a wide range of specific growth rates (0.152 to 1.04 d^{-1}; **Table S4**). It is important to note that these growth rates are not only influenced by C_i treatment, but also *Microcystis* strain, media type, light, and temperature (see the beginning of the section on *Functional diversity*). Several studies have used *Microcystis* sp. NIES 843 to explore the role of C_i availability, and synthesis of this data indicates that C_i concentration influences *Microcystis* growth rates (**Table S4**), suggesting a higher growth rate at low pCO_2 concentrations (pCO_2: 100 – 300 ppm). While these results do not conform with other studies examining C_i availability on phytoplankton (Wu *et al.*, 2010; Gao *et al.*, 2012; Errera *et al.*, 2014; Visser *et al.*, 2016; Raven *et al.*, 2020), they do fall in the middle range of growth rates for C_i/pH *Microcystis* studies. The physiological response to C_i availability is likely due to differences in carbon concentrating mechanism (CCM) between *Microcystis* strains, as NIES 843 has a low binding affinity for HCO_3^-, but a high flux rate, which has been suggested to favor low C_i water conditions (Sandrini *et al.*, 2014). Acidification from increased CO_2 can also induce oxidative stress in cyanobacteria (Wu *et al.*, 2021).

Strain-level adaptation to C_i stems largely from variation in the genes encoding the CCM, an assortment of enzymes and transporters that acquire bicarbonate (HCO_3^-) from the environment, actively transport it across the cell membrane, and concentrate CO_2 in the presence of Rubisco (Raven *et al.*, 2012). Variations in the use and effectiveness of CCM in algae have been identified at the genus, species, and strain level of phytoplankton (Giordano *et al.*, 2005; Beardall *et al.*, 2009; Reinfelder, 2011) including across *Microcystis* strains (Sandrini *et al.*, 2015; Van de Waal *et al.*, 2019). Of the five identified CCM transporters for cyanobacteria (Omata *et al.*, 1999; Shibata *et al.*, 2001, 2002; Price *et al.*, 2004; Price,

2011) the HCO_3^- uptake systems may provide the best insight into strain success during carbon replete and deplete conditions. Each transporter has a different affinity for HCO_3^- and flux rate, suggesting that the presence of one transporter over another could provide a competitive advantage depending on C_i concentrations within the system, as suggested by the review of NIES 843 (see Price, 2011; Sandrini *et al.*, 2014, 2015; Visser *et al.*, 2016 for additional information). Specifically, *bicA* and *sbtA*, which are located on the same operon, provide insight into a *Microcystis* strain's ability to uptake C_i. These HCO_3^- plasma membrane transporters (Price, 2011) have been identified in *Microcystis* strains as four possible genotypes (Sandrini *et al.*, 2014; Visser *et al.*, 2016). The C_i generalist, which has both *bicA* and *sbtA*, is able to take advantage of C_i across a wide range of concentrations. Other genotypes may be constrained through either substrate affinity or flux, including high HCO_3^- affinity specialists (*sbtA* only); high bicarbonate flux specialists (*bicA* only); and the strains that lack both of these bicarbonate transporters. In addition to the presence and absence of genes for bicarbonate transport, differences in their expression may underpin phenotypic differences among strains (Sandrini *et al.*, 2015).

Figure 2 shows the variability in the presence of CCM genes *sbtA* and *bicA* across *Microcystis* strains. 70% of strains analyzed are equipped with high HCO_3^- substrate affinity (*sbtA*), 26% of strains have a high flux transporter (*bicA*), and only 20% of strains have both. In contrast, the vast majority of all strains contain genes for both low and high affinity CO_2 uptake, ATP dependent HCO_3^- transporters (BCT), and carbonic anhydrase (CA) enzymes (Figure S1). The genotypic variability of *sbtA* and *bicA* CCM genes is not congruent with phylogeny, consistent with frequent gene gain and loss. However, there do appear to be interesting correlations between the presence-absence of these genes and others. For example, of the 36 strains that contain neither *bicA* or *sbtA*, 97% contain genes for biosynthesis of microcystins, perhaps reflecting a role for microcystins in carbon fixation (Zilliges *et al.*, 2011; Barchewitz *et al.*, 2019). Inorganic carbon availability can also influence the carbon-nitrogen balance (C:N), leading to shifts in microcystin concentrations and congener profiles. Liu et al. (2016) suggested that high C_i conditions increased the microcystin congeners with high C:N ratio in three *Microcystis* strains examined

(NIES1099, HUB524, PCC7820), although microcystin concentrations decreased under these conditions. Potential connections between toxin content, toxicity (*i.e.*, microcystin congener profile), and carbon metabolism are also apparent in the field. Blooms in Lake Erie shift from a dominance of *Microcystis* strains capable of producing microcystin to a community dominated by non-toxic strains (Davis *et al.*, 2009, 2010; Chaffin *et al.*, 2018) suggesting that ability to produce toxins and strain shifts during the bloom may also be connected to C_i uptake ability, as C_i availability decreases (*i.e.*, pH increase) over the course of the bloom. While more research is needed to understand these potential links, the data available now are consistent with inorganic carbon availability shaping the composition and toxicity of *Microcystis* strains during bloom formation and development.

Salinity

Recent expansion of *Microcystis* blooms into the estuarine and coastal marine environment has drawn attention to the dynamics of blooms along the continuum from freshwater to saltwater (Paerl *et al.*, 2018). Thus, understanding the effect of salinity on *Microcystis* growth, persistence, and toxin production is critical for managing and predicting ecosystem impacts of bloom expansion and transport towards marine estuaries and coasts. Studies on the tolerance of *Microcystis* to salinity have produced highly variable results, with some studies indicating growth and microcystins production in low-salinity waters (e.g., estuaries) and persistence in seawater for substantial periods of time (Lehman *et al.*, 2005; Miller *et al.*, 2010), while others indicate that *Microcystis* is highly sensitive to even low salinity conditions (Sellner *et al.*, 1988). At least some of this variation appears to be due to strain-specific adaptations to salinity (Preece *et al.*, 2017). Lab studies conducted after acclimation found distinct salt tolerances and stress responses to salinity for two different strains that originated in environments with contrasting salinity, confirming strain-specific adaptations (des Aulnois *et al.*, 2019).

Community Interactions

In addition to environmental and bottom-up controls discussed above, understanding the ecology of *Microcystis* strains in the context of their phenotypic traits and genes requires consideration of interactions with the broader community. This includes top-down controls such as predation by viruses, fungi, and consumers as well as interactions with other bacteria, both positive and negative.

Secondary metabolites and allelopathic interactions

Cyanobacteria are renowned for their production of a wide range of secondary metabolites, compounds that are not involved in primary metabolism and that often have roles in communication, sensing, or inhibition of other organisms (Ehrenreich *et al.*, 2005; Kehr *et al.*, 2011; Dittmann *et al.*, 2015). In terms of biosynthetic capacity, *Microcystis* is one of the most prolific cyanobacterial genera (Dittmann *et al.*, 2015), producing many cyclic peptides including aeruginosins, anabaenopeptins, cyanobactins, cyanopeptolins, microginins, microviridins, and microcystins (Carmichael, 1992; Pérez-Carrascal *et al.*, 2019). Microcystins in particular have been intensively studied due to their toxic effects on humans, livestock, and other animals, and the threat they pose to recreation and drinking water supplies (Steffen *et al.*, 2017). Indeed, this has led to a microcystin-centric view of *Microcystis*, with strains defined as "toxic" or "non-toxic" on the basis of their ability to produce microcystins. However, as discussed below, this practice is not accurate in terms of toxicity, it does not always reflect phylogeny, and the extent to which it is meaningful for the broader ecology of *Microcystis* strains is still unclear. The secondary metabolites produced by *Microcystis* and their role in allelopathic interactions have been reviewed extensively (Kehr *et al.*, 2011; Dittmann *et al.*, 2015; Pearson *et al.*, 2016; Huisman *et al.*, 2018). Here we focus on how the repertoire of secondary metabolites varies across *Microcystis* strains and its implications for the ecological differentiation of these strains.

The suite of secondary metabolites varies across strains of *Microcystis*, both in terms of the classes of metabolites and the chemical diversity of metabolites within classes. Both levels of diversity are apparent

to some extent in the genes encoding biosynthesis of these compounds, which typically occur in biosynthetic gene clusters (BGCs). Genomic BGC content is highly variable across *Microcystis* strains (**Fig. 2**) (Pérez-Carrascal *et al.*, 2019). Substantial diversity in the presence, sequence, orientation, and order of genes within BGCs also encodes structural and biochemical diversity in the products (Welker *et al.*, 2006; Tooming-Klunderud *et al.*, 2008). Many compounds are produced by non-ribosomal peptide synthetase (NRPS) and/or polyketide synthase (PKS) complexes, and on average there are about 5 NRPS/PKS BGCs present in each genome (Shih *et al.*, 2013; Otten and Paerl, 2015). In addition to BGCs encoding known compounds, many BGCs have not been linked to a known product (Shih *et al.*, 2013; Dittmann *et al.*, 2015), raising the possibility of unknown/emerging toxins and allelochemicals. The biochemical and ecological implications of varying BGC content per genome and gene content per BGC are largely unknown.

The microcystins illustrate current knowledge and remaining frontiers for one of the most intensively studied classes of secondary metabolites in cyanobacteria. Microcystins are produced by an NRPS-PKS multi-enzyme complex that is encoded in the *mcy* operon, which contains 10 genes spanning roughly 55 kb (Tillett *et al.*, 2000). The presence of *mcy* genes is scattered across the genus in a polyphyletic distribution (**Fig. 2**). However, their pattern of presence is congruent with core *Microcystis* phylogeny on the scale of sub-clades, indicating cohesion of this trait among closely related strains and suggesting that gain and loss and horizontal transfer of these genes is relatively rare. This is consistent with co-evolution of *mcy* genes and core genes (Rantala *et al.*, 2004)**.** The sequence and structure of the *mcy* operon is also highly diverse, and this variation can lead to production of different structural variants of microcystins (congeners) (Mikalsen *et al.*, 2003; Tooming-Klunderud *et al.*, 2008). There are over 270 of these microcystin congeners with varying degrees of toxicity and health effects (Bouaïcha *et al.*, 2019). While *mcy* operon structure and gene sequence influence which microcystin congeners are produced, genetics is not the only determinant; individual *Microcystis* strains can produce multiple congeners (Mikalsen *et al.*, 2003; Otten *et al.*, 2017). Environmental conditions such as availability of nitrogen (Puddick *et al.*, 2016),

external amino acids, (Tonk *et al.*, 2008) and C:N ratio (Liu *et al.*, 2016) also affect which congeners are synthesized, consistent with regulation of toxins according to ecological stoichiometry theory (Van De Waal *et al.*, 2009; Van de Waal *et al.*, 2014; Wagner *et al.*, 2019). Despite intensive study for over 50 years, the ecological and/or physiological functionality of microcystins is still unclear. Hypotheses on the role of microcystins include allelopathy, photosynthesis and light adaptation, grazer defense, oxidative stress protection, quorum sensing, iron acquisition, and have been reviewed extensively elsewhere (Omidi *et al.*, 2018).

Large gaps in knowledge also remain on the functions of other secondary metabolites in *Microcystis*, but substantial evidence supports the hypothesis that they enhance its competitiveness through allelopathic interactions (Briand *et al.*, 2016, 2019; Chia *et al.*, 2018). These compounds can inhibit the photosynthetic capacity, growth, and regulatory processes of competitors or predators (Huisman *et al.*, 2005; Chia *et al.*, 2018). While they may inhibit a wide variety of prokaryotic and eukaryotic competitors and predators (Van Wichelen *et al.*, 2016), there is also evidence that they play a role in competition between *Microcystis* strains (Schatz *et al.*, 2005; Zhai *et al.*, 2013) thus may be an important factor in shaping the strain composition of blooms. Other proposed functions for secondary metabolites include nutrient storage, grazer defense, and quorum sensing (Kaebernick *et al.*, 2001; Pflugmacher, 2002; Chia *et al.*, 2018; Omidi *et al.*, 2018; Burberg *et al.*, 2019).

The abundance and strain-based diversity of secondary metabolites in *Microcystis* confounds efforts to ascribe biochemical activities to particular compounds and confuses terminology associated with these secondary metabolites. Common use of “toxic” and “non-toxic” to refer to production of microcystins fails to address the diversity of microcystin congeners as well as the diverse suite of other *Microcystis* BGCs, including many of unknown function, which may pose risks to aquatic communities and human health. These issues extend to allelopathic interactions. For example, some results suggested that microcystins can inhibit competitors (Legrand *et al.*, 2003) but other studies have shown that this effect is

independent of microcystins (Briand *et al.*, 2012; Lei *et al.*, 2015; Dong *et al.*, 2019; Schmidt *et al.*, 2020). Indeed, other secondary metabolites may be more important in allelochemical interactions with other phytoplankton species, such as linoleic acid in the case of strain FACHB-905 (Song *et al.*, 2017). Interestingly, Perez-Carascel et al. (2019) observed a pattern in which *Microcystis* strains that lack complete *mcy* operons often contain another BGC instead. This may suggest that each strain has a suite of compounds in its arsenal that are tuned to the environment, competitors, and predators with which it co-evolved. Overall, extensive genome mining efforts, metabolomic profiling studies, and experiments all suggest that the paradigm of microcystin-based toxic and non-toxic strains is outdated both in terms of allelopathic capabilities and health effects of compounds produced by *Microcystis.* A more holistic perspective is needed.

The Microcystis *microbiome*

Microcystis harbors bacteria in the phycosphere within and around their colonies, and these microbial communities are distinct from those in the surrounding environment and associated with other phytoplankton (Smith *et al.* submitted; Cai *et al.*, 2014; Xu *et al.*, 2018; Yang *et al.*, 2018; Batista *et al.*, 2019). Thus, differences in observed traits between *Microcystis* strains are potentially due to differences in their associated microbiomes. While our understanding of this microbiome and its effect on *Microcystis* are still in their infancy, one notable example of how microbiomes affect host traits is that colony formation can be induced by introduction of bacteria to axenic *Microcystis* cells (Shen *et al.*, 2011).

Studies of the phycosphere of other phytoplankton provide insights into potential interactions between *Microcystis* and its microbiome. Many bacteria in the phycosphere, which are predominantly heterotrophic, engage in mutualistic interactions with their phytoplankton hosts. Phytoplankton provide organic carbon and sulfur to heterotrophic bacteria while the heterotrophs supply vitamins and other cofactors (Croft *et al.*, 2005; Amin *et al.*, 2015; Durham *et al.*, 2015, 2017), regenerate nutrients from

organic matter (Van Mooy *et al.*, 2012; Amin *et al.*, 2015; Arandia-Gorostidi *et al.*, 2017; Christie-Oleza *et al.*, 2017), fix nitrogen (Cook *et al.*, 2020), and detoxify ROS (Morris *et al.*, 2011; Ma *et al.*, 2018) for their phytoplankton hosts. Other interactions with bacteria may harm phytoplankton; some bacteria act as obligate parasites (Caiola and Pellegrini, 1984; Rendulic *et al.*, 2004; Seyedsayamdost *et al.*, 2011; Soo *et al.*, 2015; Agha *et al.*, 2016). In some cases, a single bacterium can either improve or hinder phytoplankton growth depending on growth conditions (Grossart and Simon, 2007; Seyedsayamdost *et al.*, 2011; Hennon *et al.*, 2018).

Similar to this spectrum of interactions observed in other phytoplankton, the exudates of heterotrophic bacteria can both positively and negatively impact *Microcystis* growth rates (Akins *et al.*, 2020). While exchange of specific metabolites is currently poorly characterized, some evidence suggests the mucilage layer of *Microcystis* colonies mediates interactions between *Microcystis* and other microbes. Many bacteria adhere to the exopolysaccharide mucilage of *Microcystis* colonies (Smith *et al.* submitted; Worm and Søndergaard, 1998; Brunberg, 1999; Agha *et al.*, 2016) and can degrade it, presumably using it as a carbon and energy source (Li *et al.*, 2009; Shen *et al.*, 2011). The chemical composition of the mucilage, which can vary between *Microcystis* strains (Forni *et al.*, 1997), may attract specific bacterial populations (Smith *et al.* submitted) or influence the outcomes of microbial interactions (Agha *et al.*, 2016). Identification of specific compounds exchanged between *Microcystis* strains and their microbiomes will be valuable in revealing the currency and variation of these interactions.

Emerging evidence suggests that the taxonomic composition of the *Microcystis* microbiome may have some strain specificity. Some of the same taxa are regularly observed during *Microcystis* blooms (Cook *et al.*, 2020), but its microbial community composition varies considerably as a function of time (Parveen *et al.*, 2013; Berry *et al.*, 2017; Chun *et al.*, 2020; Jankowiak and Gobler, 2020), location (Jankowiak and Gobler, 2020), or the dominating genotype of *Microcystis* (Chun *et al.*, 2020). Analysis of communities associated with *Microcystis*, either via culturing or direct sequencing of single colonies, shows that

different *Microcystis* strains have distinct microbiomes at a taxonomic level (Smith *et al.* submitted; Jackrel *et al.*, 2019; Pérez-Carrascal *et al.*, 2020). This is in line with species-specific microbiome assembly experimentally observed for other phytoplankton (Jackrel, Yang, *et al.*, 2020). Sequence data from single colonies of *Microcystis* show that several taxa are prevalent but none universally present across colonies, and more closely related genotypes, either based on *Microcystis* oligotypes or whole genomes, harbored more similar microbiomes (Smith *et al.* submitted; Pérez-Carrascal *et al.*, 2020). Despite this taxonomic correlation, the relationship between host functions and microbiomes functions is weak, and microbiomes broadly converge at a functional level (Jackrel *et al.*, 2019; Cook *et al.*, 2020). Multiple studies have observed that microbiomes added functions and sometimes complemented steps in metabolic pathways missing in the *Microcystis* host genome (Jackrel *et al.*, 2019; Pérez-Carrascal *et al.*, 2019; Cook *et al.*, 2020). This is in line with Black Queen dynamics, by which significant gene losses are compensated through provision of 'public goods' by community members (Morris *et al.*, 2012; Morris, 2015), as seen in the case of oxidative-stress genes in *Prochlorococcus* (Morris *et al.*, 2011; Ma *et al.*, 2018). There is some evidence that heterotrophic bacteria protect *Microcystis* from hydrogen peroxide (Kim *et al.*, 2019), but whether this is relevant at naturally occurring hydrogen peroxide concentrations remains to be demonstrated. Nonetheless, differences between *Microcystis* strains in content of genes involved in detoxification of oxidative stress **(Fig. 2)** may in part be explained by differential reliance on public goods or exudates from the non-cyanobacterial bacterial community.

The functional implications of strain-specific microbiomes are mostly unknown in the case of *Microcystis*. In other phytoplankton species, outcomes of interactions between bacteria and phytoplankton are dependent on the specific strains and species involved (Sison-Mangus *et al.*, 2014; Amin *et al.*, 2015). An interesting example for *Microcystis* is the interaction with another cyanobacterium, *Pseudanbaena*, which is commonly associated with *Microcystis* colonies (Smith et al., submitted), where it grows epiphytically and produces cyanotoxins (Rangel *et al.*, 2014) that may alter the toxicity of blooms.

