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The dual role of nitrogen supply in controlling the growth *and* toxicity of cyanobacterial blooms

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Abstract:

Historically, phosphorus (P) has been considered the primary limiting nutrient for phytoplankton assemblages in freshwater ecosystems. This review, supported by new findings from Lake Erie, highlights recent molecular, laboratory, and field evidence that the growth *and* toxicity of some non-diazotrophic blooms of cyanobacteria can be controlled by nitrogen (N). Cyanobacteria such as *Microcystis* possess physiological adaptations that allow them to dominate low-P surface waters, and in temperate lakes, cyanobacterial densities can controlled by N availability. Beyond total cyanobacterial biomass, N loading has been shown to selectively promote the abundance of *Microcystis* and *Planktothrix* strains capable of synthesizing microcystins over strains that do not possess this ability. Among strains of cyanobacteria capable of synthesizing the N-rich microcystins, cellular toxin quotas have been found to depend upon exogenous N supplies. Herein, multi-year observations from western Lake Erie are presented demonstrating that microcystin concentrations peak in parallel with inorganic N, but not orthophosphate, concentrations and are significantly lower ($p<0.01$) during years of reduced inorganic nitrogen loading and concentrations. Collectively, this information underscores the importance of N as well as P in controlling toxic cyanobacteria blooms. Furthermore, it supports the premise that management actions to reduce P in the absence of concurrent restrictions on N loading may not effectively control the growth and/or toxicity of non-diazotrophic toxic cyanobacteria such as the cosmopolitan, toxin-producing genus, *Microcystis*.

Introduction

Toxic cyanobacteria are of worldwide concern because persistent blooms threaten drinking water supplies, recreation, tourism, and fisheries (Chorus and Bartram, 1999; World Health Organization, 2011, and references therein). Such blooms are commonly promoted by excessive nutrient loading (Wetzel, 1983, 2001; Paerl 1988; O'Neil et al., 2012). Thus, there is significant interest in implementing improved management actions to control the nutrients responsible for promoting blooms. The paradigm that primary production in freshwater is controlled by phosphorus (P) (USNAS, 1969; Schindler, 1974) was established decades ago within oligotrophic lakes in Canada (e.g. Dillan and Rigler, 1974; Jones and Bachmann, 1976; Schindler, 1977). It is based largely on the premise that when inorganic nitrogen (N_i) levels are low, diazotrophic or N_2-fixing cyanobacteria balance ecosystem N deficiencies (Schindler, 2008, 2012; Scott and McCarthy, 2010). As the total P concentration in many freshwater bodies has increased and total N:P ratios have decreased, a shift has been reported in phytoplankton assemblages toward cyanobacteria dominance (Smith, 1980; Trimbee and Prepas, 1987; Watson et al., 1997).

Over the past several decades, many lakes have been driven increasingly out of stoichiometric balance due to disproportionate anthropogenic inputs of N and P, or management efforts targeting reduction of one nutrient (usually P in freshwaters) but not the other (Conley et al., 2009; Glibert et al., 2011; Burkholder and Glibert, 2013; and references therein). Concurrently, thought has evolved from consideration of only one limiting nutrient to recognition of the importance of ecological stoichiometry in directly and/or indirectly controlling phytoplankton assemblage structure and productivity

71 (Conley et al., 2009; Glibert et al., 2011; Burkholder and Glibert, 2013, and references
72 therein). Consequently, the literature is rich with examples of the importance of P
73 (Schindler, 1977; Wetzel, 2001; Sterner et al., 2008; and references therein), and N (e.g.
74 Gobler et al., 2007; Davis et al., 2010; Beversdorf et al. 2013, 2015) in controlling
75 cyanobacteria blooms as well as with examples of N and P co-limitation (Elser et al 1990,
76 2007; Lewis and Wurtsbaugh 2008; Xu et al., 2010; Chaffin et al., 2013; Chaffin &
77 Bridgeman, 2013; Davis et al., 2015).

78 N limitation in freshwater systems has been most commonly reported during
79 warmer months when planktonic cyanobacteria blooms are most common (Gobler et al.,
80 2007; Xu et al., 2010; Chaffin et al., 2013; Chaffin & Bridgeman, 2013; Davis et al.,
81 2015). Although N_2 fixation by cyanobacteria has been thought to minimize the role of N
82 in controlling blooms, various physiological and ecological lines of evidence have
83 indicated that the energetic demands of diazotrophy can restrict the extent to which N_2
84 fixation can offset N demands and limitation, particularly when concurrent rates of
85 denitrification are considered (Scott and McCarthy, 2010). Moreover, some of the most
86 common toxigenic genera of cyanobacteria, such as *Microcystis* and *Planktothrix*
87 (Chorus and Bartram, 1999; World Health Organization, 2011), are not diazotrophs but,
88 rather, depend on exogenous N supplies for growth and toxin synthesis (Berman and
89 Chava, 1999; Vézie et al., 2002; Davis et al., 2010; Monchamp et al., 2014, and
90 references therein). A strong relationship between the growth of non-diazotrophic
91 cyanobacteria and exogenous dissolved N supplies has commonly been reported. For
92 example, in laboratory studies increased N_i has promoted the growth and toxicity of
93 *Microcystis* (Watanabe and Oishi, 1985; Codd and Poon, 1988; Orr and Jones, 1998) and

94 enhanced input of N_i (inorganic N) to systems with elevated P has led to succession from
95 diazotrophs to non-diazotrophs (Bunting et al., 2007; Davis et al., 2010; Chaffin et al.,
96 2013; Harke et al., 2015).

97 This manuscript reviews recent information regarding the role of N and P in
98 supporting the growth and toxicity of cyanobacteria blooms, emphasizing non-
99 diazotrophs. Conditions that render ecosystems prone to cyanobacterial blooms are
100 considered, as well as recent molecular, laboratory, and field studies that support the
101 premise that the toxicity, and sometimes the biomass, of cyanobacterial blooms is
102 influenced by N_i availability. Finally, open questions and research priorities are identified
103 toward the goal of strengthening insights regarding nutrient controls on cyanobacterial
104 blooms and toxin production.

105
106 **Seasonal cycles in N loading, P loading, and cyanobacterial blooms**

107 Due to the seasonality of N and P inputs into freshwater ecosystems in temperate
108 late summer cyanobacteria blooms occur when N_i delivery from rivers sources is often at
109 an annual minimum (Turner et al 2003) and, thus, most likely to control the growth of
110 primary producers. Similar trends have been observed in smaller lakes more influenced
111 by groundwater flow than riverine input (Gobler et al., 2007). In Lake Erie, which has
112 sustained major cyanobacterial blooms during the past two decades (Brittian et al., 2000;
113 Conroy et al., 2005; Stumpf et al., 2012; Wynne and Stumpf, 2015), one of the dominant
114 nutrient sources, the Maumee River, has an annual TN:TP minimum during the summer
115 months when cyanobacteria blooms are most likely and summer N limitation has been
116 demonstrated (Stow et al. 2015, Chaffin et al 2013, 2014).

