DR. JUSTIN D. CHAFFIN (Orcid ID : 0000-0002-5372-4577)

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Nutrient addition effects on chlorophyll *a*, phytoplankton biomass, and heterocyte formation in Lake Erie's central basin during 2014-2017: Insights into diazotrophic blooms in high nitrogen water

Running Head: Lake Erie central basin nutrient limitation

Justin D. Chaffin[1]*, Keara Stanislawczyk[1], Douglas D. Kane[1,2,3], Madeline M. Lambrix[1,4]

1 F.T Stone Laboratory and Ohio Sea Grant, The Ohio State University, 878 Bayview Ave. Put-in-Bay, OH 43456

2 Natural Science, Applied Science, and Mathematics Division, Defiance College, Defiance, OH

3 – present address - Department of Biology and Environmental Science and National Center for Water Quality Research, Heidelberg University, Tiffin OH

4 – present address - United States Environmental Protection Agency, Region 2, 290 Broadway New York, NY 10007

* Corresponding author

Chaffin.46@osu.edu; 419-285-1800

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Abstract

1. Phosphorus (P) usually is the primary limiting nutrient of phytoplankton biomass, but attention towards nitrogen (N) and trace nutrients, like iron (Fe), has surfaced. Additionally, N-fixing cyanobacterial blooms have been documented to occur in N-rich, P-poor waters, which is counterintuitive from the paradigm that low N and high P promotes blooms. For example, Lake Erie's central basin has *Dolichospermum* blooms when nitrate concentrations are high, which raises questions about which nutrient(s) are selecting for *Dolichospermum* over other phytoplankton and why an N-fixer is present in high N waters?
2. We conducted a four-year (2014-2017) study in Lake Erie's central basin to determine which nutrient (P, N, or trace nutrients such as Fe, molybdenum (Mo), and boron (B)) constrained chlorophyll concentration, phytoplankton biovolume, and nitrate assimilation using nutrient enrichment bioassays. The enriched lake water was incubated in 1-L bottles in a growth chamber programmed at light and temperatures of *in situ* conditions for 4 to 7 days. We also quantified heterocytes when N-fixing cyanobacteria were present.
3. Compared to the non-enriched control, the P-enriched (+P) treatment had significantly higher chlorophyll and phytoplankton biovolume in ~ 75% of experiments. Combination enrichments of P with ammonium-N (+P&NH_4^+), nitrate-N (+P&NO_3^-), iron (+P&Fe), molybdenum (+P&Mo), and boron (+P&B) were compared to the +P treatment to determine secondary limitations. +P&NH_4^+ and +P&NO_3^- resulted in higher chlorophyll in 50% of experiments but higher phytoplankton biovolume in only 25% of experiments. These results show that P was the primary limiting nutrient, but there were times when N was secondarily limiting.
4. Chlorophyll concentration indicated N secondary limitation in half of the experiments, but biovolume indicated only N secondary limitation in 25% of the experiments. To make robust conclusions from nutrient enrichment bioassays, both chlorophyll and phytoplankton biovolume should be measured.

5. The secondary effects of Fe, Mo, and B on chlorophyll were low (< 26 % of experiments), and no secondary effects were observed on phytoplankton biovolume and nitrate assimilation. However, +P&Fe resulted in more chlorophyll than +P in experiments conducted during *Dolichospermum* blooms, and +P&B significantly increased the number of heterocytes in *Dolichospermum*. These results indicate that low Fe availability might select for *Dolichospermum,* and low B constrains heterocyte formation in the central basin of Lake Erie. Furthermore, these results could apply to other lakes with high N and low P where diazotrophic cyanobacterial blooms occur.

1 Introduction

The concept that nutrient availability limits primary production has been around since the 1850s when Liebig introduced the Law of the Minimum (de Baar, 1994). Under Liebig's law, the nutrient in shortest supply constrains primary production. Freshwater phytoplankton growth has typically been considered constrained by phosphorus (P) availability (Schindler, 1974; Hecky & Kilham, 1988), but recent evidence is highlighting the importance of other nutrients, such as nitrogen (N) (Conley *et al.*, 2009; Paerl *et al.*, 2016; Scott, McCarthy & Paerl, 2019), iron (Fe) (North *et al.*, 2007; Havens *et al.*, 2012; Sorichetti, Creed & Trick, 2016), and other trace metals (Sterner *et al.*, 2004). However, which nutrient limits production is not usually as straightforward as was proposed by Liebig, and therefore, terminology needs to be clarified (Davidson & Howarth, 2007; Saito, Goepfert & Ritt, 2008). The "primary limiting nutrient" is the nutrient in shortest supply relative to demand, and increasing the availability of that one nutrient will increase production. If the primary limiting nutrient and productivity are increased to a level that results in another nutrient to become limiting, that second nutrient is described as a "secondary limiting nutrient;" however, increasing the secondary limiting nutrient alone will not increase productivity. For example, if P was the primary limiting nutrient, the addition of P would result in higher phytoplankton biomass, but the addition of P and the second limiting nutrient would result in more biomass than P alone.

A secondary nutrient limitation is different from a "colimitation" (Saito *et al.*, 2008). In a "strict colimitation," the primary and secondary limiting nutrients are in equally low supply so that simultaneous increases of both are needed in order to increase biomass, and an increase in one nutrient without an increase in the other will have no effect on biomass (Elser *et al.*, 2009).

Another type of colimitation occurs when one nutrient has a biochemical dependence on another. For example, growth on nitrate is dependent on Fe and molybdenum (Mo) co-factors because nitrate must be intracellularly reduced to ammonia in order to build nitrogenous organic molecules (Flores & Herrero, 2005; Saito *et al.*, 2008). In waters with low Fe and/or Mo and low levels of ammonium regeneration, a Fe/Mo and N colimitation will occur because nitrate will be unable to be reduced and unavailable for growth. Cyanobacteria have an advantage in low Fe waters due to their high ability to scavenge low concentrations of Fe using siderophores (Sorichetti, Creed & Trick, 2014; Sorichetti *et al.*, 2016). Diazotrophic cyanobacteria have an additional need for Fe as a co-factor for the nitrogenase enzyme used in N-fixation. N-fixation occurs in specialized cells called heterocytes (Yema, Litchman & de Tezanos Pinto, 2016), and heterocyte differentiation is dependent on boron (B) for the synthesis of the cell wall (Bonilla, Garcia-González & Mateo, 1990). Furthermore, B is required by diatoms for cell wall formation (Lewin, 1966). Also, nutrient-poor waters can have cascading effects when the primary limiting nutrient is increased, such as enrichment of P (primary) resulting in a drawdown of Fe (secondary) to a level that N metabolism becomes colimited (North *et al.*, 2007).

The central basin of Lake Erie, which has been considered P-limited (Twiss *et al.*, 2005; Moon & Carrick, 2007), commonly has blooms of the diazotrophic cyanobacterium *Dolichospermum* in late June to early July (Chaffin *et al.*, 2019). *Dolichospermum* and other N-fixing taxa are usually associated with N-depleted, P-rich waters (i.e., low N to P ratios) (Smith, 1983); however, *Dolichospermum* blooms in the central basin of Lake Erie occur in waters with high nitrate concentrations (> 50 µmol/L) and low total P (< 0.5 µmol/L total P) (Chaffin *et al.*, 2019). There are many other examples of *Dolichospermum* blooms in lakes with high N availability (as reviewed by Li, Dreher & Li (2016)). The dominance of an N-fixer in high nitrate waters provides evidence that nutrients besides P and N may be selecting for *Dolichospermum* over other phytoplankton. Furthermore, *Dolichospermum* filaments in the central basin of Lake Erie lack heterocytes (Chaffin personal observation, see results), suggesting another possible nutrient deficiency.

The goal of this research was to provide insights into why diazotrophic cyanobacterium blooms in the N rich, P poor waters of the central basin of Lake Erie, with specific attention to the role of nutrients beside P constraining phytoplankton growth, ambient nitrate assimilation, and heterocyte number (heterocyte expression in *Dolichospermum* is a good proxy for N-

fixation; Yema *et al.* (2016)). Nutrient enrichment bioassays were conducted with enrichments of P and combination enrichments of P, N (nitrate and ammonium), Fe, Mo, and B. In this study, we assumed P to be the primary limiting nutrient and concluded a secondary nutrient limitation was occurring if the combination enrichment resulted in higher biomass, nitrate assimilation, or heterocyte number than the P-only enrichment. In the final year of this study, we replaced the Mo enrichment with Si because Si has been documented to be a constraining factor for diatoms in the central basin (Moon & Carrick, 2007). We hypothesized that phytoplankton growth and nitrate assimilation would be stimulated by the addition of P, indicating a primary limitation of P. We hypothesized that growth would be further stimulated by the combination enrichments of P with N, and P with Fe, Mo, and B, and that nitrate assimilation would be further stimulated by combination enrichments of P with Fe and Mo.

