An evolutionary ecological mechanism of CyanoHABs: unavoidable consequences when low nutrient requirements meet eutrophication

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Abstract

The mechanism of cyanobacterial harmful algal blooms (CyanoHABs) is complicated and confusing. One major reason is they are studied primarily from an ecological perspective and on bloom-forming species only. This narrow angle loses a broader evolutionary and ecological context in which CyanoHABs occur and fails to provide information on relevant components to achieve a wholistic understanding.

To derive a comprehensive mechanism of CyanoHABs, we examine CyanoHABs through the overlooked evolutionary and ecological lenses: evolutionary radiation, ecological comparison with co-living algae, and recently identified genomic functional repertoire between blooming and non-blooming species. We found four key factors: (1) elaborate diverse functional repertoire and low nutrient requirement in cyanobacteria molded by early adaptive evolution, (2) cyanobacteria having lower nutrient requirements than co-living algae, (3) there is no directed evolution in biological functions toward water eutrophication in cyanobacteria, (4) the CyanoHAB-associated functional repertoire are more abundant and complete in blooming than non-blooming species. These factors lead us to postulate a preliminary mechanism of CyanoHABs as a synergistic quad: superior functional repertoire, established with long adaptive radiation under nutrient-deficient conditions and not evolved toward eutrophic conditions, enables cyanobacteria to efficiently utilize elevated nutrients under current eutrophic levels for excess growth and CyanoHABs thereof, due to their lower nutrient requirements than co-living algae. This working comprehensive mechanism without doubt needs further empirical testing, which can be undertaken with more comparative studies of multiple species using integrated systems biology approaches.

Key words:

Cyanobacterial blooms, evolutionary radiation, green algae, genomic function, mechanism, natural selection, nutrient requirement, water eutrophication

I. Introduction

CyanoHABs are one of the most profound environmental hazards in modern human history in terms of their global geographical scale (Paerl et al., 2020), longstanding duration (over a century) (Francis, 1878; Lathrop & Carpenter, 1992), and tremendous economic loss (Steffensen, 2008). The mere fact that they are still intensifying and expanding under the global climate change (Chapra et al., 2017; Gobler, 2020; Paerl et al., 2020) attests CyanoHABs are still not fully understood such that their ecology remains 'complicated and confusing' (Wilhelm et al., 2020).

Are CyanoHABs an evolutionary consequence or ecological outcome in the era of water eutrophication? Nearly all research on CyanoHABs until now has focused on ecological factors, including nutrients, temperature, and climate change (Adrian et al., 2009; Beaulieu et al., 2013b; O'Neil et al., 2012). A major recent advance is the identification of genomic functional repertoire enabling the elevated nutrients for cyanobacterial growth and bloom formation (Cao et al., 2020a; Du et al., 2020). Now it becomes clear that CyanoHABs are immediate outcomes of synergistic interactions between superior cyanobacterial functions and eutrophic conditions. Is this all there is to it? In any eutrophic water, why it is primarily cyanobacteria, not other algae, that form blooms? Apparently, there ought to be more factors involved in causing this differential dominance in phytoplankton communities. One fact is the current eutrophic levels are sufficient to support CyanoHABs but not blooms of other algae.

To address this question of differential dominance, one can take a step back and use a wholistic approach by considering the fundamental principles of biology. For example, many seemingly complex genomic questions are often reconciled in the light of evolution—"Nothing in biology makes sense except in the light of evolution" (Dobzhansky, 1973)—as shown previously (Avise & Ayala, 2007; Lynch, 2007; Meyer, 2008). Cyanobacteria, the most ancient autotrophs (Boden et al., 2021) with the broadest geographical distribution on modern Earth (Schopf, 2002), have come to this success through various trajectories of adaptive evolution. By examining these

trajectories of geological timescales in the context of each niche, one can deduce the key biological functions for their success. More importantly, comparing the early adaptive radiation and present-day ecological dominance of cyanobacteria in eutrophic conditions enables identification of different factors contributing to either type of success, respectively.

Following this line of reasoning, we first reexamined the biological abilities of early cyanobacteria to survive and thrive in habitats which are physically harsh and nutritiously deficient during evolution on geological timescales. Next, we revisited the water eutrophication processes to establish elevated nutrients as the necessary condition for CyanoHABs and highlighted the overlooked fact that cyanobacteria have lower nutrient requirements than green algae, which have never bloomed in the current regime of eutrophication. Third, we summarized the biological functions driving bloom formation in eutrophic waters and showed the genes encoding these functions are not under positive (directed) selection, but purifying selection instead. This finding suggests their biological capabilities are well established before the era of water eutrophication. All these considerations above led us to postulate an evolutionary ecological mechanism of CyanoHABs. This new synthesis requires expanding biological dimensions in future CyanoHAB research.

II. Cyanobacteria: evolutionary champions

Originating in an anoxic biosphere of early Earth, cyanobacteria are likely to evolve in scarcity of macronutrients such as phosphorus and nitrogen (Bjerrum & Canfield, 2002; Falkowski, 1997; Kipp & Stüeken, 2017; Reinhard et al., 2017) and trace metals (Anbar & Knoll, 2002; Saito et al., 2003). This humble beginning of life leads to primitive metabolic networks (Goldford & Segrè, 2018), which suggests that cyanobacteria could only obtain minimal energy and matter in both variety and amount to support cell survival and division (Schopf, 2002). Consequently, their nutrient requirements for life are likely to be very low. Besides nutrient deficiency, early cyanobacteria also faced various types of stresses which are ‘unforeseeable’ for them. One type is the rising oxygen level they caused themselves through oxygenic photosynthesis, for which they

62 developed various counterattack strategies observed in modern cyanobacteria (Moore
63 et al., 2017; Raymond & Segrè, 2006). Ultraviolet light is another early stress which has
64 persisted until now, and some cyanobacteria evolved against it by producing
65 sunscreens (Garcia-Pichel et al., 2019). These global stresses were also intertwined
66 with landscape changes through geophysical processes (e.g., volcanism and climatic
67 shifts). Here, we demonstrate the remarkable adaptive ability of cyanobacteria by
68 showcasing their evolution of biological capability and morphology under the rising
69 oxygen levels.
70
71 Since origin, particularly after the Great Oxidation Event (GOE) (Figure 1), ancestral
72 cyanobacteria quickly acquired versatile antioxidant capabilities against reactive oxygen
73 species (ROSs), derived from O_2 produced as a result of oxygenic photosynthesis.
74 Different classes of superoxide dismutases (SODs) and other types of antioxidant
75 enzymes are evolved to deal with ROSs throughout their entire history (Boden et al.,
76 2021). Based on the availability of metal cofactors (another case of adaptation), three
77 types of SODs—NiSOD, CuZnSOD, and MnSOD/FeSOD—have been found in four
78 types of aquatic environments (Figure 1A). Besides direct enzymatic removal of ROSs,
79 cyanobacteria have also evolved other strategies, e.g., in photosynthetic reaction center
80 (RC) which split into two types as a response to the rising oxygen (Orf et al., 2018).
81 Another adaptation lies in the redundancy (heavy investment) of key functions. For
82 example, in photosystem I, cyanobacteria have two electron transporters that evolved
83 separately: copper-dependent plastocyanin (PC) and iron-dependent cytochrome c_6
84 ($Cytc_6$). Distinct in the primary sequence and tertiary structure of protein/amino acid,
85 their expression is controlled by a BlaI/CopY-family transcription factor (PetR) and a
86 BlaR-membrane protease (PetP), depending on the availability of the metal cofactors
87 (García-Cañas et al., 2021).
88
89 With a continuous rise in oxygen level, morphological diversification was called upon to
90 provide an extra dimension for functional expansion. It has been confirmed that the
91 evolution of multicellular morphotypes and the rate of morphological diversification
92 coincide with the GOE onset (Schirrmeister et al., 2013). Heterocyst became a

specialized cell form for nitrogen fixation, in which the oxygen level is reduced to pre-GOE levels (Figure 1B). Nitrogen fixation is viewed as a leap forward in promoting marine primary production and contributed to increased O_2 levels, coinciding with the rise of animals (Lyons et al., 2014). Similarly, cyanobacteria also obtained a specific photosynthetic structure for carbon fixation, carboxysome, in which CO_2 is increased from 10-15μM to 40mM outside and greatly improved the efficiency of enzymatic fixation of carbon dioxide (Mangan & Brenner, 2014). After the evolution of geological timescales (Figure 1C and 1D), modern cyanobacteria are among the most diverse prokaryotic phyla, with morphotypes ranging from unicellular to multicellular filamentous forms, including those able to terminally (i.e., irreversibly) differentiate in form and function (Hammerschmidt et al., 2020; Schirrmeister et al., 2013).