117 Species and sources of N present in lakes can differ in their seasonal dynamics,
118 which may also influence cyanobacterial blooms. Nitrate concentrations tend to be
119 highest in winter-spring and decline to low levels as summer progresses in many north
120 temperate lakes (Reynolds, 1984; Wetzel, 2001; Chaffin et al., 2011; Bridgeman and
121 Chaffin, 2013). These low N conditions can be alleviated by periodic summer storms
122 that deliver "new" N and/or by diazotrophic cyanobacteria that release ("leak") amino
123 acids and ammonia during N_2 fixation (Wetzel, 2001 and references therein) although
124 nitrogen fixation has been shown to not offset N ecosystem level demands (Scott and
125 McCarthy, 2010).
126 In contrast to NO_3^- dynamics, ammonia (NH_3) and ionized ammonia (NH_4^+) are
127 released from sediments and some benthic fauna during warmer months through
128 decomposition processes (Wetzel, 2001, and references therein; Zhang et al., 2008).
129 Substantial water-column NH_4^+ supplies from benthic sources in summer have also been
130 reported in areas with moderate to high densities of bivalve molluscs (Burkholder and
131 Shumway, 2011). For example, the western basin of Lake Erie has sustained major
132 invasions of dreissenid mussels capable of delivering large amounts of NH_4^+ to the water
133 column (Higgins et al., 2006, and references therein; Zhang et al., 2008). In lakes
134 showing symptoms of N limitation during late summer, cyanobacteria such as
135 *Microcystis* have been shown to become dominant by rapidly assimilating recycled
136 ammonium (e.g. Takamura et al., 1987; Ferber et al. 2004, Chaffin et al., 2011). Further,
137 *Microcystis* has been shown to have high affinity for NH_4^+ and, thus, is highly
138 competitive for recycled, reduced N (McCarthy et al., 2009; Glibert et al., 2015, and
139 references therein). Reduced N forms are rapidly recycled; increased loads of reduced N,

such as ammonia in partially treated or untreated sewage, and high NH_4^+/NO_3^- ratios, tend to promote cyanobacteria such as *Microcystis* over diatoms in phytoplankton assemblages (McCarthy et al., 2009; Glibert et al., 2015).

Lake sediments and porewaters are generally enriched in inorganic P (P_i) relative to the water column, although the extent to which sediments retain or export these nutrients varies seasonally. The PO_4^{-3} ion binds preferentially with ferric oxides in sediments under oxygenated conditions, but during summer months as temperatures warm and microbial degradation of sedimentary organic matter accelerates, sediment and near-sediment oxygen levels are progressively depleted and often become anoxic (Wetzel, 2001, and references therein; Hupfer and Lewandowski, 2008). Under such conditions, PO_4^{-3} dissociates from ferric oxides and is released to the overlying water (Carlton and Wetzel, 1984) making anoxic sediments a substantial source of P_i during warm months, particularly in systems where benthic fluxes of P_i reach surface waters. Phosphate release from organic matter directly depends on rates of microbial decomposition which are typically temperature-dependent and, thus, also maximal during summer (Wetzel, 2001; Reitzel et al., 2007; Hupfer and Lewandowski 2008). Although NH_4^+ is also released from organic matter decomposition in sediments during warm periods, the N:P ratio of sedimentary fluxes can be enriched in P relative to N, particularly in eutrophic lakes where cyanobacteria blooms are common and anoxic sediments can promote P release and denitrification (Fukishima et al., 1991; Downing and McCauley, 1992; Søndergaard et al., 2003). Hence, maximal benthic fluxes during summer can be a stronger source of P relative to N, contributing toward N limitation, particularly in shallow, well-mixed systems.

163

164 **Bloom-forming, toxic non-diazotrophic cyanobacteria in low P_i waters**

165 While P can limit cyanobacterial growth in freshwater systems, some common
166 non-diazotrophs such as *Microcystis* thrive in low P_i surface waters, conditions common
167 in many temperate lakes during late summer (Heron, 1961; Bertram, 1993; Wilhelm et
168 al., 2003; Harke et al., 2015). Such low water-column P_i conditions, of course, "mask"
169 the fact that TP in biomass typically is high in eutrophic lakes (Wetzel, 2001, and
170 references therein). Within an ecosystem setting, afore-mentioned benthic P sources may
171 be especially important to cyanobacteria such as *Microcystis* which can manipulate its
172 internal cell pressure via gas vesicles to migrate down through the water column at night,
173 then back near the surface at dawn (Reynolds, 1975), a process that facilitates both light
174 and nutrient access as well as herbivore avoidance (Wetzel, 2001, and references therein).
175 In shallow and/or stratified lakes, this vertical migration allows cyanobacteria such as
176 *Microcystis* to access benthic nutrient sources more than phytoplankton without such
177 migratory capabilities (Barbiero and Welch, 1992; Brunberg and Bostrom, 1992; Visser
178 et al., 1997; Verspagen et al., 2004; Xie, 2006; Cottingham et al., 2015, and references
179 therein) and partly accounts for the ability of *Microcystis* to form a bloom in the absence
180 or near-absence of surface water P_i (Heron, 1961; Bertram, 1993; Wilhelm et al., 2003;
181 Harke et al., 2015). In shallow, well-mixed lakes, by contrast, buoyancy regulation by
182 cyanobacteria may be less of a competitive advantage.

183 Beyond accessing sedimentary P, at a cellular level, culture studies of *Microcystis*
184 have demonstrated that this cyanobacterium has the ability to maintain rapid growth rates
185 under low or no P_i conditions. For example, dense batch culture of *Microcystis* have

been shown to grow equally well across a wide range of P_i concentrations, with comparable growth rates at starting media concentrations of 1.75 and 175 µM P_i (~56 and 560 µg L^{-1}; Saxton et al. 2012). Others have reported that cultured *Microcystis* clones continue exponential growth for at least 8 days in P-deplete media (Sbiyyaa et al., 2009). In experiments with *M. aeruginosa* clone LE-3 from Lake Erie, cultures transferred to media lacking P_i (–P_i) have displayed growth rates that exceeded those of cultures in P_i-replete media for more than two weeks before experiencing slowed growth (Fig 1).

The ability of cyanobacteria such as *Microcystis* to grow well under low Pi conditions is specifically facilitated by a series of key physiological traits that include a high-affinity PO_4^{-3} uptake system that is activated at low P_i concentrations (Harke et al., 2012), high cellular storage capacity for P (Whitton et al., 1991; Harke et al., 2012), and the production of extracellular polyphosphatase enzymes to access organic P or DOP (Healey, 1982; Harke et al., 2012). Although the reported substrate affinity (K_s) for P uptake by *Microcystis* of ~0.6 µM (19 µg PO_4^{-3}-P L^{-1}; Baldia et al., 2007) is comparable to that of other algae (e.g., Reynolds, 1984; Smayda, 1997), *Microcystis* responds to P limitation by a rapid increase in P_i uptake and increasing maximal uptake rates (V_{max}; Jacobson and Halmann, 1982; Kromkamp et al., 1989). Additionally, phosphoesters dominate DOP pools in aquatic environments (Kolowith et al., 2001) and the degradation of these compounds requires phosphatases ranging from acidic to alkaline (Wetzel, 2001, Dyhrman et al., 2006 and references therein). High rates of alkaline phosphatase activity have been reported for cultured *Microcystis aeruginosa* both on a population and a per-cell basis (Giraudet et al 1997, 1998; Strojsova et al., 2005; Harke et al., 2012).

208 Gene expression studies on cultured *Microcystis* have revealed the molecular
209 pathways that facilitate rapid growth under low P_i. In $-P_i$ media, *Microcystis* has been
210 shown to strongly upregulate (by 50- to 400-fold) two high-affinity, phosphate-binding
211 proteins (*pstS* and *sphX*) and an alkaline phosphatase (*phoX*), allowing it to maintain
212 rapid growth under extremely low or no P_i conditions (Harke et al., 2012). These genes
213 were highly conserved among ten cultures of *M. aeruginosa* isolated from different
214 geographic regions, and the expression of *phoX* was significantly correlated with alkaline
215 phosphatase activity (Harke et al., 2012). A broader, whole transcriptome study
216 demonstrated that when deprived of P_i, *Microcystis* differentially expressed nearly one-
217 fourth of its genome (Harke and Gobler, 2013). In addition to the *phoX*, *sphX*, and *pstS*
218 genes, transcript levels of many other genes within the Pst-P transport system (*pstSCAB*)
219 and *phoU* increased (Harke and Gobler, 2013). The degree of up-regulation of these P_i
220 scavenging genes varied, suggesting different affinities among transporters as observed
221 for other cyanobacteria (Pitt et al., 2010), which may extend the dynamic range over
222 which *Microcystis* can incorporate P_i. In addition, sulfate-binding and permease proteins
223 were up-regulated under low external P_i (Harke and Gobler, 2013), suggesting that
224 *Microcystis* may switch to sulfolipids in place of P-based membrane lipids to reduce
225 cellular P quotas as another adaptation to the low-P_i conditions (Van Mooy et al., 2006).
226 While these culture studies identified physiological mechanisms by which
227 *Microcystis* might form blooms under low P conditions, recent field studies of
228 *Microcystis* blooms in Lake Erie provide ecosystem-based evidence. Harke et al. (2015)
229 performed surveys and experiments from one of the largest tributary P sources in Lake
230 Erie, the Maumee River, out to the distal regions of the western basin, where P_i levels