2 Methods

2.1 Water collection sites and methods

Surface water was collected from a sample location that was 20-m deep and 25 km north of the city of Avon Lake, Ohio, USA 17 times between 24 June 2014 and 25 August 2017 (Fig. 1). Additional experiments were conducted with water collected about 17 km north of Huron, Ohio, USA, on 8 July 2014 and at Fairport Harbor on 13 July 2016 in response to a *Dolichospermum* surface bloom reported by a local agency. Surface water was collected with a P-free-detergent-cleaned 19 L bucket and poured into clean a 30-L or 45-L carboy. The bucket and carboys were first rinsed three times with lake surface water before filled for experimental water. The carboy was covered with a dark towel while in transportation back to the laboratory, which took between 2 and 6 hours. Water temperature was recorded with a YSI 6600v2 or EXO2 multi-probe sonde. Other limnological parameters were measured and presented in a parallel study (Chaffin *et al*., 2019). Field filtered (0.45 µm) water samples from a depth of 1.5 m were collected with a trace metal-free Kemmerer sample for analysis of total dissolved iron (Fe), molybdenum (Mo), and boron (B) concentrations (as in Chaffin *et al*., 2019).

Upon returning to the laboratory, the carboy was inverted 20 times to mix plankton, and 2 L was poured from the carboy for initial samples of chl *a*, dissolved concentrations of nitrate,

nitrite, ammonium, dissolved reactive phosphorus (DRP), silicate, and total dissolved concentrations of Fe, Mo, and B (analytical methods below).

2.2 Enrichment experiments methods

Nutrient enrichment bioassays were conducted to determine the effects of nutrient enrichment on chl *a*, phytoplankton biovolume, and nitrate uptake. In this study, we assumed that P was the primary limiting nutrient of phytoplankton growth, and we tested for secondary limitations of other nutrients. Ten different treatments were conducted during this investigation, but not every treatment was executed in each trial (Supplemental Table 1). The following treatments were conducted in every experiment: a no enrichment control, phosphate (+P), ammonium-only enrichment ($+NH_4^+$), phosphate and ammonium ($+P\&NH_4^+$), phosphate and ferric iron (+P&Fe), phosphate and boron (+P&B), and a treatment including all above nutrients (+All). In most experiments, phosphate and molybdenum (+P&Mo), and phosphate and nitrate ($+P\&NO_3^-$) enrichments were conducted. In the final year of the study (2017), the +P&Mo enrichment was substituted for phosphate and silicate (+P&Si) enrichment. All stock solutions were made with grade ACS certified chemicals. While ACS chemicals contain trace levels of Fe (<0.005%), the concentrations of Fe added inadvertently to the non +P&Fe treatment were 2 to 3 orders of magnitude lower (< 0.1 nmol/L, Supplemental Table 2) than concentrations known to stimulate phytoplankton growth (20 nmol/L, Twiss, Auclair & Charlton (2000)). Only the +P&Si treatment (6.4 nmol/L Fe) could have added enough impure Fe to result in a response. Enrichments of only Fe, Mo, B, and Si were not conducted and any difference between the +P and P with a secondary nutrient was concluded to be due to the presence of the secondary nutrient. The $+NH_4^+$ treatment was conducted to determine if N was a primary limiting nutrient.

To commence the experiments, clear polycarbonate 1-L bottles were rinsed with sample water from the carboy and then filled with sample water that was passed through a 300-µm mesh to remove large zooplankton. The bottles were filled in random order, and the carboy was inverted 20 times after filling every fourth bottle to ensure consistency. After filling all bottles, the carboy was emptied, cleaned with phosphate-free detergent, and stored with deionized water. All treatments were replicated with three separate 1-L bottles, and up to nine different treatments were tested per experiment. Nutrient enrichments were as follows: phosphate 1 µmol/L (KH_2PO_4), ammonium 25 µmol/L (NH_4Cl), nitrate 25 µmol/L ($NaNO_3$), iron 0.5 µmol/L

($FeCl_3$), molybdenum 0.1 µmol/L ($NaMoO_4$), boron 0.5 µmol/L (HBO_3), and silicate 25 µmol/L ($NaSiO_3$). The 1 µmol/L phosphate enrichment was selected because that approximately doubled the total P concentration we measured during the 2013 central basin bloom, the year prior to the onset of this study (Chaffin *et al*., 2019). The 25 N µmol/L enrichments were selected to have a 25:1 N:P ratio. All bottles were incubated in a temperature- and light-controlled chamber (Geneva Scientific) that was programmed to the surface water temperature recorded at the time of collection and under a light intensity of 300-350 µmol photons/m^2/s (which matches the mean light intensity throughout the mixing depth at site Avon) on light:dark cycles to match sunrise and sunset. Bottles were inverted once daily throughout incubation. Experiments were terminated after 7 days or earlier if there was a noticeable color difference among treatments, and final samples were collected for chl *a*, dissolved nutrient concentration, and phytoplankton biovolume.

2.3 Sample analysis

To measure chl *a* concentration, between 250 and 800 mL of water (depending on biomass) was filtered onto GFF filters (0.7 µm pore). The filters were stored on silica gel at -80°C until analysis. Chl *a* was extracted from the filters with dimethyl sulfoxide, centrifuged, and quantified by spectrophotometry (Golnick *et al.*, 2016).

To measure ambient concentrations of dissolved nitrate, nitrite, ammonium, phosphate, and silicate, a PETG bottle was rinsed twice with 10-mL of filtered (<0.45 µm) sample, and then a 30-mL sample was filtered into the PETG and was either stored frozen at -20°C or analyzed right away on a SEAL Analytical QuAAtro nutrient auto-analyzer, as in Chaffin *et al*. (2019). To measure concentrations of total dissolved Fe, B, and Mo, 50-mL Falcon tubes were rinsed with two 10-mL aliquots of filtered sample and then filled with 50 mL of filtered sample. Total dissolved Fe, Mo, and B concentrations were determined on acidified (2.0% nitric acid) samples by ICP-MS (Xseries 2, Thermo Scientific, MA, USA), as in Chaffin *et al*. (2019). Field blanks were conducted every tenth sample to check for contamination due to sample handling, filtering, and storage.

Phytoplankton in a 100-mL sample was preserved with 2% formalin and stored in the dark until analysis. In some experiments, equal 10-mL aliquots from each replicate were pooled and phytoplankton quantified with a FlowCam at 100X magnification, as in Chaffin *et al.*

(2018). 8,000 particle images were captured per sample. The FlowCam enumerates particles per mL and measures area (a 2-dimensional measurement) by collapsing all pixels of a particle into a circle, called area-based diameter (ABD). The particle area can be converted to biovolume if the relationship between area and volume are known. A recent study showed that the FlowCam method and traditional microscopy-based biovolume measurements had a very good agreement for filamentous cyanobacteria (like *Dolichospermum* and *Cuspidothrix*) and diatoms but poorer relationships for green algae and chrysophytes (Hrycik, Shambaugh & Stockwell, 2019). Due to the diverse phytoplankton community and because biovolume could not be determined for all taxa observed, total ABD was used as a surrogate for total phytoplankton biovolume, which was normalized to the volume of sample imaged (μm^2/mL). When diazotrophic cyanobacteria were present in the experiments (*Dolichospermum* and *Cuspidothrix*), those taxa were quantified separately, and each replicate was analyzed separately. Biovolume of *Dolichospermum* and *Cuspidothrix* were calculated from areal colony measurement assuming cylinder shape of the filament. Then the diazotrophic taxa were separated between filaments with and without a heterocyte (Fig. 2).