Billions of years of adaptive evolution have allowed structural elaboration and functional diversification in coping with nutrient deficiency, environmental extremes and constraints (Paerl, 2014). This evolutionary divergence has enabled cyanobacteria to occupy all geographic habitats in modern Earth, including terrestrial and aquatic ecosystems, ranging from deserts to tropical rain forests, soils and limestones, and from open oceans and brackish waters to freshwaters and hydrothermal vents (Figure 2). This broad array of biological functions underlying the adaptive radiation in cyanobacteria is recorded in their genomes (Chen et al., 2021). A large proportion of the genes in their genomes are associated with adaption to the specific habitats they occupy, but not to any other habitats. Therefore, the genomes produce a pangenome with most genes being unique and only a small set of 323 genes being common among cyanobacteria (Shi & Falkowski, 2008). These core genes are mainly involved in housekeeping functions, as opposed to interacting with environment, e.g., ribosomal proteins, photosynthetic apparatus, ATP synthesis, chlorophyll biosynthesis, and the Calvin cycle. This small stable core and large variable shell of genomes suggest most of the non-overlapping functions are specialized in adaptation to distinct habitats.

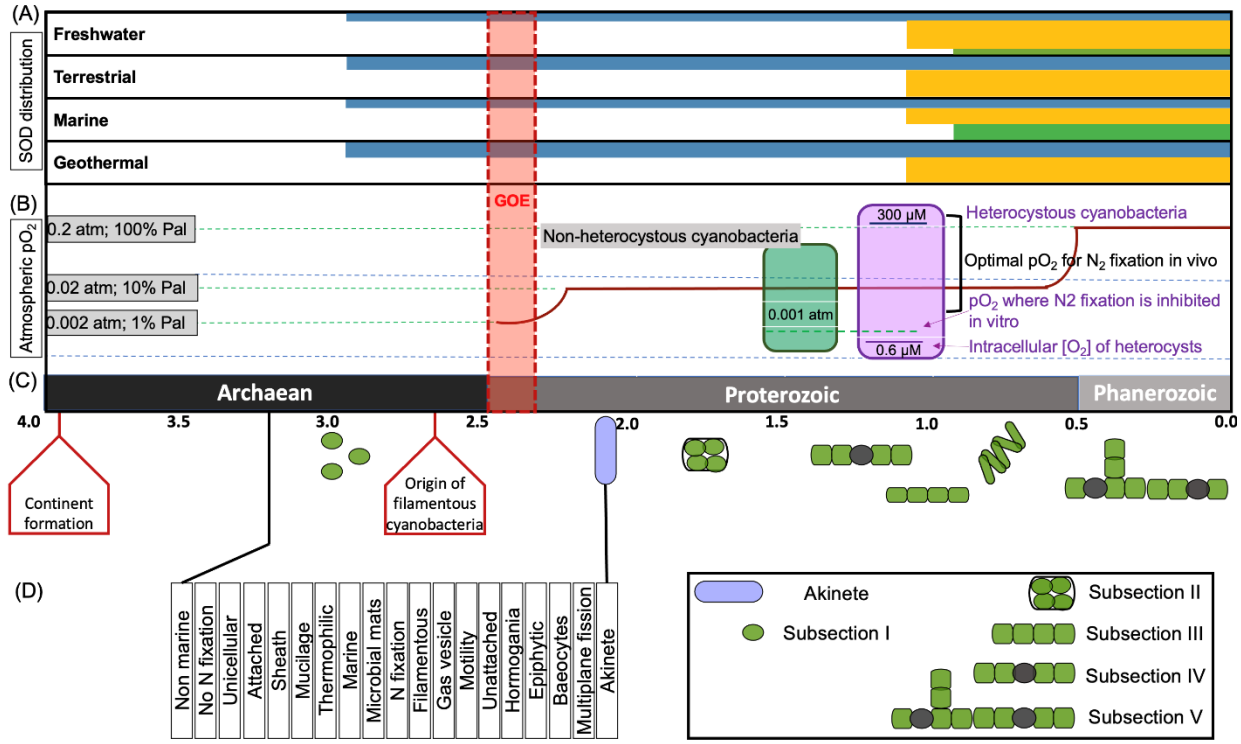

Figure 1. The evolutionary trajectory of cyanobacteria in the presence of oxygen. (A) The origin and distribution of SODs in different habitats. The timing of origin and habitat distribution of SOD genes among taxa is created based on Boden et al (Boden et al., 2021). Starting points of horizontal bars represent time of origin and color SOD types: CuZnSOD (blue), NiSOD (green), and Fe- and Mn-utilizing SODs (yellow). (B) The oxygen level in atmosphere and nitrogen fixation in heterocysts in cyanobacteria in the presence of elevated oxygen levels. The level of oxygen is based on (Tomitani et al., 2006). (C) and (D) The temporal morphological diversification cyanobacteria is based on Schirrmeistera et al (Schirrmeister et al., 2013).

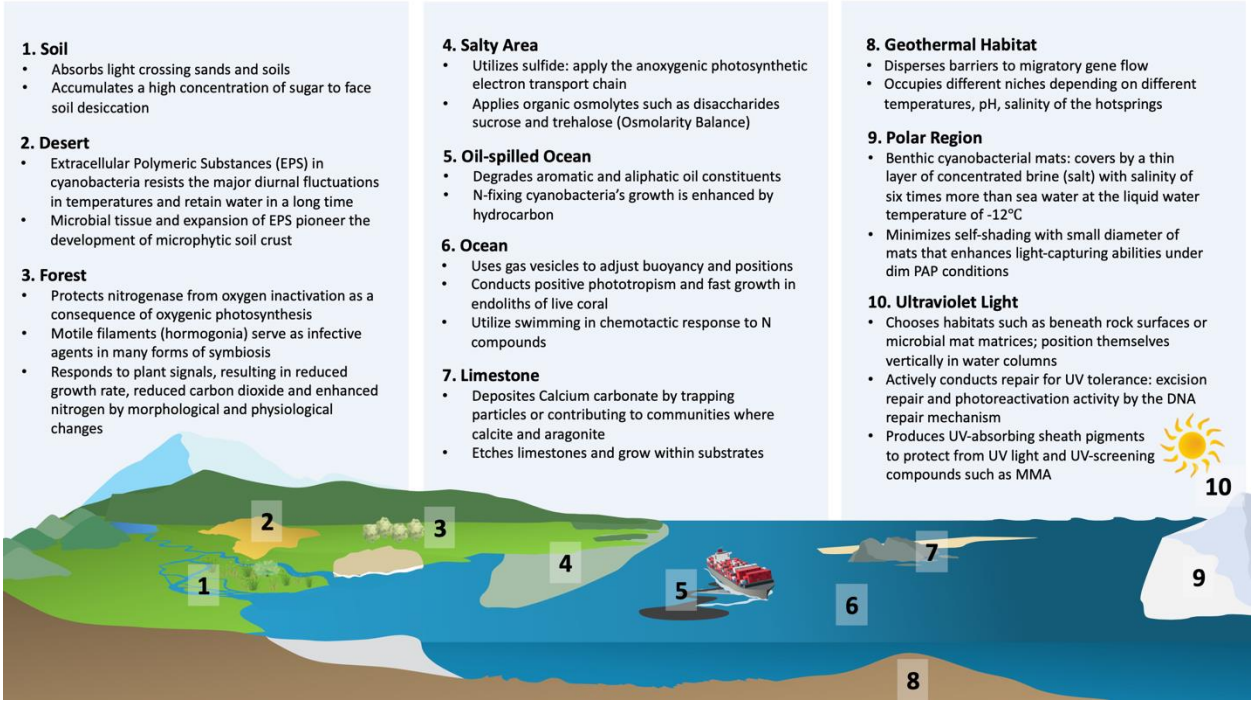

Figure 2. Cyanobacterial adaptation strategies in nine environments, namely soil, desert, forest, salty area, oil-spilled ocean, open ocean, limestone, geothermal water, and polar region. Cyanobacteria also cope with the damage caused by ultraviolet light.