231 declined from micromolar (μM) to nanomolar (nM) levels. Under high orthophosphate
232 conditions around the Maumee River mouth, *Anabaena* and *Planktothrix* were the
233 dominant cyanobacterial genera (Harke et al 2015). In contrast, in the more offshore
234 regions of the lake with low P_i concentrations, *Microcystis* became the dominate
235 cyanobacterium, as it upregulated genes associated with P scavenging (*pstSCAB*, *phoX*)
236 as well as P storage (*ppk1*; Harke et al 2015). Furthermore, experimental enrichment of
237 Lake Erie water with P_i increased the abundance of the total cyanobacterial population
238 but resulted in a decrease in the abundance of *Microcystis* (Harke et al 2015).
239 Collectively, these findings suggested that *Anabaena* is adapted to the high P regions of
240 western Lake Erie, whereas *Microcystis* dominates and persists under low-P conditions
241 (Harke et al 2015). Similar niche differentiation of diazotrophic and non-diazotrophic
242 cyanobacteria in high- and low-P environments have also been observed in the Baltic Sea
243 and Lake Taihu, China (Andersson et al. 2015; Paerl and Otten 2015).
244
245 **Evidence for N control of toxic cyanobacteria blooms**
246 Recently, N has been recognized as a key factor influencing cyanobacterial
247 blooms. Kosten et al. (2012) assessed 143 lakes along a latitudinal transect ranging from
248 subarctic Europe to southern South America, and found that temperature and TN
249 concentrations were the strongest explanatory variables for cyanobacterial biomass.
250 Similarly, Beaulieu et al. (2013) assessed cyanobacteria blooms in 1,147 lakes and
251 reservoirs of differing trophic status across the U.S. and found that the best multiple
252 linear regression model to predict these events was based on TN and water temperature.
253 This finding is also consistent with the strong positive association between N_i
254 concentrations and microcystin levels that has been reported across many U.S. lakes

255 (Yuan et al. 2014). In 102 north German lakes, Dolman et al. (2012) found that the
256 positive relationship between total cyanobacterial biovolume and P concentration
257 disappeared at high TP concentrations, but continued to increase with increasing TN
258 concentration. This may suggest that some cyanobacteria have higher N:P requirements
259 and, thus, are potentially N limited within highly P-enriched lakes. Conversely, research
260 in large experimental lake studies has shown that reduction of N_i inputs can result in a
261 decline in cyanobacterial abundance (Scott and McCarthy, 2011).

262 As other recent examples showing the importance of N in controlling
263 cyanobacteria assemblages, Davis et al. (2010) compared N versus P influence on dense
264 natural *Microcystis* blooms in a tidal (brackish) tributary and a eutrophic lake, and found
265 that in both systems during nutrient amendment experiments, all *Microcystis* populations
266 tested were stimulated by N more frequently than by P. Monchamp et al. (2014) assessed
267 three shallow, mesotrophic to hypereutrophic lakes in southwestern Quebec, Canada , and
268 found TN, NH_4^+, and DON significantly influenced the cyanobacterial assemblage
269 structure, and that the relative biomass of *Microcystis* spp. was significantly, positively
270 related to DON concentrations. Davis et al. (2015) found that in blooms dominated by
271 *Planktothrix agardhii/suspensa*, cyanobacterial growth and microcystin (MC)
272 concentrations increased as inorganic N concentrations increased, and that loading of N_i
273 combined with P_i most often lead to the highest MC concentrations.

274 Water-column N_i concentrations have also been shown to promote diazotroph-to-
275 non-diazotroph succession in cyanobacteria assemblages. For example, based on two
276 years of observations in highly eutrophic Lake Mendota, WI, USA, Beversdorf et al.
277 (2013) reported that cyanobacteria assemblage changes were strongly correlated with

dissolved N_i concentrations and that N_2-fixation by the diazotroph *Aphanizomenon* provided N supplies for toxic *Microcystis*. *Microcystis* populations increased in cell density several days after the first significant N_2-fixation rates were measured, and then *Microcystis* became dominant following a short period of low-DIN stress. In the year when N_2-fixation rates were much greater, the MC concentrations were also higher. Importantly, this system was sufficiently eutrophic to support blooms of diazotrophic or non-diazotrophic cyanobacteria depending on prevailing conditions. Within a nutrient-enriched setting, temporary low-N stress can cause an initial decrease in non-diazotrophs which can subsequently form toxic blooms when provided N from diazotrophs.

Exogenous N influences on cellular toxin composition and quotas

Beyond N influence on the *occurrence* of cyanobacteria blooms, there is evidence that the *toxicity* of blooms formed by non-diazotrophic cyanobacteria such as *Microcystis* is also highly influenced by N availability, beginning at the cellular level with toxin composition and cell quota. As noted by Glibert et al. (2015), various researchers have reported positive, direct relationships between N availability and toxin production in *Microcystis* and other toxigenic cyanobacteria (e.g., Lee et al., 2000; Vézie et al., 2002; Downing et al., 2005; Van der Waal et al., 2009; Harke and Gobler, 2013). The common cyanotoxins MCs, nodularins (NODs), cylindrospermopsins (CYNs), and saxitoxins (STXs) all either contain amino acids or require amino acid precursor(s) (Fig. 2) (Sivonen and Jones, 1999; Kellmann et al., 2008). Synthesis of amino acids, in turn, depends on N availability (Flores and Herrero, 2005; Tapia et al., 1996; Van de Waal et al., 2010). Thus, N can play a central role in determining the quantity of toxins produced by cyanobacteria.

High levels of N_i are needed to synthesize the N-rich MCs, and high levels of exogenous N_i have been shown to promote higher cellular quotas of MCs in the non-diazotrophs *Microcystis* and *Planktothrix* (Lee et al., 2000; Vézie et al., 2002; Downing et al., 2005; Harke and Gobler, 2013; Horst et al., 2014; Van der Waal et al., 2010, 2014). One of the first studies to suggest a positive relationship between MC production in *Microcystis* and available external N_i supplies was by Long et al (2001), who found a positive correlation between N-dependent growth rates and the cellular MC quota, as well as MC production rates. Later research showed that cellular MC quota depends on cellular N availability and decreases when N_i is limiting (Downing et al., 2005; Van de Waal et al., 2009, 2014; Harke and Gobler, 2013; Horst et al., 2014). At the molecular level, the microcystin synthetase gene cassette (*mcy* genes) appears to be responsive to N supply. For instance, N-deprived cultures of *Microcystis* downregulated genes involved in peptide synthesis (*mcy*ABCDE) and a decrease in cellular quota of MC under N-deplete conditions (Harke and Gobler, 2013).

In the field, addition of NH_4^+ compared with NO_3^- has led to an increase in MC concentrations and bloom maintenance for a longer duration (Donald et al., 2011). During a survey of Hirosawa-no-ike Pond, Kyoto, Japan, the strongest correlations between MCs and nutrients were found at high concentrations of NO_3^- and NH_4^+ (Ha et al., 2009). Glibert et al. (2011) noted a common phenomenon among freshwater and brackish systems that had been subjected to P reductions but not N reductions in management efforts: In systems receiving substantial NH_4^+ inputs, once the "sediment pump" of stored P began to increase P supplies to the overlying water, an interplay of P sequestration and NH_4^+ tolerance influenced shifts to new dominant taxa such as

325 *Microcystis*. Under P limitation, N-rich toxins would be expected to be favored as a
326 mechanism whereby N could accumulate in excess (Granéli and Flynn, 2006; Van der
327 Waal et al., 2014, and references therein).