2.4 Data analysis

The data were analyzed to answer the questions "Did nutrient enrichment result in higher phytoplankton biomass (as both chl *a* and biovolume) and lower nitrate concentrations than the control" and "Did enrichment of P with trace nutrients or P with N result in higher phytoplankton biomass and lower nitrate concentrations than the +P only enrichment?" The first of these questions was asked to determine the primary limiting nutrient for phytoplankton growth and nitrate assimilation, while the second question was asked to determine the secondary limiting nutrient with the assumption that P was the primary limiting nutrient. The normality of data was tested for with the Shapiro-Wilk and non-normal data was log transformed (11 of 19 experiments required transformation). Homogeneity of variances was tested for with Levene's test and differences among treatments were determined with one-way ANOVA and the Brown-Forsythe test, which uses variances around the median, was used when variances were not equal. Tukey test and differences were considered significant at $p < 0.05$. To summarize the chl *a* and nitrate concentration results, the data are presented as the percent of experiments in which the nutrient enrichment treatments resulted in differences from control and from the +P-only enrichment. The

+P&NO_3^- treatment was excluded from the nitrate data analysis because nitrate was added to this treatment. The experiments that had an initial ambient nitrate concentration of less than 5 µmol/L were also excluded from the nitrate data analysis because treatments with enriched P resulted in final nitrate concentrations below detectable concentrations (< 0.2 µmol/L).

Heterocytes were counted in experiments that had more than 100 filaments of a diazotrophic cyanobacterium imaged by the FlowCam per replicate sub-sample. Only the control, +P, +P&NH_4^+, +P&Fe, and +P&B treatments had heterocytes quantified. Filaments with heterocytes were quantified separately from filaments of the same taxa without heterocytes (Fig. 2). An ANOVA with a post-hoc Tukey test was used to determine differences among treatments.

3 Results

3.1 Ambient conditions

The initial chl *a* concentrations were less than 4.0 µg/L for 17 of the 19 experiments conducted (Table 1). The two experiments with the highest initial chl *a* concentration were collected when *Dolichospermum* was concentrated at the surface. Ambient initial NO_{2+3}^- concentrations ranged from 2.73 to 36.71 µmol/L. June and July had the highest concentrations, whereas August had lower concentrations. The initial ammonium, DRP, and total dissolved Fe (TDFe) were below detection for most experiments. TDB concentrations ranged from 1.325 to 2.327 µmol/L, and concentrations were slightly higher in 2017 than in 2016. TDMo concentrations ranged from 0.0110 to 0.0172 µmol/L and did not show a seasonal pattern. Total P concentrations ranged from 0.13 to 0.56 µmol/L, and the TN:TP molar ratio range from 63.1 to 409.2, indicating a high N environment.

3.2 Enrichment effects on chl and phytoplankton biovolume

Nutrient enrichment resulted in significant differences of chl *a* concentration among treatments in all 19 experiments (Table 2). In all experiments, the control and +NH_4^+ treatments had the lowest chl *a* concentrations (or were not significantly different from the lowest chl *a* values observed; Table 3). In general, the combination enrichments of P and N or P with a trace nutrient resulted in the highest chl *a* concentrations, and when chl *a* showed a positive response to +P&NH_4^+, there was a similar positive response to the +All treatment. Chl *a* response of the +P ranged from no response (grouping with the control and +NH_4^+), the highest response, or

intermediate response. For example, the +P treatment resulted in an intermediate chl *a* response in the first experiment conducted on 24 June 2014.

To summarize the data from the 19 experiments, the percentage of experiments with treatment averages that were significantly greater than control and greater than the +P enrichment was calculated. The +P enrichment resulted in significantly higher chl *a* concentration than the control in 73.7% of the experiments (Table 4). The $+NH_4^+$ enrichment increased chl *a* concentration in 26.3% of experiments. Enrichments of P with trace nutrients (+P&Fe, +P&Mo, +P&B) increased chl *a* concentrations in 66.7-84.6% of experiments, and P with N enrichment ($+P\&NH_4^+$, $+P\&NO_3^-$, "+All" treatments) increased chl *a* concentrations in 100% of the experiments. Overall, these results indicated that P was the primary growth-limiting nutrient for the central basin chl *a* production.

The $+NH_4^+$ enrichment did not result in more chl *a* than the +P enrichment in any experiment, but the P and N combination enrichments ($+P\&NH_4^+$, $+P\&NO_3^-$, "All" treatments) enrichment resulted in significantly higher chl *a* concentration than the +P enrichment in 47.4%-50.0% of the experiments (Table 3). The +P&Fe, +P&Mo, +P&B enrichments resulted in significantly higher chl *a* concentration than the +P enrichment in 26.3%, 7.6%, and 15.8% of the experiments, respectively. The +P&Si treatment conducted in 2017 did not result in higher chl *a* concentrations than +P in all six experiments. These results indicated that there were times when central basin phytoplankton needed N and trace nutrients, in addition to P, to reach the highest chl *a* concentrations, but there was no apparent seasonal pattern to secondary limitation.

Phytoplankton biovolume was measured in 12 experiments during the years 2015, 2016, and 2017 (Fig. 3). Phosphorus enrichment increased biovolume in nine of the 12 experiments (+P and control were similar in three experiments (Figs. 3F, 3J, and 3L)). Phosphorus and N enrichments ($+P\&NH_4^+$, +All, $+P\&NO_3^-$) resulted in more biovolume than +P in three of the 12 experiments (Figs. 3F, 3I, 3K). There were no experiments when a P and Fe, Mo, Si, or B enrichment resulted in more biovolume than the +P enrichment.

The results of a secondary limitation by N on chl *a* and biovolume did not agree for most experiments. Secondary limitation of chl *a* concentration by N was displayed in ~50% of experiments, but only 25% of the experiments did a P and N enrichment result in greater biovolume than +P. Phytoplankton biovolume significantly increased with increased chl *a* concentration ($p < 0.001$; Fig. 4) and chl *a* explained 50.3% of the variation; however, within this

variability range, a nutrient enrichment could result in a significant increase of chl *a* but no effect on biovolume. ANCOVA showed that there was no difference in the chl-biovolume relationship between the treatments with N and those without N ($p = 0.129$). There was no interaction between chl *a* concentration and treatments (with N, without N) on the chl-biovolume relationship ($p = 0.893$).

3.3 Nitrate assimilation

Nitrate concentrations were measured to determine if enrichments would stimulate nitrate assimilation (lower nitrate concentration suggests more assimilation). Seventeen experiments had an initial ambient nitrate concentration greater than 5 µmol/L (Table 1). There were significant differences among treatments in all 17 experiments (Table 3). The +P enrichment and the P with trace nutrients but without ammonium (+P&Fe, +P&Mo, +P&B, +P&Si) resulted in significantly lower ambient nitrate concentrations than the control in 92%-100% of all experiments (Table 4), which suggests that P simulated nitrate assimilation. The enrichments with ammonium ($+NH_4^+$, $+P\&NH_4^+$, and +All) resulted in lower ambient nitrate concentration than the control in 0%, 11.8%, and 5.8% of the experiments, respectively, which suggests that phytoplankton assimilated the enriched ammonium rather than ambient nitrate. When nitrate concentrations were compared to the +P enrichment, enrichments of the trace nutrients did not further decrease the ambient nitrate concentration in any experiment (Table 4). This suggests that nitrate assimilation was not increased by the addition of Fe, Mo, or B.

3.4 Diazotrophic cyanobacteria

There were five experiments with quantifiable diazotrophic cyanobacteria. *Dolichospermum* was present in four of the experiments (the four conducted during July) and *Cuspidothrix* was present in the experiment that started on 10 August 2015. Initially, the percentage of diazotrophic cyanobacterial biovolume with a heterocyte in the colony or filament ranged from 0% to 23%, indicating the majority of cyanobacteria in the central basin were not fixing N_2. Significant differences in heterocyte numbers among treatments occurred in three of the five experiments (Fig. 5A, C, and E; Table 6). In two of the three significant experiments, +P increased the number of heterocytes (compared to the control; Fig. 5C and E) and was nearly significant in the third (Fig. 5A). In all experiments, $+P\&NH_4^+$ had a similar number of

heterocytes as the control – which suggests that ammonium inhibited heterocyte formation. The +P&Fe had a similar number of heterocytes as the +P treatment. The +P&B showed increased heterocytes compared to the +P treatment in the 6 July 2015 experiment (Fig. 5A) and nearly significant in another (Fig. 5E). Regarding diazotrophic biovolume, +P significantly or nearly significantly increased biovolume over the control and initial levels, but the +P&NH_4^+, +P&Fe, and +P&B were not significantly different from +P (Fig. 5B, D, F, H, J).