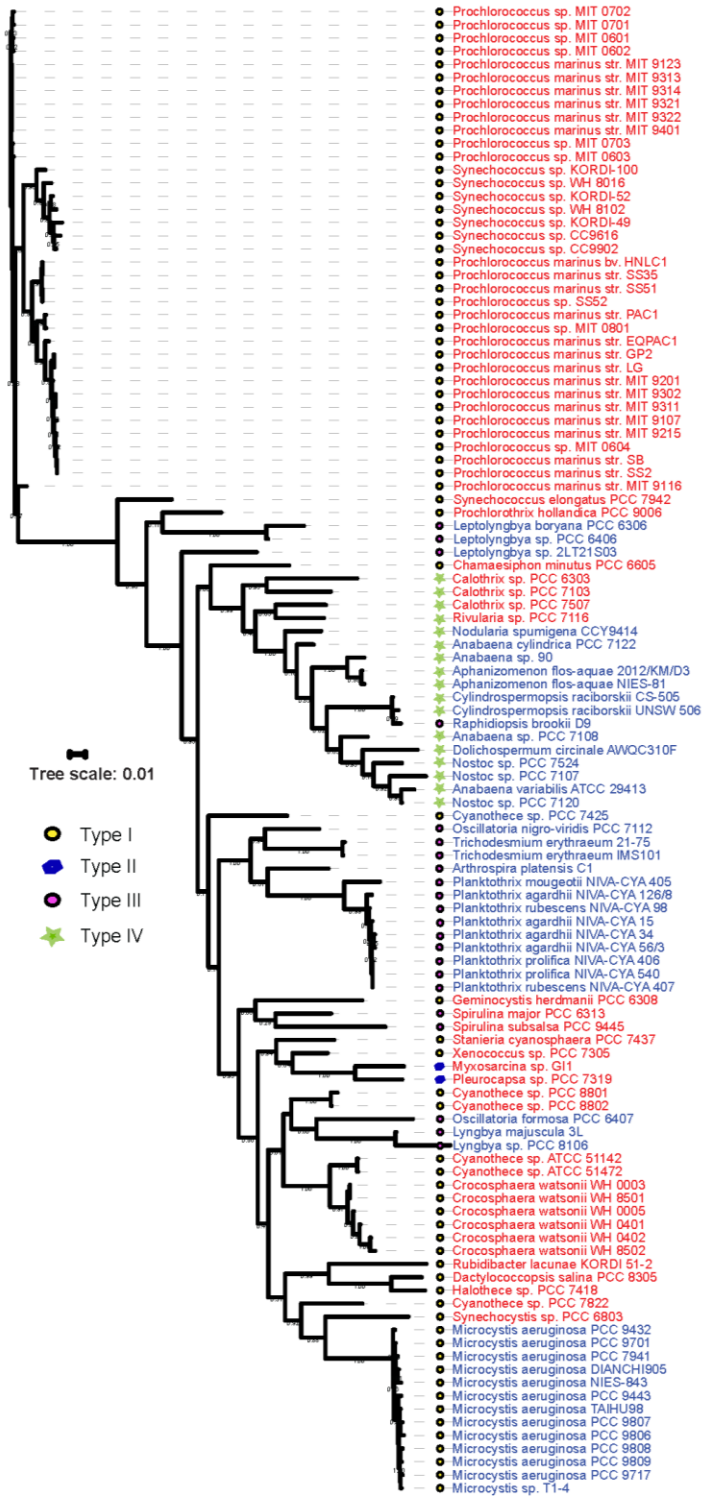

Figure 3. The phylogeny of cyanobacteria based on 16S ribosomal genes. Blue labels: bloom-forming cyanobacteria; and red labels: non-bloom-forming cyanobacteria. The shapes of different colors represent they types of morphology.

III. CyanoHABs: an unavoidable consequence when low nutrient-requiring cyanobacteria meet eutrophic waters

Anthropogenic eutrophication, elevated loading of nutrients into waters from human activities on a global scale, is an 'unforeseeable' surprise to cyanobacteria which have been evolving, but not forming blooms, in physically harsh and nutrient-deficient waters. Apparently, eutrophic waters present themselves as a new but favorable condition (a surprise), in which cyanobacteria dominate over co-living algae in phytoplankton communities and form CyanoHABs. To achieve a wholistic mechanism of CyanoHABs, three types of questions should be addressed: First, is there direct causality between eutrophication and CyanoHABs to establish eutrophication as the necessary condition for CyanoHABs? Second, why is it cyanobacteria, not other co-living algae (e.g., green algae), that form blooms? Do other algal species have higher nutrient requirements than cyanobacteria, which cannot be met by current eutrophic levels? This is another necessary condition for CyanoHABs. Last, what are the superior biological functions of blooming cyanobacteria, the sufficient condition of CyanoHABs, that take advantage of eutrophic conditions compared to the non-blooming cyanobacteria?

3.1 A first necessary factor: water eutrophication from oligotrophic state

Despite natural eutrophication, few CyanoHABs in natural waters were reported before the era of anthropogenic eutrophication. Water eutrophication is not only a result of nutrient loading but also a process during which favorable factors such as hydrology are brought about by anthropogenic landscape changes. The driving role of eutrophication in CyanoHABs is well established (Heisler et al., 2008). Its causality is manifested in two ways: fast bloom formation in response to nutrient elevation and bloom decline with de-eutrophication. Immediate bloom formation has been repeatedly observed in laboratory simulations (Lürling et al., 2018) and field studies (Molot et al., 2021; Wilkinson et al., 2018). Here we provide two examples to show that the transition from oligotrophic to eutrophic state is swift and CyanoHABs follow in a short period of time.

One example is the 'most studied lake in the world', Lake Mendota (Brock, 1985; Kitchell, 1992). Started as a clear oligotrophic lake prior to Euro-American colonization

in the 1830s in Madison, Wisconsin (Riera et al., 2001), Lake Mendota had CyanoHABs in 1882 and later 1890s (Lathrop & Carpenter, 1992) due to eutrophication from combined landscape changes of agricultural expansion in area and crops (corn in place of wheat and oats), wetlands drainage, lake-level rise and slow water flow, and soil erosion due to the damming lake outlet. Arguably the most convincing evidence for the cause of eutrophication for CyanoHABs is from Lake Erie, where CyanoHABs not only form following eutrophication but also decline with de-eutrophication, as summarized by Steffen et al (2014). Briefly, an increase in loading of phosphorus and other macronutrients to Lake Erie led to CyanoHABs (so-called autumnal maximum) dominated by filamentous cyanobacteria (*Anabaena* spp., *Aphanizomenon* spp., *Lyngbya* spp.), which quickly succeeded to *Microcystis* sp. With the phosphorus identified as the key nutrient and subsequent implementation of the Great Lakes Water Quality Agreement in 1972, the total phosphorus loading goal was reached in 1981 and continued to mid-1990s. Accordingly, a consistent decline in phytoplankton was observed. Unfortunately, a return of *Microcystis aeruginosa* blooms was observed in 1995 (Conroy et al., 2005) due to external loading of soluble reactive phosphorus (Joosse & Baker, 2011) and CyanoHABs have persisted until now.

Besides freshwater ecosystems, eutrophication-led CyanoHABs were also found in brackish waters, e.g., Baltic Sea (Bianchi et al., 2000; Gustafsson et al., 2012) and coastal waters and seas (Anderson et al., 2008; Malone & Newton, 2020; Rahav & Bar-Zeev, 2017; Sabeur et al., 2016). The fact that CyanoHABs form on the entire spectrum of salinity indicates that salinity is not an effective deterring factor, which is a confirmation that cyanobacteria have long adapted to each of these habitats under oligotrophic conditions (Figure 2).

3.2 A second necessary factor: low nutrient-requirements in cyanobacteria

As observed in the studies of causality between eutrophication and CyanoHABs, it is cyanobacteria, not green algae, that form blooms in response to nutrient addition. Considering the adaptive radiation of cyanobacteria, one can reason that cyanobacteria have lower requirements for nutrients than green algae. As nutrients increase in

originally oligotrophic waters, they meet the (lower) optimal needs of cyanobacteria first and further increases will satisfy the (higher) optimal demand of green algae to support their mass growth for bloom formation.