Interactions between *Pseudanabaena* and *Microcystis* are genotype-specific and range from neutral to antagonistic (Agha *et al.*, 2016; Chun *et al.*, 2020).

Microbiome impacts on host fitness may also impact competitive interactions between *Microcystis* strains and between *Microcystis* strains and other phytoplankton species. As the outcome of competition depends on physicochemical conditions, and as the microbiome likely alters the local environment of *Microcystis*, microbiomes could very well affect competitive outcomes. Indeed, microbiomes influence competitive interactions for plants and eukaryotic algae (Wagg *et al.*, 2011; Siefert *et al.*, 2018, 2019; Zhou *et al.*, 2018; Jackrel, Schmidt, *et al.*, 2020). The strain-specific association and effects of *Pseudanabaena* on *Microcystis* could theoretically affect intrinsic intra- and interspecific interactions among phytoplankton, though this has not been experimentally verified. In the one study where microbiome effects on competition were assessed, the effect of microbiomes on competition depended on the genotype of *Microcystis*; the ability of one *Microcystis* strain to competitively exclude a green alga was improved by heterotrophs while another strain did not require heterotrophs to do so (Schmidt *et al.*, 2020). More work is needed to understand the impacts that ubiquitous as well as strain-specific bacterial taxa have on different *Microcystis* strains.

Viruses

Viruses of microbes influence the ecology and evolution of their host populations (Fuhrman, 1999; Wommack and Colwell, 2000; Suttle, 2007; Breitbart, 2012; Brum and Sullivan, 2015). *Microcystis* is not immune to the effects of viral activity, as evidenced by viral lysis of cultures (EPA Report, 1977; Tucker and Pollard, 2005; Honjo *et al.*, 2006; Yoshida *et al.*, 2014), *in situ* predator-prey dynamics inferred from viral and host abundances and transcriptional activity (Manage *et al.*, 1999; Yoshida *et al.*, 2007; Steffen *et al.*, 2015; Mankiewicz-Boczek *et al.*, 2016), and CRISPR-spacers targeting viruses found in host genomes (Kimura *et al.*, 2018; Wang *et al.*, 2019). Viral infection of *Microcystis* may have implications for bloom fate (Bratbak *et al.*, 1993; Jacquet *et al.*, 2002), toxicity (Steffen *et al.*, 2017; McKindles *et al.*,

2020), and nutrient dynamics (Jover *et al.*, 2014; Mckindles, 2017). Further, as a strain-specific top-down control on their host populations, viruses can drive intraspecific diversification of *Microcystis*. Following the collapse from viral lysis, population density of *Microcystis* cultures may rebound, presumably due to evolved immunity (EPA Report, 1977)(Zhai *et al.*, 2013). Other studies have described the emergence of cultures with intermediate sensitivity after infection, which has been attributed to the coexistence of host populations both sensitive and resistant to the virus (Yoshida *et al.*, 2014; Mckindles, 2017). In the environment, during a season with two *Microcystis* blooms (Yoshida *et al.*, 2007), peak *Microcystis* virus marker gene abundances corresponded with the crash of the first bloom peak. Through the second bloom peak, viral gene abundances remained near-zero, presumably due to viral resistance in the late-blooming host population. Because only the second *Microcystis* population encoded genes for microcystin production, such virus-driven intraspecific diversification may have consequences for bloom toxicity. Given the limitations of marker genes and short qPCR targets, future work should move beyond marker gene analysis to resolve these coevolutionary dynamics at the population genomic level.

A long history of viral infection and subsequent CRISPR-mediated immune defense is evident in sequenced *Microcystis* genomes. The mean number of CRISPR arrays in nine sequenced *Microcystis* genomes is seven-fold greater that of the average of other publicly available microbial genomes (n=22,611 total genomes; as predicted in the CRISPRCasdb (Pourcel *et al.*, 2020)). *Microcystis* CRISPR spacer sequences target viral genomic fragments reconstructed from environmental metagenomes (Morimoto *et al.*, 2019), confirming a history of active defense against natural viral populations. The acquisition of a new virus-targeting spacer sequence will result in a novel subspecies-level *Microcystis* CRISPR-genotype (CT) that differs by 20-30 bp in length. Evidence for selective sweeps of *Microcystis* populations at the level of CT (Kimura *et al.*, 2018) means viral pressure causes competitive advantages at a genomic resolution finer than genes. Yet, the consequences of these fine scale genotypic differences for sustained impacts on bloom dynamics are unknown. Future work will clarify whether, and under

which conditions, such virus-driven selective sweeps result in phenotypic changes that influence the competitive advantage of new *Microcystis* CTs.

Due to the limited knowledge of *Microcystis* viruses, most studies have been restricted to evaluating the presence or activity of the small number of sequenced *Microcystis* viral isolates (Yoshida *et al.*, 2007; Ou *et al.*, 2013; Yang *et al.*, 2020). Future work will benefit from moving beyond viral marker gene analyses of this virus subset (eg. Yoshida *et al.*, 2007; Mankiewicz-Boczek *et al.*, 2016; Steffen *et al.*, 2017; McKindles *et al.*, 2020) towards developing a population-level understanding of both cultured and uncultured *Microcystis* viruses through community genomic sequencing (Morimoto *et al.*, 2019). Combined with the continued isolation of *Microcystis* and viral strains, cross-infection host range assays (Wang *et al.*, 2019), and experimental coevolution, these approaches are likely to reveal a breadth of viruses capable of infecting *Microcystis*, each with unique infection traits and potential fitness costs of host resistance. As arguably the least well-studied of the potential drivers of bloom dynamics, a better understanding of *Microcystis*-virus interactions has the potential to improve predictive models of toxic bloom fate.

Fungal parasites

Parasitic fungi of the phylum Chytridiomycota are widespread in aquatic environments and infect phytoplankton of harmful algal blooms (Gleason *et al.*, 2015), including cyanobacteria (Gerphagnon *et al.*, 2013; Frenken *et al.*, 2018). Infection rates in cultures suggests they could be important in modulating cyanobacterial blooms (Sime-Ngando, 2012; Gerphagnon *et al.*, 2013). This fungal parasitism can have unexpected effects on food web dynamics; fungal zoospores released from infected cyanobacterial cells can be a significant food source to zooplankton and as such compensate for the poor food source the dominant bloom-forming cyanobacterial species themselves represent (Frenken *et al.*, 2018). In addition to chytrid parasites, growth inhibition (though not parasitism) and microcystin degradation has been

shown by *Trichoderma citrinoviride*, a fungus isolated from decaying bloom biomass (Mohamed *et al.*, 2014).

While only limited documentation of chytrid infection of *Microcystis* is available (Sen, 1988), different strains of *Microcystis* appear to have varying degrees of susceptibility (Van Wichelen *et al.*, 2010). Strain-level variability also occurs for morphotypes of *Dolichospermum* (Weisbrod *et al.*, 2020) and chemotypes of *Planktothrix* (Sønstebø and Rohrlack, 2011). Thus, the strain composition of *Microcystis* blooms may be influenced by fungal parasites. However, no field observations indicating strong fungal impacts on *Microcystis* bloom population dynamics have been made (Van Wichelen *et al.*, 2016). Interestingly, secondary metabolites produced by certain *Microcystis* strains help *Daphnia* fend of its own fungal parasites (Sanchez *et al.*, 2019). Because secondary metabolites produced by *Microcystis* vary across strains they likely defend against fungal parasites in a strain-dependent way, but this remains to be tested.

Predatory bacteria

Certain bacterial species are specialized predators of other bacteria, with the best studied example being *Bdellovibrio bacteriovorus* (Sockett, 2009). Another predatory bacterium, *Vampirovibrio chlorellavorus*, has been shown to adhere to the surface of the eukaryotic green alga *Chlorella vulgaris* using specialized attachment structures, and to subsequently destroy the cell (Soo *et al.*, 2015; Kim *et al.*, 2016). While little is known about the importance of bacterial predation on *Microcystis*, one study showed that it may be as important as viral predation in the demise of blooms (Manage *et al.*, 2001). Predatory bacteria are physically associated with *Microcystis* colonies in substantial abundance (Smith *et al.* submitted)**,** and they show patterns of co-occurrence with specific *Microcystis* genotypes, hinting at the possibility that predatory bacteria may have strain-specific interactions with *Microcystis* strains or their microbiomes (Chun *et al.*, 2020).

Grazers

Grazers such as microzooplankton (*e.g.*, protists and rotifers), mesozooplankton (e.g. *Daphnia*, small cladocerans, and copepods), and mussels can have major effects on phytoplankton and cyanobacterial community composition and toxicity, as illustrated by the impacts of invasive mussels on lakes and river systems around the world (Vanderploeg *et al.*, 2001, 2002, 2009, 2013; Cataldo *et al.*, 2012; Waajen *et al.*, 2016). Selective feeding can promote or depress different components of the phytoplankton community, including *Microcystis*, and there has long has been an interest in how grazer populations control blooms as well as the reciprocal inhibitory response of *Microcystis* on grazers, which in turn affects transfer of energy up the food web (Ger and Panosso, 2014; Ger *et al.*, 2016). Several considerations suggest that interactions between grazers and *Microcystis* vary across *Microcystis* strains. First, in general, trait variation is an important predictor for interactions between herbivores and phytoplankton (Leibold, 1989; Tessier and Woodruff, 2002; Cottingham *et al.*, 2004). Second, in particular, *Microcystis* colony size/morphology and toxin content can deter grazing (Ger and Panosso, 2014; Ger *et al.*, 2016), and these traits vary across *Microcystis* strains and have been linked to grazing defense as discussed more below. Third, the literature shows conflicting results on impacts of grazer-*Microcystis* interactions, likely resulting in part from trait variation across *Microcystis* strains (Chislock *et al.*, 2013; Ger and Panosso, 2014; Ger *et al.*, 2016). Thus, grazers may play an important role in selecting for *Microcystis* strains in field populations and likely shape *Microcystis* traits involving colony size and secondary metabolites.

The large size of *Microcystis* colonies usually found in nature is a deterrent to predation, but whether this trait is based on phenotypic plasticity or genetic differences between strains is unclear (Bittencourt-oliveira *et al.*, 2001; Lürling, 2003; Sarnelle *et al.*, 2005; Vanderploeg *et al.*, 2013; White and Sarnelle, 2014). The largest size of particles ingested by crustacean zooplankton is determined by the size of the food collection apparatus and mouth of the grazer and thus increases with grazer size (Burns, 1968; Vanderploeg, 1994). For example, *Daphnia magna*, a large pond form, is able to ingest particles 80 μm in

diameter; however, in general particles (colonies) larger than ~30-40 µm in diameter are too large to be ingested by most mesozooplankton (cladocerans and copepods) found in lakes, rivers, or estuaries (Vanderploeg, 1994). Feeding experiments with a size range of colonies of a non-toxigenic strain of *Microcystis* suggest that the upper level of colony size ingested for dreissenid mussels may be 80 µm (White and Sarnelle, 2014). This implies grazers can only access *Microcystis* before they mature into large colonies often found in nature. Several studies support this conclusion. *Microcystis* consisting of unicells and small colonies < 20 µm were readily ingested in Lake IJsselmeer, Netherlands (Pires *et al.*, 2005). On the other hand, Cataldo et al. (2012) showed that the invasive mussel *Limnoperna fortunei* shifted the initial phytoplankton community comprised of a small percentage of *Microcystis* sp., present mostly as unicells, into dominance by *Microcystis* as large colonies. The importance of *Microcystis* strain and size have become important issues in the controversy that invasive mussels promote *Microcystis* blooms in some systems but not others (Vanderploeg *et al.*, 2001, 2002, 2009, 2013).

Variable toxicity across *Microcystis* strains and species also likely plays a key role in the varying interactions that grazers have with *Microcystis* (Jungman and Benndorf, 1994; Vanderploeg *et al.*, 2001; Juhel *et al.*, 2006; Schwarzenberger *et al.*, 2014). “Toxicity” here includes effects due the wide variety of congeners of microcystins, which have varying bioactivity, as well as undefined secondary metabolites, which can depress feeding rate, interfere with molting and ultimately lead to low survival of *Daphnia*, for example (Jungmann and Benndorf, 1994; Kaebernick *et al.*, 2001). Exposure of *Daphnia* to *Microcystis* can result in varying outcomes, which are dependent both on *Daphnia* and *Microcystis* genotypes (Lemaire *et al.*, 2012; Chislock *et al.*, 2013). Many clones of *Daphnia* found in eutrophic systems can detoxify microcystin (Lyu *et al.*, 2019) and readily feed on *Microcystis* along with other phytoplankton without harm (Chislock *et al.*, 2013). In contrast, calanoid copepods, not tolerant of microcystin, selectively reject *Microcystis* with high microcystin content (Ger *et al.*, 2016, 2019), thereby potentially promoting *Microcystis* dominance. Vanderploeg et al. (2001; 2014) demonstrated the complexity of mussel response to microcystin and other traits possessed by different strains by combining feeding

experiments with video observations. Mussels fed on the highly toxic (microcystin-LR) LE-3 strain (microcystin/chlorophyll a content = 0.66 µg·µg Chl a–1) at a greatly reduced feeding rate and also exhibited low overall feeding rate when offered together with preferred food. In contrast, clearance rate was normal for a non-toxic (CCAP 1450/11) and toxic strain (PCC 7820), the latter having a lower microcystin content of 0.22 µg·µg Chl a^{-1}. Strains isolated from Michigan lakes (Wilson *et al.*, 2005) that maintained their colonial integrity showed factors other than microcystin content were important (Vanderploeg *et al.*, 2013). The strain from Gilkey Lake (strain GilkeyL02), having a low microcystin content of 0.1 µg·µg Chl a^{-1} caused complete shutdown (valve closure); and when paired with a desirable food, they showed extreme sensitivity to an irritating substance in the colonies by rejecting each colony as it entered the siphon (Vanderploeg *et al.*, 2013). No ingestion occurred for non-toxic colonies of the strain from Hudson Lake (strain HudsonBD), which has heavy mucilage content. In this case the colonies were enthusiastically captured but rejected as pseudofeces.

While invasive dreissenid mussels can enhance *Microcystis*-dominated harmful cyanobacterial blooms by selective removal of non-cyanobacterial phytoplankton, declines of *Microcystis* following dreissenid mussel invasions have been reported as well and are sometimes hypothesized to be related to ambient nutrient concentration (Bastviken *et al.*, 1998; Smith *et al.*, 1998; Vanderploeg *et al.*, 2001, 2013; White *et al.*, 2011; Waajen *et al.*, 2016). While invasive dreissenid mussels can enhance *Microcystis* blooms by selective removal of non-cyanobacterial phytoplankton, declines of *Microcystis* abundance following dreissenid mussel invasions have been reported (Bastviken *et al.*, 1998; Smith *et al.*, 1998; Vanderploeg *et al.*, 2001, 2013; White *et al.*, 2011; Waajen *et al.*, 2016). These conflicting observations may be due to a trade-off between *Microcystis* adaptations to rapid growth at high nutrient levels and grazing resistance at low nutrient levels (Sarnelle *et al.*, 2005, 2012), similar to resource-defense trade-offs in plants (Züst and Agrawal, 2017). Nutrient levels, in particular nitrogen levels and N:P ratios also influence secondary metabolite production by *Microcystis* (Gobler *et al.*, 2016), further influencing the presence and expression of defense traits in *Microcystis*. Thus, distinct traits of *Microcystis* strains may help explain

why grazers have variable effects on ecosystems, depending on nutrient levels. However, this remains to be confirmed in large part because the genetic traits and gene-environment interactions that determine feeding resistance remain unclear. An additional challenge for the future will be to identify the *Microcystis* strains and traits that deter or allow grazing in a mixture with other phytoplankton species found in nature. An understanding of the roles of selective feeding mechanisms and behavior, as well as post-ingestion consequences of the different strains on grazer fitness, is clearly needed.

Conclusions and Outlook

Trait variation between species is an important predictor for the distribution and abundance of organisms (Tilman *et al.*, 1982; Leibold *et al.*, 1997; Chase and Leibold, 2003) as well as interactions between them (Leibold, 1989; Tessier and Woodruff, 2002). While diversity within species has been overlooked historically, it is now clear that intraspecific variation, both in terms of phenotypic plasticity (Lima and Dill, 1990; Agrawal, 2001; Werner and Peacor, 2003) and genetic diversity, can shape species interactions and even community composition and ecosystem processes (Agrawal and Van Zandt, 2003; Whitham *et al.*, 2006; Hughes *et al.*, 2008; Lemaire *et al.*, 2012; Van Rossum *et al.*, 2020). The extensive genetic and phenotypic diversity that has been uncovered within *Microcystis* emphasizes the need to move beyond considering it as a homogeneous entity when we try to understand, model, and predict bloom dynamics and their ecosystem impacts, particularly toxicity and trophic transfer. Trait-based theory and models developed for phytoplankton species (Litchman *et al.*, 2007; Follows and Dutkiewicz, 2011; Glibert, 2016) provide a framework to understand and model this intra-*Microcystis* diversity. This requires (i) identifying key traits that define niches within *Microcystis*; (ii) defining clades of *Microcystis* strains that share ecologically-relevant traits and thus serve as appropriate functional groups for experimental studies and modeling; (iii) measuring and quantifying these traits so that they can be parameterized within models that can be used to test and improve understanding; and (iv) developing these insights into an understanding of rules governing links and tradeoffs among traits.

The information synthesized in this review shows that these requirements have not yet been met for *Microcystis*. Functional groups and key traits have not been defined. Just a scattered subset of traits and strains have been measured (**Fig. S2, Table S5**). We have only hints of the links and tradeoffs between traits. A major shortcoming is that only a small portion of the diversity of *Microcystis* has been cultured (**Fig. 1**) and an even smaller portion of that has been characterized systematically. However, our review identifies key gaps and paths forward. While determining the traits of all strains is unrealistic, determination of these parameters for selected strains chosen in a phylogenetic context will produce a new view of how traits are distributed across taxa within the genus, allowing definition of functional groups and selection of cluster-based reference strains for further experiments (Ramos *et al.*, 2017). Coordination of this phenotypic characterization with genomic, transcriptomic, and proteomic analyses will lead to an understanding of how traits are genetically encoded, potentially enabling inference of the traits of uncharacterized and even uncultured *Microcystis* based on genetic information gleaned from field samples. This integrated phenotypic-genotypic perspective will also reveal links and trade-offs between traits, as in plant ecology (Díaz *et al.*, 2004).

Despite the current gaps and the grand challenge of filling them, we have preliminary views of the landscape of *Microcystis* diversity. Cohesive genotypic clusters are relatively conserved across methods and studies ((**Fig. 2**; Pérez-Carrascal *et al.*, 2019). While these clusters are likely to change with new data, they are a starting point to test the hypothesis that genotypic groups represent functional groups with shared traits and niches. Previous studies also suggest traits that define functional groups and point towards potential links and tradeoffs between traits (**Fig. 6**). For example, re-wiring of gene expression and physiology following spontaneous *mcy* mutants and gene knockouts provide clues to trade-offs (Hesse and Kohl, 2001; Schatz *et al.*, 2005; Van De Waal *et al.*, 2011; Zilliges *et al.*, 2011; Sandrini *et al.*, 2014; Makower *et al.*, 2015).