4 Discussion

The major finding of this research was that phytoplankton biomass (both as chl *a* and biovolume) in the central basin of Lake Erie was primarily limited by P availability from 2014 to 2017 throughout the growing seasons. In a diverse phytoplankton community, it is possible that one nutrient limits one taxon and another nutrient limits others (Lewis, Wurtsbaugh & Paerl, 2011). However, this research showed that P was the primary limiting nutrient for the total phytoplankton community and cyanobacteria during bloom conditions. Additionally, the strong responses of chl *a* and total phytoplankton biovolume to +P relative to the controls in 73.7% and 75% of the experiments overall, respectively, and low response rate to +NH_4^+ suggests that the central basin had a single primary limiting nutrient more frequently than multiple limiting nutrients. A similar study conducted during the early 2000s by Moon & Carrick (2007) showed that P was the primary limiting nutrient, and they also proposed that the central basin had been P-limited since P-abatement programs were enacted in the early 1980s (DePinto, Young & McIlroy, 1986). Collectively, this study and previous studies indicate that central basin phytoplankton growth remained P-limited throughout the onslaught of numerous stressors including the *Dreissena* mussel invasion (Nicholls, Hopkins & Standke, 1999), increasing summer-time hypoxia (Zhou *et al.*, 2013), and the eastward spread of western basin cyanobacterial blooms (Michalak *et al.*, 2013; Chaffin *et al.*, 2019).

Secondary limitation of chl *a* production by N (+P&NH_4^+, +All, +P&NO_3^-) was displayed in 50% of experiments, but only 25% of the experiments did a P and N enrichment result in greater biovolume than +P. Likewise, Moon & Carrick (2007) reported secondary N limitation in 47% of their experiments. Secondary limitations of Fe, Mo, and B were less frequent (<25% of experiments) for chl *a* concentration and were not apparent for phytoplankton biovolume. Collectively, these results confirm that P is the main limiting driver of phytoplankton

biomass in the central basin of Lake Erie, but there were times when N, and less frequently Fe and B, were needed in addition to P to achieve the greatest biomass.

Recent bioassay experiments showed that Lake Erie's western basin chl *a* and phytoplankton biovolume (Chaffin, Bridgeman & Bade, 2013; Chaffin *et al.*, 2014, 2018) were primarily limited by P during early summer and then primarily limited by N during late summer and fall. The ambient concentration of dissolved inorganic N (the sum of nitrate, nitrite, and ammonium) dictated which nutrient was limiting because enrichments of N alone significantly increased chl *a* concentration only when dissolved inorganic N concentration was less than 10 µmol/L (Chaffin *et al.*, 2014). Additionally, phytoplankton response to N or P enrichment for 90 experiments conducted in Europe and western United States showed similar results with respect to ambient N (Elser *et al.*, 2009). Chl *a* production in the central basin experiments did not show a similar threshold response to ambient N concentration because several experiments showed a secondary N limitation even when ambient nitrate concentrations were greater than 10 µmol/L. For example, the experiments on 3 September 2014, 2 June 2016, and 6 July 2016 showed a secondary N limitation, and ambient initial nitrate concentration ranged from 17.3 to 36.7 µmol/L. However, all central basin experiments with an ambient nitrate concentration less than 12 µmol/L showed a secondary N limitation of chl *a*. Denitrification, nitrate assimilation, and anammox are likely the main drivers of the low ambient nitrate concentrations and the corresponding N limitation (Scott *et al.*, 2019; Boedecker *et al.*, 2020; Loeks-Johnson & Cotner, 2020).

The overall effects of Fe and Mo on chl *a*, phytoplankton biovolume, and nitrate assimilation were low to none. These results agree with a study conducted ~15 years prior by Twiss *et al.* (2005) that concluded that the frequency of Fe limitation was low in the central basin. Additionally, Sterner *et al.* (2004) showed that the combination enrichments of P and Fe resulted in greater biomass than P alone in the oligotrophic water of Lake Superior. Fe is a critical co-factor for nitrate assimilation (Flores & Herrero, 2005), but Fe enrichment did not further increase ambient nitrate assimilation in any experiment, which suggests that the Fe stimulation effect on chl *a* observed in 26.3% experiments was not due to increased availability of nitrate. Furthermore, the +P&NO_3^- enrichment increased chl *a* to greater levels than +P in 50% of the experiments, which suggests that the ambient Fe and Mo concentrations were adequate to support nitrate assimilation. Fe is a requirement for several steps in chl biosynthesis

(Beale, 1999), which might help explain the discrepancy between the chl *a* and biovolume Fe secondary limitation. However, North *et al.* (2007) provided an example when additions of Fe increased ambient nitrate assimilation in the eastern basin of Lake Erie. The eastern basin is oligotrophic and furthest from the nutrient-rich tributaries that flow into the western basin. Collectively, these studies suggest that the likelihood of Fe limitation in the mesotrophic central basin is less than that of more oligotrophic waters of the Great Lakes.

Dolichospermum is the dominant colony-forming cyanobacteria in the central basin during early summer (June and July), yet its dominance is unexpected due to relatively high concentrations of nitrate (Chaffin *et al.*, 2019). In two experiments conducted during *Dolichospermum* blooms (6 July 2015 and 13 July 2016), the +P&Fe treatment resulted in higher chl *a* concentration than the +P treatment, which suggests Fe could have been a secondary limiting nutrient for phytoplankton growth at these times. In the lake, low Fe availability would have limited the growth of green algae and diatoms and gave the cyanobacteria a competitive advantage because they are more competitive for Fe (Sorichetti *et al.*, 2014, 2016). Increased Fe alleviated the Fe limitation of green algae and diatoms, and allowed them to increase chl *a* concentration. Furthermore, *Dolichospermum* had heterocytes at the start of these two experiments, further suggesting that ambient nitrate was not available, because *Dolichospermum* will not produce heterocytes if it is growing on nitrate (Yema *et al.*, 2016). The colimitations of Fe and N could have selected for *Dolichospermum* dominance over eukaryotic algae and *Microcystis* in the central basin of Lake Erie. Similar nutrient limitations cascades (P, N, Fe) could be playing out in other oligotrophic waters of North America and Europe that have experienced *Dolichospermum* blooms (Carey, Weathers & Cottingham, 2008; Callieri *et al.*, 2014; Salmaso *et al.*, 2015).

The stimulation of chl *a* by B enrichment was somewhat surprising, but not necessarily novel. Heterocytous cyanobacteria require B for heterocyte envelope development to prevent oxygen diffusion into the cell (Mateo *et al.*, 1986; Bonilla *et al.*, 1990; Bolaños *et al.*, 2004). Enrichments of P with B increased the percentage of *Dolichospermum* and *Cuspidothrix* that contained a heterocyte more so than the +P enrichment, but B enrichment did not stimulate the growth of either cyanobacterium over the short duration of the experiments. However, a mesocosm experiment in Lake Erken (Sweden) showed that enrichments of P, N, Fe, and B resulted in a greater abundance of the heterocytous cyanobacterium *Gloeotrichia* than did

enrichments without B (Hyenstrand *et al.*, 2001). Additionally, P and B enrichment increased chl *a* concentrations to higher levels than +P treatments in 15.8% of the experiments overall. It has been known for several decades that diatoms require B for silica metabolism and siliceous cell wall formation (Lewin, 1966; Healey, 1973).

The results about B led to the question, 'Is B a possible growth-limiting nutrient in freshwater?' In waters with pH less than 9.24 (central basin pH < 9) B occurs as the highly soluble $B(OH)_3$ (Parks & Edwards, 2005). Marine waters have an average B concentration of 425 µmol/L (Parks & Edwards, 2005), but B concentration in freshwater is several orders of magnitude less with an average of 2.7 µmol/L (Frey *et al.*, 2005). Moreover, surface waters in the eastern US have lower B concentrations than western waters (Frey *et al.*, 2005), which is likely due to the prevailing westerly winds and the distance from the Pacific Ocean. Thus, waters further from the Pacific coast but not influenced by the Atlantic Ocean could be more prone to a potential B colimitation, and B concentrations measured in the central basin during 2016 and 2017 were lower than the United States surface water average. While the overall effect of B was rather low in this study, there may be times and places when B may be important for N-fixing cyanobacteria. Currently, there is a debate amongst limnologists whether or not N-fixation can compensate for an N deficiency (Scott & McCarthy, 2010; Paterson *et al.*, 2011). Further investigation is needed to determine if B may be an underlying mechanism for that disagreement.