To test this hypothesis, four comparisons were performed. First, we compared the recipes of synthetic media for cyanobacteria and green algae and found those for green algae have much higher ionic concentrations than those for cyanobacteria (Figure 4). Second, we summarized the results which directly compared the growth of cyanobacteria and green algae in the field, laboratory, and cosmos by supplying external nutrients (Table 1). Five groups of studies support our hypothesis: low nutrients (TP, TN, DIN, urea-N, Ca, etc.) support dominance of cyanobacteria and high nutrients support dominance of green algae and even brown algae, as summarized in Table 1. Third, cyanobacteria have fewer genes, about 50% of those encoded in green algae. More importantly, based on the genome-scale metabolic networks (GSMs), the size of the GSMs of cyanobacteria is about half of those of green algae, having on average 780 metabolites and 820 reactions, which are about half of those in green algal GSMs (Table 2). Last, cyanobacteria tend to have a higher stress tolerance to N limitation than green algae. For example, *M. aeruginosa* can increase their photosynthetic performance which helps maintain their population size during seasonal N limitation, while photosynthetic performance is decreased in green alga *Porphyridium Cruentum* under the same N limitation (Jin *et al.*, 2020). All these evidence clearly shows that cyanobacteria indeed have low requirements than green algae and thus bloom first when nutrient levels increase.

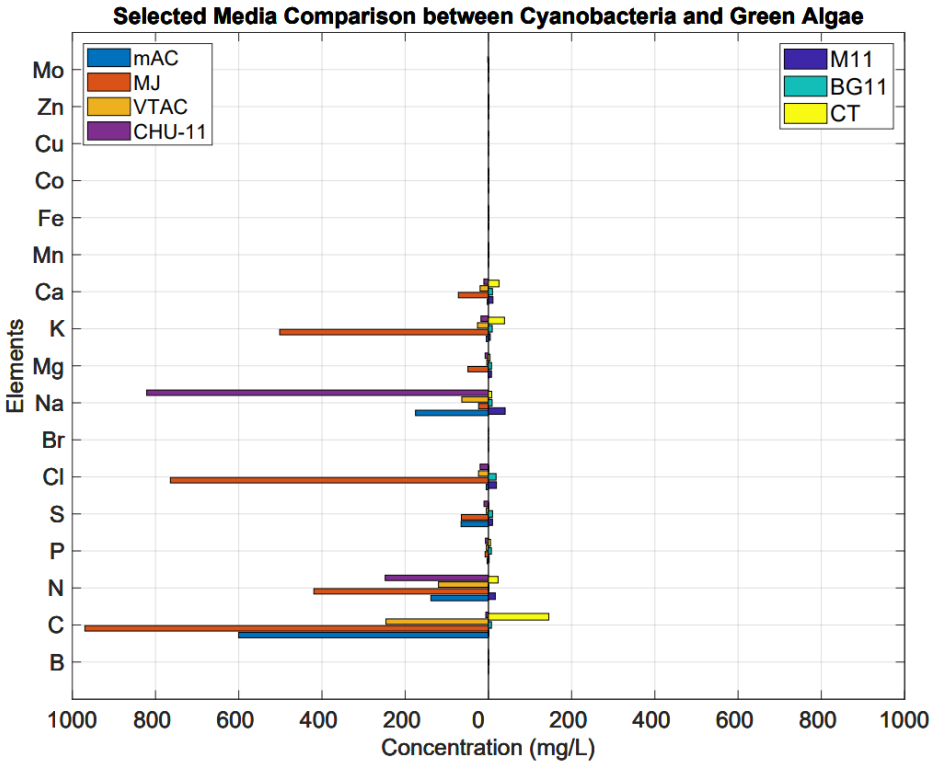

Figure 4. Lower nutrient requirements by cyanobacteria than green algae in synthetic culture media. Among seven selected media, the recipes of mAC, MJ, VTAC, and CHU-11 (left panel) are used for culturing green algae and the recipes of M11, BG11 and CT (right panel) are used for culturing cyanobacteria.

231 Table 1. A list of case studies which show cyanobacteria have lower nutrient requirements than green algae. B: cyanobacteria; G:
232 green algae. DIN: dissolved inorganic N; SRP: soluble reactive phosphorus; TN: total nitrogen; and TP: total phosphorus.

	Study Type	Study Site(s)	Indicator(s)	Concentration(s)	Dominance	Dominant Species	References
Group 1	Mesocosm	Wascana Lake, Canada	Urea-N	1-8 mg N L^{-1}	B	Colonial cyanobacteria	(Bogard *et al.*, 2020)
				>8 mg N L^{-1}	G	Chlorophytes	
	Laboratory	N/A	NH_4^+ and NO_3^-	32μM (NO_3) after 48h; 32μM NH_4^+ after 48h	B	*Synechococcus 6301*	(Hyenstrand *et al.*, 2000)
				32μM NO_3 after 25h; 32μM NH_4 after 18h	G	*Scenedesmus sp.*	
Group 2	*In situ*	210 Danish lakes	TP	0.25-0.8 mg P L^{-1}	B	Non-heterocystous cyanobacteria	(Jensen *et al.*, 1994)
				>1.0 mg P L^{-1}	G	Chlorophytes	
Group 3	*In situ*	Vela Lake, Central Portugal	DIN and SRP	0.66-2.42 mg L (DIN); 0-0.24 mg L (SRP)	B	*Aphanizomenon flos-aquae, Ch roococcus limneticus, M. aeruginosa* and *Pseudanabaena sp.*	(Figueiredo *et al.*, 2006)
				0.7-3.3 mg L (DIN); 0.19-0.93 mg L (SRP)	G	*Coelastrum reticulatum* var. *reticulatum, Kirchneriella lunaris, Monoraphidium contortum, Scenedesmus acuminatus* var. *acuminatus* and *Pediastrum boryanum* var. *boryanum*	
	In situ	Murchison Bay, Lake Victoria	TN and TP	1127 ug L (TN); 91 ug L(TP)	B	*Anabaena sp., M. wesenbergii, Gomphosphaeria aponina and Microcystis aeruginosa, Planktolyngbya circumcreta*	(Haande *et al.*, 2011)
	Laboratory and *in situ*	Lake Tai, China	N and P	3 mg/L (TN) and 0.2 mg/L (TP)	B	*M. aeruginosa*	(Zhu, Wan, & Zhao, 2010)
				10, 15, or 20 mg/L (TN), and 1 mg/L (TP)	G	*Scenedesmus quadricauda*	
Group 4	Mesocosm	Archipelago Sea	N:P Ratio	1N:1P 1.7 μg L^{-1}: 1.7 μg L^{-1}	B	*Aphanizomenon* sp., *Nodularia spumigena*, *Anabaena* spp., *Synechococcus* spp.	(Vuorio *et al.*, 2005)
				7N:1P 12 μg L^{-1}:1.7 μg L^{-1}	G	*Dictyosphaerium subsolitarium*, *Monoraphidium contortum*, and *Monoraphidium contortum, Oocystis* spp..	
	Mesocosm	Wascana Lake, Canada	N:P ratio	15:1 to >24:1	G	*Micractinium, Oocystis*	(Finlay *et al.*, 2010)
Group 5	*In situ* and laboratory	Taipei Feitsui Reservoir	Ca	7-9 mg L^{-1}	B	*Microcystis* spp. and *Aphanocapsa delicatissima*.	(Wu & Kow, 2010)
				9,11,13,15,18 mg L^{-1}	G	*Eutetramorus*, *Coelastrum*, *Coenocystis*, and *Dictyosphaerium*	

233

234 Table 2. Comparison of genome-scale metabolic networks of cyanobacteria and green
235 algae.

	Organism	Genes	Model Genes	Metabolites	Reactions	Reference
Cyanobacteria	Arthrospira platensis	6631	620	673	746	(Yoshikawa et al., 2015)
	Cyanothece sp. ATCC 51142	4912	806	651	667	(Vu et al., 2012)
	Spirulina platensis C1	6153	692	837	875	(Klanchui et al., 2012)
	Synechococcus elongatus	2723	785	768	850	(Broddrick et al., 2016)
	Synechococcus sp PCC 7002	3179	821	777	792	(Qian et al., 2017)
	Synechocystis sp. PCC6803	3221	678	795	863	(Nogales et al., 2012)
	Synechocystis sp. PCC6803	3221	669	790	882	(Montagud et al., 2010)
Green algae	Chlamydomonas reinhardtii	16709	866	1862	1725	(Gomes de Oliveira Dal'Molin et al., 2011)
	Chlamydomonas reinhardtii	16709	1080	1068	2190	(Chang et al., 2011)
	Chlorella variabilis	9879	526	1236	1455	(Juneja et al., 2016)
	Chlorella vulgaris	7100	843	1770	2286	(Zuñiga et al., 2016)

236

237 **3.3 A sufficient factor: superior functional repertoire without prior local adaptation**

