

# Phytoplankton N<sub>2</sub>-fixation efficiency and its effect on harmful algal blooms

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**Abstract:** Toxin production during harmful algal blooms (HABs) depends on N availability. However, the role of N<sub>2</sub> fixation as a mechanism to fuel 'new' N into HABs and increase their toxicity has not been well studied. We quantified the effects of N : P supply ratios on N<sub>2</sub>-fixation efficiency in HABs from 3 warm-temperate man-made reservoirs. We enriched mesocosms with the same concentration of P (112 µg P/L) but differing amounts of N (50–2500 µg N/L) labeled with a <sup>15</sup>N tracer to simulate HABs growing along a large molar N : P gradient (1–50). N<sub>2</sub> fixation increased significantly at low N : P but generally did not alleviate N limitation and lead to accumulating N and phytoplankton biomass efficiently unless the magnitude of stoichiometric imbalance was low. Furthermore, microcystin concentrations >1.0 µg/L occurred only in mesocosms receiving N : P = 50 supply and only in the reservoir with detectable concentrations of microcystin at the beginning of the experiment. These results suggest that HABs in P-rich reservoirs may yield significantly more biomass and potentially become more toxic when reactive N is plentiful in the water column relative to P. Thus, reducing N concentrations can be useful as a supplement to the primary P reduction strategies used to minimize the harmful effects of algal blooms.

**Key words:** nitrogen fixation, nutrient limitation, supply ratios, cyanobacteria, HABs

The ability of some species of cyanobacteria to fix atmospheric N<sub>2</sub> into biologically reactive forms gives them a competitive advantage over other phytoplankton under N-limiting conditions (Horne and Goldman 1972, Howarth et al. 1988a, Paerl et al. 2001). However, many factors, such as water-column turbulence, temperature, light availability, and micronutrients can constrain phytoplankton N<sub>2</sub> fixation regardless of N-limitation (Howarth et al. 1988b, Paerl 1990, Paerl et al. 2001). Perhaps most important, N acquisition via N<sub>2</sub> fixation is a highly energy-intensive process compared to the direct uptake of NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup> because of the costs of the biochemical reaction (16 ATP/N fixed; Herrero et al. 2004) and because many cyanobacterial taxa must differentiate and maintain heterocytes in which the nitrogenase enzyme can function (Turpin et al. 1985). Therefore, cyanobacteria relying on N<sub>2</sub> fixation as their major source of N may experience decreased yield (per unit P or other growth-limiting resource) relative to the yield of cyanobacteria using NO<sub>3</sub><sup>-</sup> or NH<sub>4</sub><sup>+</sup> as their primary N source.

Proliferation of cyanobacteria is undesirable in lakes and reservoirs because of their tendency to produce aesthetically unpleasing surface scums, odors, and potentially harmful toxins, such as microcystin (Paerl et al. 2001). N cycling plays an important, but complicated and not yet well understood, role in cyanobacterial toxin production. Gobler et al. (2016) recently summarized the mounting evidence suggesting that both cyanobacterial growth and toxin production requires significant N input because toxins themselves contain a large number of amino acids or have amino acid precursors with large N demands. For example, microcystin-LR has 10 N atoms in every molecule resulting in a C : N ratio of 4.2 by mass. Evidence is mounting that cyanobacterial toxicity increases with N availability (Rolland et al. 2005), which is influenced by both concentration and form, as has been demonstrated in cyanobacterial cultures (Long et al. 2001, Harke and Gobler 2013), cyanobacterial blooms in individual lakes (Donald et al. 2011), and across many lakes at continental scales (Scott et al. 2013, Yuan et al. 2014). In particular, the production of microcystin by non-N<sub>2</sub>-fixing *Microcystis*

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*aeruginosa* has been linked directly to N availability across multiple scales of investigation (Horst et al. 2014).

What is less obvious is whether N<sub>2</sub>-fixing cyanobacterial blooms can become toxic. Beversdorf et al. (2013) demonstrated an indirect link whereby N<sub>2</sub>-fixing cyanobacteria blooms were not toxic but were followed seasonally by toxic non-N<sub>2</sub>-fixing cyanobacterial blooms after the N concentration of surface waters had increased. van de Waal et al. (2014) suggested that the stoichiometric requirements of cyanobacterial toxin production probably produce a large N demand. The potential lack of toxicity in cyanobacterial populations that are actively fixing N<sub>2</sub> suggests the existence of stoichiometric or energetic tradeoffs between assimilated N apportioned for growth and assimilated N apportioned for toxin production. Thus, the efficiency of N<sub>2</sub> fixation in fueling 'new' production during harmful algal blooms (HABs) may be an important constraint on toxin production when N is in short supply relative to P or other growth-limiting resources.

