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The effectiveness of cyanobacteria nitrogen fixation: Review of bench top and pilot scale nitrogen removal studies and implications for nitrogen removal programs

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Abstract: One of the primary goals of eutrophication management of freshwater systems is to lower the risk of cyanobacterial blooms. This is typically accomplished at the watershed scale by placing controls on phosphorus (P) discharge from point sources. However, several researchers have questioned the predominance of the P management paradigm, arguing that dual nitrogen (N) and P controls would be more effective at preventing cyanobacteria blooms than P controls alone. This hypothesis is predicated in part on the hypothesis that if cyanobacteria are starved of N, which is an essential nutrient, cyanobacteria N\textsubscript{2} fixation rates will not be high enough to maintain growth rates and biomass yields at or near previous levels. However, several single species cultures of heterocystous cyanobacteria directly examining the effect of removing N show that, when deprived of ammonium and nitrate, N\textsubscript{2} fixing cyanobacteria compensate biochemically for the high energy cost of fixation when supplied with sufficient nutrients other than N. Biomass and growth rates were only slightly different under N\textsubscript{2} than when grown under ammonium and nitrate, which is consistent with observations from the long-term experimental fertilization of Lake 227. Collectively, these bench top and pilot scale studies suggest that N control programs will not have a major impact on the magnitude of freshwater cyanobacteria blooms, although cyanobacteria species composition and toxin production might be affected.

Keywords: eutrophication, nitrogen fixation, cyanobacteria, nutrient management, proof of concept
Introduction: The link between excessive phosphorus (P) loading to aquatic systems and cyanobacteria blooms, low water clarity and depleted dissolved oxygen in hypolimnetic waters is well documented and widely recognized, providing the basis for generally successful management actions via P controls, primarily from point sources (Dolan and Chapra 2012; Schindler et al. 2016). Indeed, limnologists and managers consider P management to be of paramount importance. However, not all P management programs have successfully mitigated cyanobacterial blooms, at least in the short term (Schindler et al. 2016), and several authors have questioned the predominance of the P management paradigm, arguing that dual nitrogen (N) and P controls (i.e., supplementing P controls with N controls) would be more effective at preventing cyanobacterial blooms than P controls alone (Lewis and Wurtsbaugh 2008; Paerl 2009; Scott and McCarthy 2010; Paerl et al. 2011; Hellweger et al. 2016). In many cases, this means adding N controls to existing point source P control programs.

While external and internal P loads are not supplemented by biological inputs of P from a gas phase, N inputs are supplemented by N$_2$ fixation if supplies of ammonia and nitrate limit growth. The idea that adding N to existing P control programs will mitigate the severity of blooms is predicated on the argument that if cyanobacteria are starved of N, cyanobacteria N$_2$ fixation rates will not be high enough to maintain cyanobacteria biomass at or near previous levels (Lewis and Wurtsbaugh 2008; Scott and McCarthy 2010; Paerl et al. 2014). The dual or co-limitation idea has been disputed by Schindler and his colleagues (Schindler et al. 2008; Schindler 2012; Schindler et al. 2012, 2016) who argue that N controls will have little impact, other than to shift the dominant cyanobacteria to N$_2$-fixing taxa if the system becomes N-limited.
Despite criticisms of the dual nutrient hypothesis, support for dual nutrient controls has grown (e.g., United States Environmental Protection Agency 2015), an idea which could influence expenditure of public funds.

This brief review examines the claim that N control is an effective method to mitigate cyanobacteria blooms with a focus on adaptation by heterocystous N\textsubscript{2} fixers to N removal in laboratory cultures and a long-term lake fertilization experiment. The potential for N control to affect toxin production by cyanobacteria (Gagnon and Pick 2012; Harke et al. 2015; Gobler et al. 2016; Pick 2016), marine coastal eutrophication (Smith 2003; Scavia and Bricker 2006; Diaz and Rosenberg 2008) and other issues (Schindler et al. 2016) are recognized as topics of concern but are not addressed here.

**Proof of concept**: Proof of concept, also known as proof of principle, is defined as a small scale demonstration with the aim of determining whether a concept will work as proposed (Dym et al. 2014) - the idea being to test a product before bringing it to market. Since every new concept, or hypothesis, comes with an unknown risk of failure, controlled tests at bench top and larger pilot scales can help determine if the concept has the potential to achieve the desired objective at an industrial scale (or watershed scale in this case), whether the benefits are worth the costs and thus whether the concept should be employed at the watershed scale. Proof of concept is an approach widely used in product development in areas as diverse as engineering (Dym et al. 2014) and drug development (Corr and Williams 2009).
Eutrophication managers will want to know if an investment in N controls will likely result in declines in cyanobacteria bloom magnitude or duration that are large enough to justify the cost. The most valid proof of concept test in this case is direct N removal from experimental systems because it most closely resembles N control programs at a watershed scale. Inferences from relatively indirect evidence not employing N removal, such as descriptive correlations of field observations, N addition bioassays, computational modeling, etc., on the hypothesized effectiveness of N controls (Lewis and Wurtsbaugh 2008; Paerl 2009; Scott and McCarthy 2010; Paerl et al. 2011; Hellweger et al. 2016) can be useful in the early stages of developing an idea, but the risk of misinterpreting indirect evidence is much larger than the risk of misinterpreting direct tests of N removal. Therefore, direct N removal tests should be seen as critical elements of the decision making process because they lower uncertainty.