Understanding links and tradeoffs between traits (Fig. 6) is a promising route towards the ability to model bloom toxicity. Identifying whether and how these traits are correlated, and their ecophysiological importance, will be key for models that incorporate different biological, chemical, and physical factors to predict biomass and toxicity of *Microcystis* in water. As described in this review, the composition of toxigenic and non-toxigenic strains within blooms may be due to resource competition for light, inorganic carbon, bioavailable nitrogen and phosphorus, interactions with grazers, buoyancy and vertical migration, or a combination of these factors. However, it remains unresolved whether the presence of the microcystin synthetase genes is a driving factor behind observed patterns, or rather other traits that vary across strains. Further research, including competition experiments and examination of physiological traits beyond production of microcystins, are necessary to understand the links between genotype, phenotype, and competitive outcomes. Likewise, integration of genetic data with information on habitat will link *Microcystis* genotypes with environmental conditions from which strains are derived.

Patterns of diversity within *Microcystis* stand in stark contrast to other cyanobacteria such as *Prochlorococcus*, providing valuable opportunities for comparison. However, functional groups within *Microcystis* are likely underpinned by a subset of trait dimensions that define broader phytoplankton functional groups, *i.e.*, the "phytoplankton mandala" (e.g., Glibert, 2016). Piecing together this more holistic view will inform controls on bloom toxicity but also move us beyond the dichotomy of toxigenic vs. non-toxigenic strains defined by a single trait, ushering in a new era in the understanding and management of *Microcystis*-dominated cyanobacterial harmful algal blooms.

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Figure Captions

Figure 1: Comparison of diversity of uncultured *Microcystis* strains with diversity of cultured strains with genome sequences. Outer circle: phylogenetic tree of 16S-23S internal transcribed spacer (ITSc region) from *Microcystis* strains. Included are 3,874 ITSc amplicon sequences from uncultured strains in the field and 128 ITSc sequences that could be identified from 159 cultured *Microcystis* isolates for which whole genome data was available. Cultured isolates are highlighted by filled circles, colored based on phylogenetic groups of Pérez-Carrascal et al. (2019), modified as described in Fig. 2. Insert: distribution of average nucleotide identities (ANI) between pairs of the 159 available Microcystis genomes. See **Supporting Information** for methods.

Figure 2: Phylogenomic analysis of *Microcystis* genomes. Colored bars indicate genotypic groups as defined by Perez-Carrascal et al. 2019, modified with additional sub-groups (Mfl_ae3-5) to designate sub-clades recognized here. **A.** Dendrogram based on gene presence/absence. **B.** Phylogenetic tree based on alignment of concatenated sequences from 161 single copy core genes shared by all genomes. **C.** Presence/absence (black/white) of key genes involved in biosynthesis of secondary metabolites, carbon concentrating mechanisms (CCM), detoxification of reactive oxygen species (ROS), and acquisition of nitrogen and phosphorus. Only genes with variable presence are shown here; see **Supporting Information** for methods, complete figure (**Fig. S1**), and information on strains used (**Table S1**).

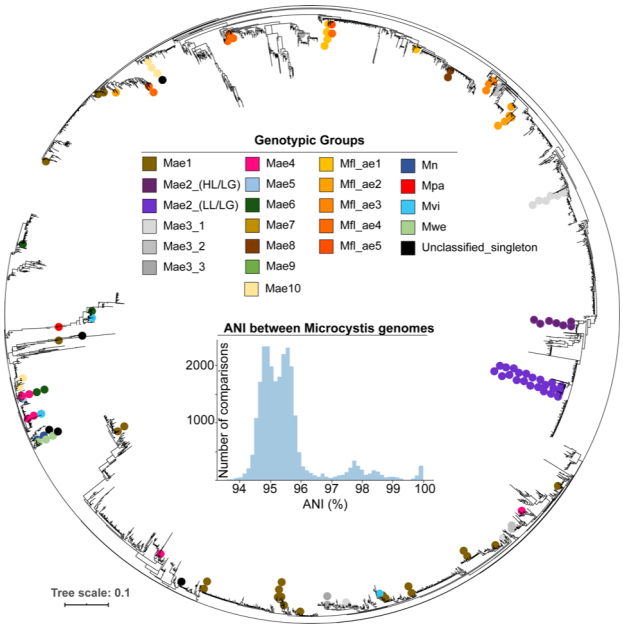
Figure 3. Summary of biomass nitrogen and phosphorus content in Microcystis strains. Panels A and B depict nutrient content irrespective of growth conditions and panels C and D depict the relationship between N and P content and instantaneous growth rate.

Figure 4. Growth rate of *Microcystis* strains as function of temperature. Color of points and line indicates the light intensity at which experiments were conducted. Strain names are positioned at the temperature of maximum growth for each strain (indicated with star), with ability to produce microcystins indicated by +/- where this information is available. See **Table S2** for details of strains and data. "NA" indicates that no strain name was given.

Figure 5. Growth rate of *Microcystis* strains as function of light intensity. Color of points and lines indicates the temperature at which experiments were conducted. Light intensity at which maximum growth was measured for each strain is indicated with a star. See **Table S3** for details of strains and data.

Figure 6. Hypothesized links and tradeoffs between traits within *Microcystis* strains. Tradeoffs include: (1) tradeoff between defense traits (especially grazing resistance) and nutrient resources due to nutrient availability determining colony size and/or capability for production of secondary metabolites; (2) defenses against reactive oxygen species have a metabolic cost (e.g., synthesis of enzymes); (3) tradeoff between growth and productivity on the one hand, and maintenance and efficiency of resource acquisition on the other, which emerges from underlying biochemical traits of transporters, etc.; (4) plasticity of nutrient quota drops with growth rate (Hillebrand et al. 2013). Links between traits include: (5) growth rate is linked to rate of nutrient uptake; (6) light adaptation is linked to nutrient availability (through pigment content) and to defense from reactive oxygen species; (7) ROS defense is linked to nutrient availability. [a]These traits may be different for each form of nutrient; and the form of nutrient used may in itself be a differentiating trait. [b]Refers to the suite of secondary metabolites present in each strain. [c]Refers to acquired resistance to particular phage, e.g. via CRISPR or cell-surface proteins. These traits and

tradeoffs can be organized in the Competitor-Ruderal-Stress tolerator framework (Krause et al. 2014), which is not shown here.



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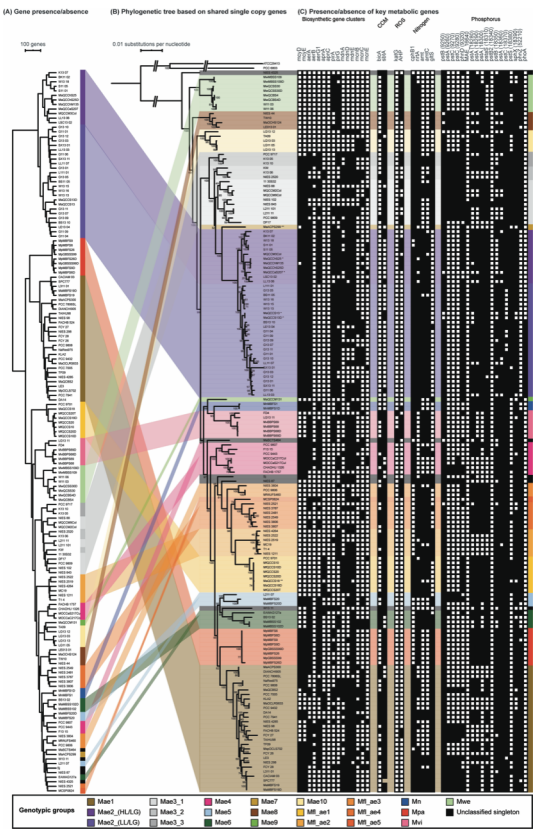

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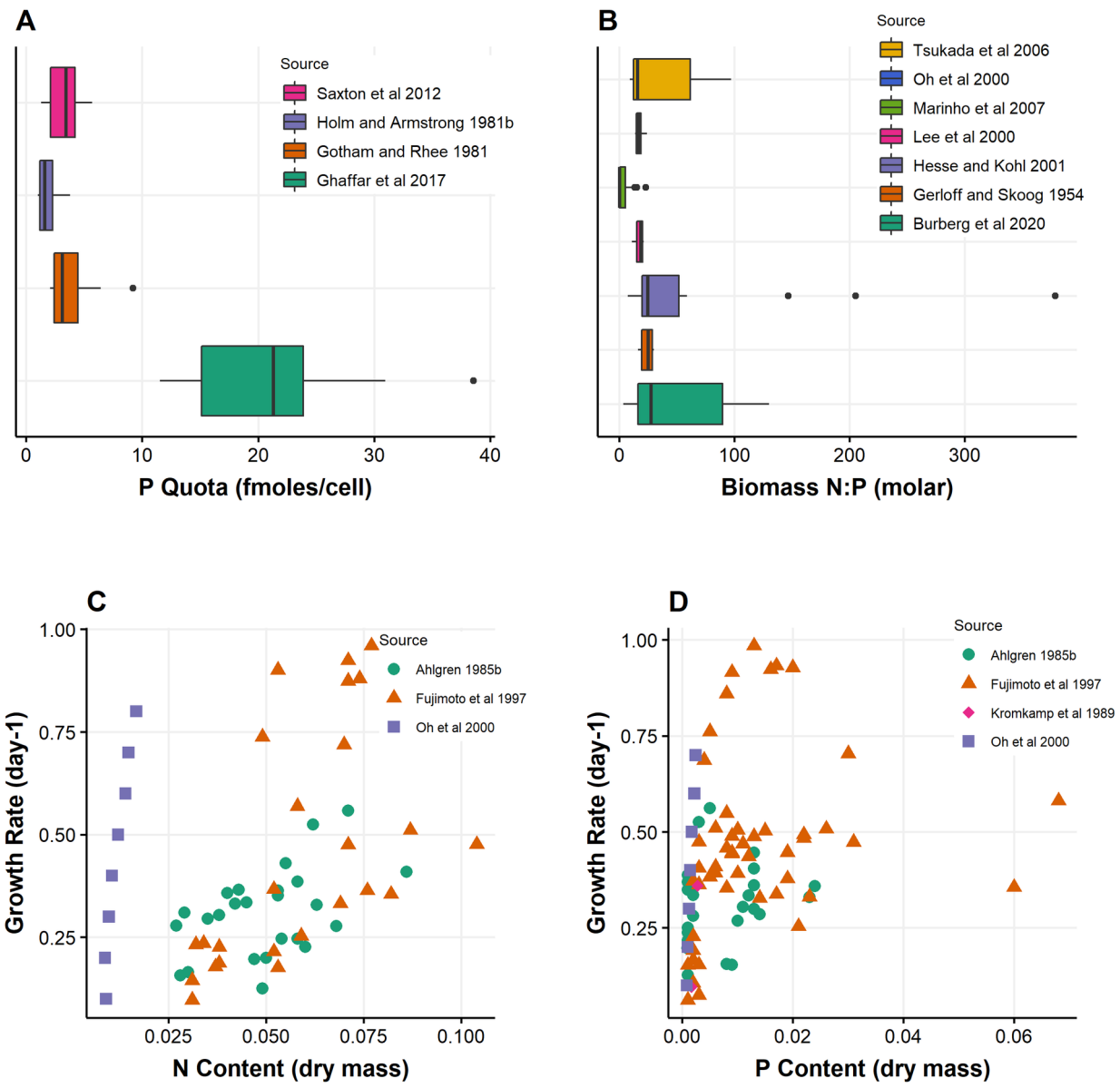

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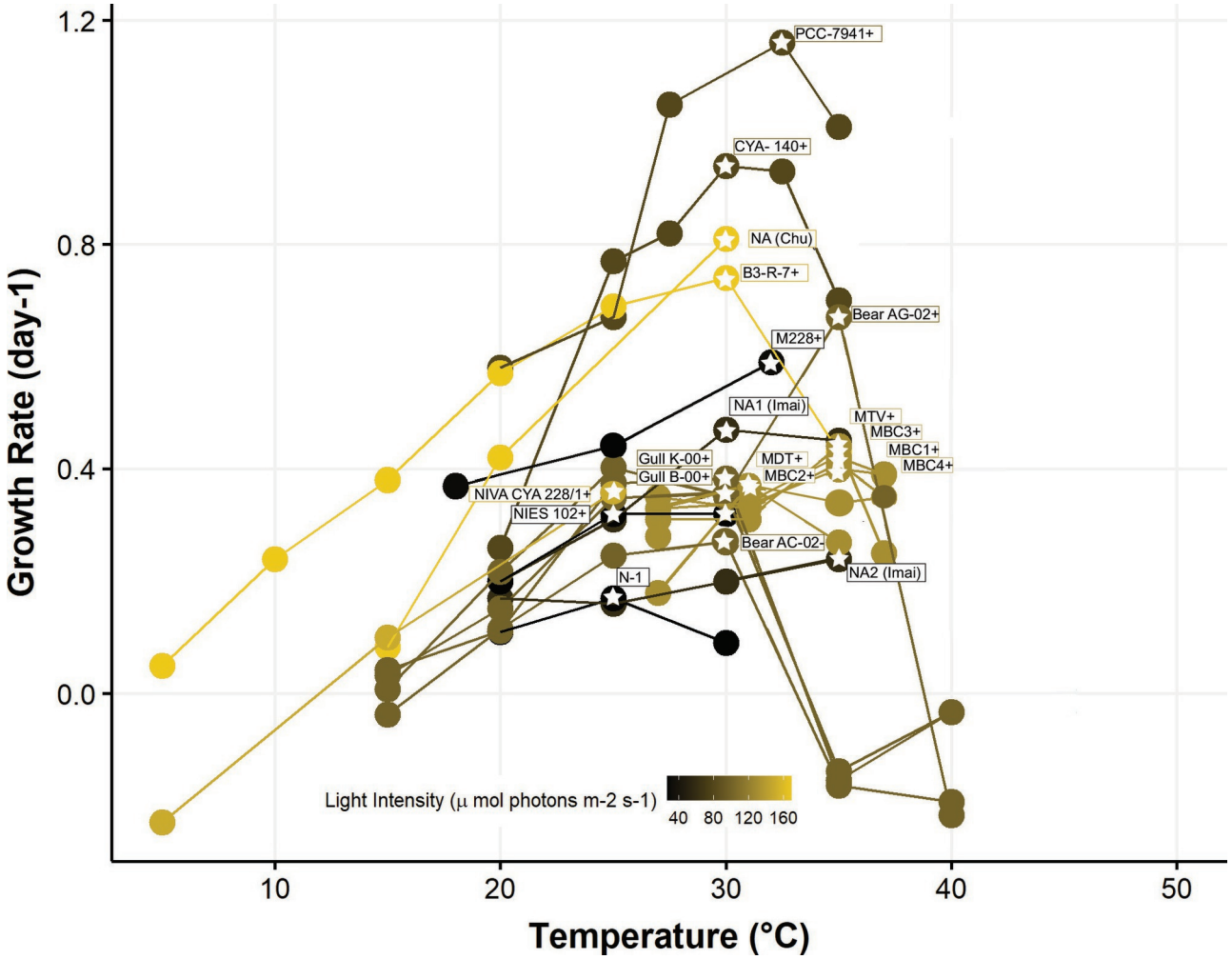

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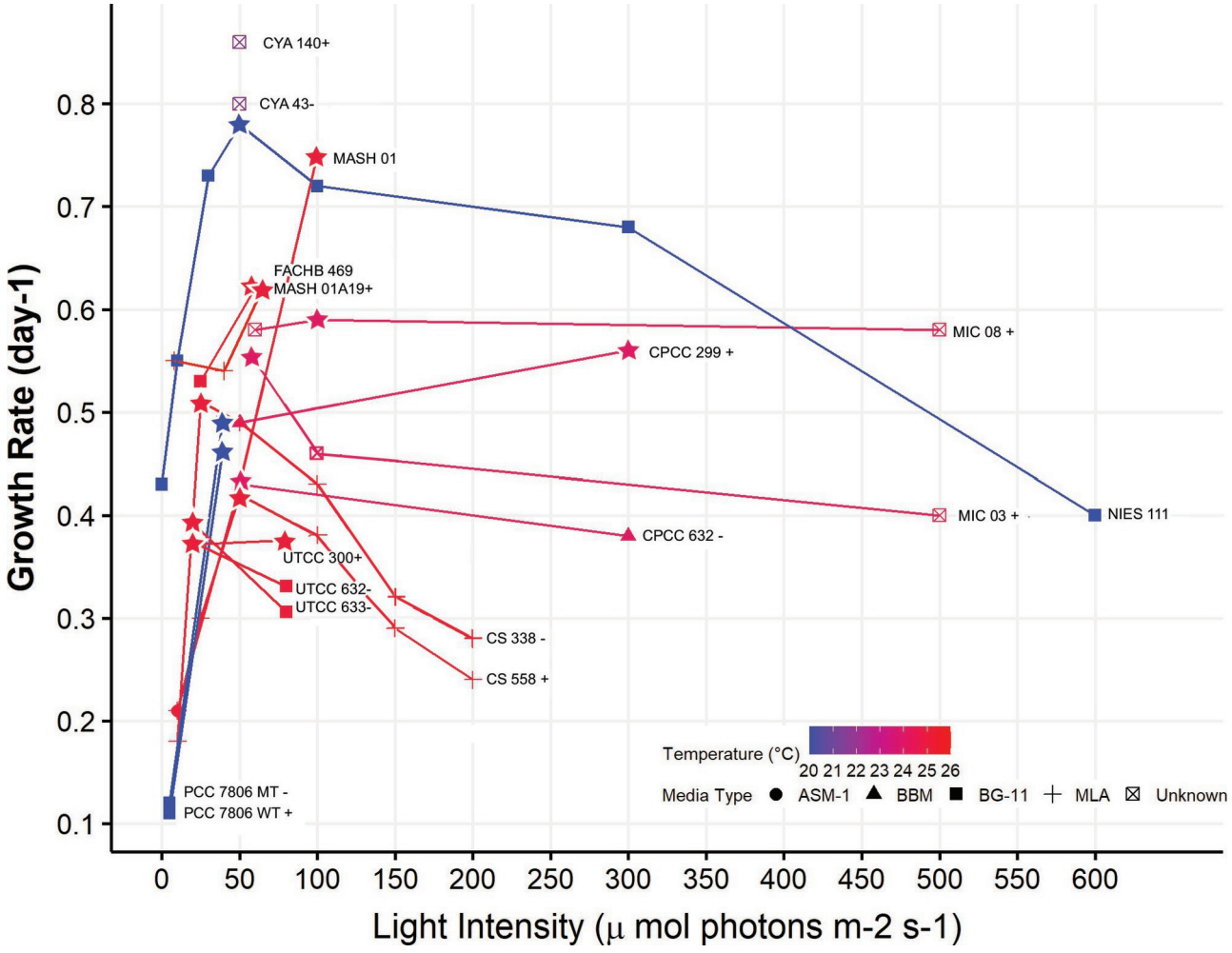