N<sub>2</sub>-fixation efficiency may be a useful indicator of how much cyanobacteria rely on N<sub>2</sub> during bloom formation, but it can be defined in a number of ways. We define gross N<sub>2</sub> fixation as the amount of N accumulated from fixation and N<sub>2</sub>-fixation efficiency as the amount of phytoplankton biomass achieved with active N<sub>2</sub> fixation relative to the amount of biomass accumulated from cyanobacteria growth on reactive N sources (i.e., NH<sub>4</sub><sup>+</sup> or NO<sub>3</sub><sup>-</sup>). For example, consider a hypothetical situation in which lake water was enriched with P and various amounts of N to stimulate algal blooms across a large N : P gradient. The response ratio (RR) of phytoplankton biomass (presented in our study as particulate C [PC]; RR<sub>PC</sub> = final PC/initial PC) would indicate the efficiency by which fixed N<sub>2</sub> contributed to PC accumulation relative to existing PC. If the phytoplankton were given sufficient time (days to weeks) under ideal conditions for bloom formation and without other constraining factors (e.g., grazing or excessive turbulence), a 0 slope of the RR<sub>PC</sub> vs N : P would indicate complete N<sub>2</sub>-fixation efficiency.

We measured gross N<sub>2</sub> fixation and experimentally tested phytoplankton N<sub>2</sub>-fixation efficiency across a wide range of N : P supply ratios in mesocosms simulating conditions favorable for HABs. We used a mass-balance-constrained <sup>15</sup>N stable-isotope-tracer method in mesocosms placed in 3 small, temperate reservoirs to test the degree to which N<sub>2</sub> fixation alleviated N limitation during bloom formation, and quantified microcystin production across the N : P gradient. We hypothesized that phytoplankton biomass would be least in low N : P and greatest at high N : P treatments, and that RR<sub>PC</sub> (and RR total N [RR<sub>TN</sub>]) would be positively correlated with experimental N : P because water-column N concentrations (relative to P) would control the biomass of the bloom. We also hypothesized that N<sub>2</sub>-fixation rates and efficiencies would vary among reservoirs because of different initial conditions and that microcystin production

would be least in phytoplankton assemblages with greatest N<sub>2</sub> fixation because of a metabolic tradeoff between nitrification and toxin synthesis.

## METHODS

### Study sites

We conducted mesocosm experiments in 3 small (<0.2 km<sup>2</sup>) monomictic reservoirs in the Springfield Plateau region of northwestern Arkansas. Lake Brittany (lat 36°28'08"N, long 94°12'04"W), Lake Norwood (lat 36°28'45"N, long 94°14'44"W), and Lake Rayburn (lat 36°27'43"N, long 94°14'21"W) are all steeply sloped reservoirs with mean depths of 7.6 to 8.8 m and maximum depths of 21.0 to 23.3 m. All 3 reservoirs have similar watershed landuse characteristics, with primarily forested (64–78%) and urban (14–25%) watersheds. We monitored the study reservoirs approximately weekly from May–October 2012. We used a 4-L Van Dorn horizontal sampler to collect vertically integrated water samples from the euphotic zone (typically 0–<5 m depth) at 5 locations equally spaced along the thalwegs of each reservoir. The 5 samples were mixed to create a whole-reservoir composite sample and returned to the laboratory on ice.

Within 24 h of collection, we filtered a subsample from each composite sample onto a precombusted (4 h at 450°C) GF/F filter (Whatman, Maidstone, UK) for particulate C (PC) and particulate N (PN) and onto an acid-washed GF/F filter for particulate P (PP). We froze the filters for later analysis. Filtrate from filtered samples (GF/F) was retained, frozen, and later analyzed for NO<sub>3</sub><sup>-</sup> + NO<sub>2</sub><sup>-</sup>-N (henceforth NO<sub>3</sub><sup>-</sup>-N), total dissolved N (TDN), and total dissolved P (TDP). We preserved a subsample of the composite sample with M3 fixative (final concentrations: 0.06 M I<sub>2</sub>, 0.02 M KI, 0.67 M glacial acetic acid, and 19% formalin; Shubert and Gärtner 2015) for phytoplankton enumeration with the aid of an inverted microscope method (Utermöhl 1958). We identified phytoplankton based on the method outlined by Olrik et al. (1998). We allowed 5 mL of sample to settle in an Utermöhl chamber overnight and identified and enumerated a minimum of 200 cells at 100× magnification. We used taxon-specific geometric formulae recommended by Rott (1981) and Olrik et al. (1998) to calculate phytoplankton biovolumes. Last, we filtered a subsample of the composite sample from each reservoir onto GF/F filters, froze the filters, and then analyzed them for microcystin on 26 July and 17 August 2012.