Chlorophyll *a* concentration (either fluorescence or filter-extracted) is a standard metric to indicate biomass in nutrient enrichment bioassay studies. In this study, we observed chl *a* concentration indicating N secondary limitation in half of the experiments, but biovolume indicated N secondary limitation in only 25% of the experiments, and there was a weak correlation between chl *a* and biovolume. Chlorophyll is an N-rich molecule, and the phytoplankton in our experiments increased chl *a* content per cell disproportionately when enriched with N, which has been shown elsewhere when N limitation was alleviated (Krasikov *et al.*, 2012; Harke & Gobler, 2015; Wagner *et al.*, 2019). Additionally, the discrepancy could be due to photo-acclimation as the phytoplankton altered chl *a* content per cell in response to the new light regime of the incubator (MacIntyre *et al.*, 2002). Therefore, a flawed conclusion could be drawn from the N enrichments if biovolume had not been measured. Future bioassay experiments are highly recommended to include measurements of biovolume.

In conclusion, this 4-year project showed that P was the primary limiting nutrient of phytoplankton growth in the central basin of Lake Erie. However, combination enrichments of P and N resulted in higher chlorophyll *a* concentration and phytoplankton biovolume than the P-only enrichment in 50% and 25% of experiments, respectively, which suggests that N was a secondarily constraining nutrient. Iron was secondarily limiting for chlorophyll *a* concentration at times of *Dolichospermum* blooms and suggested that low Fe availability may be a factor in selecting for *Dolichospermum* dominance during early summer in Lake Erie. Heterocytes in *Dolichospermum* and *Cuspidothrix* were also primarily constrained by P, but B had a secondary limiting effect. Additionally, B may also have a secondary limiting effect on diatoms. Overall, this research showed that P has remained the primary limiting nutrient in the central basin of Lake Erie despite increased cyanobacterial biomass and hypoxia, but other nutrients play secondary roles in constraining phytoplankton biomass. Moreover, these results could apply to other meso- to oligotrophic bodies of water with high N and low P concentrations (high N:P ratios) where diazotrophic cyanobacterial blooms occur.

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Tables

698 Table 1. Initial concentrations of chl *a* (µg/L) and nutrients (µmol/L) and incubation temperature (°C). The abbreviations are defined
699 in the text. ND = No data. Values with a < symbol indicate concentrations below the method detection limit.

Site	Date	Chl *a*	NO_{2+3}^-	NH_4^+	DRP	Si	TDFe	TDB	TDMo	TP	TN	TN:TP	Temp °C
Avon	24 Jun '14	1.7	19.65	<0.55	<0.04	1.62	<0.005	ND	ND	0.56	35.29	63.14	20.2
SOFF	8 Jul '14	3.4	26.56	<0.55	<0.04	7.56	<0.005	ND	ND	0.23	51.18	220.59	22.6
Avon	29 Jul '14	2.5	7.56	<0.55	<0.04	2.43	<0.005	ND	ND	0.23	25.10	111.06	21.7
Avon	3 Sep '14	2.5	17.31	3.23	0.14	3.80	<0.005	ND	ND	0.29	41.67	146.22	23.0
Avon	13 Jun '15	2.1	22.50	<0.55	<0.04	7.57	<0.005	ND	ND	0.30	41.87	141.94	16.9
Avon	6 Jul '15	3.8	15.80	0.89	<0.04	35.78	<0.005	ND	ND	0.54	35.15	64.98	20.9
Avon	10 Aug '15	3.1	11.41	<0.55	<0.04	6.10	<0.005	ND	ND	0.38	30.72	80.84	23.7
Avon	2 Jun '16	0.3	24.66	<0.55	<0.04	7.35	<0.005	1.481	0.0110	0.13	53.61	409.21	17.3
Avon	25 Jun '16	1.1	22.68	<0.55	<0.04	9.33	<0.005	1.456	0.0111	0.14	35.94	249.55	21.3
Avon	6 Jul '16	2.8	36.71	<0.55	<0.04	4.87	<0.005	1.365	0.0138	0.24	50.51	214.00	23.1
FPH	13 Jul '16	6.9	32.08	<0.55	<0.04	6.92	<0.005	1.535	0.0140	0.30	54.11	182.19	26.6
Avon	19 Jul '16	2.7	28.05	<0.55	<0.04	6.99	<0.005	1.325	0.0117	0.25	48.83	192.23	24.6
Avon	26 Aug '16	3.9	2.73	<0.55	<0.04	5.06	<0.005	1.478	0.0122	0.29	24.08	81.90	25.2
Avon	2 Jun '17	1.5	9.51	<0.55	<0.04	1.39	<0.005	1.695	0.0146	0.23	25.46	112.67	15.5
Avon	21 Jun '17	1.1	19.94	<0.55	<0.04	9.08	<0.005	1.927	0.0128	0.36	38.89	108.93	20.5
Avon	3 Jul '17	2.0	24.43	<0.55	<0.04	8.61	<0.005	1.932	0.0127	0.19	45.10	237.38	21.7
Avon	11 Jul '17	14.1	16.11	<0.55	<0.04	2.13	<0.005	1.921	0.0130	0.32	41.24	129.26	22.1
Avon	31 Jul '17	2.1	5.58	<0.55	ND	9.28	<0.005	1.924	0.0172	0.29	31.73	109.81	23.7
Avon	25 Aug '17	3.0	2.79	<0.55	<0.04	4.64	<0.005	2.327	0.0129	0.35	26.89	77.27	22.9

Table 2. Summary of statistics for final chlorophyll concentrations for 17 experiments conducted at site Avon and one experiment each from site SOFF and Fairport Harbor (FPH). All significant P values are in italics. Log transformations were used when the test for normality failed ($P < 0.05$). The Brown-Forsythe ANOVA P value was used when the test for equal variances failed ($P < 0.05$), and the P value used from the ANOVA is bolded. The between group degrees of freedom was 7 for the first three experiments conducted during 2014 and 8 for the rest of the experiments.

		Normality Test		Homogeneity of Variances		ANOVA		
		Shapiro-Wilk		Levene			ANOVA	Brown-Forsythe
Date	Site	Statistic	P value	Statistic	P value	F value	P value	P value
24 June 2014	Avon	0.955	0.518	3.083	*0.029*	49.205	*<0.001*	***<0.001***
8 July 2014	SOFF	0.856	*0.010*	4.373	*0.007*	139.562	*<0.001*	***<0.001***
29 July 2014	Avon	0.909	0.084	4.824	*0.004*	191.289	*<0.001*	***<0.001***
3 Sept.2014	Avon	0.914	0.103	3.711	*0.010*	38.477	*<0.001*	***<0.001***
13 June 2015	Avon	0.844	*0.007*	2.937	*0.027*	17.206	*<0.001*	***<0.001***
6 July 2015	Avon	0.919	0.125	1.495	0.227	35.761	***<0.001***	*<0.001*
10 August 2015	Avon	0.670	*<0.001*	3.297	*0.017*	16.693	*<0.001*	***0.001***
2 June 2016	Avon	0.902	0.063	6.858	*<0.001*	20.736	*<0.001*	***0.013***
25 June 2016	Avon	0.921	0.133	4.367	*0.004*	14.014	*<0.001*	***0.002***
6 July 2016	Avon	0.792	*0.001*	4.049	*0.007*	31.828	*<0.001*	***0.003***
13 July 2016	FHP	0.850	*0.009*	6.334	*0.001*	56.088	*<0.001*	***0.002***
19 July 2016	Avon	0.803	*0.002*	7.185	*<0.001*	64.911	*<0.001*	***<0.001***
26 August 2016	Avon	0.690	*<0.001*	3.272	*0.017*	49.985	*<0.001*	***0.001***

2 June 2017	Avon	0.756	*<0.001*	2.233	0.075	388.326	***<0.001***	*<0.001*
21 June 2017	Avon	0.874	*0.020*	1.479	0.233	160.002	***<0.001***	*<0.001*
3 July 2017	Avon	0.921	0.136	9.300	*<0.001*	13.549	*<0.001*	***0.035***
11 July 2017	Avon	0.926	0.163	4.997	*0.002*	13.606	*<0.001*	***0.003***
31 July 2017	Avon	0.726	*<0.001*	3.366	*0.015*	15.188	*<0.001*	***0.001***
25 August 2017	Avon	0.747	*<0.001*	4.562	*0.004*	15.855	*<0.001*	***0.001***

Table 3. Summary of the effects of nutrient enrichment on chl *a* concentration for 19 experiments. The mean chl *a* concentration for each treatment are ordered lowest to highest from left to right, and treatments joined by an underline were not significantly different as determined by Tukey test. Site abbreviations are as follows: A = Avon, S = SOFF, F = Fairport Harbor. Initial ambient nitrate concentration (µmol/L), incubation temperature (°C), and duration of incubation in days (d) are listed.