238 Given their global presence and persistence for over a century (Francis, 1878; Lathrop
239 & Carpenter, 1992), one may wonder whether there is prior adaptive evolution in
240 cyanobacteria to form bloom in eutrophic waters, or CyanoHABs are simply ecological
241 consequences of synergistic interaction between pre-equipped (before eutrophication
242 era) superior biological functions and elevated nutrients. We think the latter is more
243 likely than the former. First, short-time whole-lake experiments in fertilization or nutrient

244 reduction (Molot et al., 2021; Schindler et al., 2016; Wilkinson et al., 2018) have
245 indisputably shown the causality of eutrophication for CyanoHABs on an ecological time
246 scale, allowing no time for evolution. Second, anthropogenic eutrophication is
247 unforeseeable to cyanobacteria, and therefore they could not proactively prepare
248 themselves through directed evolution.
249
250 Third, we confirmed there is no directed evolution toward CynaoHABs, by calculating
251 the metric, dN/dS (ratio of non-synonymous to synonymous mutations), for all
252 homologous genes in 20 blooming species of *M. aeruginosa* genomes from different
253 countries. Indeed, almost all homologous genes display dN/dS < 0.8, with a median of
254 0.32 (Figure 5A). When grouped into the functional pathways curated previously (Cao et
255 al., 2020b), the homologous genes associated with CyanoHABs displayed the levels of
256 purifying selection pressure as those in core metabolism and housekeeping processes
257 (e.g., translation and transcription regulation) (Figure 5B). This strong purifying selection
258 suggests these gene functions not only are important functions such that cells cannot
259 afford them to be comprised by mutations. The levels of function are sufficient in the
260 metabolic networks for blooming cyanobacteria to thrive in oligotrophic conditions and
261 dominate in eutrophic conditions.
262
263 Last, the timescales observed between eutrophication and first blooms permit no
264 evolution. We can calculate the positive mutation generated by mutations in one annual
265 cycle. The spontaneous mutation rate (μ) in cyanobacteria is estimated at 10^{-7} per
266 genome per generation (García-Villada et al., 2004; López-Rodas et al., 2007; Osburne
267 et al., 2011; Segawa et al., 2018). For a lake of a million m^3, with cyanobacterial 20
268 cells/L growing to 10^8 cells/L after 25 doublings (t) at a growth rate 0.05-1.1 d^{-1} in an
269 annual cycle bottlenecked in fall and winter, this gives an effective population size (N_e)
270 of 10^8 cells, based on the equation 15 in (Wilson Alan et al., 2006). So, the number of
271 beneficial mutations to be fixed can be approximated by, assuming a beneficial rate (s)
272 at 0.01 and a rate of fixation ($2s$) at 0.05, $N_e \mu t 2s$ ($10^8 \times 10^{-7} \times 25 \times 2 \times 0.05 = 0.12$).
273 Therefore, each year there is less than one beneficial mutant will occur, which makes
274 prior adaptive evolution highly unlikely.

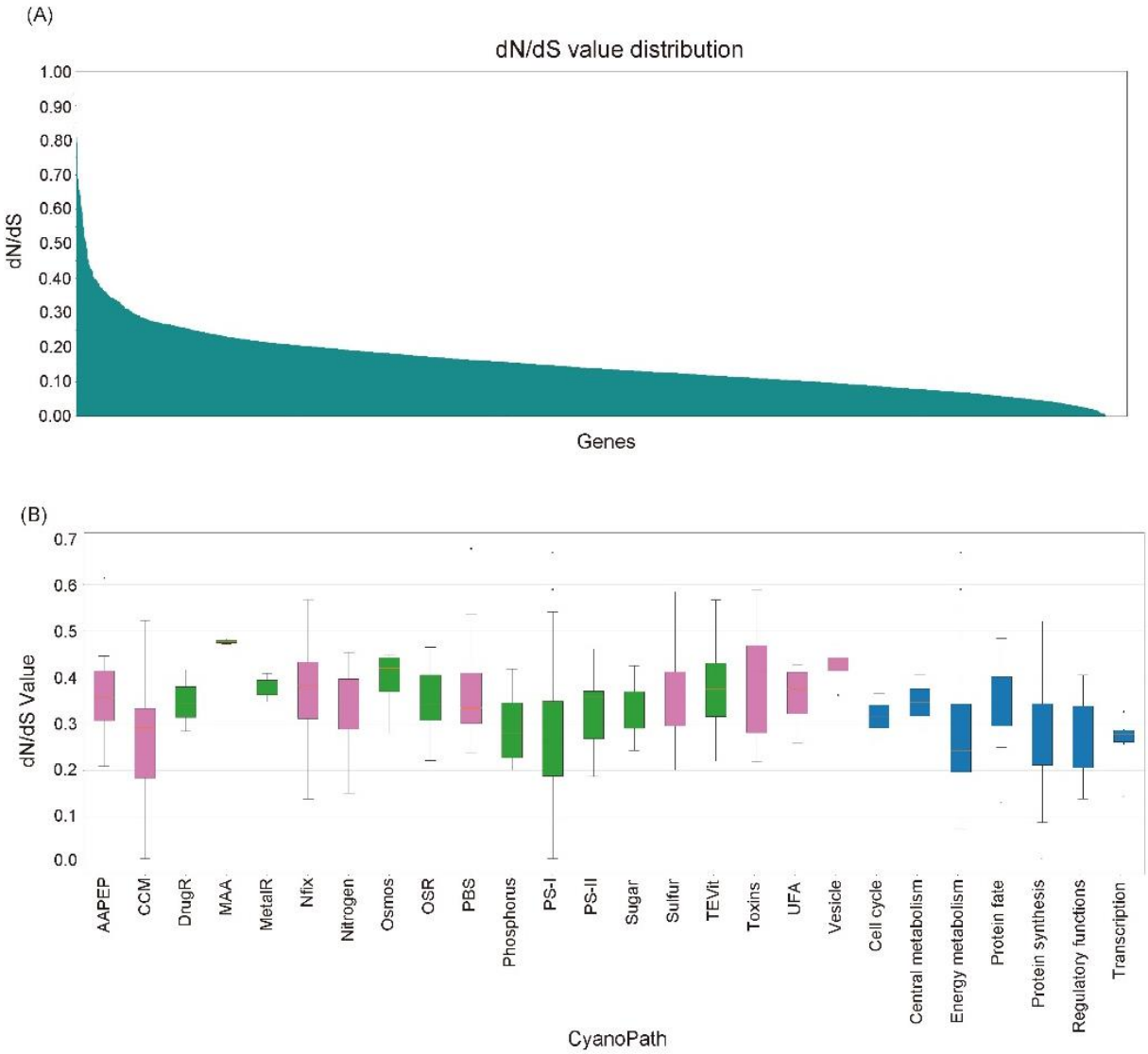

Figure 5. dN/dS for all homologous genes in 20 genomes of *M. aeruginosa*. (A) An overall distribution of dN/dS for all homologous genes; and (B) the dN/dS for genes in different groups of functions. dN/dS values were calculated by CODEML from the PAML 4.9j package with default settings (Yang, 2007). AAPEP: uptake of amino acids and peptides; CCM: CO_2-concentrating mechanism; DrugR: antibiotics resistance; MAA: UV radiation; MetalR: heavy metal resistance; Nfix: nitrogen fixation; Nitrogen: nitrogen utilization; Osmos: osmos homeostasis; OSR: oxidative stress resistance; PBS: phycobilisome; Phosphorus: inorganic/organic utilization; PS-I/PS-II: photosystem I and photosystem II; Sugar: sugar assimilation; TEVit: assimilation of trace metals and vitamins; Toxins: cyanotoxins; UFA: unsaturated fatty acids; Vesicle: gas vesicles. Purple bars are pathways strongly associated with CyanoHABs; green bars are pathways not specifically related to CyanoHABs; the last seven blue bars represent essential biological functions (Rubin *et al.*, 2015).

IV. Biological functions associated with CyanoHABs

The relevant biological functions assimilating and converting them into biomass have been recently identified by comparative genomics between blooming and non-blooming species (Cao et al., 2020b). They are also made available as a database, CyanoPATH (Du et al., 2020) (Figure 6). These pathways have one-on-one correspondence with nutrients or stressors, which also show varying degrees of hierarchical structure. Below we detail the major pathways associated with CyanoHABs.