### Mesocosm experiment

We conducted a mesocosm experiment from 31 July to 7 September 2012 in each of the 3 reservoirs to address how N : P supply ratios affected N<sub>2</sub> fixation, TN and biomass accumulation, and microcystin concentrations, with respect to initial conditions. Mesocosms were white, 166-L polyeth-

ylene containers (Brute®; Rubbermaid, Atlanta, Georgia) that were closed at the bottom and open to the atmosphere. We submerged all mesocosms in each reservoir for 4 wk to allow leaching of any potentially harmful chemicals and cleaned them prior to the experiment. We attached 12 mesocosms to a floating polyvinylchloride frame on 31 July in each reservoir (36 mesocosms total). We filled mesocosms with 130 L of vertically integrated euphotic-zone water by slowly lowering a sump pump through the photic zone. We randomly assigned an N : P (molar) treatment of 1, 10, 25, or 50 to each mesocosm (3 mesocosms per treatment per reservoir). All mesocosms received some level of N and P enrichment so that there was no true control in the experiment. The same amount of P was added to all mesocosms, and N was manipulated according to the N : P treatment. We added nutrients approximately biweekly, and by the end of the experiment, all mesocosms had received a total of 112 µg P/L as NaH<sub>2</sub>PO<sub>4</sub> and, depending on the N : P treatment, either 50, 500, 1250, or 2500 µg N/L as KNO<sub>3</sub> enriched with <sup>15</sup>N (δ<sup>15</sup>N = 962‰) so that we could distinguish it from N supplied by N<sub>2</sub> fixation, which is isotopically light (~0‰). Upon addition of nutrients, we stirred mesocosms thoroughly to allow distribution of N and P throughout the water column.

We collected seston samples just before the first nutrient addition (day 0) and on the last day of the experiment (day 37) for biological and chemical analyses. We collected initial samples from mesocosm source water for each reservoir. We collected a final sample from each mesocosm after homogenizing the contents of the mesocosm, including material accumulated at bottom and loosely attached periphyton that may have grown on the sides during the experiment. Samples were collected in acid-washed dark bottles, stored on ice, taken back to the laboratory, and filtered within 48 h. Upon returning to the laboratory, each mesocosm sample was filtered as described previously for PC, PN, and PP, with an additional subsample filtered onto a Pall tissue quartz filter for particulate δ<sup>15</sup>N analysis. In addition, we combined water samples from each mesocosm for each treatment and filtered a subsample onto a GF/F filter. We froze filters for later microcystin analysis. We retained the filtrate, froze it, and later analyzed it for NO<sub>3</sub><sup>-</sup>-N, TDN, and TDP.

### Laboratory analyses

We thawed and dried frozen filters for 24 h (50°C) and analyzed them for PC and PN on a Thermo Flash 2000 Organic Elemental Analyzer (CE ElanTech, Lakewood, New Jersey). We digested PP filters in a persulfate solution and analyzed them colorimetrically based on the ascorbic acid method (APHA 2005). We analyzed filtrate for TDN and TDP simultaneously on a Skalar San Plus (Skalar Inc., Buford, Georgia) following a persulfate digestion (APHA

2005). We analyzed filtrate subsamples from routine monitoring for NO<sub>3</sub><sup>-</sup>-N colorimetrically using the Cd-reduction method (APHA 2005). We conducted PP and NO<sub>3</sub><sup>-</sup>-N colorimetric analyses on a Trilogy Lab Fluorometer (Turner Designs, San Jose, California) with a spectrophotometer adaptor containing 800- and 600-nm filters, respectively. We quantified microcystin in particulate matter with enzyme-linked immunosorbent assay (ELISA; An and Carmichael 1994) after extraction from filters with acidified 75% aqueous methanol. All <sup>15</sup>N filters were freeze-dried and then analyzed at the University of Arkansas Stable Isotope Laboratory using a Finnigan Delta Plus mass spectrometer (Thermo Electron, Bremen, Germany) following combustion in a Carlo Erba NC2500 elemental analyzer (connected via a Finnigan ConFlo II interface [Thermo Electron]). The <sup>15</sup>N : <sup>14</sup>N ratio was expressed in δ notation relative to air (Peterson and Fry 1987).

### Calculations and statistical analyses

We used an approach similar to that of Vrede et al. (2009) to calculate N<sub>2</sub> fixation in the mesocosms based on the <sup>15</sup>N stable isotope. The δ<sup>15</sup>N of the final sample (δ<sup>15</sup>N<sub>final</sub>) was defined by a multisource-mixing model as:

$$\delta^{15}\text{N}_{\text{final}} = (\delta^{15}\text{N}_{\text{initial}} \times f_{\text{initial}}) + (\delta^{15}\text{N}_{\text{added}} \times f_{\text{added}}) + (\delta^{15}\text{N}_{\text{fix}} \times f_{\text{fix}}), \quad (\text{Eq. 1})$$