To what extent do N₂ fixing cyanobacteria adapt to loss of N? One argument for assuming that N₂ fixation rates can be safely ignored by N control programs is based on the high energy cost of N₂ fixation (Stam et al. 1987), a cost high enough that it might be presumed to significantly limit growth rates relative to growth in the presence of ammonium and nitrate if a large fraction of photosynthetic energy is diverted into N₂ fixation at the expense of biosynthesis of other compounds. Total costs stem from the cost of the fixation pathway itself and the cost of minimizing the dissolved oxygen concentration inside the heterocyst. Heterocysts use several passive and active strategies to deal with the latter including a thick impermeable membrane barrier around the cell, not using photosystem II, increased cell size and enhanced respiration rate (Berman-Frank et al. 2007; Flores and Herrero 2010; Inomura et al. 2017). A total cost high
enough to lower growth rate could mean a delay in reaching the maximum biomass which is limited by availability of a nutrient other than N$_2$.

Cyanobacteria physiologically adapt to the absence of nitrate and ammonium in laboratory cultures. The gross cellular composition of the N$_2$ fixer *Nostoc paludosum* shifted when the sole source of extracellular N was N$_2$ (Vargas et al. 1998) with the proportion of N-rich protein decreasing and the proportion of N-poor, energy-rich lipids and carbohydrates increasing (Table 1). The shift to less protein when the only N source was N$_2$ was also observed in *Anabaena variabilis* (Sanz et al. 1995) and *Nostoc muscorum* (Bagchi et al. 1985). This strategy could conserve N and provide energy for dark N$_2$ fixation when photosynthesis-generated ATP is unavailable. Dark fixation appears common in cyanobacteria (Fay 1976 and 1992; Jensen and Cox 1983; Oh et al. 1991; Bebout et al. 1993).

Several bench top studies have shown that N starvation results in very modest changes in growth rates and biomass yields in single species laboratory cultures of heterocystous N$_2$ fixing cyanobacteria. Growth rates and yields in nutrient-rich batch and semi-continuous cultures without ammonium and nitrate were only slightly different, about 10%, than in media with ammonium and nitrate (Table 1) (Allen and Arnon 1955; Schlangstedt et al. 1987; Bagchi et al. 1985; Vargas et al. 1998). In fact, growth rates and yields were higher in some experiments using only N$_2$, for example, when measured as dry weight by Allen and Arnon (1955) and cell number by Bagchi et al. (1985). Major declines in growth rate can, however, be achieved by depriving cyanobacteria of cobalt (Holm-Hansen et al. 1954; Reisenauer 1960) and molybdenum (Allen and Arnon 1955), which are essential for fixation. Kratz and Myers (1955) showed that depriving
*Nostoc muscorum* of cobalt lowered the growth rate in batch culture by 26% when the only N source was \( \text{N}_2 \). Presumably, growth still occurred because cobalt was added as an impurity in other reagents.

*Cylindrospermopsis raciborskii* biomass in steady state (flow through) chemostats was 5 to 70% lower under \( \text{N}_2 \) than when grown with nitrate and ammonium at dilution rates below 0.75 d\(^{-1}\) with negligible biomass at dilution rates \( \geq 1.0 \text{ d}^{-1} \) unless supplied with ammonium or nitrate (Kenesi et al. 2009). This study suggests that, when starved of N at high dilution rates, *Cylindrospermopsis raciborskii* diverts a significant fraction of its energy into \( \text{N}_2 \) fixation instead of organic carbon synthesis. Note that unlike batch cultures, growth rates in chemostats are forced to match the dilution rates making direct comparison between these two methods difficult.

These laboratory results suggest that energy compensation mechanisms are efficient and \( \text{N}_2 \) fixation can supply most, if not all, of the cyanobacteria N requirements in cultures if fixation is not limited by essential micronutrients. \( \text{N}_2 \) fixation rates may not be able to support very high growth rates (Kenesi et al. 2009), but the batch and semi-continuous culture studies cited herein (Allen and Arnon 1955; Schlangstedt et al. 1987; Bagchi et al. 1985; Vargas et al. 1998) indicate that fixation can probably support lower growth rates more common in eutrophic lakes (e.g., Robarts and Zohary 1987).