328 Recent research conducted by Beversdorf et al. (2015) is germane in this regard,
329 and indicates that N supply and speciation can control MC synthesis: In Lake Mendota
330 (WI, USA), the toxic phase of the annual cyanobacterial blooms occurred during a
331 transition of high NO_3^- but declining NH_4^+ concentrations, coinciding with upregulation
332 of the MC synthetase gene operon, and leading to high MC levels in the ecosystem. In
333 addition, concentrations of MCs peaked at the same time as the TN/TP ratios, suggesting
334 the importance of an elevated N supply in supporting MC production. These findings are
335 consistent with prior laboratory studies, wherein MC production was tightly coupled to
336 N-dependent growth rates (Long et al. 2001; Harke and Gobler, 2013) and field studies
337 showing that N enrichment enhanced MC levels and the expression of peptide synthesis
338 genes involved in MC production in *Microcystis* (*mcyBEG*; Harke et al., 2015).

339 Compared to the plethora of laboratory and field studies showing the strong link
340 between MC synthesis and elevated nitrogen levels, two studies have reported an
341 *increase* in cell quota of MCs, and/or MC synthetase gene expression under N limitation
342 of *Microcystis* (Ginn et al., 2010; Pimentel and Giani, 2014). Such an apparently
343 counterintuitive increase of MC synthesis may be linked to the putative role of MCs in
344 protection against increased oxidative stress (Pimentel and Giani, 2014; Zilliges et al.,
345 2011; Meissner et al., 2013, 2015). Thus, cellular MC quota depends on the relative
346 availability of external bioavailable N, as N is needed for MC synthesis, but this

dependency may in turn be affected by the function of MCs which determines when the compounds are required.

There are more than 90 known MC congeners (Schmidt et al. 2014) that differ in two variable amino acids (Welker and von Döhren, 2006). There is high variation in the structure and C:N composition of MCs (Van der Waal et al., 2009, and references therein) and MCs may be important in redox control within cyanobacterial cells (Neilan et al., 2013; Glibert et al., 2015). It has also been suggested that toxin production may be associated with pathways of energy balance or cellular stoichiometric rebalancing (e.g., Glibert and Burkholder, 2011; Van der Waal et al., 2014, and references therein; Glibert et al., 2015). Two common MC variants are MC-LR and MC-RR which consist of a leucine (i.e. L) and arginine (i.e. R), or of two arginine molecules (RR) on the two variable positions. These variants differ in toxicity, with respective LD_{50} values (i.p. on mice) of 33-73 and 310-630 µg kg^{-1} (Sivonen and Jones, 1999; Chen et al., 2006) and N:C ratios (0.20 and 0.27, respectively). N availability can alter the synthesis of specific MCs, with a shift from MC-LR to the more N-rich MC-RR at higher N availability (Van de Waal et al., 2009, 2010). The extent to which such shifts may occur in an ecosystem setting have yet to be evaluated.

Cyanobacteria NODs, CYNs, and STXs are generally produced by N_2-fixing cyanobacteria (Chorus and Bartram, 1999; Sivonen and Jones, 1999). Early work on CYN production by *Cylindrospermopsis raciborskii* revealed that N species differentially influenced CYN production (Saker and Neilan, 2001). Subsequent studies that have investigated the molecular response of CYN synthetase genes to N, however, suggested that CYN synthesis does not depend on N availability or species (e.g. Shalev-Malul et al.,

370 2008). Other laboratory (Davis et al., 2014) and field (Burford et al., 2014) experiments
371 clarified that CYN is constitutively produced; therefore, changes in CYN concentrations
372 likely are due to changes in ratios of CYN-producing and non-CYN-producing genotypes
373 (i.e., intraspecific or strain differences; Orr et al., 2010 – and see below). The synthesis
374 of STXs and NODs in N_2-fixing cyanobacteria has also shown counterintuitive responses
375 to N additions, and appears to be inhibited by NH_4^+ (Kabir and El-Shehawy, 2012;
376 Stucken et al., 2010). For example, Stucken et al. (2014) quantified intra- and extra-
377 cellular toxin content in cultured *C. raciborskii* (CYN producer) and *Raphidiopsis brookii*
378 (STX producer) at early stages of growth under NO_3^-, NH_4^+, urea, and N-free media, and
379 showed that the N source did not influence either CYN or STX production in the strains
380 tested. In media without N added, however, precursor toxins decreased, and *R. brookii*
381 also produced less STX under growth with NH_4^+. These observations for cyanobacteria
382 differ markedly from the strong, predictable dependencies on N availability that have
383 been observed in the STX-producing dinoflagellate *Alexandrium* (Boyer et al., 1987;
384 Anderson et al., 1990; Flynn et al., 1994; John and Flynn, 2000; Hattenrath et al., 2010;
385 van de Waal et al., 2013).
386
387 **Intraspecific differences in N influence: toxic versus nontoxic strains**
388 Consistent with studies of marine toxigenic algae, within a given genus of
389 cyanobacteria, strains differ in toxin composition and cellular toxin quota, and nontoxic
390 strains not only co-occur but can be major components of blooms (Burkholder and
391 Glibert, 2006; and references therein). In natural cyanobacteria populations, total cellular
392 toxin quotas resemble average values for an entire population and strongly depend on the
393 contribution of toxigenic versus nontoxic genotypes (Janse et al., 2005; Kardinaal et al.,

394 2007; Briand et al., 2008; Davis et al., 2009, 2010; Orr et al., 2010; Burford et al., 2014).
395 Shifts in the genotypic composition of a population will cause changes in both the
396 average cellular toxin quota but also the toxin composition (Bittencourt-Oliveira et al.,
397 2001; Ame and Wunderlin, 2005; Zurawell et al., 2005; Monchamp et al., 2014). While
398 N can strongly influence the relative abundance of toxic versus nontoxic strains of
399 cyanobacteria, intraspecific variation (strain differences) in toxin production are poorly
400 understood but of paramount importance in the dynamics of overall bloom toxicity and N
401 controls.

402 While environmental drivers such as N have been shown to influence cell quotas
403 of MCs, most studies have shown only up to four-fold changes in such quotas (Sivonen
404 and Jones, 1999; Horst et al., 2014; Harke and Gobler, 2013). During cyanobacterial
405 blooms, however, changes in MCs and other cyanotoxins can often vary many times
406 greater than four-fold (Chorus and Bartram, 1999; Zurawell et al., 2005; and references
407 therein). Therefore, changes in community composition between cells with the genetic
408 ability to produce cyanotoxins (i.e. toxigenic cells), and those lacking that capability
409 (nontoxic cells; Davis et al., 2009) are likely to play a key role in influencing bloom
410 toxicity.