where δ<sup>15</sup>N<sub>initial</sub>, δ<sup>15</sup>N<sub>added</sub>, and δ<sup>15</sup>N<sub>fix</sub> are the isotopic signatures of seston at the beginning of the experiment, <sup>15</sup>N-enriched fertilizer (962‰), and N<sub>2</sub> gas from the atmosphere (0‰), respectively. The variables *f*<sub>initial</sub>, *f*<sub>added</sub>, and *f*<sub>fix</sub> are the fractional contributions of initial seston N, N fertilizer, and fixed N to the final N concentration in each mesocosm. The sum of *f*<sub>initial</sub>, *f*<sub>added</sub>, and *f*<sub>fix</sub> was assumed to equal 1. We also assumed that the isotopic composition of PN and TDN were equal and that no fractionation occurred during cycling of N from the particulate to dissolved pool.

A 3-source mixing model (Eq. 1) cannot have a single solution because it has 3 unknown variables that cannot be solved with simultaneous equations. Therefore, we used IsoSource (version 1.3; US Environmental Protection Agency, Western Ecology Division, Corvallis, Oregon) to calculate a range of solutions for *f*<sub>fix</sub> following the procedure developed by Phillips and Gregg (2003). IsoSource is a software package used to calculate a range of feasible source combinations that satisfy a mixing model with >1 unknown variable. The user supplies all known δ<sup>15</sup>N values (mixture and sources), a source increment that determines the interval of source-combination iterations, and a mass-balance tolerance that defines how similar a predicted mixture δ<sup>15</sup>N has to be to the actual mixture signature. We used a source increment of 1% and a mass-balance tol-