		Mean chl *a* and Tukey Test	Ambient		
Date	Site	Lowest Highest	Nitrate	Temp	d
24 June '14	A	NH_4^+ C PNH_4^+ PMo ALL P PFe PB	19.65	20.2	7
8 July '14	S	C NH_4^+ PFe ALL P PMo PB PNH_4^+	26.56	22.6	4
29 July '14	A	C NH_4^+ P PMo PFe PB PNH_4^+ ALL	7.56	21.7	7
3 Sept '14	A	C NH_4^+ P PFe PMo PB PNO_3^- PNH_4^+ ALL	17.31	23.0	7
13 June '15	A	C NH_4^+ P PNH_4^+ PFe PB PMo ALL PNO_3^-	22.50	16.9	7
6 July '15	A	C NH_4^+ P PNO_3^- PMo PB ALL PNH_4^+ PFe	15.80	20.9	4
10 Aug. '15	A	NH_4^+ P PB PFe PMo C PNH_4^+ PNO_3^- ALL	11.41	23.7	7
2 June '16	A	C P NH_4^+ PNO_3^- PB PMo PFe PNH_4^+ ALL	24.66	17.3	6
25 June '16	A	C NH_4^+ PFe PMo ALL PNO_3^- PB P PNH_4^+	22.68	21.3	6
6 July '16	A	C NH_4^+ PB P PFe PNO_3^- PMo ALL PNH_4^+	36.71	23.1	5
13 July '16	F	C NH_4^+ P PNH_4^+ PMo ALL PFe PB PNO_3^-	32.08	26.6	5
19 July '16	A	C NH_4^+ PNH_4^+ PMo PNO_3^- PB ALL P PFe	28.05	24.6	6
26 Aug. '16	A	PMo PFe NH_4^+ P PB C PNO_3^- PNH_4^+ ALL	2.73	25.2	3
2 June '17	A	C NH_4^+ PSi PFe PB P PNO_3^- PNH_4^+ ALL	9.51	15.5	7
21 June '17	A	C NH_4^+ PFe ALL PSi PNO_3^- PB PNH_4^+ P	19.94	20.5	7
3 July '17	A	C NH_4^+ ALL PNH_4^+ PFe PNO_3^- PSi PB P	24.43	21.7	7
11 July '17	A	C NH_4^+ PNO_3^- PFe P PSi PB PNH_4^+ ALL	16.11	22.1	3
31 July '17	A	C PFe PB NH_4^+ PSi P PNH_4^+ PNO_3^- ALL	5.58	23.7	7
25 Aug. '17	A	P PSi PB C NH_4^+ PFe PNO_3^- ALL PNH_4^+	2.79	22.9	6

Table 4. Summary of results of nutrient enrichment experiments as the percentage of experiments that enrichment treatment resulted in significantly greater chl *a* concentration than the control and greater than the P-only enrichment as indicated by Tukey test and significantly lower nitrate concentration than the control and the P-only enrichment. The number of experiments conducted with each treatment is listed.

	Chl *a* concentration			Nitrate concentration		
Treatment	% > Control	% > +P	#	% < Control	% < +P	#
+P	73.7%	-	19	100.0%	-	17
+NH_4^+	26.3%	0.0%	19	0.0%	0.0%	17
+P&NH_4^+	100.0%	47.4%	19	11.8%	0.0%	17
+P&Fe	84.2%	26.3%	19	100.0%	0.0%	17
+P&Mo	84.6%	7.7%	13	91.7%	0.0%	12
+P&Si	66.7%	0.0%	6	100.0%	0.0%	5
+P&B	78.9%	15.8%	19	94.1%	0.0%	17
+All	100.0%	47.4%	19	5.9%	0.0%	17
+P&NO_3^-	100.0%	50.0%	16			

731 Table 5. Summary of statistics for final nitrate concentrations for experiments with initial nitrate concentration greater than 5 µmol/L.
732 15 experiments were conducted at site Avon and one experiment each from site SOFF and Fairport Harbor (FPH). All significant P
733 values are in italics. Log transformations were used when the test for normality failed (P < 0.05). The Brown-Forsythe ANOVA P
734 value was used when the test for equal variances failed (P < 0.05), and the P value used from the ANOVA is bolded. The between
735 group degrees of freedom was 7 for all experiments.

		Normality Test		Homogeneity of Variances		ANOVA		
		Shapiro-Wilk		Levene			ANOVA	Brown-Forsythe
Date	Site	Statistic	P value	Statistic	P value	F value	P value	P value
24 June 2014	Avon	0.688	*<0.001*	9.774	*<0.001*	7.998	<0.001	**0.038**
8 July 2014	SOFF	0.717	*<0.001*	5.346	*0.003*	628.896	<0.001	**<0.001**
29 July 2014	Avon	0.746	*<0.001*	4.909	*0.004*	37.974	<0.001	**0.001**
3 Sept. 2014	Avon	0.767	*<0.001*	1.293	0.315	100.954	**<0.001**	<0.001
13 June 2015	Avon	0.702	*<0.001*	9.907	*<0.001*	34.683	<0.001	**0.001**
6 July 2015	Avon	0.680	*<0.001*	14.843	*<0.001*	43.093	<0.001	**<0.001**
10 August 2015	Avon	0.695	*<0.001*	11.864	*<0.001*	10.175	<0.001	**<0.001**
2 June 2016	Avon	0.816	*0.001*	5.283	*0.003*	10.416	<0.001	**0.061**
25 June 2016	Avon	0.857	*0.003*	6.517	*0.001*	34.545	<0.001	**0.001**
6 July 2016	Avon	0.803	*<0.001*	6.218	*0.001*	35.491	<0.001	**0.001**
13 July 2016	FPH	0.791	*<0.001*	7.596	*<0.001*	150.424	<0.001	**<0.001**
19 July 2016	Avon	0.717	*<0.001*	7.855	*<0.001*	35.245	<0.001	**0.002**
2 June 2017	Avon	0.736	*<0.001*	9.269	*<0.001*	48.723	<0.001	**0.005**

21 June 2017	Avon	0.802	*<0.001*	6.251	*0.001*	60.479	<0.001	**0.004**
3 July 2017	Avon	0.811	*<0.001*	5.291	*0.003*	44.229	<0.001	**0.003**
11 July 2017	Avon	0.875	*0.006*	5.943	*0.002*	11.254	<0.001	**0.008**
31 July 2017	Avon	0.807	<0.001	13.031	<0.001	82.239	<0.001	**<0.001**

737 Table 6. Summary of statistics for five experiments with heterocystous cyanobacteria present. Four experiments were conducted at site
738 Avon and one experiment from Fairport Harbor (FPH). All significant P values are in italics. Log transformations were used when the
739 test for normality failed ($P < 0.05$). The Brown-Forsythe ANOVA P value was used when the test for equal variances failed ($P <$
740 0.05), and the P value used from the ANOVA is bolded. The between group degrees of freedom was 4 for all experiments.