411 Laboratory studies of *Microcystis* have shown that toxigenic (MC+) strains yield
412 faster growth rates than nontoxic (MC–) strains at high N_i concentrations (Vézie et al.,
413 2002, Zurawell et al., 2005). In contrast, MC– strains of *Microcystis* require lower N_i
414 concentrations to achieve maximal growth rates in comparison to MC+ strains (Vézie et
415 al., 2002) and nontoxic *Microcystis* strains have been shown to outcompete MC+ strains
416 when N_i concentrations are low (Vézie et al., 2002; Davis et al., 2010). In field research,

417 bloom populations of *Microcystis* in a temperate, tidal (brackish) tributary and a
418 eutrophic lake shifted from dominance of MC+ strains to MC- strains as N_i
419 concentrations decreased through the summer (Davis et al., 2010). Other researchers
420 working in various lakes have observed a similar seasonal succession of toxic to nontoxic
421 *Microcystis* populations (Fastner et al., 2001; Welker et al., 2007; Briand et al., 2009,
422 Otten et al., 2012, Singh et al., 2015; Beversdorf et al., 2015) or have noted the
423 dominance of MC– strains during the peak of a *Microcystis* bloom event (Welker et al.,
424 2003, 2007; Kardinaal et al., 2007). Since inorganic nutrient levels are generally depleted
425 by dense algal blooms (Wetzel, 2001, Sunda et al., 2006, and references therein), the
426 predominance of MC– strains in established (and senescing) blooms has been
427 hypothesized to be a function of their ability to outcompete MC+ strains when nutrient
428 levels are lower (Vézie et al., 2002; Davis et al., 2010). Thus, under low N conditions,
429 MC+ strains would be succeeded by MC– strains, and/or MC synthesis would be down-
430 regulated. Overall, toxic *Microcystis* cells appear to have a higher N requirement than
431 nontoxic cells (Vézie et al., 2002; Davis et al., 2010), likely related at least in part to the
432 additional N requirements associated with the enzymes involved in MC synthesis (Tillet
433 et al., 2000) and perhaps with additional light-harvesting pigments (Hesse et al., 2001).
434 MC is a N-rich compound (average of 10 N atoms per molecule) and MC can represent
435 up to 2% of cellular dry weight of toxic *Microcystis* cells (Nagata et al., 1997).
436 Accordingly, in many eutrophic systems MC concentrations have more commonly been
437 reported to increase in response to increasing N than increasing P (Gobler et al., 2007;
438 Donald et al., 2011, Chaffin et al., 2013; Chaffin & Bridgeman 2013, Davis et al., 2015).

439 Interactions between N and other environmental factors further influence
440 cyanotoxin concentrations (Chorus and Bartram, 1999; Zurawell et al., 2005; and
441 references therein). For example, when MC+ strains dominate assemblages in the early
442 bloom phase when N concentrations are high, there is lower overall biomass and, thus,
443 higher average light intensities. MC+ strains have been shown to grow well under higher
444 light intensities, better than their MC- counterparts (Zilliges et al., 2011), while MC–
445 strains are better competitors at low light intensities characteristic of dense blooms
446 (Kardinaal et al. 2007). Hence, while N plays a primary role in shaping the relative
447 abundance of MC-producing cells in an ecosystem setting, other biotic and abiotic factors
448 likely act and interact to influence these populations as well.
449
450 **The dynamics of N, P, *Microcystis,* and micocystins in western Lake Erie**
451 In support of prior studies showing the importance of exogenous N in controlling
452 the toxicity of cyanobacterial blooms, data from western Lake Erie, a region long known
453 for cyanobacterial blooms and recently 160experiencing a re-intensification of these
454 events (Stumpf et al., 2012; Obenour et al., 2014), has been synthesized for this review.
455 Inter-annual differences in the duration, intensity, and toxicity of cyanobacterial blooms
456 were considered in relationship to in-lake and tributary nutrient concentrations.
457 Monitoring surveys totaling 14, 19, and 21 events in 2012, 2013, and 2014, respectively,
458 were conducted across an area of ~300 km^2 (mean depth ~5 m, total volume 1.5 km^3)
459 wherein sampling occurred at four fixed stations weekly (July – September) to biweekly
460 or monthly (May, June, October). Discharge for the Maumee River was obtained as
461 USGS daily averages (Waterville gaging station 4193500;

462 http://waterdata.usgs.gov/usa/nwis/uv?site_no=04193500, last accessed in July 2015).
463 River nutrient concentrations were obtained from the Water Quality Laboratory at
464 Heidelberg College, Tifton (OH, USA) and were averaged as daily means when more
465 than one sample was generated on a given day.

466 Maumee River discharge was significantly lower in 2012 than in the other two
467 years, especially during spring and summer when flows are likely to have the greatest
468 potential influence on the size and intensity of western Lake Erie cyanobacterial blooms
469 (Stumpf et al., 2012). The river discharge for that period in 2012 (0.42 km^3) was only 16
470 and 17% of that observed in 2013 (2.71 km^3) and 2014 (2.47 km^3; Fig. 3A). Similarly in
471 the lower Maumee River, the mean NO_3^- concentration in April – September 2012 (1.03
472 mg L^{-1}) was 28% of that in 2013 and 2014 (3.82 and 3.60 mg L^{-1}, respectively; Fig. 3B).
473 NO_3^- concentrations in June and July 2012 were significantly lower than those present in
474 during those months in 2013 and 2014 (ANOVA; $p<0.01$). Average PO_4^{-3} in the lower
475 Maumee River in April – September 2012 (26 µg L^{-1}) was lower than in 2013 and 2014
476 (50.2 and 57.4 µg L^{-1}, respectively; Fig. 3C) although extended periods of significant
477 differences were not detected.

478 Reflective of the tributary loads, in-lake NO_3^- concentrations varied substantially
479 among years both in terms of the timing and magnitude of peak levels, as well as, the
480 rates of decline during the bloom season (Fig 4). Maximum NO_3^- levels in 2012 were 25
481 and 30% of levels seen in 2013 and 2014, with minimum concentrations present 1 - 2
482 months earlier in the season (Fig. 4A). NO_3^- concentrations reached their seasonal
483 minima of < 0.1 mg L^{-1} several weeks before the onset of the 2012 cyanobacterial blooms
484 in August and remained below that level during the ~6 weeks of elevated biomass (Fig.

485 4A). The concentrations of NO_3^- during the May, June, and July were significant lower
486 in 2012 compared to the same period during 2013 and 2014 (ANOVA; $p<0.01$) whereas
487 PO_4^{-3} concentrations were not significantly different among years during this time period.
488 Levels of the cyanobacterial pigment, phycocyanin, varied seasonally and
489 annually but showed clear seasonal maxima from mid-July to September (Fig. 4C). The
490 highest cyanobacterial biomass in this area of the lake was measured in 2012 when river
491 flows and nutrient inputs were lower (Fig. 3, 4C). Maximum levels of phycocyanin (200-
492 300 µg L^{-1}) were about twice as high in 2012 than in the other two years (Fig. 4C), a
493 finding emphasizing the ability of *Microcystis* (the dominant genera present during
494 blooms) to form dense blooms under low P conditions (Wilhelm et al., 2003; Harke et al.,
495 2015). Regarding MC, however, in the summer of 2012, there was almost no detectable
496 increase in whole-cell (particulate) MC (Fig. 4D), suggesting that nontoxic *Microcystis*
497 strains comprised a significant portion of the bloom biomass and that the toxic strains that
498 were present likely had low MC cellular quotas, conclusions supported by prior sections
499 of this manuscript. At the beginning of September 2012, when the bloom appeared to
500 wane and NO_3^- increased slightly and MC finally increased but remained relatively low
501 (maximum, 2.5 µg L^{-1}; Fig. 4D). In contrast, during summers of 2013 and 2014, NO_3^-
502 levels remained higher for a longer period (over 2.0 and 0.6 mg L^{-1} at the end of July) and
503 peak MC concentrations exceeded 13 and 21 µg L^{-1}, respectively. MC concentrations
504 during August 2013 and 2014 were significantly higher than the levels present during
505 August of 2012 (ANOVA; $p<0.01$). NH_4^+ dynamics were similar to those for nitrate,
506 higher in 2013 and 2014 compared to 2012, and depleted in August but slightly elevated
507 before and after that month (data not shown). Except for one sampling date in each of the

508 latter two years, particulate MC concentrations declined once NO_3^- decreased to < 0.5 mg
509 L^{-1}. In contrast, there was no apparent relationship between particulate MC
510 concentrations and SRP (P_i; Fig 4). Hence, the significantly lower overall MC
511 concentrations in 2012 compared to 2013 and 2014, as well as declines in MC in those
512 years, coincided with lower NO_3^- levels.