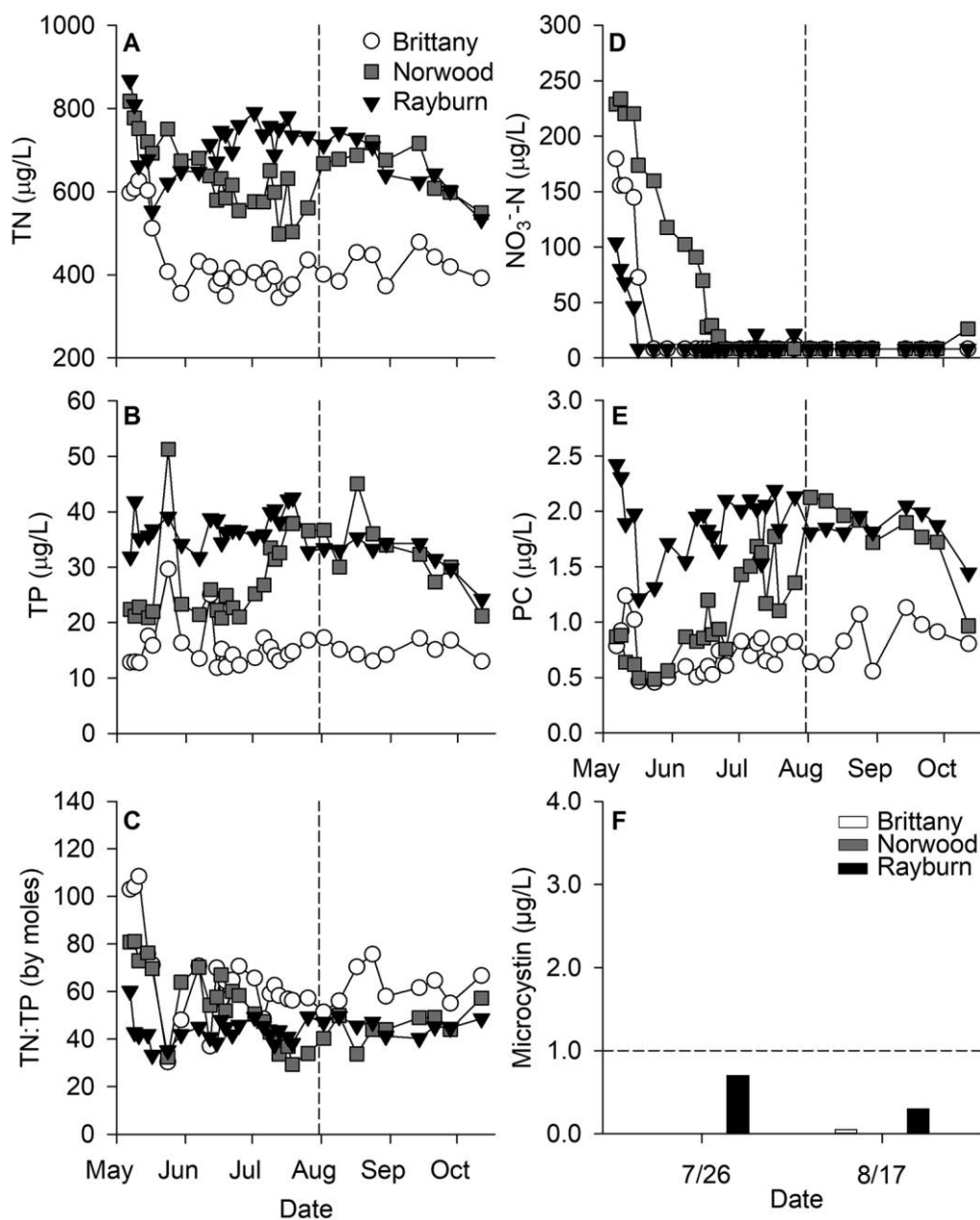


Figure 1. Total N (TN) (A), total P (TP) (B), molar TN : TP (C),  $\text{NO}_3^-$ -N (D), particulate C (PC) (E), and microcystin (F) in Lakes Brittany, Norwood, and Rayburn in May–October 2012 (33 sampling dates). The  $y$ -axis in panel F is scaled to match that in Fig. 6. The vertical dashed line represents the beginning of the mesocosm experiment on 31 July 2012. Microcystin was below detection limit on both sampling dates in Lake Norwood.

erance equal to the average within-treatment standard deviation (SD) for IsoSource computations. TN data were used to constrain the range of values calculated to only those solutions that satisfied the mass-balance equation described as:

$$\text{TN}_{\text{final}}/\text{TN}_{\text{initial}} = (f_{\text{initial}} + f_{\text{added}} + f_{\text{fix}})/f_{\text{initial}}, \quad (\text{Eq. 2})$$

where  $\text{TN}_{\text{final}}$  is the mass of TN of the final sample and  $\text{TN}_{\text{initial}}$  is the mass of TN present at the beginning of

the experiment. For each treatment in each reservoir, we used the mean and SD of the  $\text{TN}_{\text{final}}$  values to estimate a 95% confidence interval (CI) by generating 1000 random values (normal distribution; SigmaPlot 11; Systat, San Jose, California). We calculated the  $\text{TN}_{\text{final}} : \text{TN}_{\text{initial}}$  ratio for each of the randomly generated  $\text{TN}_{\text{final}}$  values based on the  $\text{TN}_{\text{initial}}$  measured for each reservoir. The upper and lower limits of the range of  $\text{TN}_{\text{final}} : \text{TN}_{\text{initial}}$  values were defined as the 97.5<sup>th</sup> and 2.5<sup>th</sup> percentiles of the distribution. We used these upper and lower limits to constrain the so-



lutions generated using IsoSource after calculating the  $F$  ratio (right-hand term) in Eq. 2. The minimum, median, and maximum amount of total fixed N ( $TN_{fix}$ ) was calculated as the product of the 5<sup>th</sup>, 50<sup>th</sup>, and 95<sup>th</sup> percentiles of  $f_{fix}$  and the final TN concentrations of each mesocosm.

We derived  $N_{fix}$  from  $TN_{fix}$  values calculated from the median value of  $f_{fix}$  and expressed as  $\mu\text{g N L}^{-1} \text{h}^{-1}$ . Each  $N_{fix}$  value was considered to represent the average  $N_2$ -fixation rate for that mesocosm over the course of the experiment (assuming a photoperiod of 13 h for each day of the experiment). We conducted ordinary least squares (OLS) regression analyses in SAS (version 9.1; SAS Institute, Cary, North Carolina) to estimate the effect of N : P supply on  $\delta^{15}\text{N}$ ,  $f_{fix}$  (median),  $N_{fix}$ ,  $RR_{TN}$ , and  $RR_{PC}$ . RRs were the final concentration divided by the initial concentration in each mesocosm (accounting for volume loss throughout the course of the experiment). We used RRs to estimate gross  $N_2$  fixation ( $RR_{TN}$ ) and the efficiency of  $N_2$  fixation ( $RR_{PC}$ ) across N : P supply in each reservoir because a slope  $> 0$  indicates that added reactive N yielded more biomass than  $N_2$  fixation. Therefore, any slopes in OLS regressions for  $RR_{PC}$  that were significant ( $p < 0.05$ ) were considered to indicate that  $N_2$  fixation was not efficient in terms of biomass accumulation, respectively, for that reservoir.

To test for effects of initial conditions, we compared the slopes between each variable ( $\delta^{15}\text{N}$ ,  $f_{fix}$ ,  $N_{fix}$ ,  $RR_{TN}$ , and  $RR_{PC}$ ) vs N : P supply among reservoirs and subsequently tested for differences in  $y$ -intercepts with analysis of covariance (ANCOVA) in cases where slopes were equal among reservoirs. We used generalized linear models (GLMs) in Systat (version 13.1) to compare the slopes of the N : P and each variable among reservoirs by including the N : P  $\times$  reservoir interaction term. In cases where the slopes were not statistically different among reservoirs ( $p > 0.05$  for N : P  $\times$  reservoir for a given variable), we reran the GLM without the interaction term to generate the ANCOVA.