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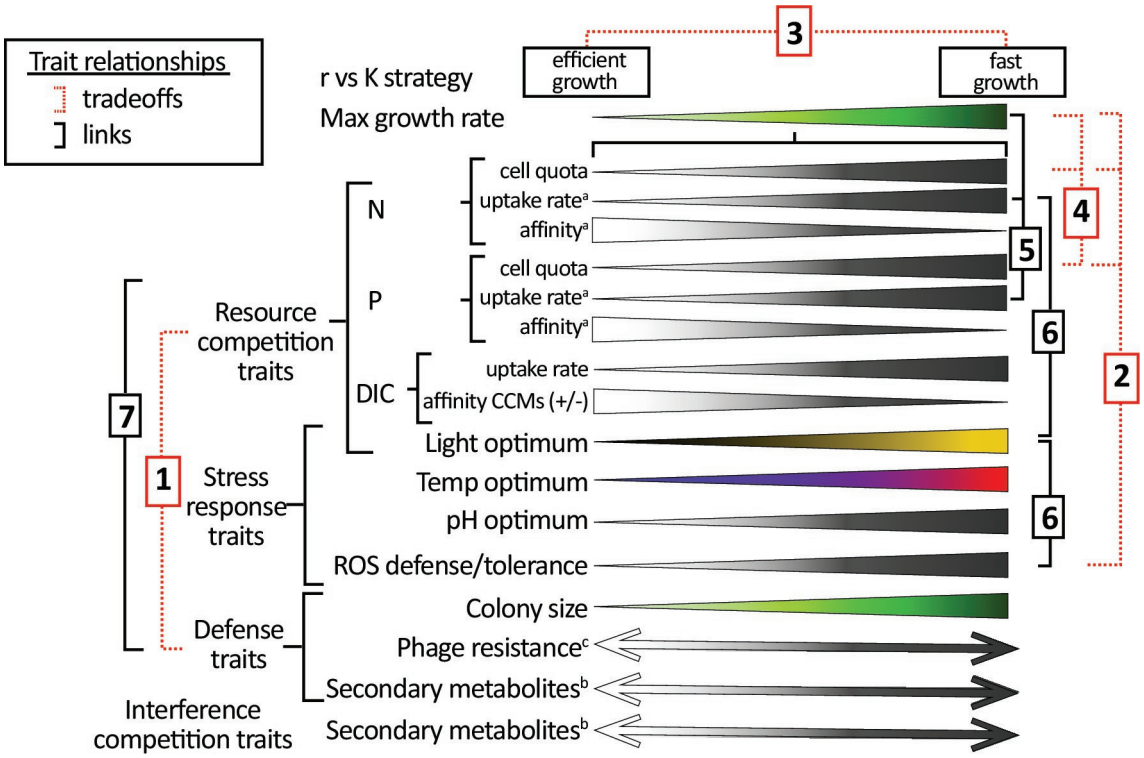

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The genetic and ecophysiological diversity of *Microcystis*

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Originality Significance Statement

Toxic cyanobacterial blooms are proliferating globally due to cultural eutrophication, climate change, and ecosystems made vulnerable by invasive species. Among the most prevalent concerns is *Microcystis*, which threatens freshwater ecosystems and drinking water supplies by formation of dense blooms and production of microcystins, a diverse class of liver toxins. Diverse *Microcystis* strains exhibit remarkable variation in their physiology, habitat, and ability to produce toxins. This strain-level variation is recognized as a key determinant of the ecological success of *Microcystis* and the toxicity of its blooms. However, the links between genotype and phenotype and how they underpin the environmental distribution of strains and production of toxins remain unclear. This review synthesizes current knowledge on the environmental distribution and genetic and phenotypic diversity of *Microcystis* strains and identifies knowledge gaps and promising paths for future research.

Summary

Microcystis is a cyanobacterium that forms toxic blooms in freshwater ecosystems around the world. Biological variation among taxa within the genus is apparent through genetic and phenotypic differences between strains and via the spatial and temporal distribution of strains in the environment, and this fine-scale diversity exerts strong influence over bloom toxicity. Yet we do not know how varying traits of *Microcystis* strains govern their environmental distribution, the tradeoffs and links between these traits, or how they are encoded at the genomic level. Here we synthesize current knowledge on the importance of diversity within *Microcystis* and on the genes and traits that likely underpin ecological differentiation of taxa. We briefly review spatial and environmental patterns of *Microcystis* diversity in the field and genetic evidence for cohesive groups within *Microcystis*. We then compile data on strain-level diversity regarding growth responses to environmental conditions and explore evidence for variation of community interactions across *Microcystis* strains. Potential links and tradeoffs between traits are identified and discussed. The resulting picture, while incomplete, highlights key knowledge gaps that need to be filled to enable new models for predicting strain-level dynamics, which influence the development, toxicity, and cosmopolitan nature of *Microcystis* blooms.

Introduction

Toxic cyanobacterial blooms degrade drinking water supplies and freshwater ecosystems around the world (Huisman *et al.*, 2018). Driven mainly by cultural eutrophication, these blooms are expected to become even more intense, frequent, and widespread with climate change as increasing temperature and water stratification favor bloom-forming cyanobacteria (Paerl and Huisman, 2008, 2009; O'Neil *et al.*, 2012; Michalak *et al.*, 2013; Paerl and Otten, 2013b; Visser *et al.*, 2016; Ho *et al.*, 2019). Among the most common and harmful of all bloom-forming cyanobacteria is *Microcystis*, which is widespread in temperate and subtropical lakes, reservoirs, and rivers in at least 108 countries on six continents (Harke, Steffen, *et al.*, 2016). *Microcystis* produces a variety of natural products, with the main focus on microcystins due to their environmental abundance and known toxic effects (Welker *et al.*, 2006; Kehr *et al.*, 2011; Dittmann *et al.*, 2015; Lezcano *et al.*, 2019; Pérez-Carrascal *et al.*, 2019; Pearson *et al.*, 2020). Recent years have seen *Microcystis* blooms in large lakes that were among the largest in recorded history (Michalak *et al.*, 2013), that shut down drinking water supplies in large cities (Qin *et al.*, 2010; Steffen *et al.*, 2017), and that expanded into lakes, rivers, and estuaries that were previously unaffected (Kramer *et al.*, 2018).

What factors have contributed to the rise of *Microcystis* blooms in recent decades? Phosphorus loading is a key determinant of bloom size, *i.e.*, *Microcystis* biomass (Stumpf *et al.*, 2012; Michalak *et al.*, 2013), thus management strategies are often focused on P (Ohio EPA, 2013). However, in some cases blooms have become more intense and frequent without apparent increases in phosphorus loading, highlighting the complexity of the issue and the important role of factors such as top-down and bottom-up ecological controls (Vanderploeg *et al.*, 2001; Sarnelle *et al.*, 2005), the form of phosphorus (Kane *et al.*, 2014), nitrogen availability (Paerl *et al.*, 2016), and competitive interactions that may be shifting along with climate change (Paerl and Huisman, 2008; Sandrini *et al.*, 2016). Furthermore, the factors that drive bloom formation and those that drive toxicity may be uncoupled owing to intracellular regulation of toxin

production as well as the relative proportion of toxic and non-toxic *Microcystis* cells within blooms (Rinta-Kanto *et al.*, 2009; Wilhelm and Boyer, 2011).

Remarkably, *Microcystis* dominates phytoplankton communities across a wide range of physicochemical conditions that vary both between lakes around the world and within lakes over time and seasonal changes. Several key traits appear to underpin the success of *Microcystis* over such wide-ranging conditions. First, *Microcystis* can sustain high biomass despite low concentrations of inorganic phosphorus, in part because of its ability to access benthic nutrients by vertical migration (Gobler *et al.*, 2016). Second, increased availability of nitrogen from anthropogenic sources likely helps *Microcystis*, which does not fix its own nitrogen, compete with other phytoplankton (Gobler *et al.*, 2016; Paerl *et al.*, 2016; Nolan and Cardinale, 2019). Third, *Microcystis* produces a variety of natural products that can deter grazers and inhibit competitors (Van Wichelen *et al.*, 2016). Fourth, *Microcystis* forms gas vesicles that allow it to effectively migrate vertically in the water column in order to find optimal light intensity and quality, and to form surface scums that reduce light availability to other phototrophs (Komárek, 2003). Fifth, by forming large colonies of cells, *Microcystis* can evade predation by key zooplankton taxa (Ger *et al.*, 2016). Finally, *Microcystis* effectively overwinters on the sediments, allowing rapid initiation of blooms during favorable conditions (Humbert *et al.*, 2005; Welker *et al.*, 2007; Kitchens *et al.*, 2018).

Another explanation for the widespread ecological success of *Microcystis* is its phenotypic diversity across taxa within the genus. The question of whether there are truly different species of *Microcystis* is considered in the section on genetic diversity below. There are also various definitions of “strain”; here we use the term in a broad sense to refer to natural intra-species variants with distinct genotypes and/or phenotypes, including both cultured isolates as well as uncultured entities observed through molecular methods, recognizing that thresholds to delineate strains are undefined (Van Rossum *et al.*, 2020). The diversity of *Microcystis* strains is apparent both through observations of spatial and temporal patterns in the field and the widely varying traits of strains studied in the laboratory. Such intraspecific diversity

enables rapid shifts in strains optimized to varying environmental conditions and biotic interactions while maintaining dominance at a coarser phylogenetic level (Muraille, 2018; Willis and Woodhouse, 2020), and likely plays a key role in the widespread occurrence and persistence of *Microcystis* blooms.

Much of the prior work on strain variability within *Microcystis* has focused on the fact that some strains have genetic capability to produce microcystins ("toxigenic") whereas others do not ("non-toxigenic"). This trait receives attention because microcystin is a primary concern for human and animal health and the relative abundance of toxigenic and non-toxigenic cells within *Microcystis* blooms is a key determinant of microcystins concentrations in blooms (Carmichael and Gorham, 1981; Ohtake *et al.*, 1989; Vezie *et al.*, 1998; Davis *et al.*, 2009; Gobler *et al.*, 2016) together with the amount of microcystins produced per cell (Chorus, 2001; Jahnichen *et al.*, 2001). Indeed, *Microcystis* strains are often referred to as "toxic" and "non-toxic" based on ability to produce microcystins. In fact, this simplified classification scheme hides the fact that collectively, *Microcystis* produces a large array of secondary metabolites with possible toxic properties including over 200 (Puddick *et al.*, 2014; Taranu *et al.*, 2019) different microcystin variants (congeners), which range in their toxicity (Rinehart *et al.*, 1994; Chernoff *et al.*, 2020). These toxin congener profiles differ between strains (Puddick *et al.*, 2014), and environmental conditions (Amé and Wunderlin, 2005; Taranu *et al.*, 2019). Understanding and modeling the ecology and physiology of these strains will be critical to inform forecasts and management of toxic blooms (Hellweger *et al.*, 2019). However, despite the important role of strain diversity in shaping the ecological success of *Microcystis* and toxicity of blooms, and the prevalence of genotypic and phenotypic variability within *Microcystis* (Harke, Steffen, *et al.*, 2016; Pérez-Carrascal *et al.*, 2019) (and bacterial species in general (Van Rossum *et al.*, 2020), a complete understanding of how genetic and phenotypic traits are distributed across the *Microcystis* genus, and what trade-offs may exist between toxin production and other traits key to their ecological success, remains elusive.

With the recent acceleration of genomics-enabled research, this review aims to provide a timely synthesis of our current knowledge and to identify critical knowledge gaps in the genetic and phenotypic diversity of *Microcystis* strains. We focus on traits likely to underpin a strain's fitness in response to environmental parameters or community interactions (Litchman *et al.*, 2007; Zwart *et al.*, 2015). In the interest of exposing links between genotype and phenotype, we draw on data from studies of pure cultures. However, inherent limitations of cultures should be recognized. Strains in culture are biased toward those collected from eutrophic conditions because of difficulty in isolating and culturing strains that are not adapted to the usually high concentration of nutrients found in typical culture media (Wilson *et al.*, 2006). Investigators tend to work with axenic unicellular cultures available from culture collections, whereas in nature *Microcystis* usually grows in multicellular colonies. Although the colonial form likely influences several key traits, it is usually lost over time and is rarely maintained for more than a year or two in culture. Toxin profiles can change over time because of spontaneous mutations (Kaebernick *et al.*, 2001). These factors, along with differences in culture conditions between studies, can result in different trait values even for the same isolate. Finally, laboratory strains are often characterized from a small subset of perspectives, thus holding incomplete information on physiological properties, genome sequence, and toxin content (microcystin congeners or other secondary compounds), and thus concealing potential strain-dependent links between these traits.

Spatial and temporal patterns of strain diversity suggest ecologically distinct strains

Various methods have been used to study the diversity of *Microcystis* taxa in the environment, ranging from microscopy to characterization of isolated cultures to cultivation-independent molecular analyses. Cultivation methods impart major biases on which strains of *Microcystis* are retrieved (Wilson *et al.*, 2006) and morphology does not accurately reflect phylogeny or function (Kondo *et al.*, 2000; Otsuka *et al.*, 2001; Harke, Steffen, *et al.*, 2016), thus much work has focused on cultivation-independent genetic methods that can quantify the diversity and abundance of *Microcystis* (Baker *et al.*, 2002; Rinta-Kanto *et*

al., 2005; Rinta-Kanto and Wilhelm, 2006). Given the practical importance of understanding controls on bloom toxicity (*i.e.*, toxin concentration relative to biomass), many studies on the diversity of *Microcystis* have focused on the relative abundance of toxigenic and non-toxigenic strains, which often coexist in *Microcystis* blooms. The proportion of toxigenic cells in a *Microcystis* population, which is often estimated by quantifying the number of cells carrying *mcy* genes as a percentage of total *Microcystis* cells (*e.g.*, via 16S rRNA gene copies), can range from essentially 0% to 100% (Yoshida *et al.*, 2007; Davis *et al.*, 2009). This proportion often varies spatially within a water body (Otten *et al.*, 2012) and even within a single water sample according to colony size/morphology, with larger colonies enriched in toxigenic cells in some cases (Kurmayer *et al.*, 2003; Wang *et al.*, 2013). In lakes around the world, a temporal succession of strains is often observed, with toxigenic strains dominating in early/peak blooms and non-toxigenic strains dominating in later stages of blooms (Park *et al.*, 1998; Welker *et al.*, 2007; Briand *et al.*, 2009; Davis *et al.*, 2009, 2010; Bozarth *et al.*, 2010; Singh *et al.*, 2015; Gobler *et al.*, 2016). This succession can explain dynamics of the toxicity of blooms, but in some cases it does not (Kardinaal *et al.*, 2007; Rinta-Kanto *et al.*, 2009). Other water bodies show distinct patterns of strain composition altogether (Yoshida *et al.*, 2007; Joung *et al.*, 2011). Regardless, the oft-observed succession of strains, even in low resolution terms of toxigenic vs non-toxigenic, suggests that there are ecologically distinct strains of *Microcystis*, with specific adaptations to bloom stage in terms of nitrogen requirements, resistance to oxidative stress, and optimal light and temperature conditions for growth (see sections on these topics below). This inference is supported by experiments in which manipulation of environmental conditions results in changes in the composition of toxigenic/non-toxigenic cells in blooms (Davis *et al.*, 2009, 2010).

Despite the importance of strain composition in bloom toxicity and dynamics, the genomic and ecophysiological traits that delineate ecologically cohesive groups of strains are unknown. Because *mcy* genes are variably present in *Microcystis* strains and polyphyletic (Tillett *et al.*, 2000, 2001; Rantala *et al.*, 2004), they cannot serve as a phylogenetically accurate or comprehensive marker of the strain

composition of blooms. The 16S rRNA gene is not sufficient to resolve phylogeny of strains; oligotyping of 16S sequences has shown some promise in distinguishing strains, though with significant caveats (Berry *et al.*, 2017). Genes and intergenic regions with higher levels of sequence divergence are required to more accurately profile strain composition in a sample. The intergenic spacer of genes for phycocyanin biosynthesis (*cpcBA* IGS) and the internal transcribed spacer (ITS) of the rRNA operon have been used for this purpose (Janse *et al.*, 2004; Moisander *et al.*, 2009; Bozarth *et al.*, 2010; Wang *et al.*, 2013; Guan *et al.*, 2018; Ho *et al.*, 2019; Chun *et al.*, 2020). Such studies have tracked dynamics of strain composition of *Microcystis* populations across time and space (Bozarth *et al.*, 2010; Otten *et al.*, 2017), identified sources and transport of *Microcystis* cells in watersheds (Otten *et al.*, 2015), and revealed relationships between strain abundance and nutrient levels (Guan *et al.*, 2018) and bacterial and eukaryotic communities (Chun *et al.*, 2020). Finally, the CRISPR-cas loci may provide even greater resolution of strains (Kimura *et al.*, 2018)**,** but does not reflect phylogeny due to frequent horizontal transfer (Varble *et al.*, 2019).

Whole genome sequences provide a complete picture of the gene content of *Microcystis* strains (Humbert *et al.*, 2013; Meyer *et al.*, 2017; Pérez-Carrascal *et al.*, 2019). While *de novo* assembly of genomes from the environment remains challenging due to the highly complex and repetitive nature of the *Microcystis* genome (Humbert *et al.*, 2013), shotgun metagenomics is already providing insights into the environmental distribution and expression of strain-specific genes (Steffen *et al.*, 2012; Meyer *et al.*, 2017), including those encoding toxins and taste and odor compounds (Otten *et al.*, 2016). Even without assembly, shotgun metagenomic approaches offer a range of applications for studying the dynamics of strains in the environment (Van Rossum *et al.*, 2020). Higher resolution reconstruction of strain-specific genomes awaits improvements to assembly and binning software, and especially continued advances in long sequence read technologies. For now, the main value of whole-genome sequencing lies in revealing the genetic diversity of cultured *Microcystis* strains, and in assessing how informative single markers such as the ITS region are relative to whole genome-based phylogenetic relationships.

Genetic diversity of *Microcystis* strains

The rapidly growing number of whole genome sequences provides new views of the genetic diversity of *Microcystis* strains (Humbert *et al.*, 2013; Harke, Davis, *et al.*, 2016; Jackrel et al., 2019; Pérez-Carrascal *et al.*, 2019; Willis and Woodhouse, 2020). To assess how well these cultured strains represent the environmental diversity of *Microcystis,* we constructed a phylogenetic tree of the 16S-23S internal transcribed spacer (ITSc) from all publicly available high-quality draft whole genome sequences of cultured isolates and sequences retrieved by PCR from uncultured *Microcystis* in the field (**Fig. 1**). 128 of the 159 *Microcystis* genomes available on NCBI (accessed on June 1, 2020) had a readily identifiable ITS region in the assembled contigs. Others were likely missing due to known issues with assembly of rRNA operons. The sequences from genomes of cultured *Microcystis* represent clades across the tree of environmental diversity, but they are unevenly distributed, with some narrow clades represented by many genomes (e.g., Mae2 LL/LG) and other clades having no representatives (**Fig. 1**). Thus, the known diversity of *Microcystis* is still sparsely represented by genome sequences, and likely also by cultured isolates. The phylogeny based on ITSc sequences is reasonably consistent with clusters defined by phylogeny of core genes (Pérez-Carrascal *et al.*, 2019). However, in some cases ITSc does not resolve clusters that were resolved by core genes (*e.g*., Mae4, Mae6), and in other cases ITSc sequences show more phylogenetic divergence/dispersion than clusters formed by core genes (*e.g.*, Mae1).