			Normality Test		Homogeneity of Variances		ANOVA		
Parameter	Date	Site	Shapiro-Wilk Statistic	P value	Levene Statistic	P value	F	ANOVA P value	Brown-Forsythe P value
% Heterocysts	6 July 2015	Avon	0.878	0.044	2.473	0.112	13.122	***0.001***	*0.003*
Biovolume	6 July 2015	Avon	0.939	0.374	1.006	0.449	2.381	**0.121**	0.145
% Heterocysts	10 Aug. 2015	Avon	0.824	0.008	3.808	*0.039*	37.343	*<0.001*	***0.004***
Biovolume	10 Aug. 2015	Avon	0.929	0.264	2.871	0.080	2.583	**0.102**	0.155
% Heterocysts	6 July 2016	Avon	0.910	0.136	3.016	0.071	46.501	***<0.001***	*<0.001*
Biovolume	6 July 2016	Avon	0.907	0.123	4.217	*0.030*	2.011	0.169	**0.253**
% Heterocysts	13 July 2016	FPH	0.825	0.008	2.333	0.126	0.511	**0.729**	0.731
Biovolume	13 July 2016	FPH	0.911	0.139	2.959	0.075	0.682	**0.620**	0.632
% Heterocysts	11 July 2017	Avon	0.897	0.085	2.429	0.116	0.619	**0.659**	0.675
Biovolume	11 July 2017	Avon	0.969	0.839	2.092	0.157	5.522	***0.013***	0.023

742 **Data availability statement**

743 All data will be made available on Ohio Sea Grant's research website following publication at

744 https://ohioseagrant.osu.edu/research/live/water

745 **Conflict of Interest Statement**

746 All authors have no conflict of interests.

747

748 **Figures**

749

750 Figure 1. Map of Lake Erie showing water collection sites for the experiments. Site Avon,

751 marked by the star in the center of the map, was sampled 17 times, while the circled-X sites

752 SOFF and Fairport Harbor (FPH) were sampled just once. The inset map in the lower left shows

753 the entire Great Lakes basin.

754

755 Figure 2: FlowCam images (100x) of *Dolichospermum* with a heterocyte (marked by an arrow)

756 on the left and one without a heterocyte. Print readers are referred to the online copy for a color

757 image.

758

759 Figure 3: Post incubation chl *a* concentrations (bars) and phytoplankton biovolume (circles) of

760 12 enrichment experiments with both parameters were quantified. The values are the mean of

761 three replicates (± 1 standard error) or the measured value of three equal volume pooled aliquots

762 of the three replicates where error bars are not present.

763

764 Figure 4: Correlation between chl *a* and phytoplankton biomass concentration from the 12

765 experiments presented in Figure 2. Filled circles and the solid line are treatments that received

766 phosphorus but no nitrogen (+P, +P&Fe, +P&Mo, +P&B, +P&Si) and the open circles and the

767 dashed line are the treatments that received nitrogen ($+NH_4^+$, $+P\&NH_4^+$, $+P\&NO_3^-$, +ALL).

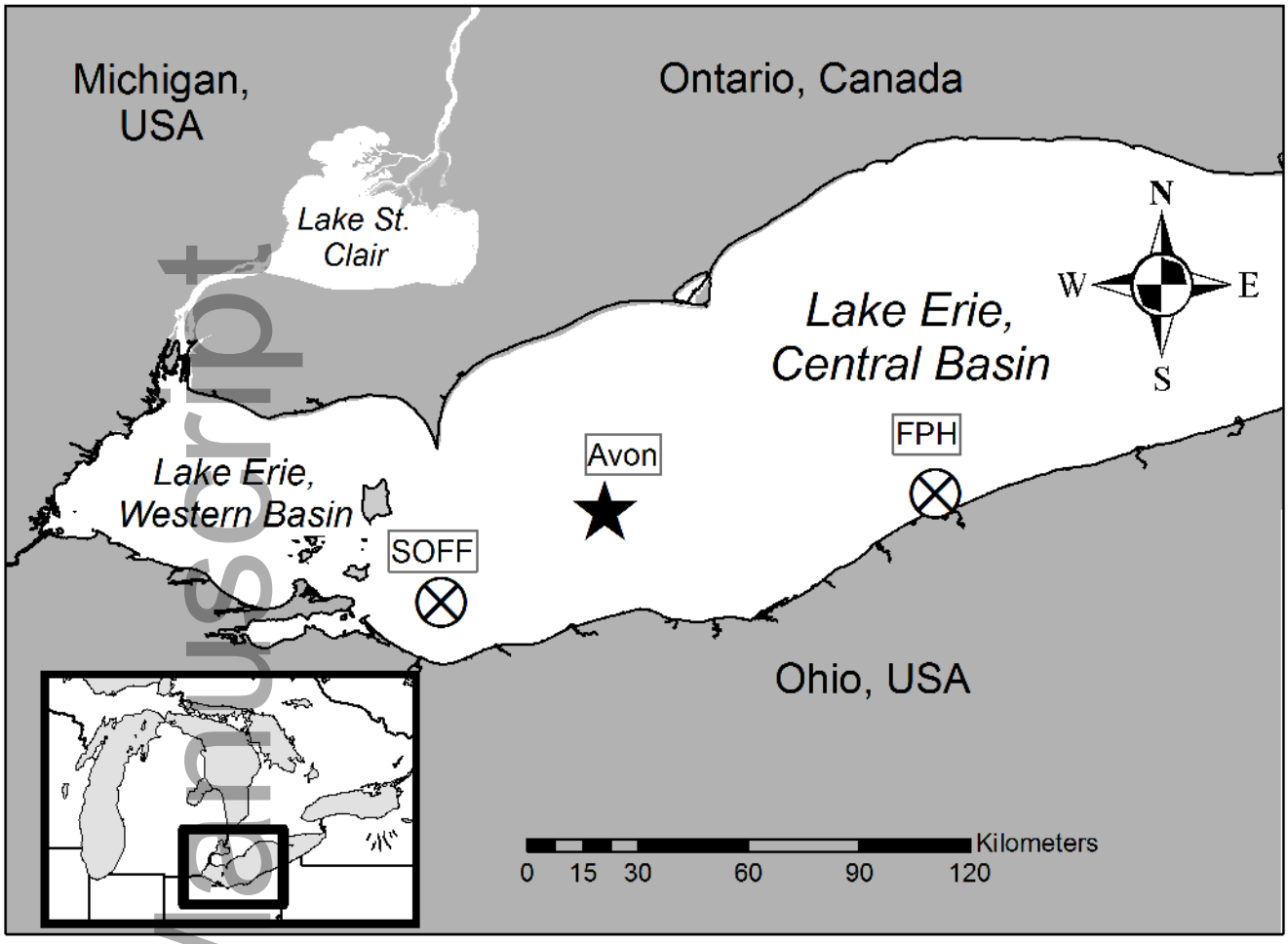
768 There was no significant difference between the two groups.

769

770 Figure 5. Percentage of biovolume with at least one heterocyte in the colony or filament (A, C,

771 E, G, I) and total diazotroph biovolume (B, D, F, H, J) in five enrichment experiments.

772 *Dolichospermum* was present in the 4 experiments that started in July (A, B, E – J) and
773 *Cuspidothrix* was present in the 10 August 2015 experiment. Bars are mean ± 1 standard error.



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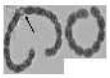

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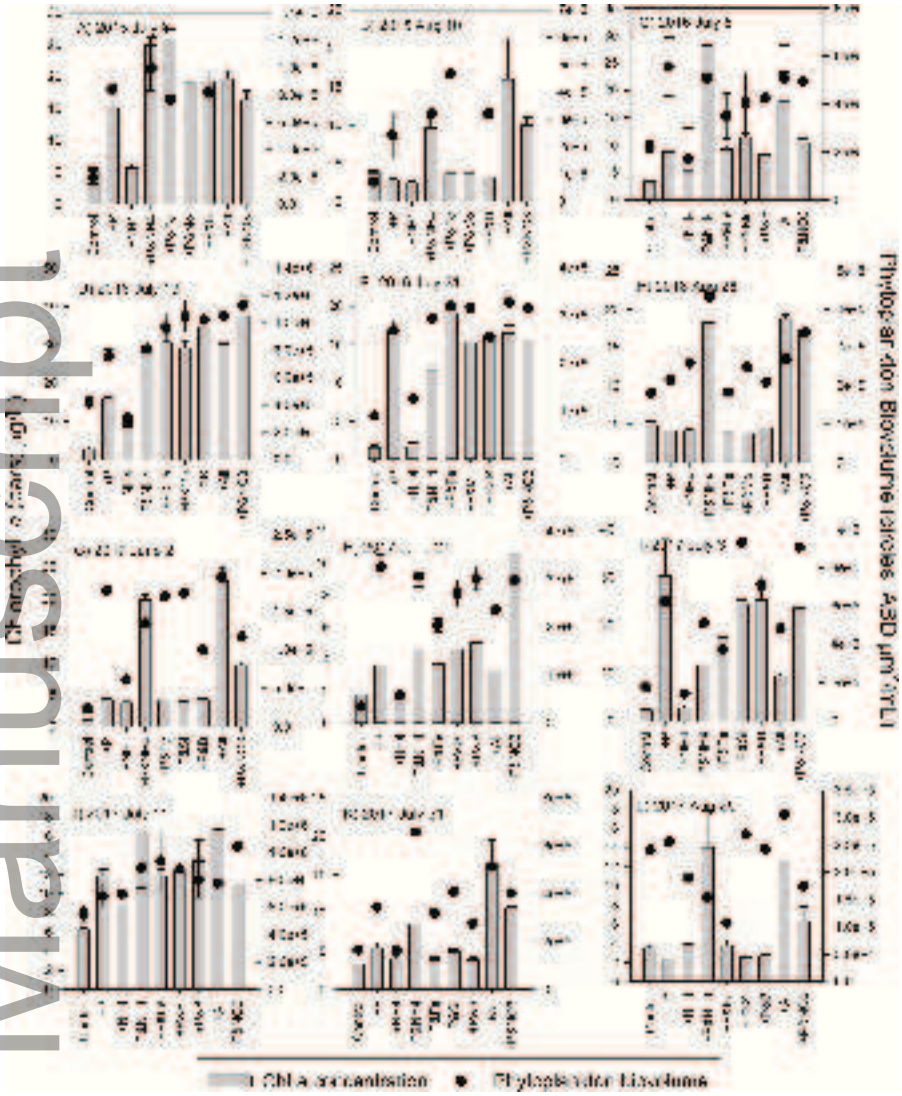