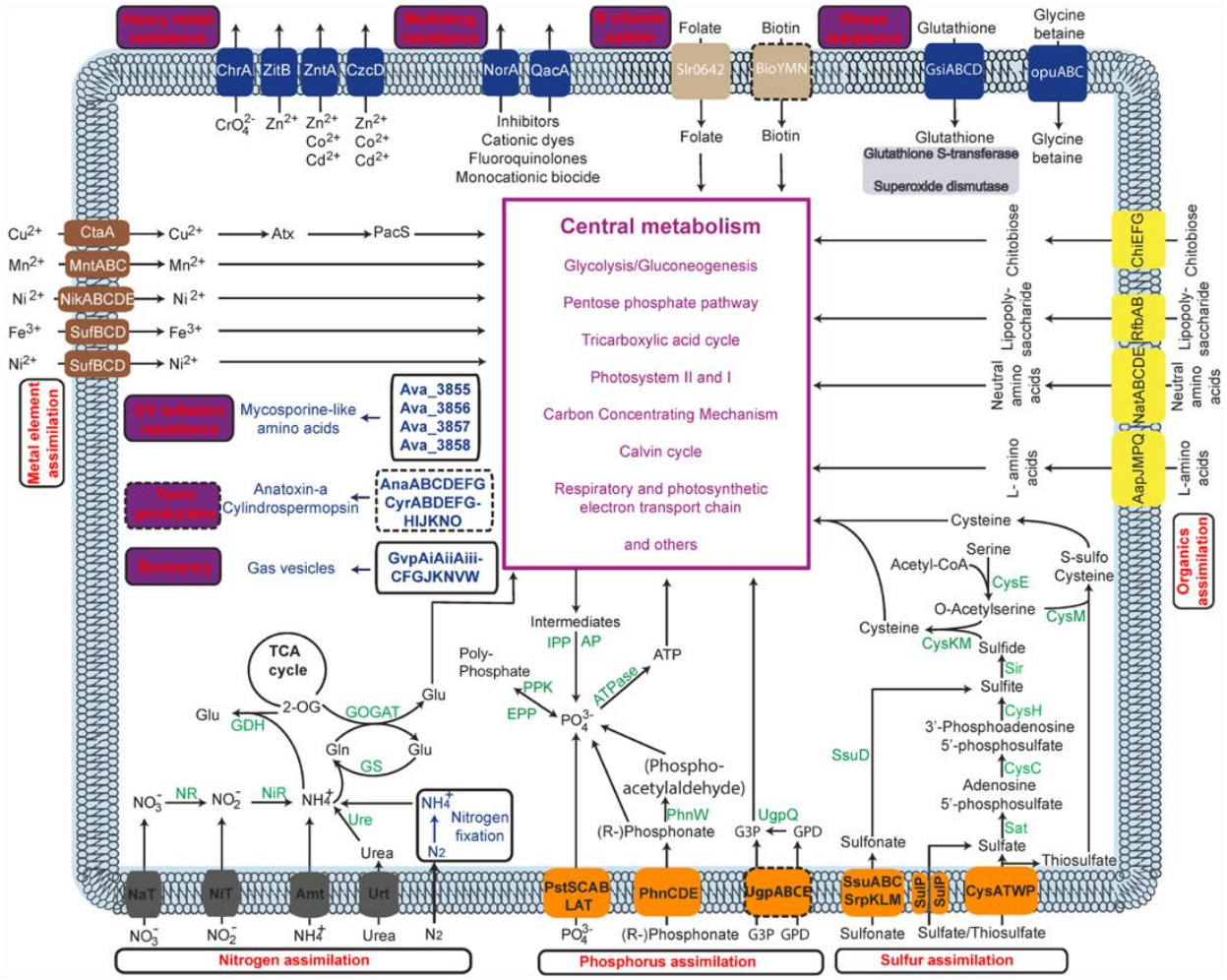

Figure 6. The core and query pathways in *Aphanizomenon flos-aquae* NIES-81. The pathways labeled with dashed borders are not complete (due to the absence of required components) in this strain but may be complete in others.

4.1 Gas vesicles

Gas vesicles are hollow protein structures made of gene products of *gvpA* and *gvpC* of the *gvp* gene cluster, consisting of 14 genes in several operons (Walsby, 1972, 1994). Gas vesicles are well-known for their roles in buoyancy regulation, allowing cyanobacteria to position themselves in the water column for optimal resource utilization (Carey et al., 2012; Walsby et al., 1997). This pathway is more enriched in blooming than non-blooming species and correlated with the blooming incidence of cyanobacteria recorded in the literature (Cao et al., 2020b): nearly all blooming species have at least one copy of the *gvpA* and *gvpC* genes, and the genes *gvpFGJKNVWX*, while all but one non-blooming species do not have any of these genes (Figure 7A). This pathway operates independently of other pathways, without a direct association with central metabolism.

4.2 Functions for macronutrient utilization

Phosphorus and nitrogen are recognized as the top two macronutrients for CyanoHABs by empirical studies (Heisler et al., 2008; Molot et al., 2021; Schindler et al., 2016) and statistical analyses (Beaulieu et al., 2013a). Indeed, the reconstructed pathways show that bloom-forming cyanobacteria invest heavily in these two functions, as they are limiting in the history of Earth (Kipp & Stüeken, 2017; Lyons et al., 2014; Reinhard et al., 2017). For phosphorus, both organic and inorganic phosphorus can be utilized. Inorganic phosphate can be internalized via ABC transporter *pstSCAB* or low facilitator affinity transporter *pitA*/*pitB* (Harris et al., 2001). Strikingly, nearly all cyanobacteria have one or two *pstSCAB* operons, and the globally dominant species *M. aeruginosa* has three. Organic phosphonate is imported via an ABC transporter PhnCDE and converted into phosphate by aminotransferase PhnW. The phosphate obtained has two destinations: incorporated into ATP as an energy molecule or converted to polyphosphate by polyphosphate kinase (PPK). Additionally, phosphate can be internally recycled, released from phosphate-containing compounds by inorganic pyrophosphatase and alkaline phosphatase. Interestingly, despite its verified driving role in CyanoHABs, the phosphate utilization pathway is not significantly more enriched (abundant or complex) in blooming than non-blooming species (Cao et al., 2020b). This

underscores two points. First, phosphorus has been a global limiting factor historically (Elser et al., 2007; Maberly et al., 2020; Schindler, 1977) such that all cyanobacteria have at least one and often two ABC transporters; phosphate appears to be only a necessary but not sufficient driver for CyanoHABs, this is probably why total nitrogen rather than total phosphorus is a better CyanoHAB predictor (Beaulieu et al., 2013a). Second, this suggests the biological machinery for CyanHABs is complicated and more likely to operate in a systems manner (Kirschner, 2005).

Total nitrogen and water temperature are recognized as the best predictors of cyanobacterial biomass (Beaulieu et al., 2013a). Indeed, as the nitrogen utilization pathway reconstructed with transporters and enzymes shows, cyanobacteria can use four forms of nitrogen: nitrogen gas, inorganic nitrate/nitrite, ammonium, and organic urea (Figure 7C). Key transporters for nitrate/nitrite (*nrtABCD*), ammonium (amt), urea (*urtABCDE*) and key nitrate/nitrite reductive enzymes *narB*, *nirB*, and urease (*ureABCDE*) are more enriched in blooming than non-blooming cyanobacteria (Figure 7D). Interestingly, the genes involved in incorporating nitrogen into amino acids (e.g., gdhA or gltB/gltD) are not significantly more enriched in blooming species; this suggests that superior nitrogen utilizing ability mainly lies in the uptake from the extracellular environment, other than intracellular incorporation. Other metabolic pathways for macronutrients include those for sulfur (Figure 6), which is also enriched in blooming than non-blooming species (Cao et al., 2020b).