## RESULTS

### Reservoir conditions

Lakes Brittany, Norwood, and Rayburn are mesotrophic to slightly eutrophic reservoirs with average TN and TP ranging from 435 to 699  $\mu\text{g N/L}$  and 15 to 35  $\mu\text{g P/L}$ , respectively (Fig. 1A, B) and do not typically experience HABs. Lakes Norwood and Rayburn had similar nutrient concentrations, which were generally greater than in Lake Brittany. However, molar TN : TP was similar across reservoirs (average across reservoirs: 44.7 to 65.1; Fig. 1C). A clear trend in  $\text{NO}_3^-$ -N drawdown was observed in all 3 reservoirs as summer thermal stratification progressed. The date on which  $\text{NO}_3^-$ -N was reduced below the detection limit (16.5  $\mu\text{g N/L}$ ) differed substantially among reservoirs. Lake Norwood experienced drawdown  $\sim 1$  mo after Lakes Brittany and Rayburn (Fig. 1D). An apparent distinc-

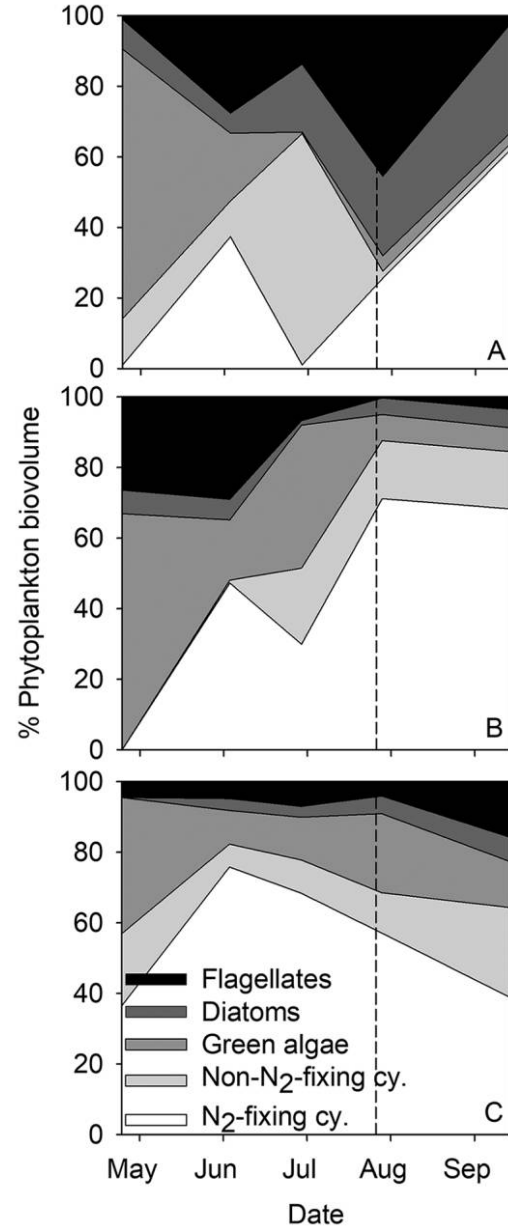


Figure 2. Relative phytoplankton biomass in Lakes Brittany (A), Norwood (B), and Rayburn (C) on 6 sampling dates in May–September 2012. Taxonomic groups were identified based on keys published by Olrik et al. (1998), but cyanobacteria (cy.) were split into 2 categories:  $N_2$ -fixing and non- $N_2$ -fixing cyanobacteria based on identified species' capability for carrying out  $N_2$  fixation. Vertical dashed line represents the beginning of the mesocosm experiment on 31 July 2012.

tion in PC (used here as a proxy for phytoplankton biomass) concentrations between Lake Brittany and the other reservoirs indicated that Lake Brittany was less productive and possibly more nutrient limited than Lakes Norwood or Rayburn (Fig. 1E).

Both Lakes Rayburn and Brittany had measurable concentrations of microcystin on the 2 dates it was analyzed

(before and in the middle of the mesocosm experiments; Fig. 1F). The concentrations on both dates in each reservoir were below the World Health Organization's (WHO) provisional guideline value of 1 µg/L, although microcystin was higher in Lake Rayburn (0.70 µg/L) than in Lake Brittany (0.01 µg/L) just before the mesocosm experiments began in late July (Fig. 1F). Each reservoir experienced an increase in N<sub>2</sub>-fixing and non-N<sub>2</sub>-fixing cyanobacteria from late May to late June following NO<sub>3</sub><sup>-</sup>-N drawdown (Fig. 2A–C). These taxa subsequently decreased in Lakes Brittany and Rayburn, and another relative increase in N<sub>2</sub>-fixing cyanobacteria biomass was observed in Lake Brittany in September. Lake Norwood experienced cyanobacteria dominance (with N<sub>2</sub>-fixers accounting for nearly 70% of total phytoplankton biomass) sustained late into the growing period following NO<sub>3</sub><sup>-</sup>-N drawdown (Fig. 2B).