Pairwise comparisons of *Microcystis* genomes showed they all have > 93.6 % average nucleotide identity (ANI) across the shared parts of their genomes with every other *Microcystis* genome, with 99.8 % of comparisons > 94 % ANI and 70 % of comparisons > 95 % ANI (**Fig. 1**, histogram insert). This high ANI relatedness among sequenced *Microcystis* isolates has been consistently observed (Harke, Davis, *et al.*, 2016; Pérez-Carrascal *et al.*, 2019; Willis and Woodhouse, 2020). Based on a commonly used genomic definition of species, *i.e.*, a monophyletic group sharing > 94-95% ANI (Konstantinidis *et al.*, 2006; Chan

et al., 2012)**,** this implies that most sequenced *Microcystis* strains belong to a single species. However, 19 named *Microcystis* species exist, named primarily based on colony morphology (morphospecies). Considering the plasticity observed in morphology within single isolates (Yang *et al.*, 2006), it has long been clear that colony morphology alone does not accurately delineate species within the *Microcystis* genus, and regrouping of multiple morphospecies has been suggested before (Kondo *et al.*, 2000; Otsuka *et al.*, 2001; Harke, Steffen, *et al.*, 2016). Comparison of morphospecies delineations with genomic information shows that distinct clusters of *Microcystis* genomes sharing > 96% ANI only partially correspond to current *Microcystis* morphospecies, and the monophyletic morphospecies often correspond to highly related strains sampled at a single location (Pérez-Carrascal *et al.*, 2019; Willis and Woodhouse, 2020). It has been argued that a strict species definition based on an ANI cutoff of 94-95 % may not match the reality of species delineations across bacterial and archaeal life, and instead that levels of recombination relative to mutation among strains should be evaluated to group strains into species (Bobay and Ochman, 2017; Arevalo *et al.*, 2018; Shapiro, 2018). For *Microcystis*, inferred recombination rates between some of the > 96 % ANI clusters are lower than within some of these genetic clusters, making the case that speciation events have occurred or are ongoing within the *Microcystis* genus (Pérez-Carrascal *et al.*, 2019).

These observations for *Microcystis* stand in contrast to the genus of Earth's most abundant cyanobacterium, *Prochlorococcus*. The ANI among sequenced strains of *Prochlorococcus* can be as low as 65% (Paul *et al.*, 2019), yet genomic subclusters are referred to as 'ecotypes' of a single species, *Prochlorococcus marinus*, rather than different species of *Prochlorococcus* (Biller *et al.*, 2015). Ecotypes, a concept originating from botany, are groups of genetically distinct strains within a species that are adapted to specific environmental conditions (Turesson, 1922). Translating this concept to bacteria poses challenges and the term has been interpreted in various ways. Considering the genome-based species cutoff of 94-95 % ANI, the choice for ecotype rather than species delineations within *Prochlorococcus* seems contrary to genomic evidence. However, a recent analysis inferred substantial homologous

recombination between distantly related strains of *Prochlorococcus*, suggesting the absence of strict species boundaries (Bobay and Ochman, 2017). Going back to the initial definitions of the term ecotype in sexually reproducing organisms, ecotypes are still interfertile with other ecotypes of the same species, but gene flow between ecotypes is limited (though not eliminated) by ecological barriers, i.e., lack of spatial or temporal co-occurrence (Turrill, 1946). Thus, considering the limited evolutionary divergence of core genes among sequenced *Microcystis* genomes, the variable levels of genetic exchange across and within genetic subclusters of *Microcystis* does not necessarily imply that these should be considered distinct species rather than ecotypes that are a product of the ongoing process of speciation. Further sampling of the genomic diversity of *Microcystis* and analyses of the extent to which recombination maintains cohesive subclusters within the genus will help determine whether multiple species of *Microcystis* should be delineated. Alternatively, all genomic subclusters of *Microcystis* may be considered as 'ecotypes' of the most commonly named species, *Microcystis aeruginosa*, which currently already comprises a polyphyletic group of strains distributed across the *Microcystis* phylogeny (Pérez-Carrascal *et al.*, 2019; Willis and Woodhouse, 2020) (**Fig. 2**), as proposed previously (Harke, Davis, *et al.*, 2016).

In the case of *Prochlorococcus*, ecotypes have been linked to various environmental factors, with adaptations to light conditions along vertical layers of the ocean delineating the primary separation between phylogenetic clusters. Adaptations to different levels of iron, temperature, and N and P availability are conserved only at finer phylogenetic scales (Biller *et al.*, 2015; Martiny *et al.*, 2015)**.** *Synechococcus*, another common marine cyanobacterium, has a biogeography that is more evident horizontally, driven by temperature, macronutrients, and iron availability (Sohm *et al.*, 2016). In the case of *Microcystis*, limited mapping of ecological niche to genomic clusters has been carried out, with the exception of the low phosphorus/high phosphorus dichotomy observed in a set of 46 isolates originating from Michigan lakes (Berry *et al.*, 2017; Jackrel *et al.*, 2019). This lack of mapping of environmental niche to genomic clusters highlights an important knowledge gap that should be explored. Conveniently, the presence of a *Microcystis* colony in a water body indicates fitness of the strain in this environment, as

collected colonies are frequently composed of 10^4 to 10^5 cells (Costas *et al.*, 2008). Thus, mapping of environmental conditions of the water body from which a culture originated onto phylogenetic trees may reveal additional environmental optima for genomic subclusters.

In the absence of extensive environmental data to identify ecotypes, we can also resort to a reverse ecology approach, inferring ecology from genomic information. This has been done either by identifying genes specific to each genomic subcluster, as recently done for *Microcystis* (Willis and Woodhouse, 2020), or by determining the levels of recent gene flow to delineate ecologically relevant populations (Arevalo *et al.*, 2018)(Pérez-Carrascal *et al.*, 2019). The latter study found some genomic subclusters to correspond to higher levels of gene flow within than between clusters, but this was not universally the case. The former approach, *i.e.*, inferring functions from genes specific to genotypic clusters, identified genes of potential interest involved in nutrient adaptation, viral defense, membrane biosynthesis, DNA repair, chaperones, and Ca^{2+} transport (Willis and Woodhouse, 2020). We find patterns in the presence of genes involved in key processes between genotypic clusters, suggesting variable adaptations to conditions related to these traits (**Fig. 2**, **Fig. S1**). For example, in the super-clade including Mwe, Mae8, and Mae10 (top of phylogenetic tree) the absence of *mcy* genes correlates with absence of genes for ammonium transporters (*amt*), consistent with a link between microcystin production and uptake of ammonium (Chaffin *et al.*, 2018), but this linkage is not universal across clades. Taken together, this mix-and-match of various genes (and presumably traits) across the sub-species clusters within *Microcystis* suggests a diversity of multi-dimensional niches defined by various combinations of traits. Full implementation of the reverse ecology approach awaits clear delineation of ecologically relevant genotypic clusters and the environments to which they are adapted. Jackrel and colleagues illustrated the promise of this approach by showing that isolates from low-nutrient lakes have genome-wide reductions in nitrogen use and an expansion of phosphorus assimilation genes (Jackrel *et al.*, 2019).

Comparison of genotypic relatedness based on gene content versus phylogeny of core genes shows similarity in some clustering but differences in others (**Fig. 2**). Such discrepancies are expected given that the consensus phylogeny of core genes represents a mixture of many different evolutionary histories of single genes (Bapteste *et al.*, 2007; Thiergart *et al.*, 2014), whereas gene content may be better at differentiating phenotypes of sub-species clusters shaped by horizontal gene transfer (Van Rossum *et al.*, 2020) but does not necessarily reflect the phylogeny of the evolutionary core. Closer examination of geographic and environmental drivers of these evolutionary patterns is needed. In addition, current analyses have not yet taken into account smaller differences in sequence composition of shared genes with functional impact (*e.g.*, variation in microcystin congener biosynthesis) (Mikalsen *et al.*, 2003; Pearson *et al.*, 2004; Tooming-Klunderud *et al.*, 2008) nor evolution of gene expression of shared genes, which has been found to be a driving factor in ecological divergence between closely related organisms (Whitehead and Crawford, 2006; Denef *et al.*, 2010), including *Microcystis* (Srivastava *et al.*, 2019). There are also high levels of genomic variation within genomic clusters, driven by large levels of horizontal gene transfer between *Microcystis* genomes as well as external sources (Humbert *et al.*, 2013; Meyer *et al.*, 2017; Willis and Woodhouse, 2020), further underscoring the extensive genomic diversity within *Microcystis*. Despite these caveats and remaining gaps in knowledge, it is clear that there is a genetic clustering of strains within *Microcystis*, with each cluster having a unique set of flexible genes, suggesting that they are ecologically distinct. Testing this hypothesis will ultimately require phenotypic characterization of isolates from the different clusters; we consider the current state of this endeavor in the next section.

Functional diversity of *Microcystis* strains

Distinct temporal and spatial patterns of strain abundance and genetic clustering of strains suggest ecologically distinct sub-species groups of *Microcystis*, but higher-order and neutral processes also contribute to community assembly and genomic evolution and thus care should be taken not to

automatically assume selection for specific genotypes to explain these observations (Lynch, 2007; Nemergut *et al.*, 2013; Dick, 2018). Direct examination of the distribution of traits across the *Microcystis* phylogeny can further inform the ecological relevance of observed genetic clusters; differentiated traits may represent adaptations to specific environmental conditions. Studies of multiple traits across have shown significant differences between *Microcystis* strains (López-Rodas *et al.*, 2006; Wilson *et al.*, 2006), but to our knowledge these data have not been comprehensively synthesized across studies or been put into phylogenetic context. While we consider each trait independently, in reality they are intertwined. For example, the response to light is temperature-dependent and the response to temperature and oxidative stress depends on nutrient availability (Thomas and Litchman, 2016; Sandrini *et al.*, 2020). Similarly, the response to oxidative stress is also light dependent (Drábková *et al.*, 2007, 2007; Mikula *et al.*, 2012; Piel *et al.*, 2019) and the impact of inorganic carbon availability depends on other co-stressors (Gao *et al.*, 2012; Harvey *et al.*, 2013; Glibert, 2020). The effect of environmental factors, such as stimulation of microcystins production by nitrogen for example, may be linked to growth rate rather than specific metabolic processes (Orr *et al.*, 1998). Further, comparisons across studies may be influenced by conditions that were unreported or difficult to control for, such as subtle differences between light sources or growth media. Finally, growth rates may be influenced by whether strains form colonies or are single celled (Wilson *et al.*, 2006), a property that can change during lab culture. In some cases we can constrain such effects whereas in others there is insufficient information, and these limitations highlight the need for future work, especially studies that specifically assess traits across strains representative of the diversity within the genus.

Colony morphology and buoyancy

Microcystis cells clump together within an exopolysaccharide-rich mucilage matrix to form colonies of various sizes and shapes. Recent evidence from metagenomic sequencing of single colonies or cultures suggests that colonies initiated from clonal expansion from single cells are more prevalent than clumping

of distinct genotypes (Jackrel *et al.*, 2019; Pérez-Carrascal *et al.*, 2020). Although colony morphology was once used for species identification (Otsuka *et al.*, 2000), this trait is now known to change with environmental conditions and season, and genetic data supports the unification of morphospecies into a single species (Harke, Davis, *et al.*, 2016; Xiao *et al.*, 2018; Pérez-Carrascal *et al.*, 2019). In addition, colony morphology varies across strains under the same culturing conditions (Wilson *et al.*, 2006), and has important implications for surface area to volume ratio and thus nutrient uptake and growth rate (Wilson *et al.*, 2010), avoidance of grazing (Webster and Peters, 1978; Fogg, 1991; Yang *et al.*, 2006; see grazing section below), and light competition or availability to individual cells (Reynolds, 1984), suggesting that it could shape the strain composition of *Microcystis* blooms (Wilson *et al.*, 2006). Colony size and morphology also shows a relationship with presence of *mcy* genes for biosynthesis of microcystins (Kurmayer *et al.*, 2003; Via-Ordorika *et al.*, 2004).

Buoyancy regulation is an interplay of vesicle synthesis, protein and carbohydrate production, colony size, and light intensity (Xiao *et al.*, 2012). Like the seasonal succession of morphospecies, gas vesicle formation changes with the season in response to environmental factors (Xiao *et al.*, 2018)**.** Different laboratory strains of *Microcystis* have different gas vesicle production characteristics that are regulated by the *gvp* gene, which varies with light intensity. For example, strains that may not float at typical lab culture light intensities become buoyant at high light intensities and floating strains may sink at high light intensities (Xiao *et al.*, 2012). The extent to which buoyancy varies among strains and how this variation is genetically encoded remains unclear.

Nutrient requirements and biomass stoichiometry

Microcystis blooms are manifestations of excess nutrients, particularly nitrogen (N) and phosphorus (P). Therefore the N and P content of *Microcystis* biomass is a critical trait for understanding how elevated N and P concentrations become blooms (Gerloff and Skoog, 1954), how *Microcystis* competes with other phytoplankton (Olsen, 1989; Olsen *et al.*, 1989), and how the stoichiometry of these elements shapes

toxin production (Van de Waal *et al.*, 2014). Like other phytoplankton, *Microcystis* exhibits substantial plasticity in the quantity of N and P in its biomass (**Fig. 3A-B**). The median strain can vary its P quota (atoms P per cell) by 4-fold, biomass P content by 8-fold, and molar N:P ratio in biomass by 6-fold. While some of the differences among strains in Figure 3A are attributable to differences in culture conditions, the two studies that compared multiple strains indicate that strains differ in both their P content and plasticity (Hesse and Kohl, 2001; Saxton *et al.*, 2012). Some of this intra and inter-strain variability in N and P content is attributable to two determinants of elemental content within phytoplankton: nutrient availability (Rhee, 1973) and growth rate (Droop, 1973), which can act both individually and interactively (Flynn *et al.*, 2010; Hillebrand *et al.*, 2013). Figure 3 (C-D) shows that, within individual strains, biomass N and P content are positively correlated with growth rate, but the differences among strains are substantial. Wide differences in response to N limitation between strains (Brandenburg *et al.*, 2018) further supports strain-dependent quotas. Additional experiments with multiple strains would help to determine the extent of intraspecific variability in these traits. Further, the outcome of competition depends on whether nutrients are supplied constantly or in pulses (Suominen *et al.*, 2017), so the capacity for some *Microcystis* strains to assimilate N and P beyond their immediate needs for growth underscores the need for care when extrapolating from laboratory experiments to dynamics in the field.

Whereas the nutrient content of *Microcystis* influences the amount of biomass that potentially results from excess nutrients, development of a bloom also requires that the concentrations of available nutrients are sufficient for *Microcystis* to maintain a positive net growth rate despite competition from other species (Holm and Armstrong, 1981; Marinho and De Oliveira E Azevedo, 2007). However, no study to date has compared the Monod kinetics (*i.e.*, growth rate as a saturating function of N or P concentration) or Droop kinetics (growth rate as a function of internal quota) for more than a few strains of *Microcystis*. Available data among studies show that the initial slope of the Monod function (d^{-1} μM^{-1}, (Healey, 1980)) ranges from 1.3 to 24 for P (Ahlgren, 1985; Baldia *et al.*, 2007) and 0.014 to 1.3 for N (Kappers, 1985; Tan *et*

al., 2019). While some of this variation in the relationship between resource N and P and growth rate is attributable to differences in experimental design, there is also evidence that strains differ in their affinity for acquiring these elements. Comparison of P uptake kinetics in eight strains of *Microcystis* and showed that the half-saturation constant for phosphate uptake ranged from 9.5-19.5 µM and the apparent maximum uptake rate ranged from 0.27-0.57 µM mg^{-1} h^{-1} (Shen and Song, 2007). Given the variation among strains in both resource use kinetics and biomass elemental content, and the apparent sorting of genotypes based on resource availability (Jackrel *et al.*, 2019), further experiments that systematically characterize this variation would be especially useful.

Nitrogen uptake and metabolism

Nitrogen plays an important role in stimulating and sustaining *Microcystis* blooms, and nitrogen availability appears to strongly influence microcystin cellular quotas (Harke and Gobler, 2013, 2015; Horst *et al.*, 2014). Furthermore, nitrogen addition to natural communities promotes blooms with significantly higher microcystins concentrations to a greater extent than phosphorus additions (Donald *et al.*, 2011; Davis *et al.*, 2015; Jankowiak *et al.*, 2019). The regulation of microcystins production likely involves both C and N metabolism (see below), and the highest concentrations of microcystins correlated with high N:P ratios, in both the lab and the field (Downing *et al.*, 2005; Beversdorf *et al.*, 2015). The rate of microcystins production is tightly coupled to cell division rates, suggesting potential for indirect effects of environmental factors on microcystin production (Orr *et al.*, 1998, Lyck *et al.*, 2004). However, in some cases microcystins production rate is strongly positively correlated with nitrogen uptake rate (and negatively correlated with phosphorus uptake and carbon fixation rates) independent of growth rate (Downing *et al.*, 2005). To our knowledge, no study to date has examined the effect of growth rate and nitrogen availability on microcystins production or cellular content across multiple *Microcystins* strains using a common approach, thus the extent to which these different results can be explained by different strains is unclear.

Both field and lab culture data suggest that there are strain-specific differences in nitrogen metabolism, but the driving mechanisms are still largely unknown. Most strains harbor similar genetic capabilities for nitrogen assimilation and transport (**Fig. 2; Fig. S1**), thus observed phenotypic differences likely indicate differences in regulation of gene expression and/or functional differences (*e.g.*, affinity, kinetics) associated with alleles. This is consistent with differential expression of several proteins involved in nitrogen uptake and metabolism between strains, under both N-replete and N-limited conditions (Alexova *et al.*, 2011, 2016). Ammonium transporters (*amt*, *amtc*) are among the most variably present N-related genes across *Microcystis* strains (**Fig. 2**). Increased availability of inorganic nitrogen sources leads to production of more microcystins by toxigenic strains (Orr and Jones, 1998), which apparently have a higher nitrogen requirement than non-toxigenic strains, and require higher nitrogen concentrations to achieve their maximum growth rate (Vézie *et al.*, 2002). Co-culture competition experiments further suggest that toxigenic strains grow better at high combined (N and P) nutrient concentrations, whereas non-toxigenic strains grow better at low concentrations (Vézie *et al.*, 2002). However, it is unclear whether these conclusions apply broadly to toxigenic and non-toxigenic strains across the diversity of *Microcystis*, or rather are confined to the few strains tested.