513 These ecosystem observations are consistent with studies outlined above which
514 have shown that MC concentrations during toxic *Microcystis* and *Planktothrix* blooms in
515 western Lake Erie have been controlled by the availability of water-column N_i (Horst et
516 al., 2014; Davis et al., 2015; Harke et al., 2015). Furthermore, these findings suggest the
517 intriguing possibility that declines in N loading in western Lake Erie (Stow et al., 2015)
518 could yield blooms of lower toxicity, even if P_i levels rise or remain stable. Therefore, in
519 order to manage Lake Erie towards smaller blooms of lower toxicity, managing N and P
520 should be considered. The response of Lake Erie to changes in nutrient conditions as
521 management actions are implemented will offer an opportunity to learn more about these
522 processes in the context of a large-scale ecosystem manipulation. It will be important to
523 develop rigorous, testable hypotheses, and execute a well-conceived research and
524 monitoring program to take advantage of this opportunity, and refine management
525 priorities as the lake responds.

526

527 **Conclusions and challenges ahead**

528 Although P has traditionally been considered the primary nutrient influencing
529 harmful cyanobacterial blooms in freshwater systems, this review of recent findings from
530 laboratory studies and lakes throughout the world demonstrates that N can be important

531 in in controlling the timing, density, *and* toxicity of some non-diazotrophic
532 cyanobacterial blooms (Fig 5). Moreover, some non-diazotrophic cyanobacteria seem
533 well-adapted to low P_i environments (Fig 5). Finally, it seems clear that high N_i
534 environments favor cyanobacterial capable of synthesizing MC and permit those cells to
535 maximize the amount of toxin synthesized per cell (Fig 5). Hence, while cyanobacteria
536 dominate high N and high P environments, reducing levels of P, but not N, could result in
537 a shift in cyanobacterial diversity, but not necessarily toxicity (Fig 5).

538 There exist important gaps in the understanding of how N influences
539 cyanobacterial blooms. Information is often lacking regarding N speciation within
540 freshwater bodies, especially separate data for NO_3^- and NH_4^+ (Glibert et al., 2011, and
541 references therein), as management efforts often focus on TN (e.g. U.S. Environmental
542 Protection Agency, 2000). This practice can prohibit gaining insights about the influence
543 of specific N species on cyanobacteria and other phytoplankton groups, particularly given
544 that the TN pool can include mostly N contained within cyanobacteria cells and other
545 plankton. In many aquatic ecosystems, a poor understanding of external and internal N
546 loading and biological N-fixation rates prevents accurate assessment of the relative
547 importance of N versus P in controlling cyanobacteria toxicity and blooms. In systems
548 where external N loading rates are constrained, assessments of how bloom toxicity is
549 related to N loading, as presented above for Lake Erie, are needed.

550 Laboratory and ecosystem studies provide many examples of N controlling the
551 biomass and toxicity of cyanobacteria. The extent to which N and/or P controls the
552 density and toxicity of cyanobacterial blooms is likely to vary as a function of watershed
553 N and P loads, lake geomorphology, and resident cyanobacterial assemblages. The

554 relative importance of N_i species versus key components of DON in controlling bloom
555 toxicity remains poorly understood, especially considering the DON concentrations in
556 freshwaters are rarely quantified, despite representing the largest dissolved N pool in
557 aquatic systems (Berman and Bronk, 2003). Research is needed to strengthen insights
558 regarding the competitive outcomes between diazotrophs, non-diazotrophs, and
559 eukaryotic algae under varying N and P regimes and influences of physical and biological
560 interactions such as temperature, mixing, parasitism, and grazing. While Figure 5 is
561 presented as a framework for understanding the extent to which varying N and P
562 concentrations may influence such competition, there remain significant knowledge gaps
563 on this topic. The precise role of nutrients in driving MC congener production and total
564 bloom toxicity also remains to be determined (e.g., Van de Waal et al., 2009, 2010;
565 Monchamp et al. 2014). For example, do differing N sources and concentrations result in
566 differing MC congeners or overall MC concentrations in an ecosystem setting, or does N
567 affect congener production only indirectly through cyanobacteria assemblage structure?
568 It is unclear what causes the differing dependencies of toxins synthesis on N
569 availability in N_2-fixing cyanobacteria versus non-N_2 fixing cyanobacteria or other STX-
570 producing algae. Thus far, studies on N regulation of toxin production in cyanobacteria
571 have been limited and, thus, further studies will be required to elucidate the intriguing
572 interplay between N_2-fixing capability and the synthesis of cyanotoxins. A substantial
573 portion of cellular MC and NOD can bind to proteins, particularly when cells are under
574 oxidative stress, suggesting a role of these toxins in protection from free radicals and the
575 potential for oxidative stress to influence their production in conjunction with N (Zilliges
576 et al., 2011; Meissner et al., 2013). Approaches are needed to assess the extent to which

577 shifts in toxin speciation, cellular toxin quotas, and total cellular toxicity result from
578 changes N concentrations and speciation, changes in toxin binding, and/or additional
579 exogenous forcing factors (Meissner et al., 2013).

580 Finally, the information presented in this review has implications for the
581 management of freshwater bodies. During the past decade, compelling information has
582 accumulated in support of a need to control both N and P to mitigate algal blooms in
583 freshwater and estuarine ecosystems (GEOHAB 2006; Conley et al., 2009; Glibert et al.
584 2011; U.S. Environmental Protection Agency, 2015, Woodland et al., 2015). Although
585 the U.S. Environmental Protection Agency (2000) required U.S. states to develop
586 numeric nutrient criteria to reduce TN and TP loads to surface waters, most states have
587 not yet developed numeric criteria for N species (Barvenik et al., 2009, U.S.
588 Environmental Protection Agency [EPA] 2014; and see http://cfpub.epa.gov/wqsits/nnc-
589 development/). For example, present management practices in states bordering the
590 western basin of Lake Erie call only for reductions in P, not N (Ohio Environmental
591 Protection Agency 2013, Great Lakes Water Quality Agreement Annex 4 Report, 2015).
592 How reductions in N, as well as P, will shape cyanobacterial blooms at an ecosystem
593 level is not yet fully clear, and will depend on a range of abiotic and biotic factors. This
594 review has demonstrated that key, non-diazotrophic cyanobacteria such as *Microcystis*
595 can sustain high biomass even when P_i is near or below detection limits, and that
596 populations can become more toxic within high-N_i environments (Fig. 5). Hence,
597 reductions of both N and P loading will be required to lessen the intensity *and* toxicity of
598 blooms caused by *Microcystis* and other non-diazotrophs.
599

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605

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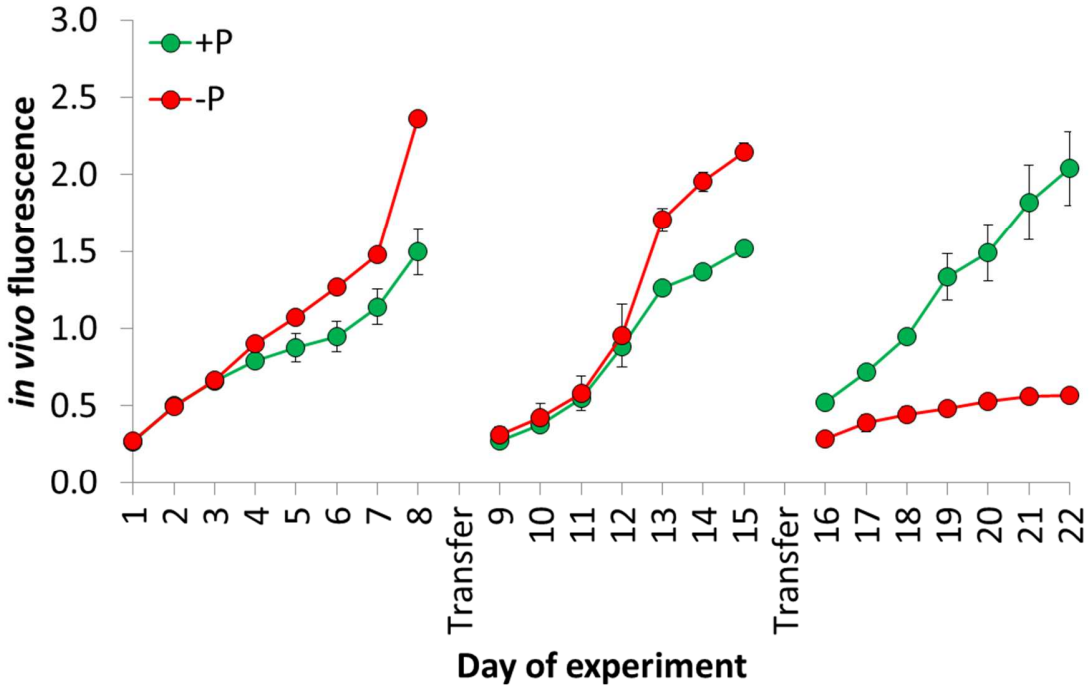