These laboratory studies are small scale, having taken place in glassware without sediments, hypoxic bottom waters, algal competitors or grazers over a period of days to a few weeks. While it would not be wise to predict the outcome of long-term, lake-scale N removal programs based
solely on these experiments, they provide a clear answer to a specific physiological issue
germaine to the debate, demonstrating that N removal does not have a large impact on growth
rate and biomass (yield) in single species systems under controlled conditions. Factors operating
at the lake scale over longer periods of time may, however, interact to affect N\textsubscript{2} fixation rates
necessitating investigation at the whole-lake scale.

Cyanobacteria compensate for the high cost of N\textsubscript{2} fixation in experimentally fertilized Lake 227.
This long-term whole-lake fertilization experiment began in 1969 with additions of nitrate and
phosphate during the ice-free seasons (Schindler 2008). While phosphate additions have
remained constant since 1969, nitrate was lowered in 1975 and then stopped completely in 1990.
There were no significant trends in the annual average chlorophyll \(a\) or phytoplankton biomass
over a 41 year period between 1969 and 2009, regardless of the N:P ratio used in loading. There
were no significant declines in cyanobacteria biomass over the entire fertilization period or since
1990 (Paterson et al. 2011), although cyanobacteria biomass appears to have been more variable
prior to 1990 (Schindler et al. 2008). After 1990, the phytoplankton community was dominated
by N\textsubscript{2}-fixing \textit{Aphanizomenon} and the apparent annual N\textsubscript{2} fixation rate began to increase based on
increasing heterocyst counts (Paterson et al. 2011), presumably in response to gradual depletion
of legacy N stored in sediments. As pointed out by Schindler et al. (2016), “nitrogen fixation in
2011 exceeded the sum of fertilizer plus nitrogen fixation in any year before 1990.” These
observations clearly show that the amount of N\textsubscript{2} fixed during the bloom period was not impaired
and was enough to allow continued cyanobacteria bloom formation.
Conclusions: The efficiency of N$_2$ fixation has been one of the focal points of argument in the N removal debate. Collectively, the bench top and pilot scale direct proof of concept tests demonstrate that heterocystous N$_2$ fixers readily adapt to removal of N with at most a small cost to themselves when provided with adequate nutrients to fix N$_2$. This contrasts with claims based on indirect evidence that cyanobacteria fixation is inefficient and cannot sufficiently compensate for removed N.

If less than 100% of anthropogenic N was removed, a likely scenario for a watershed management program, the predicted impact on cyanobacteria should be less than 10%. In many lakes, biomass decreases of 10% or less would be well within year to year variation, for example, in Lake Erie (Bertani et al. 2016) and, therefore, would be undetectable. Based on direct N removal proof of concept tests, cyanobacteria bloom control does not provide a valid basis for N removal programs unless future N removal studies demonstrate otherwise.

A final point worth mentioning is that the definition of what constitutes an adequate body of evidence varies with the specific issue at hand. In a debate on N controls, single species cultures provide valuable evidence on the efficiency of growth of heterocystous cyanobacteria under N$_2$ as the sole source of N and should be an important part of the body of evidence under consideration. On the other hand, single species experiments that withhold (or limit) P from culture media would not be as meaningful in a debate on P controls because there are no biological gas phase inputs.

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Table 1. Effect of nitrogen source on specific growth rate and biochemical composition of the N$_2$ fixer *Nostoc paludosum* grown in semi-continuous culture at 1380 µmol photon m$^{-2}$ s$^{-1}$.

Biochemical composition is expressed as a % of dry weight. Values are means ± sd; n is shown in parentheses. Data from Vargas et al. (1998).

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<th>Air</th>
<th>Air + nitrate</th>
<th>Air + ammonium</th>
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<td>growth rate (d$^{-1}$)</td>
<td>0.69 ± 0.11 (4)</td>
<td>0.76 ± 0.05 (4)</td>
<td>0.74 ± 0.08 (4)</td>
</tr>
<tr>
<td>% protein</td>
<td>43.1 ± 2.5 (5)</td>
<td>53.6 ± 0.8 (3)</td>
<td>55.5 ± 2.0 (3)</td>
</tr>
<tr>
<td>% carbohydrate</td>
<td>27.1 ± 2.0 (4)</td>
<td>22.5 ± 2.2 (3)</td>
<td>16.9 ± 1.3 (3)</td>
</tr>
<tr>
<td>% lipid</td>
<td>8.5 ± 0.4 (4)</td>
<td>6.0 ± 0.7 (3)</td>
<td>4.9 ± 0.7 (3)</td>
</tr>
<tr>
<td>% lipid + carbohydrate</td>
<td>35.6 ± 2.0 (4)</td>
<td>28.5 ± 2.3 (3)</td>
<td>21.8 ± 1.5 (3)</td>
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