Observations of natural communities also suggest that the form and concentration of nitrogen influences the abundance of *Microcystis* strains/species (Monchamp *et al.*, 2014). Nitrogen enrichment, particularly inorganic forms of N like nitrate and ammonia, promotes toxigenic strains over non-toxigenic strains (Davis *et al.*, 2009, 2010). Non-toxigenic strains were stimulated by organic forms of N like urea and glutamic acid (Davis *et al.*, 2010). This could explain in part why community shifts from mostly toxigenic to non-toxigenic strains are often observed midway through the bloom in seasonally stratified systems, as the primary available nitrogen source often changes from inorganic to organic forms (Davis *et al.*, 2010; Beversdorf *et al.*, 2015; Gobler *et al.*, 2016). However, the relative importance of inorganic nitrogen vs organic nitrogen sources in driving *Microcystis* strain composition of blooms remains poorly understood, especially considering that dissolved organic nitrogen concentrations in freshwaters are rarely quantified.

Temperature

Microcystis blooms rarely occur at cooler temperatures (< 20°C). Water temperature affects *Microcystis* in many ways including colony size, metabolic rate, buoyancy, chlorophyll-a concentration, toxin concentration, and growth rate (Wantabe and Oishi, 1985; Staehr and Birkeland, 2006; Bouchard and Purdie, 2011; Li and Reidenbach, 2014; Duan *et al.*, 2018; You *et al.*, 2018). Temperature also influences competition, including between *Microcystis* and other bloom forming algae (Chu *et al.*, 2007; Lürling *et al.*, 2013; Thomas and Litchman, 2016) and between different *Microcystis* strains (Xiao *et al.*, 2017). Cyanobacteria generally have a higher temperature of optimal growth than other freshwater phytoplankton, thus there is an apparent link between increasing temperatures and global proliferation of cyanobacterial blooms (Paerl and Huisman, 2008).

As reported previously and compiled here, different *Microcystis* species/strains have distinct growth responses to temperature (Xiao *et al.*, 2017; Bui *et al.*, 2018)(**Fig. 4**). Optimum temperature for growth ranges from 25℃ to 35℃, and this effect appears to be independent of light levels and other experimental conditions, suggesting that optimal growth temperature is a key trait that may differentiate strain niches. Unfortunately, growth rate data at different temperatures are sparse, and thus many of the temperature optima are imprecise. Moreover, there is little data on non-toxigenic strains, and **Fig. 4** does not clarify the relationship between production of microcystins and temperature adaptations. However, taken together with the strong effect of temperature on *Microcystis* physiology, these results suggest that temperature influences bloom occurrence, intensity, and strain composition. Supporting this hypothesis, higher temperature favored toxigenic strains in some studies (Davis *et al.*, 2009; Dziallas and Grossart, 2011). However, in other lab culture experiments, a non-toxigenic strain had higher optimal growth temperature than toxic strains (Thomas and Litchman, 2016). The microcystins quota per cell decreases with increasing temperature (Bui *et al.*, 2018; Peng *et al.*, 2018), counteracting the effect of growth rate and strain selection, which further complicates the effect of temperature on bloom toxicity. However, there is also some evidence that the cell quota of microcystins increases with temperature, thus the

response and threshold of how temperature influences microcystin quota may be strain dependent (van der Westhuizen and Eloff, 1985; Wantabe and Oishi, 1985; Mowe *et al.*, 2015). These inconsistent conclusions from field and lab data highlight persistent gaps in knowledge; we have a highly incomplete picture of how temperature response maps onto genotype and phylogeny.

Light

Light intensity impacts the growth rate (Wiedner *et al.*, 2003), toxin production (Wiedner *et al.*, 2003; Salvador *et al.*, 2016), and buoyancy (Brookes, 2001; Straub *et al.*, 2011) of *Microcystis*, and it is a major factor in determining the growth and cell density of *Microcystis* blooms (Huisman *et al.*, 1999; Tomioka *et al.*, 2011; Verspagen *et al.*, 2014). Responses to light include both short-term cellular acclimation as well as evolutionary adaptation of strains. Studies of gene expression offer insight into the molecular encoding of light acclimation and adaptation (Srivastava *et al.*, 2019). High light intensity causes oxidative stress (Zilliges *et al.*, 2011), presenting a challenge for buoyant scums at the water-air interface (Brookes *et al.*, 2003). The effect of light on *Microcystis* is complex because it depends on several processes and factors, including light quality (*i.e.*, wavelength), turbulence, tolerance of oxidative stress, photoacclimation, and colony formation, which themselves may be dependent on water depth and clarity, temperature, media composition, and adaptation to laboratory culturing conditions. The effect of light is also complex in the context of a community – different cells and colonies (and perhaps strains) in a single habitat can experience vast differences in intensity at a given time, depending on factors such as water depth and microenvironment. Light limitation may increase the phenotypic heterogeneity within *Microcystis* communities, reducing overlap in traits between cells and therefore increasing the probability of individual success (Fontana *et al.*, 2019). These findings suggest that competition, both interspecies and intraspecies, is light-dependent (Fontana *et al.*, 2019).

The diversity of light adaptations across *Microcystis* strains has long been of interest in terms of how it shapes the relative success of toxigenic and non-toxigenic strains of *Microcystis*. In some cases, toxigenic

strains are less competitive under light limitation, perhaps due to the cost of microcystins production (Kardinaal *et al.*, 2007; Renaud *et al.*, 2011; Van De Waal *et al.*, 2011; Briand *et al.*, 2012). Consistent with this conclusion, some toxigenic strains are adapted to high light conditions and non-toxigenic strains to low light conditions (Deblois and Juneau, 2012), and UV-B radiation may inhibit growth of non-toxigenic strains more than toxigenic strains (Yang *et al.*, 2015). Many studies have found that non-toxigenic strains dominate blooms later in the summer, when light and nutrient limitation are highest, indicating that non-toxigenic strains are better at competing for light in the field (Janse *et al.*, 2004; Bozarth *et al.*, 2010). It is also interesting to note that all strains isolated from Michigan lakes with low total phosphorus (which have clear water and thus high light availability) are toxigenic (the Mae2 LL/LG clade in **Fig. 2**), supporting the link between high light and toxicity. However, other studies observed toxigenic strains that dominate under low light, which undermines the link of microcystin production to light adaptation (Schatz *et al.*, 2005; Renaud *et al.*, 2011; Zhai *et al.*, 2013). As indicated in **Fig. 2** and discussed in the conclusion, the ability to produce microcystins is scattered throughout the *Microcystis* phylogeny and it is not directly correlated with presence/absence of many functional genes, raising the question of whether traits are coordinated (co-vary). Production of microcystins is just one trait, which may or may not be linked to other traits. From this viewpoint it is not surprising to see conflicting conclusions for light adaptation of toxigenic and non-toxigenic strains.

A compilation of the growth response of *Microcystis* strains to light intensity shows several interesting trends (**Fig. 5**). First, while *Microcystis* growth occurs across a broad range of light intensities, the optimum for growth for all strains (where apparent) occurs within a relatively narrow range of 25-100 μmol photons m^{-2} s^{-1}. Second, there appear to be differences in optimal light level between strains, independent of temperature and media composition. However, in many cases the optimum is poorly defined due to the limited number of measurements. Third, the shape of the curve for growth response to light varies, indicating that some strains are photoinhibited (*e.g.*, NIES 111, CS 338, CS 558) at high light whereas others are not (*e.g.*, CPCC 299, MIC 08). In part this may be explained by colony-forming traits;

single-cells reach light saturation at lower light levels than colony-forming strains (Chorus, 2001). Unfortunately, colony-forming traits are not widely reported for the strains (**Table S3**). No evidence of a relationship between growth response to light intensity and capability of producing microcystins was identified in our review (**Fig. 5**). While these and other data support the conclusion that light adaptation may be a key trait for differentiating the physiology of *Microcystis* strains, more precise and controlled measurements are needed to directly compare across strains and evaluate links and tradeoffs with other traits.

Oxidative stress

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) are ubiquitous in aquatic ecosystems and may influence microbial community composition because of varying sensitivity among taxa due to variable presence of genes for catalase and peroxidase enzymes that decompose H_2O_2 (Perelman *et al.*, 2003; Morris *et al.*, 2008, 2011; Kim *et al.*, 2016). This includes large differences in sensitivity between genera (Ostrowski *et al.*, 2001; Perelman *et al.*, 2003; Morris *et al.*, 2008, 2011; Kim *et al.*, 2016, 2019; Ma *et al.*, 2018; Bayer *et al.*, 2019) as well as subtle differences among different strains (Morris *et al.*, 2011; Bayer *et al.*, 2019), suggesting that ROS may affect community composition at multiple taxonomic levels. Cyanobacteria are particularly sensitive to H_2O_2, enabling treatment of cyanobacterial blooms with H_2O_2, which kills the cyanobacteria, leaves algae and other organisms intact, and decomposes to water and O_2 (Matthijs *et al.*, 2012). H_2O_2 at natural concentrations may also impact the strain composition of *Microcystis* blooms (Paerl and Otten, 2013a; Schuurmans *et al.*, 2018). This hypothesis is supported by findings that microcystins covalently bind to photosynthetic proteins that are sensitive to oxidative damage (Zilliges *et al.*, 2011), thus protecting toxigenic strains and favoring them over non-toxigenic strains in blooms under oxidative stress (Dziallas and Grossart, 2011; Zilliges *et al.*, 2011; Paerl and Otten, 2013a).

To date, the differential impact of H_2O_2 on growth of *Microcystis* strains has only been explored along the toxigenic vs. non-toxigenic dichotomy (Dziallas and Grossart, 2011; Zilliges *et al.*, 2011; Paerl and Otten, 2013a; Schuurmans *et al.*, 2018), yielding conflicting results. In one culturing study, three microcystins producing strains had lower reductions in chlorophyll content than two non-toxigenic strains when treated with H_2O_2 (Dziallas and Grossart, 2011). However, the opposite result was found in a study where the mutant strain unable to produce microcystins degraded H_2O_2 faster than the toxic wild type, and a natural non-toxic strain could recover from large additions of H_2O_2 at low light intensities (Schuurmans *et al.*, 2018). This may indicate that the H_2O_2 sensitivity of *Microcystis* strains varies, albeit not in the previously hypothesized toxigenic vs. nontoxigenic manner. However, the conflicting results could be due to lack of standardized conditions between experiments. The effect of H_2O_2 on cyanobacteria is dependent on light intensity (Drábková *et al.*, 2007; Morris *et al.*, 2011; Piel *et al.*, 2019) and wavelength (Sandrini *et al.*, 2020), deviation from optimal growth temperature (Ma *et al.*, 2018), cell density (Morris *et al.*, 2008, 2011), and associated heterotrophic bacteria (Kim *et al.*, 2019). Therefore, it is important to standardize these parameters in experiments comparing the H_2O_2 sensitivities of cyanobacteria. The aforementioned studies were performed at different light and temperature regimes, which makes interpreting and comparing their results difficult. In addition, Schuurmans et al. largely measured growth inhibition at large H_2O_2 doses, orders of magnitude above concentrations observed in natural waters (Cooper *et al.*, 1989; Cory *et al.*, 2016), and used light levels far below those typical of natural sunlight (Sagert and Schubert, 2000). The Dziallas and Grossart and Zilliges et al. studies did not measure background H_2O_2 concentrations of the growth media during their experiment, so the total H_2O_2 exposure in their experiments is unknown. Therefore, the extent to which naturally occurring H_2O_2 concentrations impact the growth of different *Microcystis* strains is still uncertain.

The distribution of genes encoding catalases and peroxidases among publicly available *Microcystis* genomes is similar to that of *Prochlorococcus* and *Nitrosopumilus* (Morris *et al.*, 2012; Kim *et al.*, 2016, 2019); the majority lack heme catalases and peroxidases and contain multiple peroxiredoxin genes (**Fig.**

2). This suggests that like *Prochlorococcus* and *Nitrosopumilus*, many *Microcystis* strains are sensitive to naturally occurring H_2O_2 concentrations. However, three strains of a nontoxic *Microcystis* clade have the catalase-peroxidase *katG*, the canonical enzyme for degradation of high extracellular H_2O_2 concentrations (Seaver and Imlay, 2001; Perelman *et al.*, 2003; Cosgrove *et al.*, 2007; Morris *et al.*, 2012). In addition, several *Microcystis* genomes have genes annotated as animal haem peroxidases (AHPs), cell surface enzymes that oxidize manganese through superoxide production and H_2O_2 decomposition (Wariishi *et al.*, 1992; Scheeline *et al.*, 1997; Schlosser and Höfer, 2002; Anderson *et al.*, 2009; Andeer *et al.*, 2015). The dual ROS production and decay activity of these enzymes makes it unclear whether they influence oxidative stress in microbes or if the ROS are merely transient intermediates in the oxidation of manganese and other metals (Zinser, 2018). Overall, these genomic insights support the physiological evidence for variation in resistance to ROS across *Microcystis* strains.

Inorganic carbon/pH

Microcystis strains also have distinct adaptations to availability of dissolved inorganic carbon (C_i) (Van De Waal *et al.*, 2011; Sandrini *et al.*, 2016), raising the question of how availability of C_i influences the strain composition, and thus toxicity, of *Microcystis* blooms. Dynamics of C_i play out across several time scales of interest including daily (diel cycles associated with photosynthesis), seasonal (*e.g.*, bloom development), and decadal and longer time scales associated with rising atmospheric CO_2 due to anthropogenic emissions. In all cases, C_i availability is intertwined with pH based on the chemical relation between pH and C_i system; shifts in pH determine the C_i availability for photosynthetic organisms, and photosynthesis alters the pH of the environment. pH can vary by up to 0.5 units on diel cycles (Krausfeldt *et al.*, 2019). On the time scale of bloom development, the initiation of *Microcystis* blooms is typically characterized by neutral or slightly acidic conditions (high pCO_2 concentrations), while dense blooms have pH levels that can exceed 9.5, at which pCO_2 concentrations in the water are essentially negligible, leading to potential carbon limitation (Bañares-España *et al.*, 2006; Gu *et al.*, 2011; Krausfeldt *et al.*, 2019). Lab experiments show that increased C_i availability induces changes in the strain

composition of blooms, suggesting that rising atmospheric CO_2 will shape the strain composition of blooms and evolution of *Microcystis* (Sandrini *et al.*, 2016). Further, both C_i availability and pH have a profound impact on biota at the community and ecosystem levels, likely leading to complex effects that must be considered in addition to the *Microcystis* strain-level effects that we focus on here.

A handful of experiments have explored the impact of C_i availability (pCO_2 of 100 – 1250 ppm) on a variety of *Microcystis* strains, showing a wide range of specific growth rates (0.152 to 1.04 d^{-1}; **Table S4**). It is important to note that these growth rates are not only influenced by C_i treatment, but also *Microcystis* strain, media type, light, and temperature (see the beginning of the section on *Functional diversity*). Several studies have used *Microcystis* sp. NIES 843 to explore the role of C_i availability, and synthesis of this data indicates that C_i concentration influences *Microcystis* growth rates (**Table S4**), suggesting a higher growth rate at low pCO_2 concentrations (pCO_2: 100 – 300 ppm). While these results do not conform with other studies examining C_i availability on phytoplankton (Wu *et al.*, 2010; Gao *et al.*, 2012; Errera *et al.*, 2014; Visser *et al.*, 2016; Raven *et al.*, 2020), they do fall in the middle range of growth rates for C_i/pH *Microcystis* studies. The physiological response to C_i availability is likely due to differences in carbon concentrating mechanism (CCM) between *Microcystis* strains, as NIES 843 has a low binding affinity for HCO_3^-, but a high flux rate, which has been suggested to favor low C_i water conditions (Sandrini *et al.*, 2014). Acidification from increased CO_2 can also induce oxidative stress in cyanobacteria (Wu *et al.*, 2021).

Strain-level adaptation to C_i stems largely from variation in the genes encoding the CCM, an assortment of enzymes and transporters that acquire bicarbonate (HCO_3^-) from the environment, actively transport it across the cell membrane, and concentrate CO_2 in the presence of Rubisco (Raven *et al.*, 2012). Variations in the use and effectiveness of CCM in algae have been identified at the genus, species, and strain level of phytoplankton (Giordano *et al.*, 2005; Beardall *et al.*, 2009; Reinfelder, 2011) including across *Microcystis* strains (Sandrini *et al.*, 2015; Van de Waal *et al.*, 2019). Of the five identified CCM transporters for cyanobacteria (Omata *et al.*, 1999; Shibata *et al.*, 2001, 2002; Price *et al.*, 2004; Price,

2011) the HCO_3^- uptake systems may provide the best insight into strain success during carbon replete and deplete conditions. Each transporter has a different affinity for HCO_3^- and flux rate, suggesting that the presence of one transporter over another could provide a competitive advantage depending on C_i concentrations within the system, as suggested by the review of NIES 843 (see Price, 2011; Sandrini *et al.*, 2014, 2015; Visser *et al.*, 2016 for additional information). Specifically, *bicA* and *sbtA*, which are located on the same operon, provide insight into a *Microcystis* strain's ability to uptake C_i. These HCO_3^- plasma membrane transporters (Price, 2011) have been identified in *Microcystis* strains as four possible genotypes (Sandrini *et al.*, 2014; Visser *et al.*, 2016). The C_i generalist, which has both *bicA* and *sbtA*, is able to take advantage of C_i across a wide range of concentrations. Other genotypes may be constrained through either substrate affinity or flux, including high HCO_3^- affinity specialists (*sbtA* only); high bicarbonate flux specialists (*bicA* only); and the strains that lack both of these bicarbonate transporters. In addition to the presence and absence of genes for bicarbonate transport, differences in their expression may underpin phenotypic differences among strains (Sandrini *et al.*, 2015).

Figure 2 shows the variability in the presence of CCM genes *sbtA* and *bicA* across *Microcystis* strains. 70% of strains analyzed are equipped with high HCO_3^- substrate affinity (*sbtA*), 26% of strains have a high flux transporter (*bicA*), and only 20% of strains have both. In contrast, the vast majority of all strains contain genes for both low and high affinity CO_2 uptake, ATP dependent HCO_3^- transporters (BCT), and carbonic anhydrase (CA) enzymes (Figure S1). The genotypic variability of *sbtA* and *bicA* CCM genes is not congruent with phylogeny, consistent with frequent gene gain and loss. However, there do appear to be interesting correlations between the presence-absence of these genes and others. For example, of the 36 strains that contain neither *bicA* or *sbtA*, 97% contain genes for biosynthesis of microcystins, perhaps reflecting a role for microcystins in carbon fixation (Zilliges *et al.*, 2011; Barchewitz *et al.*, 2019). Inorganic carbon availability can also influence the carbon-nitrogen balance (C:N), leading to shifts in microcystin concentrations and congener profiles. Liu et al. (2016) suggested that high C_i conditions increased the microcystin congeners with high C:N ratio in three *Microcystis* strains examined

(NIES1099, HUB524, PCC7820), although microcystin concentrations decreased under these conditions. Potential connections between toxin content, toxicity (*i.e.*, microcystin congener profile), and carbon metabolism are also apparent in the field. Blooms in Lake Erie shift from a dominance of *Microcystis* strains capable of producing microcystin to a community dominated by non-toxic strains (Davis *et al.*, 2009, 2010; Chaffin *et al.*, 2018) suggesting that ability to produce toxins and strain shifts during the bloom may also be connected to C_i uptake ability, as C_i availability decreases (*i.e.*, pH increase) over the course of the bloom. While more research is needed to understand these potential links, the data available now are consistent with inorganic carbon availability shaping the composition and toxicity of *Microcystis* strains during bloom formation and development.