4.3 Utilization of organic compounds

Organic compounds were not initially considered, even though cyanobacteria were known to utilize organic nutrients under light (Baalen et al., 1971; Rippka, 1972; Stanier, 1973). As CyanoHAB research progresses, organics gained some attention but were limited to organic nitrogen and phosphorus in a few species (Berman & Chava, 1999; Chaffin & Bridgeman, 2014). The transporters internalizing organic compounds, such as amino acids and sugars, have been identified systematically (Figure 6) and ABC amino acid transporters are found more enriched in blooming than non-blooming species (Cao et al., 2020b). Two transporters, AaPJMPQ for L-amino acids and NatABCDE for neutral

363 amino acids, are found to be encoded in nearly all blooming species. Strikingly, the top
364 blooming species, *M. aeruginosa* have additional transporters, HisJMPQ (for histidine),
365 ArtIJMQP (arginine), DppABCDF (d,d-dipeptide), GlnHPQ (Glutamine), GltIKJL
366 (Glutamate/aspartate), LivFGHKM (leucine, isoleucine, and valine), GluABCD
367 (glutamate), and AotJMPQ (arginine/ornithine). Experimentally, this has been verified:
368 glutamic acid, aspartic acid, leucine, alanine and arginine can be assimilated by *M.*
369 *aeruginosa* at varying rates (Dai et al., 2009). Besides, some sugars can also be
370 internalized by blooming cyanobacteria, e.g., lipopolysaccharides internalized by
371 transporter RfbAB and chitobiose by ChiEFG.
372
373 **4.4 CO_2-Concentrating Mechanism (CCM)**
374 CCM concentrates CO_2 in carboxysomes to high levels (~ 40μM) for effective
375 incorporation into ribulose bisphosphate in the Calvin cycle. CCM is more enriched in
376 blooming than non-blooming species (Cao et al., 2020b) *et al.*, 2020). The CCM genes
377 encode four types of functions: *ccmKLMNO* (structural proteins forming the
378 carboxysome), *icfA* (carbonic anhydrase), *bicA* and *sbtA* (bicarbonate facilitator),
379 *cdnF$_3$D$_3$chY*, *cdnF$_4$D$_4$chX*, and *cmpABCD* (ABC transporter for HCO_3^-), and *rbcLS*
380 (ribulose bisphosphate carboxylase/oxygenase). All but one (*bicA*) have more gene
381 copies in blooming than non-blooming species (Figure 7E). This enrichment is
382 understandable because heavy drawdown of CO_2 can easily lead to carbon limitation
383 during a bloom, particularly when a diffusion limitation effect is associated with high
384 population density of cyanobacteria in the water. Meanwhile, elevated CO_2 level has
385 been found to intensify CyanoHABs (Verspagen et al., 2014) and CCM transporters and
386 regulators respond quickly (Ma et al., 2019; Sandrini et al., 2016).

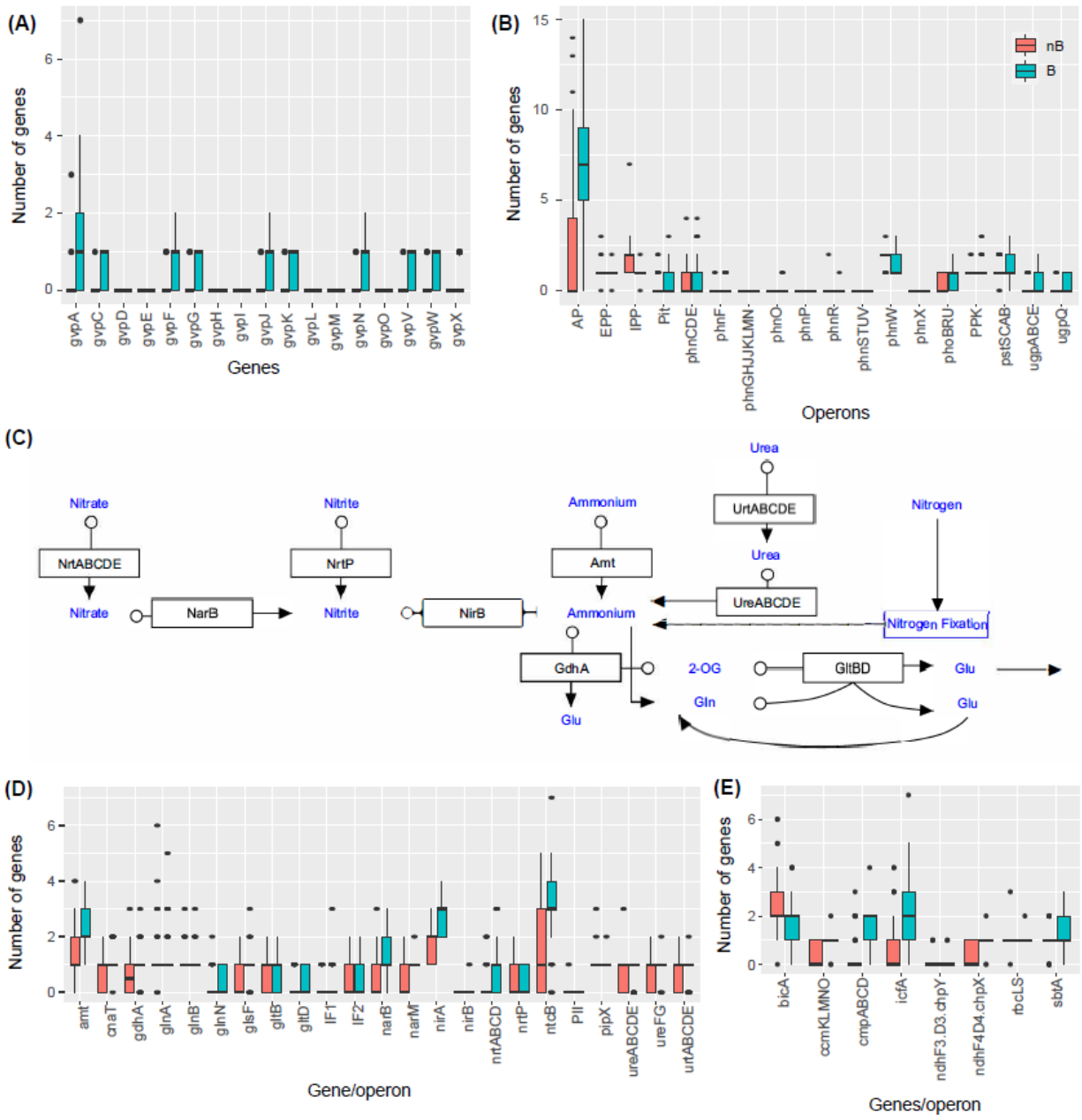

Figure 7. Some important CyanoPATHs associated with CyanoHABs in blooming vs. non-blooming cyanobacteria. (A): gas vesicle genes; (B): genes for phosphorus utilization; (C): nitrogen utilization pathway; (D): nitrogen utilization genes; and (E): CCM genes.

4.5 Disputed roles of cyanotoxins

Cyanotoxins are conventionally viewed as deterrents for anti-predation/anti-grazing of cyanobacteria (Zanchett & Oliveira-Filho, 2013). Recent research suggests their

396 functions are not limited to deterrents: their toxicity may as well be a coincidence, and
397 its real roles are rather different. First, a new study suggests a direct role in antioxidant
398 response leading to reduced ROSs outside and inside of *M. aeruginosa* cells (Malanga
399 et al., 2019). Second, a protein-binding role has been identified for microcystin-LR (MC-
400 LR), which binds several enzymes of the Calvin cycle, phycobiliproteins and two
401 NADPH-dependent reductases. The binding, strongly enhanced under high irradiance
402 and oxidative stress, is a protection and increases cell growth (Zilliges et al., 2011).
403 Third, they act as a signaling molecule in maintaining algal colonies and the dominance
404 of *Microcystis* spp. (Gan et al., 2012). Last, some cyanotoxins, e.g., microcystins, has a
405 deep evolutionary origin, which predates that of predators/grazers and eliminates the
406 possibility of directed evolution for cyanotoxins toward intoxicating animals (Rantala et
407 al., 2004).
408
409 **4.6 Other functions**
410 There are two additional types of functions associated with CyanoHABs: assimilation of
411 trace elements and stress resistance.
412
413 ***Trace elements and vitamins***. This pathway mainly consists of various classes of
414 transporters for their uptake. The utilization pathways are for magnesium, ferric/ferrous
415 ions, copper, nickel, manganese, and cobalt (Ahern et al., 2008; Kosakowska et al.,
416 2007). Among them, iron (ferric and ferrous) has the most types of transporters for
417 siderophore-based high-affinity iron-uptake *fecBCDE*, *feoABC* (for ferrous iron),
418 *fhuBCD*, *futABC*, *sfuABC*, and *sufBCD*. Despite these driving roles reported for some
419 trace elements (Berman-Frank et al., 2001; Facey et al., 2019; Gu et al., 2020; Molot et
420 al., 2014), there are no significant changes in gene copy number between blooming and
421 non-blooming species (Cao et al., 2020b). For vitamins, the transporters are for
422 riboflavin, nicotinamide riboside, biotin (vitamin B-7), and cobalamin (vitamin B12). Most
423 bloom-forming species are vitamin B1 and B12 auxotrophs and their transporters have
424 been identified BioYMN (biotin), YbaA/PnuX/ImpX (for riboflavin), PnuC (for
425 nicotinamide riboside), and BtuFCD (cobalamin).
426