Figure 1. Phosphorus limitation experiment wherein cultures of *Microcystis aeruginosa* LE-3 harvested from P-replete media were grown under 0 µM inorganic P (-P) and 1,200 µM inorganic P (+P). Cultures were repeatedly diluted back to starting densities during the "Transfer" for this time series in order to fully limit the population. *In vivo* fluorescence levels of this culture were highly correlated with cell densities (Harke et al, 2012).

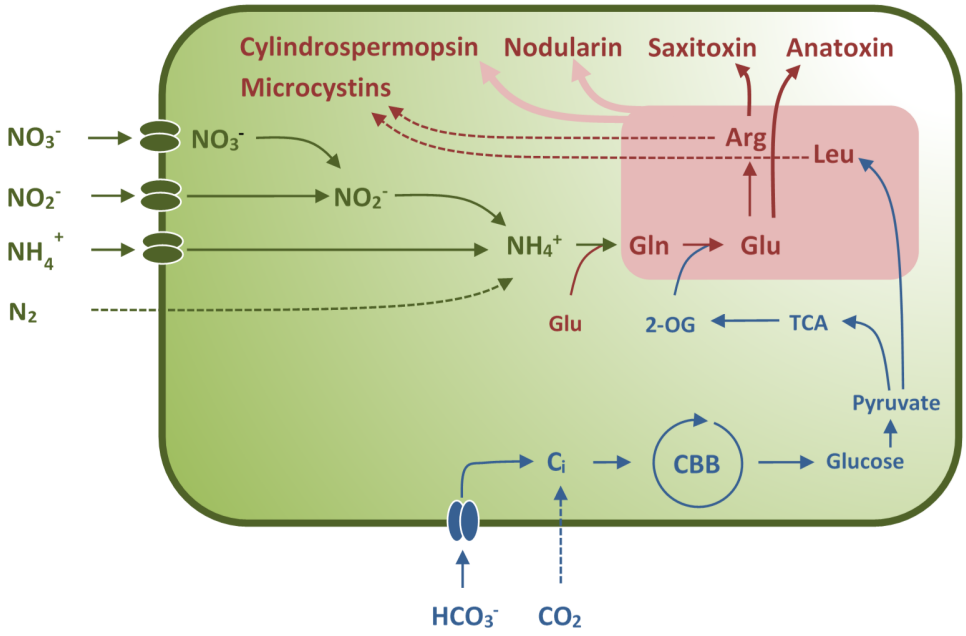
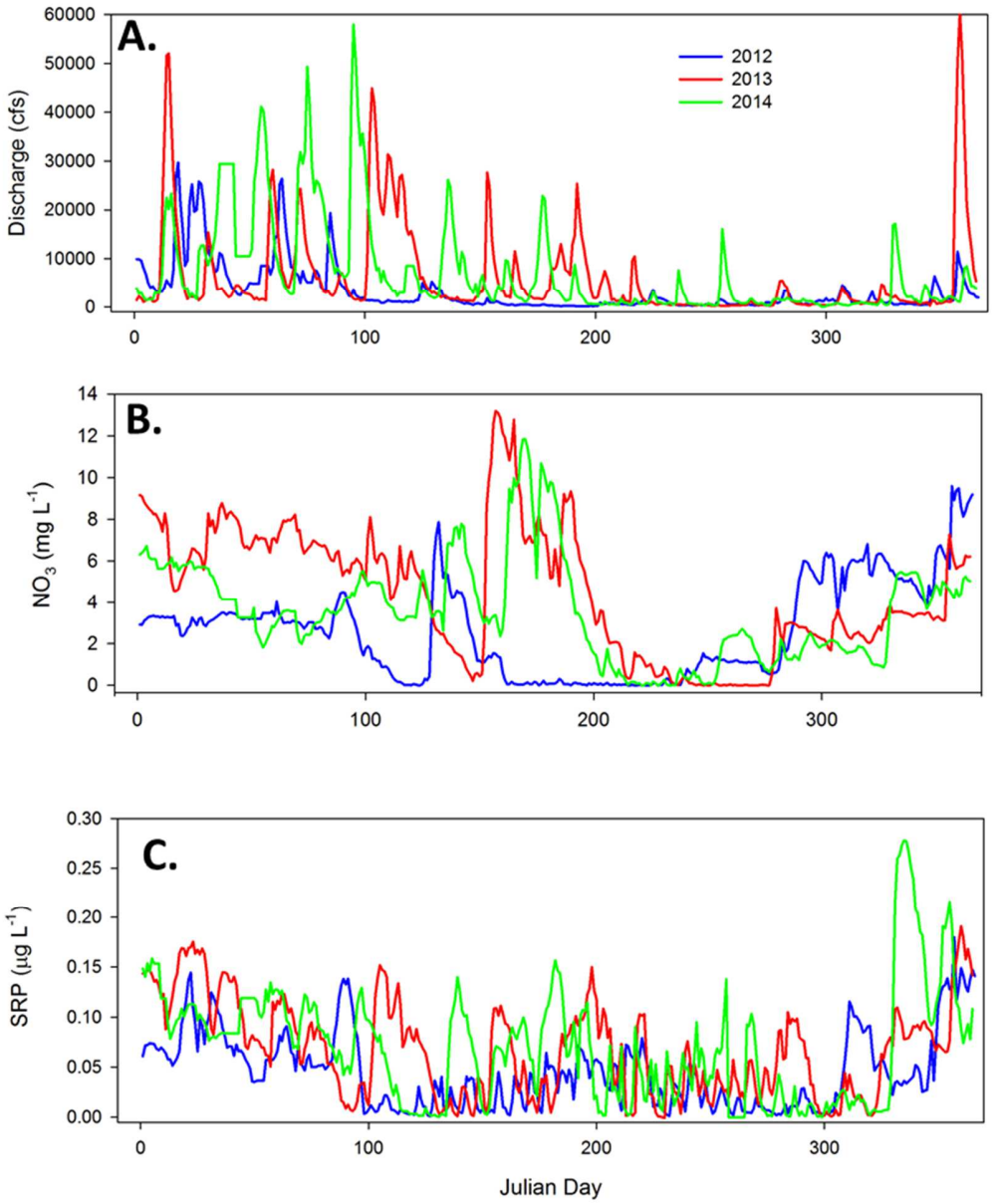


Figure 2. Cell model describing the coupling of C and N assimilation with synthesis of some cyanobacterial toxins, and showing the importance of N in toxin production. N_i is assimilated to amino acids by the incorporation of cellular NH_4^+ into C skeletons through the glutamine synthetase-glutamate synthase pathway. The toxins are synthesized from the cellular amino acid pool (light grey arrows), or from distinct amino acids (dark grey arrow). C_i, cellular inorganic carbon; CBB, Calvin-Benson-Bassham cycle; TCA, tricarboxylic acid; 2-OG, 2-oxoglutarate; Gln, glutamine; Glu, glutamate; Arg, arginine; Leu, leucine. Note, N_2 fixation occurs in a heterocyte, and that toxins are produced by different cyanobacteria species. Here, all cyanobacteria are shown in the same cell model for simplicity. Modified after Van de Waal et al. (2010).