Salinity

Recent expansion of *Microcystis* blooms into the estuarine and coastal marine environment has drawn attention to the dynamics of blooms along the continuum from freshwater to saltwater (Paerl *et al.*, 2018). Thus, understanding the effect of salinity on *Microcystis* growth, persistence, and toxin production is critical for managing and predicting ecosystem impacts of bloom expansion and transport towards marine estuaries and coasts. Studies on the tolerance of *Microcystis* to salinity have produced highly variable results, with some studies indicating growth and microcystins production in low-salinity waters (e.g., estuaries) and persistence in seawater for substantial periods of time (Lehman *et al.*, 2005; Miller *et al.*, 2010), while others indicate that *Microcystis* is highly sensitive to even low salinity conditions (Sellner *et al.*, 1988). At least some of this variation appears to be due to strain-specific adaptations to salinity (Preece *et al.*, 2017). Lab studies conducted after acclimation found distinct salt tolerances and stress responses to salinity for two different strains that originated in environments with contrasting salinity, confirming strain-specific adaptations (des Aulnois *et al.*, 2019).

Community Interactions

In addition to environmental and bottom-up controls discussed above, understanding the ecology of *Microcystis* strains in the context of their phenotypic traits and genes requires consideration of interactions with the broader community. This includes top-down controls such as predation by viruses, fungi, and consumers as well as interactions with other bacteria, both positive and negative.

Secondary metabolites and allelopathic interactions

Cyanobacteria are renowned for their production of a wide range of secondary metabolites, compounds that are not involved in primary metabolism and that often have roles in communication, sensing, or inhibition of other organisms (Ehrenreich *et al.*, 2005; Kehr *et al.*, 2011; Dittmann *et al.*, 2015). In terms of biosynthetic capacity, *Microcystis* is one of the most prolific cyanobacterial genera (Dittmann *et al.*, 2015), producing many cyclic peptides including aeruginosins, anabaenopeptins, cyanobactins, cyanopeptolins, microginins, microviridins, and microcystins (Carmichael, 1992; Pérez-Carrascal *et al.*, 2019). Microcystins in particular have been intensively studied due to their toxic effects on humans, livestock, and other animals, and the threat they pose to recreation and drinking water supplies (Steffen *et al.*, 2017). Indeed, this has led to a microcystin-centric view of *Microcystis*, with strains defined as "toxic" or "non-toxic" on the basis of their ability to produce microcystins. However, as discussed below, this practice is not accurate in terms of toxicity, it does not always reflect phylogeny, and the extent to which it is meaningful for the broader ecology of *Microcystis* strains is still unclear. The secondary metabolites produced by *Microcystis* and their role in allelopathic interactions have been reviewed extensively (Kehr *et al.*, 2011; Dittmann *et al.*, 2015; Pearson *et al.*, 2016; Huisman *et al.*, 2018). Here we focus on how the repertoire of secondary metabolites varies across *Microcystis* strains and its implications for the ecological differentiation of these strains.

The suite of secondary metabolites varies across strains of *Microcystis*, both in terms of the classes of metabolites and the chemical diversity of metabolites within classes. Both levels of diversity are apparent

to some extent in the genes encoding biosynthesis of these compounds, which typically occur in biosynthetic gene clusters (BGCs). Genomic BGC content is highly variable across *Microcystis* strains (**Fig. 2**) (Pérez-Carrascal *et al.*, 2019). Substantial diversity in the presence, sequence, orientation, and order of genes within BGCs also encodes structural and biochemical diversity in the products (Welker *et al.*, 2006; Tooming-Klunderud *et al.*, 2008). Many compounds are produced by non-ribosomal peptide synthetase (NRPS) and/or polyketide synthase (PKS) complexes, and on average there are about 5 NRPS/PKS BGCs present in each genome (Shih *et al.*, 2013; Otten and Paerl, 2015). In addition to BGCs encoding known compounds, many BGCs have not been linked to a known product (Shih *et al.*, 2013; Dittmann *et al.*, 2015), raising the possibility of unknown/emerging toxins and allelochemicals. The biochemical and ecological implications of varying BGC content per genome and gene content per BGC are largely unknown.

The microcystins illustrate current knowledge and remaining frontiers for one of the most intensively studied classes of secondary metabolites in cyanobacteria. Microcystins are produced by an NRPS-PKS multi-enzyme complex that is encoded in the *mcy* operon, which contains 10 genes spanning roughly 55 kb (Tillett *et al.*, 2000). The presence of *mcy* genes is scattered across the genus in a polyphyletic distribution (**Fig. 2**). However, their pattern of presence is congruent with core *Microcystis* phylogeny on the scale of sub-clades, indicating cohesion of this trait among closely related strains and suggesting that gain and loss and horizontal transfer of these genes is relatively rare. This is consistent with co-evolution of *mcy* genes and core genes (Rantala *et al.*, 2004)**.** The sequence and structure of the *mcy* operon is also highly diverse, and this variation can lead to production of different structural variants of microcystins (congeners) (Mikalsen *et al.*, 2003; Tooming-Klunderud *et al.*, 2008). There are over 270 of these microcystin congeners with varying degrees of toxicity and health effects (Bouaïcha *et al.*, 2019). While *mcy* operon structure and gene sequence influence which microcystin congeners are produced, genetics is not the only determinant; individual *Microcystis* strains can produce multiple congeners (Mikalsen *et al.*, 2003; Otten *et al.*, 2017). Environmental conditions such as availability of nitrogen (Puddick *et al.*, 2016),

external amino acids, (Tonk *et al.*, 2008) and C:N ratio (Liu *et al.*, 2016) also affect which congeners are synthesized, consistent with regulation of toxins according to ecological stoichiometry theory (Van De Waal *et al.*, 2009; Van de Waal *et al.*, 2014; Wagner *et al.*, 2019). Despite intensive study for over 50 years, the ecological and/or physiological functionality of microcystins is still unclear. Hypotheses on the role of microcystins include allelopathy, photosynthesis and light adaptation, grazer defense, oxidative stress protection, quorum sensing, iron acquisition, and have been reviewed extensively elsewhere (Omidi *et al.*, 2018).

Large gaps in knowledge also remain on the functions of other secondary metabolites in *Microcystis*, but substantial evidence supports the hypothesis that they enhance its competitiveness through allelopathic interactions (Briand *et al.*, 2016, 2019; Chia *et al.*, 2018). These compounds can inhibit the photosynthetic capacity, growth, and regulatory processes of competitors or predators (Huisman *et al.*, 2005; Chia *et al.*, 2018). While they may inhibit a wide variety of prokaryotic and eukaryotic competitors and predators (Van Wichelen *et al.*, 2016), there is also evidence that they play a role in competition between *Microcystis* strains (Schatz *et al.*, 2005; Zhai *et al.*, 2013) thus may be an important factor in shaping the strain composition of blooms. Other proposed functions for secondary metabolites include nutrient storage, grazer defense, and quorum sensing (Kaebernick *et al.*, 2001; Pflugmacher, 2002; Chia *et al.*, 2018; Omidi *et al.*, 2018; Burberg *et al.*, 2019).

The abundance and strain-based diversity of secondary metabolites in *Microcystis* confounds efforts to ascribe biochemical activities to particular compounds and confuses terminology associated with these secondary metabolites. Common use of “toxic” and “non-toxic” to refer to production of microcystins fails to address the diversity of microcystin congeners as well as the diverse suite of other *Microcystis* BGCs, including many of unknown function, which may pose risks to aquatic communities and human health. These issues extend to allelopathic interactions. For example, some results suggested that microcystins can inhibit competitors (Legrand *et al.*, 2003) but other studies have shown that this effect is

independent of microcystins (Briand *et al.*, 2012; Lei *et al.*, 2015; Dong *et al.*, 2019; Schmidt *et al.*, 2020). Indeed, other secondary metabolites may be more important in allelochemical interactions with other phytoplankton species, such as linoleic acid in the case of strain FACHB-905 (Song *et al.*, 2017). Interestingly, Perez-Carascel et al. (2019) observed a pattern in which *Microcystis* strains that lack complete *mcy* operons often contain another BGC instead. This may suggest that each strain has a suite of compounds in its arsenal that are tuned to the environment, competitors, and predators with which it co-evolved. Overall, extensive genome mining efforts, metabolomic profiling studies, and experiments all suggest that the paradigm of microcystin-based toxic and non-toxic strains is outdated both in terms of allelopathic capabilities and health effects of compounds produced by *Microcystis.* A more holistic perspective is needed.

The Microcystis *microbiome*

Microcystis harbors bacteria in the phycosphere within and around their colonies, and these microbial communities are distinct from those in the surrounding environment and associated with other phytoplankton (Smith *et al.* submitted; Cai *et al.*, 2014; Xu *et al.*, 2018; Yang *et al.*, 2018; Batista *et al.*, 2019). Thus, differences in observed traits between *Microcystis* strains are potentially due to differences in their associated microbiomes. While our understanding of this microbiome and its effect on *Microcystis* are still in their infancy, one notable example of how microbiomes affect host traits is that colony formation can be induced by introduction of bacteria to axenic *Microcystis* cells (Shen *et al.*, 2011).

Studies of the phycosphere of other phytoplankton provide insights into potential interactions between *Microcystis* and its microbiome. Many bacteria in the phycosphere, which are predominantly heterotrophic, engage in mutualistic interactions with their phytoplankton hosts. Phytoplankton provide organic carbon and sulfur to heterotrophic bacteria while the heterotrophs supply vitamins and other cofactors (Croft *et al.*, 2005; Amin *et al.*, 2015; Durham *et al.*, 2015, 2017), regenerate nutrients from

organic matter (Van Mooy *et al.*, 2012; Amin *et al.*, 2015; Arandia-Gorostidi *et al.*, 2017; Christie-Oleza *et al.*, 2017), fix nitrogen (Cook *et al.*, 2020), and detoxify ROS (Morris *et al.*, 2011; Ma *et al.*, 2018) for their phytoplankton hosts. Other interactions with bacteria may harm phytoplankton; some bacteria act as obligate parasites (Caiola and Pellegrini, 1984; Rendulic *et al.*, 2004; Seyedsayamdost *et al.*, 2011; Soo *et al.*, 2015; Agha *et al.*, 2016). In some cases, a single bacterium can either improve or hinder phytoplankton growth depending on growth conditions (Grossart and Simon, 2007; Seyedsayamdost *et al.*, 2011; Hennon *et al.*, 2018).

Similar to this spectrum of interactions observed in other phytoplankton, the exudates of heterotrophic bacteria can both positively and negatively impact *Microcystis* growth rates (Akins *et al.*, 2020). While exchange of specific metabolites is currently poorly characterized, some evidence suggests the mucilage layer of *Microcystis* colonies mediates interactions between *Microcystis* and other microbes. Many bacteria adhere to the exopolysaccharide mucilage of *Microcystis* colonies (Smith *et al.* submitted; Worm and Søndergaard, 1998; Brunberg, 1999; Agha *et al.*, 2016) and can degrade it, presumably using it as a carbon and energy source (Li *et al.*, 2009; Shen *et al.*, 2011). The chemical composition of the mucilage, which can vary between *Microcystis* strains (Forni *et al.*, 1997), may attract specific bacterial populations (Smith *et al.* submitted) or influence the outcomes of microbial interactions (Agha *et al.*, 2016). Identification of specific compounds exchanged between *Microcystis* strains and their microbiomes will be valuable in revealing the currency and variation of these interactions.

Emerging evidence suggests that the taxonomic composition of the *Microcystis* microbiome may have some strain specificity. Some of the same taxa are regularly observed during *Microcystis* blooms (Cook *et al.*, 2020), but its microbial community composition varies considerably as a function of time (Parveen *et al.*, 2013; Berry *et al.*, 2017; Chun *et al.*, 2020; Jankowiak and Gobler, 2020), location (Jankowiak and Gobler, 2020), or the dominating genotype of *Microcystis* (Chun *et al.*, 2020). Analysis of communities associated with *Microcystis*, either via culturing or direct sequencing of single colonies, shows that

different *Microcystis* strains have distinct microbiomes at a taxonomic level (Smith *et al.* submitted; Jackrel *et al.*, 2019; Pérez-Carrascal *et al.*, 2020). This is in line with species-specific microbiome assembly experimentally observed for other phytoplankton (Jackrel, Yang, *et al.*, 2020). Sequence data from single colonies of *Microcystis* show that several taxa are prevalent but none universally present across colonies, and more closely related genotypes, either based on *Microcystis* oligotypes or whole genomes, harbored more similar microbiomes (Smith *et al.* submitted; Pérez-Carrascal *et al.*, 2020). Despite this taxonomic correlation, the relationship between host functions and microbiomes functions is weak, and microbiomes broadly converge at a functional level (Jackrel *et al.*, 2019; Cook *et al.*, 2020). Multiple studies have observed that microbiomes added functions and sometimes complemented steps in metabolic pathways missing in the *Microcystis* host genome (Jackrel *et al.*, 2019; Pérez-Carrascal *et al.*, 2019; Cook *et al.*, 2020). This is in line with Black Queen dynamics, by which significant gene losses are compensated through provision of 'public goods' by community members (Morris *et al.*, 2012; Morris, 2015), as seen in the case of oxidative-stress genes in *Prochlorococcus* (Morris *et al.*, 2011; Ma *et al.*, 2018). There is some evidence that heterotrophic bacteria protect *Microcystis* from hydrogen peroxide (Kim *et al.*, 2019), but whether this is relevant at naturally occurring hydrogen peroxide concentrations remains to be demonstrated. Nonetheless, differences between *Microcystis* strains in content of genes involved in detoxification of oxidative stress **(Fig. 2)** may in part be explained by differential reliance on public goods or exudates from the non-cyanobacterial bacterial community.

The functional implications of strain-specific microbiomes are mostly unknown in the case of *Microcystis*. In other phytoplankton species, outcomes of interactions between bacteria and phytoplankton are dependent on the specific strains and species involved (Sison-Mangus *et al.*, 2014; Amin *et al.*, 2015). An interesting example for *Microcystis* is the interaction with another cyanobacterium, *Pseudanbaena*, which is commonly associated with *Microcystis* colonies (Smith et al., submitted), where it grows epiphytically and produces cyanotoxins (Rangel *et al.*, 2014) that may alter the toxicity of blooms.

Interactions between *Pseudanabaena* and *Microcystis* are genotype-specific and range from neutral to antagonistic (Agha *et al.*, 2016; Chun *et al.*, 2020).

Microbiome impacts on host fitness may also impact competitive interactions between *Microcystis* strains and between *Microcystis* strains and other phytoplankton species. As the outcome of competition depends on physicochemical conditions, and as the microbiome likely alters the local environment of *Microcystis*, microbiomes could very well affect competitive outcomes. Indeed, microbiomes influence competitive interactions for plants and eukaryotic algae (Wagg *et al.*, 2011; Siefert *et al.*, 2018, 2019; Zhou *et al.*, 2018; Jackrel, Schmidt, *et al.*, 2020). The strain-specific association and effects of *Pseudanabaena* on *Microcystis* could theoretically affect intrinsic intra- and interspecific interactions among phytoplankton, though this has not been experimentally verified. In the one study where microbiome effects on competition were assessed, the effect of microbiomes on competition depended on the genotype of *Microcystis*; the ability of one *Microcystis* strain to competitively exclude a green alga was improved by heterotrophs while another strain did not require heterotrophs to do so (Schmidt *et al.*, 2020). More work is needed to understand the impacts that ubiquitous as well as strain-specific bacterial taxa have on different *Microcystis* strains.

Viruses

Viruses of microbes influence the ecology and evolution of their host populations (Fuhrman, 1999; Wommack and Colwell, 2000; Suttle, 2007; Breitbart, 2012; Brum and Sullivan, 2015). *Microcystis* is not immune to the effects of viral activity, as evidenced by viral lysis of cultures (EPA Report, 1977; Tucker and Pollard, 2005; Honjo *et al.*, 2006; Yoshida *et al.*, 2014), *in situ* predator-prey dynamics inferred from viral and host abundances and transcriptional activity (Manage *et al.*, 1999; Yoshida *et al.*, 2007; Steffen *et al.*, 2015; Mankiewicz-Boczek *et al.*, 2016), and CRISPR-spacers targeting viruses found in host genomes (Kimura *et al.*, 2018; Wang *et al.*, 2019). Viral infection of *Microcystis* may have implications for bloom fate (Bratbak *et al.*, 1993; Jacquet *et al.*, 2002), toxicity (Steffen *et al.*, 2017; McKindles *et al.*,

2020), and nutrient dynamics (Jover *et al.*, 2014; Mckindles, 2017). Further, as a strain-specific top-down control on their host populations, viruses can drive intraspecific diversification of *Microcystis*. Following the collapse from viral lysis, population density of *Microcystis* cultures may rebound, presumably due to evolved immunity (EPA Report, 1977)(Zhai *et al.*, 2013). Other studies have described the emergence of cultures with intermediate sensitivity after infection, which has been attributed to the coexistence of host populations both sensitive and resistant to the virus (Yoshida *et al.*, 2014; Mckindles, 2017). In the environment, during a season with two *Microcystis* blooms (Yoshida *et al.*, 2007), peak *Microcystis* virus marker gene abundances corresponded with the crash of the first bloom peak. Through the second bloom peak, viral gene abundances remained near-zero, presumably due to viral resistance in the late-blooming host population. Because only the second *Microcystis* population encoded genes for microcystin production, such virus-driven intraspecific diversification may have consequences for bloom toxicity. Given the limitations of marker genes and short qPCR targets, future work should move beyond marker gene analysis to resolve these coevolutionary dynamics at the population genomic level.

A long history of viral infection and subsequent CRISPR-mediated immune defense is evident in sequenced *Microcystis* genomes. The mean number of CRISPR arrays in nine sequenced *Microcystis* genomes is seven-fold greater that of the average of other publicly available microbial genomes (n=22,611 total genomes; as predicted in the CRISPRCasdb (Pourcel *et al.*, 2020)). *Microcystis* CRISPR spacer sequences target viral genomic fragments reconstructed from environmental metagenomes (Morimoto *et al.*, 2019), confirming a history of active defense against natural viral populations. The acquisition of a new virus-targeting spacer sequence will result in a novel subspecies-level *Microcystis* CRISPR-genotype (CT) that differs by 20-30 bp in length. Evidence for selective sweeps of *Microcystis* populations at the level of CT (Kimura *et al.*, 2018) means viral pressure causes competitive advantages at a genomic resolution finer than genes. Yet, the consequences of these fine scale genotypic differences for sustained impacts on bloom dynamics are unknown. Future work will clarify whether, and under

which conditions, such virus-driven selective sweeps result in phenotypic changes that influence the competitive advantage of new *Microcystis* CTs.