427 ***Stress resistance***. Some stressors in eutrophic waters include heavy metals,
428 antibiotics, and dyes (Devarajan et al., 2015; Guo et al., 2020; Li et al., 2020). Despite
429 the expected inhibitive effects of heavy metals on cyanobacterial growth (Richardson et
430 al., 2018), some antibiotics are found to be promoting in two recent studies (Li et al.,
431 2021; Xu et al., 2021). Here, three types of stress resistance are summarized in
432 CyanoPATH: heavy metal and antibiotics, and mycosporine-like amino acids (MAAs) to
433 shield ultraviolet light (Fig. 6). For heavy metals and antibiotics resistance, the main
434 metabolic functions identified so far are either ATP-powered efflux ABC transporters or
435 simple facilitator outward pumps relying on cross-membrane gradients as drive. The
436 identified efflux transporters include ChrA (chromate efflux), CnrABC (cobalt/nickel),
437 CopABC (copper efflux ATPase), CueO (Multicopper oxidase), CusABCF (Cu^{+}/Ag^{+}
438 efflux), CzcABC and ZitB (Co^{2+}, Zn^{2+}, Cd^{2+}, and Ni^{2+} efflux), CzcD (Co^{2+} and Zn^{2+} efflux),
439 NccABC and NreB (Ni^{2+}), and ZntA (Zn^{2+} and Cd^{2+} P-type ATPase exporter) (Cao et al.,
440 2020b). The antibiotic resistance genes are mostly responsible for exporting toxic
441 compounds and antibiotics out of cells. These transporters are EmrBA-TolC (uncouplers
442 and antibiotics (nalidixic acid)), LmrP (cationic dyes, tetracycline, and
443 daunomycin/macrolides), MdfA (cationic dyes, chloramphenicol/fluoroquinolone), NorA
444 (fluoroquinolones/cationic dye/inhibitors), QacA/B (monocationic biocide and dyes),
445 EmrE (lipophilic cations), AcrB/D-A-TolC, MexBA-OprM, MexDC-OprJ, and MexFE-
446 OprN (multidrugs, e.g., tetracycline, chloramphenicol, fluoroquinolones, novobiocin,
447 erythromycin), and MexYX-OprM (aminoglycosides).
448
449 Another type of stress is universal, UV light, since early Earth (Garcia-Pichel et al.,
450 2019). Cyanobacteria have been equipped with the capabilities to produce sunscreen
451 compounds MAAs (Jain et al., 2017; Sinha et al., 2001). The operons encoding the
452 biosynthetic genes have been identified in *Anabaena variabilis* and other species
453 (Balskus & Walsh, 2010; Miyamoto et al., 2014). A gene cluster of four synthetases
454 catalyzes the formation of shinorine from sedoheptulose 7-phosphate in the pentose
455 phosphate pathway. The synthetases are nonribosomal peptide synthetase (ava-3855),
456 ATP-grasp family enzyme (ava-3856), O-methyltransferase (ava_3857), and a
457 dehydroquinate synthase (ava_3858). Interestingly, only some of the blooming species

458 have one cluster whereas non-blooming species do not contain any (Cao et al., 2020b).
459

460 V. Synthesis of an CyanoHAB mechanism and outlook

461 Through the lens of adaptive radiation, four factors of the ecology of CyanoHABs stand
462 out: (1) elaborate diverse functional repertoire and low nutrient requirement in
463 cyanobacteria molded by early adaptive evolution, (2) cyanobacteria having lower
464 nutrient requirements than green algae indeed, (3) there is no directed evolution in
465 biological functions toward water eutrophication in cyanobacteria, (4) the CyanoHAB-
466 associated functional repertoire are more abundant and complete in blooming than non-
467 blooming species. Based on all the discussions above, we propose a comprehensive
468 working mechanism of CyanoHABs as a synergistic quad: superior functional repertoire,
469 established with long adaptive radiation under nutrient-deficient conditions and not
470 having evolved toward eutrophic conditions, enables cyanobacteria to efficiently utilize
471 elevated nutrients under current eutrophic regime for excess growth and CyanoHABs
472 thereof, due to their lower nutrient requirements than co-living algae. Low nutrient
473 requirement in cyanobacteria and higher requirements in green algae are necessary
474 conditions, and superior functional repertoire in cyanobacteria and water eutrophication
475 are sufficient conditions. One prominent feature of the quad mechanism is the relative
476 nutrient requirement of different algae. This boils down to the chemical composition of
477 the cellular machinery and how the composition can be achieved under various growth
478 conditions. In this sense, this quad mechanism has profound implications for essentially
479 all fields of life sciences.
480
481 The significance of the synthesized mechanism lies in the fact it places CyanoHABs in
482 the continuum of cyanobacterial evolution in aquatic environment and in the context of
483 community. This replacement underscores the wholistic requirement in fully
484 understanding occurrence and control of CyanoHABs, and in turn calls for a shift in
485 study approach. Here we identify three types of approaches. First, comparative
486 research of multiple factors in nutrient requirements and their abilities to compete for
487 available resources in the same water. Second, integrated systems biology assessment
488 of the relative roles in promoting growth in cyanobacteria, which should be based on

genome-scale metabolic networks. Generally speaking, the evolutionary ecological perspective is applicable to various of life science areas, such as pathogens.

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Figure Legend

Figure 1

Figure 1. The evolutionary trajectory of cyanobacteria in the presence of oxygen. (A) The origin and distribution of SODs in different habitats. The timing of origin and habitat distribution of SOD genes among taxa is created based on Boden et al (Boden et al., 2021). Starting points of horizontal bars represent time of origin and color SOD types: CuZnSOD (blue), NiSOD (green), and Fe- and Mn-utilizing SODs (yellow). (B) The oxygen level in atmosphere and nitrogen fixation in heterocysts in cyanobacteria in the presence of elevated oxygen levels. The level of oxygen is based on (Tomitani et al., 2006). (C) and (D) The temporal morphological diversification cyanobacteria is based on Schirrmeistera et al (Schirrmeister et al., 2013).

Figure 2

Figure 2. Cyanobacterial adaptation strategies in nine environments, namely soil, desert, forest, salty area, oil-spilled ocean, open ocean, limestone, geothermal water, and polar region. Cyanobacteria also cope with the damage caused by ultraviolet light.

Figure 3

Figure 3. The phylogeny of cyanobacteria based on 16S ribosomal genes. Blue labels: bloom-forming cyanobacteria; and red labels: non-bloom-forming cyanobacteria. The shapes of different colors represent they types of morphology.

Figure 4

Figure 4. Figure 4. Lower nutrient requirements by cyanobacteria than green algae in aquatic environment. Among seven selected media, the recipes of mAC, MJ, VTAC, and CHU-11 (left panel) are used for culturing green algae and the recipes of M11, BG11 and CT (right panel) are used for culturing cyanobacteria.

883 Figure 5

884 Figure 5. dN/dS for all homologous genes in 20 genomes of *M. aeruginosa*. (A) An overall
885 distribution of dN/dS for all homologous genes; and (B) the dN/dS for genes in different groups
886 of functions. dN/dS values were calculated by CODEML from the PAML 4.9j package with
887 default settings (Yang, 2007). AAPEP: uptake of amino acids and peptides; CCM: CO_2-
888 concentrating mechanism; DrugR: antibiotics resistance; MAA: UV radiation; MetalR: heavy
889 metal resistance; Nfix: nitrogen fixation; Nitrogen: nitrogen utilization; Osmos: osmos
890 homeostasis; OSR: oxidative stress resistance; PBS: phycobilisome; Phosphorus:
891 inorganic/organic utilization; PS-I/PS-II: photosystem I and photosystem II; Sugar: sugar
892 assimilation; TEVit: assimilation of trace metals and vitamins; Toxins: cyanotoxins; UFA:
893 unsaturated fatty acids; Vesicle: gas vesicles. Purple bars are pathways strongly associated
894 with CyanoHABs; green bars are pathways not specifically related to CyanoHABs; the last
895 seven blue bars represent essential biological functions (Rubin *et al.*, 2015).
896

897 Figure 6

898 Figure 6. The core and query pathways in *Aphanizomenon flos-aquae* NIES-81. The pathways
899 labeled with dashed borders are not complete (due to the absence of required components) in
900 this strain but may be complete in others.
901

902 Figure 7

903 Figure 7. Some important CyanoPATHs associated with CyanoHABs in blooming vs. non-
904 blooming cyanobacteria. (A): gas vesicle genes; (B): genes for phosphorus utilization; (C):
905 nitrogen utilization pathway; (D): nitrogen utilization genes; and (E): CCM genes.