### Mesocosm experiment

The δ<sup>15</sup>N<sub>initial</sub> of seston varied across reservoirs, ranging from 0.33‰ in Lake Norwood to 9.66‰ in Lake Brittany (Table 1). A strong, positive linear trend between δ<sup>15</sup>N<sub>final</sub> (seston δ<sup>15</sup>N from day 37 of mesocosm experiment) and N : P supply was observed in each reservoir (Table 2, Fig. 3A), but this relationship was not significantly different among reservoirs (Table 3). The median *f*<sub>fix</sub> values generated by IsoSource ranged from 0.08 to 0.77 across all treatments and reservoirs (Fig. 3B). A significant trend of

decreasing *f*<sub>fix</sub> as N : P supply increased was observed in each reservoir (Table 2). The slope of *f*<sub>fix</sub> vs N : P supply did not differ among reservoirs, but the ANCOVA results indicated that the intercepts were significantly different (Table 3). This result indicated that fixed N made up more of TN<sub>final</sub> at low N : P supply in Lake Brittany than in Lakes Norwood and Rayburn (Fig. 3B). Furthermore, N<sub>fix</sub> ranged from 0.61 to 4.31 µg N L<sup>-1</sup> h<sup>-1</sup> throughout the experiment, with the highest rates occurring in the lowest N : P supply treatment in Lakes Brittany and Rayburn (Fig. 4). Slopes of N<sub>fix</sub> vs N : P supply regressions did not differ among reservoirs. However, the intercept for the N<sub>fix</sub> regression was significantly lower in Lake Norwood than in Lakes Brittany and Rayburn, which were not different from each other (Table 3). Thus, N<sub>fix</sub> was lower in Lake Norwood than in Lakes Brittany and Rayburn, especially across low N : P (Fig. 4).

RR<sub>TN</sub> increased with N : P supply in all reservoirs (Fig. 5A), and the slope of this regression was significantly > 0 in each reservoir (Table 2). The RR<sub>TN</sub> increase/unit N : P supply was significantly greater in Lake Brittany than in Lakes Norwood and Rayburn, which were not different from each other (Table 3, Fig. 5A). Likewise, RR<sub>PC</sub> increased significantly with N : P supply, but the increase was statistically significant only in Lakes Brittany and Norwood (Table 2). RR<sub>PC</sub> increased with N : P supply significantly more in Lake Brittany than in Lakes Norwood and Rayburn (Tables 2, 3). Mean

Table 1. Mean (±SD) conditions of each reservoir the week that the experiment started (initial) and final conditions in mesocosms supplied with nutrients in molar total N (TN) : total P (TP) = 1, 10, 25, and 50 in 3 reservoirs. δ<sup>15</sup>N = isotopic composition of seston, PC is particulate C, and Chl *a* is chlorophyll *a*.

Reservoir/condition	δ <sup>15</sup> N (‰)	TN (mg/L)	TP (mg/L)	TN : TP	PC (mg/L)	Chl <i>a</i> (µg/L)
Lake Brittany						
Initial	9.66	0.38	0.009	93.5	0.64	9.5
1	24.6 ± 3.4	2.78 ± 0.53	0.14 ± 0.02	43.4 ± 2.7	25.9 ± 5.9	63.9 ± 42.7
10	197 ± 78.0	3.15 ± 0.92	0.17 ± 0.03	39.8 ± 5.0	31.1 ± 3.4	66.1 ± 12.7
25	498 ± 31.7	3.13 ± 0.59	0.18 ± 0.04	38.6 ± 5.3	38.2 ± 6.5	105 ± 32.4
50	697 ± 7.7	4.51 ± 0.52	0.22 ± 0.06	47.6 ± 8.1	49.3 ± 7.7	703 ± 189
Lake Norwood						
Initial	0.33	0.64	0.025	56.6	2.13	27.6
1	29.5 ± 16.0	2.51 ± 0.71	0.14 ± 0.03	40.0 ± 7.1	19.9 ± 5.6	45.0 ± 20.2
10	217 ± 33.3	2.58 ± 0.27	0.17 ± 0.03	34.4 ± 2.2	28.0 ± 5.6	64.2 ± 27.2
25	449 ± 56.2	2.84 ± 0.08	0.14 ± 0.02	45.8 ± 6.0	27.8 ± 2.8	60.3 ± 42.3
50	636 ± 9.1	3.54 ± 0.72	0.15 ± 0.04	52.0 ± 2.8	33.5 ± 7.9	193 ± 100.4
Lake Rayburn						
Initial	1.81	0.79	0.020	87.5	1.81	20.4
1	11.0 ± 2.0	3.31 ± 0.44	0.13 ± 0.01	58.5 ± 6.9	26.4 ± 1.2	147 ± 43.8
10	163 ± 25.7	3.05 ± 0.49	0.16 ± 0.04	42.5 ± 5.8	31.6 ± 4.6	147 ± 55.6
25	413 ± 36.8	3.10 ± 0.28	0.16 ± 0.03	42.9 ± 6.2	32.3 ± 6.4	124 ± 39.5
50	597 ± 22.1	4.04 ± 0.49	0.16 ± 0.04	51.0 ± 7.4	32.5 ± 4.0	244 ± 146.3