Due to the limited knowledge of *Microcystis* viruses, most studies have been restricted to evaluating the presence or activity of the small number of sequenced *Microcystis* viral isolates (Yoshida *et al.*, 2007; Ou *et al.*, 2013; Yang *et al.*, 2020). Future work will benefit from moving beyond viral marker gene analyses of this virus subset (eg. Yoshida *et al.*, 2007; Mankiewicz-Boczek *et al.*, 2016; Steffen *et al.*, 2017; McKindles *et al.*, 2020) towards developing a population-level understanding of both cultured and uncultured *Microcystis* viruses through community genomic sequencing (Morimoto *et al.*, 2019). Combined with the continued isolation of *Microcystis* and viral strains, cross-infection host range assays (Wang *et al.*, 2019), and experimental coevolution, these approaches are likely to reveal a breadth of viruses capable of infecting *Microcystis*, each with unique infection traits and potential fitness costs of host resistance. As arguably the least well-studied of the potential drivers of bloom dynamics, a better understanding of *Microcystis*-virus interactions has the potential to improve predictive models of toxic bloom fate.

Fungal parasites

Parasitic fungi of the phylum Chytridiomycota are widespread in aquatic environments and infect phytoplankton of harmful algal blooms (Gleason *et al.*, 2015), including cyanobacteria (Gerphagnon *et al.*, 2013; Frenken *et al.*, 2018). Infection rates in cultures suggests they could be important in modulating cyanobacterial blooms (Sime-Ngando, 2012; Gerphagnon *et al.*, 2013). This fungal parasitism can have unexpected effects on food web dynamics; fungal zoospores released from infected cyanobacterial cells can be a significant food source to zooplankton and as such compensate for the poor food source the dominant bloom-forming cyanobacterial species themselves represent (Frenken *et al.*, 2018). In addition to chytrid parasites, growth inhibition (though not parasitism) and microcystin degradation has been

shown by *Trichoderma citrinoviride*, a fungus isolated from decaying bloom biomass (Mohamed *et al.*, 2014).

While only limited documentation of chytrid infection of *Microcystis* is available (Sen, 1988), different strains of *Microcystis* appear to have varying degrees of susceptibility (Van Wichelen *et al.*, 2010). Strain-level variability also occurs for morphotypes of *Dolichospermum* (Weisbrod *et al.*, 2020) and chemotypes of *Planktothrix* (Sønstebø and Rohrlack, 2011). Thus, the strain composition of *Microcystis* blooms may be influenced by fungal parasites. However, no field observations indicating strong fungal impacts on *Microcystis* bloom population dynamics have been made (Van Wichelen *et al.*, 2016). Interestingly, secondary metabolites produced by certain *Microcystis* strains help *Daphnia* fend of its own fungal parasites (Sanchez *et al.*, 2019). Because secondary metabolites produced by *Microcystis* vary across strains they likely defend against fungal parasites in a strain-dependent way, but this remains to be tested.

Predatory bacteria

Certain bacterial species are specialized predators of other bacteria, with the best studied example being *Bdellovibrio bacteriovorus* (Sockett, 2009). Another predatory bacterium, *Vampirovibrio chlorellavorus*, has been shown to adhere to the surface of the eukaryotic green alga *Chlorella vulgaris* using specialized attachment structures, and to subsequently destroy the cell (Soo *et al.*, 2015; Kim *et al.*, 2016). While little is known about the importance of bacterial predation on *Microcystis*, one study showed that it may be as important as viral predation in the demise of blooms (Manage *et al.*, 2001). Predatory bacteria are physically associated with *Microcystis* colonies in substantial abundance (Smith *et al.* submitted)**,** and they show patterns of co-occurrence with specific *Microcystis* genotypes, hinting at the possibility that predatory bacteria may have strain-specific interactions with *Microcystis* strains or their microbiomes (Chun *et al.*, 2020).

Grazers

Grazers such as microzooplankton (*e.g.*, protists and rotifers), mesozooplankton (e.g. *Daphnia*, small cladocerans, and copepods), and mussels can have major effects on phytoplankton and cyanobacterial community composition and toxicity, as illustrated by the impacts of invasive mussels on lakes and river systems around the world (Vanderploeg *et al.*, 2001, 2002, 2009, 2013; Cataldo *et al.*, 2012; Waajen *et al.*, 2016). Selective feeding can promote or depress different components of the phytoplankton community, including *Microcystis*, and there has long has been an interest in how grazer populations control blooms as well as the reciprocal inhibitory response of *Microcystis* on grazers, which in turn affects transfer of energy up the food web (Ger and Panosso, 2014; Ger *et al.*, 2016). Several considerations suggest that interactions between grazers and *Microcystis* vary across *Microcystis* strains. First, in general, trait variation is an important predictor for interactions between herbivores and phytoplankton (Leibold, 1989; Tessier and Woodruff, 2002; Cottingham *et al.*, 2004). Second, in particular, *Microcystis* colony size/morphology and toxin content can deter grazing (Ger and Panosso, 2014; Ger *et al.*, 2016), and these traits vary across *Microcystis* strains and have been linked to grazing defense as discussed more below. Third, the literature shows conflicting results on impacts of grazer-*Microcystis* interactions, likely resulting in part from trait variation across *Microcystis* strains (Chislock *et al.*, 2013; Ger and Panosso, 2014; Ger *et al.*, 2016). Thus, grazers may play an important role in selecting for *Microcystis* strains in field populations and likely shape *Microcystis* traits involving colony size and secondary metabolites.

The large size of *Microcystis* colonies usually found in nature is a deterrent to predation, but whether this trait is based on phenotypic plasticity or genetic differences between strains is unclear (Bittencourt-oliveira *et al.*, 2001; Lürling, 2003; Sarnelle *et al.*, 2005; Vanderploeg *et al.*, 2013; White and Sarnelle, 2014). The largest size of particles ingested by crustacean zooplankton is determined by the size of the food collection apparatus and mouth of the grazer and thus increases with grazer size (Burns, 1968; Vanderploeg, 1994). For example, *Daphnia magna*, a large pond form, is able to ingest particles 80 μm in

diameter; however, in general particles (colonies) larger than ~30-40 µm in diameter are too large to be ingested by most mesozooplankton (cladocerans and copepods) found in lakes, rivers, or estuaries (Vanderploeg, 1994). Feeding experiments with a size range of colonies of a non-toxigenic strain of *Microcystis* suggest that the upper level of colony size ingested for dreissenid mussels may be 80 µm (White and Sarnelle, 2014). This implies grazers can only access *Microcystis* before they mature into large colonies often found in nature. Several studies support this conclusion. *Microcystis* consisting of unicells and small colonies < 20 µm were readily ingested in Lake IJsselmeer, Netherlands (Pires *et al.*, 2005). On the other hand, Cataldo et al. (2012) showed that the invasive mussel *Limnoperna fortunei* shifted the initial phytoplankton community comprised of a small percentage of *Microcystis* sp., present mostly as unicells, into dominance by *Microcystis* as large colonies. The importance of *Microcystis* strain and size have become important issues in the controversy that invasive mussels promote *Microcystis* blooms in some systems but not others (Vanderploeg *et al.*, 2001, 2002, 2009, 2013).

Variable toxicity across *Microcystis* strains and species also likely plays a key role in the varying interactions that grazers have with *Microcystis* (Jungman and Benndorf, 1994; Vanderploeg *et al.*, 2001; Juhel *et al.*, 2006; Schwarzenberger *et al.*, 2014). “Toxicity” here includes effects due the wide variety of congeners of microcystins, which have varying bioactivity, as well as undefined secondary metabolites, which can depress feeding rate, interfere with molting and ultimately lead to low survival of *Daphnia*, for example (Jungmann and Benndorf, 1994; Kaebernick *et al.*, 2001). Exposure of *Daphnia* to *Microcystis* can result in varying outcomes, which are dependent both on *Daphnia* and *Microcystis* genotypes (Lemaire *et al.*, 2012; Chislock *et al.*, 2013). Many clones of *Daphnia* found in eutrophic systems can detoxify microcystin (Lyu *et al.*, 2019) and readily feed on *Microcystis* along with other phytoplankton without harm (Chislock *et al.*, 2013). In contrast, calanoid copepods, not tolerant of microcystin, selectively reject *Microcystis* with high microcystin content (Ger *et al.*, 2016, 2019), thereby potentially promoting *Microcystis* dominance. Vanderploeg et al. (2001; 2014) demonstrated the complexity of mussel response to microcystin and other traits possessed by different strains by combining feeding

experiments with video observations. Mussels fed on the highly toxic (microcystin-LR) LE-3 strain (microcystin/chlorophyll a content = 0.66 µg·µg Chl a–1) at a greatly reduced feeding rate and also exhibited low overall feeding rate when offered together with preferred food. In contrast, clearance rate was normal for a non-toxic (CCAP 1450/11) and toxic strain (PCC 7820), the latter having a lower microcystin content of 0.22 µg·µg Chl a^{-1}. Strains isolated from Michigan lakes (Wilson *et al.*, 2005) that maintained their colonial integrity showed factors other than microcystin content were important (Vanderploeg *et al.*, 2013). The strain from Gilkey Lake (strain GilkeyL02), having a low microcystin content of 0.1 µg·µg Chl a^{-1} caused complete shutdown (valve closure); and when paired with a desirable food, they showed extreme sensitivity to an irritating substance in the colonies by rejecting each colony as it entered the siphon (Vanderploeg *et al.*, 2013). No ingestion occurred for non-toxic colonies of the strain from Hudson Lake (strain HudsonBD), which has heavy mucilage content. In this case the colonies were enthusiastically captured but rejected as pseudofeces.

While invasive dreissenid mussels can enhance *Microcystis*-dominated harmful cyanobacterial blooms by selective removal of non-cyanobacterial phytoplankton, declines of *Microcystis* following dreissenid mussel invasions have been reported as well and are sometimes hypothesized to be related to ambient nutrient concentration (Bastviken *et al.*, 1998; Smith *et al.*, 1998; Vanderploeg *et al.*, 2001, 2013; White *et al.*, 2011; Waajen *et al.*, 2016). While invasive dreissenid mussels can enhance *Microcystis* blooms by selective removal of non-cyanobacterial phytoplankton, declines of *Microcystis* abundance following dreissenid mussel invasions have been reported (Bastviken *et al.*, 1998; Smith *et al.*, 1998; Vanderploeg *et al.*, 2001, 2013; White *et al.*, 2011; Waajen *et al.*, 2016). These conflicting observations may be due to a trade-off between *Microcystis* adaptations to rapid growth at high nutrient levels and grazing resistance at low nutrient levels (Sarnelle *et al.*, 2005, 2012), similar to resource-defense trade-offs in plants (Züst and Agrawal, 2017). Nutrient levels, in particular nitrogen levels and N:P ratios also influence secondary metabolite production by *Microcystis* (Gobler *et al.*, 2016), further influencing the presence and expression of defense traits in *Microcystis*. Thus, distinct traits of *Microcystis* strains may help explain

why grazers have variable effects on ecosystems, depending on nutrient levels. However, this remains to be confirmed in large part because the genetic traits and gene-environment interactions that determine feeding resistance remain unclear. An additional challenge for the future will be to identify the *Microcystis* strains and traits that deter or allow grazing in a mixture with other phytoplankton species found in nature. An understanding of the roles of selective feeding mechanisms and behavior, as well as post-ingestion consequences of the different strains on grazer fitness, is clearly needed.

Conclusions and Outlook

Trait variation between species is an important predictor for the distribution and abundance of organisms (Tilman *et al.*, 1982; Leibold *et al.*, 1997; Chase and Leibold, 2003) as well as interactions between them (Leibold, 1989; Tessier and Woodruff, 2002). While diversity within species has been overlooked historically, it is now clear that intraspecific variation, both in terms of phenotypic plasticity (Lima and Dill, 1990; Agrawal, 2001; Werner and Peacor, 2003) and genetic diversity, can shape species interactions and even community composition and ecosystem processes (Agrawal and Van Zandt, 2003; Whitham *et al.*, 2006; Hughes *et al.*, 2008; Lemaire *et al.*, 2012; Van Rossum *et al.*, 2020). The extensive genetic and phenotypic diversity that has been uncovered within *Microcystis* emphasizes the need to move beyond considering it as a homogeneous entity when we try to understand, model, and predict bloom dynamics and their ecosystem impacts, particularly toxicity and trophic transfer. Trait-based theory and models developed for phytoplankton species (Litchman *et al.*, 2007; Follows and Dutkiewicz, 2011; Glibert, 2016) provide a framework to understand and model this intra-*Microcystis* diversity. This requires (i) identifying key traits that define niches within *Microcystis*; (ii) defining clades of *Microcystis* strains that share ecologically-relevant traits and thus serve as appropriate functional groups for experimental studies and modeling; (iii) measuring and quantifying these traits so that they can be parameterized within models that can be used to test and improve understanding; and (iv) developing these insights into an understanding of rules governing links and tradeoffs among traits.

The information synthesized in this review shows that these requirements have not yet been met for *Microcystis*. Functional groups and key traits have not been defined. Just a scattered subset of traits and strains have been measured (**Fig. S2, Table S5**). We have only hints of the links and tradeoffs between traits. A major shortcoming is that only a small portion of the diversity of *Microcystis* has been cultured (**Fig. 1**) and an even smaller portion of that has been characterized systematically. However, our review identifies key gaps and paths forward. While determining the traits of all strains is unrealistic, determination of these parameters for selected strains chosen in a phylogenetic context will produce a new view of how traits are distributed across taxa within the genus, allowing definition of functional groups and selection of cluster-based reference strains for further experiments (Ramos *et al.*, 2017). Coordination of this phenotypic characterization with genomic, transcriptomic, and proteomic analyses will lead to an understanding of how traits are genetically encoded, potentially enabling inference of the traits of uncharacterized and even uncultured *Microcystis* based on genetic information gleaned from field samples. This integrated phenotypic-genotypic perspective will also reveal links and trade-offs between traits, as in plant ecology (Díaz *et al.*, 2004).

Despite the current gaps and the grand challenge of filling them, we have preliminary views of the landscape of *Microcystis* diversity. Cohesive genotypic clusters are relatively conserved across methods and studies ((**Fig. 2**; Pérez-Carrascal *et al.*, 2019). While these clusters are likely to change with new data, they are a starting point to test the hypothesis that genotypic groups represent functional groups with shared traits and niches. Previous studies also suggest traits that define functional groups and point towards potential links and tradeoffs between traits (**Fig. 6**). For example, re-wiring of gene expression and physiology following spontaneous *mcy* mutants and gene knockouts provide clues to trade-offs (Hesse and Kohl, 2001; Schatz *et al.*, 2005; Van De Waal *et al.*, 2011; Zilliges *et al.*, 2011; Sandrini *et al.*, 2014; Makower *et al.*, 2015).

Understanding links and tradeoffs between traits (Fig. 6) is a promising route towards the ability to model bloom toxicity. Identifying whether and how these traits are correlated, and their ecophysiological importance, will be key for models that incorporate different biological, chemical, and physical factors to predict biomass and toxicity of *Microcystis* in water. As described in this review, the composition of toxigenic and non-toxigenic strains within blooms may be due to resource competition for light, inorganic carbon, bioavailable nitrogen and phosphorus, interactions with grazers, buoyancy and vertical migration, or a combination of these factors. However, it remains unresolved whether the presence of the microcystin synthetase genes is a driving factor behind observed patterns, or rather other traits that vary across strains. Further research, including competition experiments and examination of physiological traits beyond production of microcystins, are necessary to understand the links between genotype, phenotype, and competitive outcomes. Likewise, integration of genetic data with information on habitat will link *Microcystis* genotypes with environmental conditions from which strains are derived.

Patterns of diversity within *Microcystis* stand in stark contrast to other cyanobacteria such as *Prochlorococcus*, providing valuable opportunities for comparison. However, functional groups within *Microcystis* are likely underpinned by a subset of trait dimensions that define broader phytoplankton functional groups, *i.e.*, the "phytoplankton mandala" (e.g., Glibert, 2016). Piecing together this more holistic view will inform controls on bloom toxicity but also move us beyond the dichotomy of toxigenic vs. non-toxigenic strains defined by a single trait, ushering in a new era in the understanding and management of *Microcystis*-dominated cyanobacterial harmful algal blooms.

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Figure Captions

Figure 1: Comparison of diversity of uncultured *Microcystis* strains with diversity of cultured strains with genome sequences. Outer circle: phylogenetic tree of 16S-23S internal transcribed spacer (ITSc region) from *Microcystis* strains. Included are 3,874 ITSc amplicon sequences from uncultured strains in the field and 128 ITSc sequences that could be identified from 159 cultured *Microcystis* isolates for which whole genome data was available. Cultured isolates are highlighted by filled circles, colored based on phylogenetic groups of Pérez-Carrascal et al. (2019), modified as described in Fig. 2. Insert: distribution of average nucleotide identities (ANI) between pairs of the 159 available Microcystis genomes. See **Supporting Information** for methods.

Figure 2: Phylogenomic analysis of *Microcystis* genomes. Colored bars indicate genotypic groups as defined by Perez-Carrascal et al. 2019, modified with additional sub-groups (Mfl_ae3-5) to designate sub-clades recognized here. **A.** Dendrogram based on gene presence/absence. **B.** Phylogenetic tree based on alignment of concatenated sequences from 161 single copy core genes shared by all genomes. **C.** Presence/absence (black/white) of key genes involved in biosynthesis of secondary metabolites, carbon concentrating mechanisms (CCM), detoxification of reactive oxygen species (ROS), and acquisition of nitrogen and phosphorus. Only genes with variable presence are shown here; see **Supporting Information** for methods, complete figure (**Fig. S1**), and information on strains used (**Table S1**).

Figure 3. Summary of biomass nitrogen and phosphorus content in Microcystis strains. Panels A and B depict nutrient content irrespective of growth conditions and panels C and D depict the relationship between N and P content and instantaneous growth rate.

Figure 4. Growth rate of *Microcystis* strains as function of temperature. Color of points and line indicates the light intensity at which experiments were conducted. Strain names are positioned at the temperature of maximum growth for each strain (indicated with star), with ability to produce microcystins indicated by +/- where this information is available. See **Table S2** for details of strains and data. "NA" indicates that no strain name was given.

Figure 5. Growth rate of *Microcystis* strains as function of light intensity. Color of points and lines indicates the temperature at which experiments were conducted. Light intensity at which maximum growth was measured for each strain is indicated with a star. See **Table S3** for details of strains and data.

Figure 6. Hypothesized links and tradeoffs between traits within *Microcystis* strains. Tradeoffs include: (1) tradeoff between defense traits (especially grazing resistance) and nutrient resources due to nutrient availability determining colony size and/or capability for production of secondary metabolites; (2) defenses against reactive oxygen species have a metabolic cost (e.g., synthesis of enzymes); (3) tradeoff between growth and productivity on the one hand, and maintenance and efficiency of resource acquisition on the other, which emerges from underlying biochemical traits of transporters, etc.; (4) plasticity of nutrient quota drops with growth rate (Hillebrand et al. 2013). Links between traits include: (5) growth rate is linked to rate of nutrient uptake; (6) light adaptation is linked to nutrient availability (through pigment content) and to defense from reactive oxygen species; (7) ROS defense is linked to nutrient availability. [a]These traits may be different for each form of nutrient; and the form of nutrient used may in itself be a differentiating trait. [b]Refers to the suite of secondary metabolites present in each strain. [c]Refers to acquired resistance to particular phage, e.g. via CRISPR or cell-surface proteins. These traits and

tradeoffs can be organized in the Competitor-Ruderal-Stress tolerator framework (Krause et al. 2014), which is not shown here.