1117

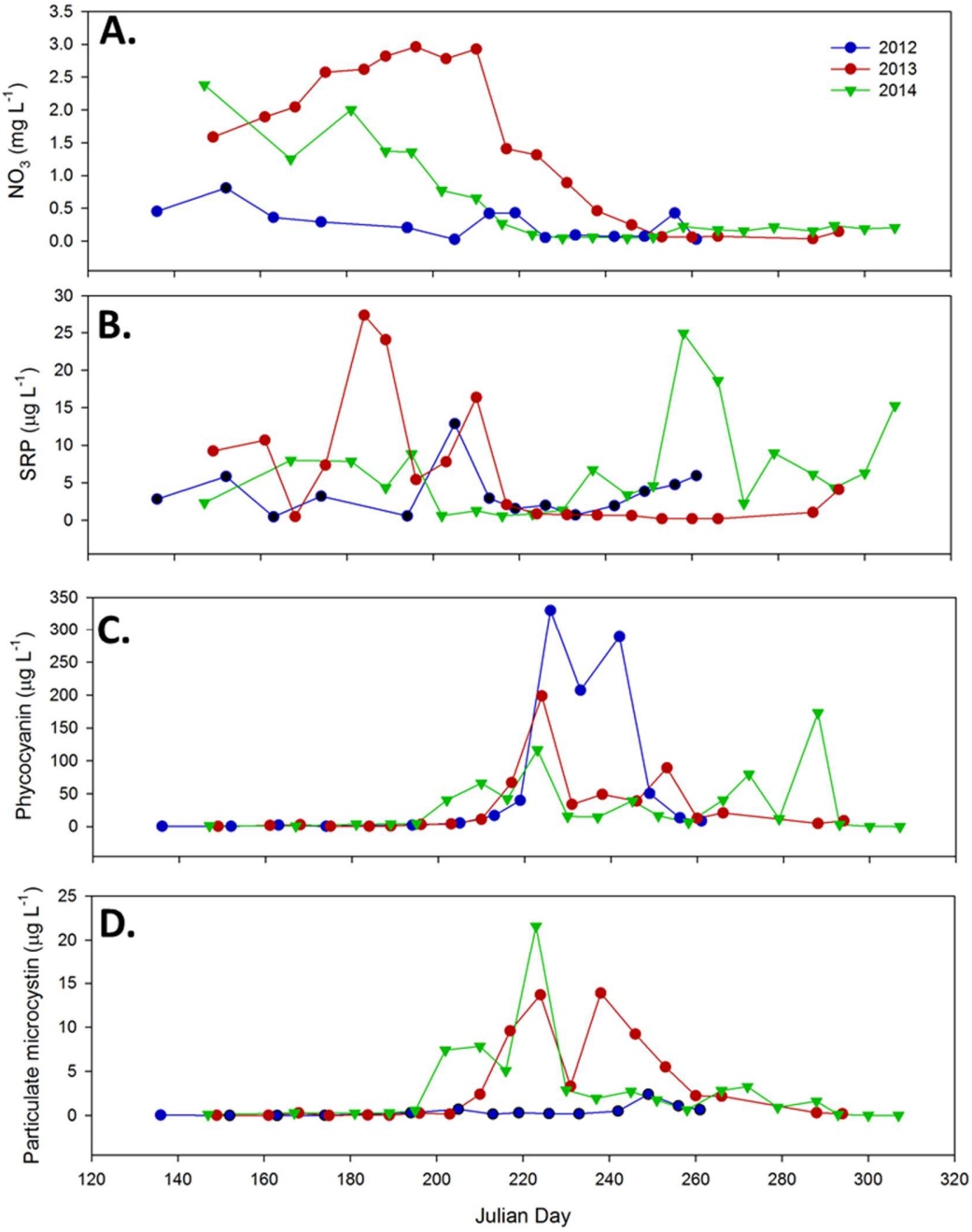
1118 **Figure 3.** (A) Maumee River discharge (cubic feet per second, cfs), from daily data taken

1119 by the USGS at gaging station (B,C), with concentrations of (B) NO_3^- (mg L^{-1}) and (C)

1120 SRP (soluble reactive phosphorus: µg L^{-1}) in the Maumee River (mg L^{-1}). Data from the

1121 NOAA Great Lakes Environmental Research Laboratory, Muskegon, MI, U.S.A.

1122



1123

1124 **Figure 4.** Concentrations of (A) nitrate (mg L^{-1}), (B) SRP (soluble reactive phosphorus;

1125 µg L^{-1}), (C) phycocyanin (µg L^{-1}), and (D) particulate microcystin (µg L^{-1}) in western

1126 Lake Erie during 2012-2014.

1127

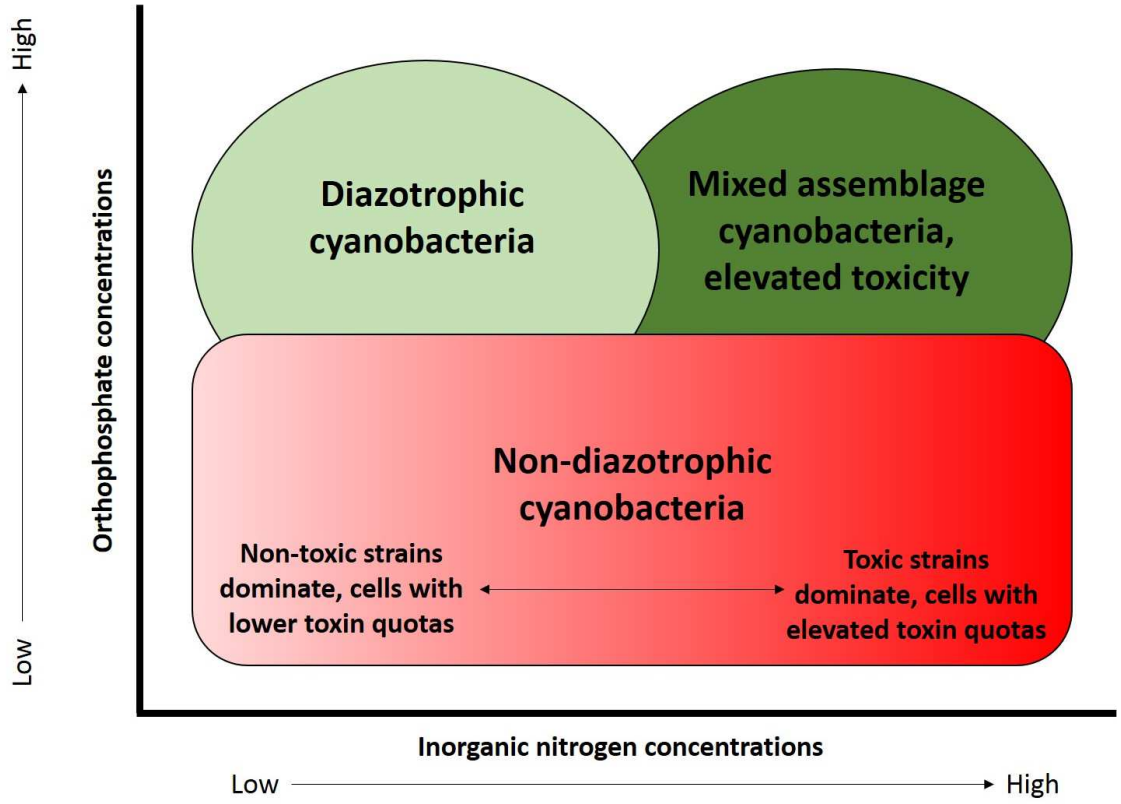


Figure 5. Conceptual diagram of shifts in cyanobacterial populations that can be facilitated by high and low levels of N and P. While ecosystem-specific exceptions to this general depiction are likely, this paper emphasizes mechanisms that control instances wherein changes in N_i and P_i concentrations facilitate shifts among cyanobacterial populations.