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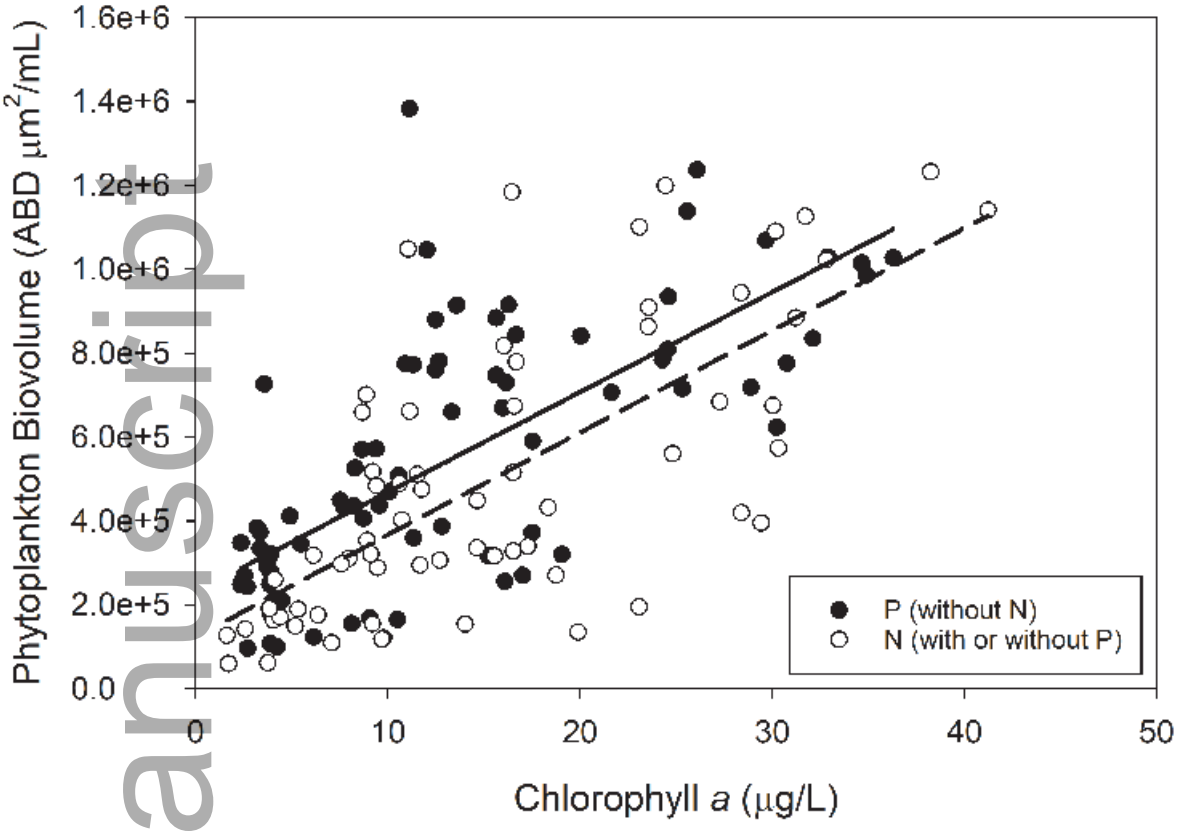

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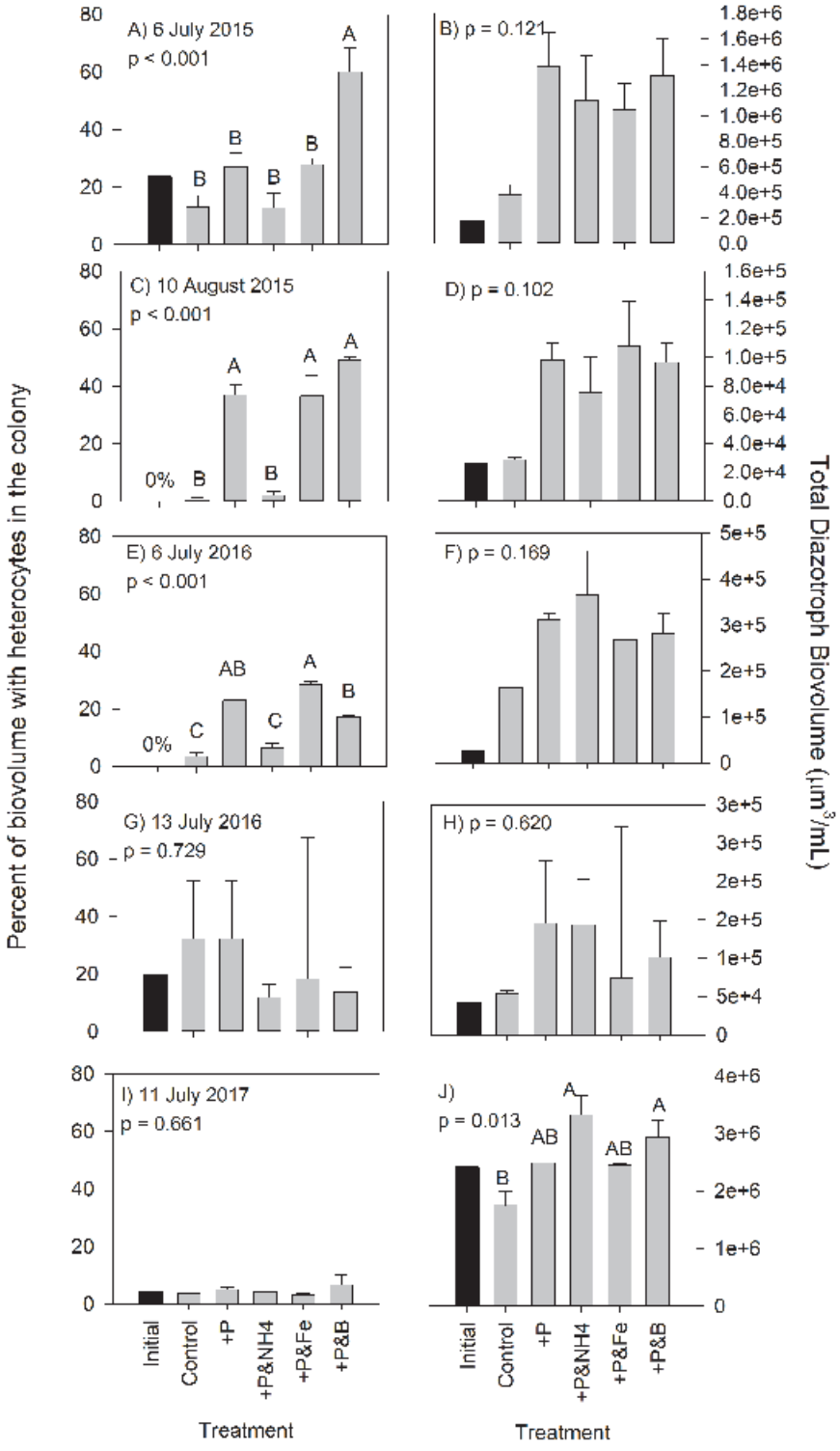

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