Table 2. Results from ordinary least squares linear regression analyses for median fraction of total N composed of fixed N ( $f_{\text{fix}}$ ), the  $\text{N}_2$ -fixation rate ( $\text{N}_{\text{fix}}$ ), the response ratio of total N ( $\text{RR}_{\text{TN}}$ ), and the response ratio of particulate C ( $\text{RR}_{\text{PC}}$ ) vs N : P supply for each reservoir ( $n = 12$ ). Slopes ( $m$ ) were considered significantly different from 0 when  $p < 0.05$ .

Variable (by reservoir)	$m$	$y_0$	$F$	$p$	$r^2$
$\delta^{15}\text{N}$ (‰)					
Lake Brittany	13.6	60.3	138	<0.001	0.93
Lake Norwood	12.1	73.7	145	<0.001	0.93
Lake Rayburn	11.9	40.6	185	<0.001	0.95
$f_{\text{fix}}$					
Lake Brittany	-0.012	0.70	69.6	<0.001	0.87
Lake Norwood	-0.009	0.51	182	<0.001	0.95
Lake Rayburn	-0.010	0.60	106	<0.001	0.91
$\text{N}_{\text{fix}}$ ( $\mu\text{g L}^{-1} \text{h}^{-1}$ )					
Lake Brittany	-0.048	3.88	13.2	0.005	0.57
Lake Norwood	-0.041	2.60	29.9	<0.001	0.75
Lake Rayburn	-0.060	3.75	37.2	<0.001	0.79
$\text{RR}_{\text{TN}}$					
Lake Brittany	0.108	4.82	18.1	0.002	0.64
Lake Norwood	0.033	2.78	10.1	0.010	0.50
Lake Rayburn	0.028	2.7	12.4	0.006	0.55
$\text{RR}_{\text{PC}}$					
Lake Brittany	0.776	29.0	29.1	<0.001	0.74
Lake Norwood	0.096	8.75	8.78	0.014	0.47
Lake Rayburn	0.045	10.1	2.97	0.116	0.23

$\text{RR}_{\text{PC}}$  increased ~66% from the 1 to 50 N : P supply in Lake Norwood, and an increase of 127% on average was observed in Lake Brittany from 1 to 50 N : P (Fig. 5B).

Microcystin was detected in each treatment across all reservoirs (Fig. 6). However, concentrations greater than the WHO's provisional guideline value of 1  $\mu\text{g/L}$  occurred only in Lake Rayburn at the highest N : P supply treatment, where they exceeded the guideline by nearly  $3\times$  (Fig. 6). Microcystin concentrations in the highest N : P supply treatment in Lake Rayburn were 4 to  $6\times$  greater than ambient reservoir concentrations measured just before the start of the mesocosm experiment in late July and in mid-August, respectively.

## DISCUSSION

$\text{N}_2$ -fixation rates were strongly related to the experimental N : P conditions across the 37-d mesocosm experiments in all 3 reservoirs, but  $\text{N}_2$  fixation was not fully efficient.  $\text{RR}_{\text{TN}}$  was positively correlated with experimental N : P in all reservoirs, a result indicating that gross  $\text{N}_2$  fixation was never great enough to match the N accumulation that occurred when cyanobacteria used reactive N from the water column. We held grazing pressure by planktivorous fish and hydrologic advection to minima in the mesocosms, so the energetic requirements of  $\text{N}_2$  fixation alone may have prohibited  $\text{N}_2$  fixation from meeting po-

tential N demands.  $\text{RR}_{\text{PC}}$  also was positively correlated with experimental N : P in Lakes Brittany and Norwood. These findings support the idea that  $\text{N}_2$  fixation may not effectively balance the N pool of HABs over short time scales and that lower N availability reduced the phytoplankton biomass yield (Scott and McCarthy 2010). However,  $\text{RR}_{\text{PC}}$  was not related to experimental N : P in Lake Rayburn, a result suggesting that the phytoplankton biomass yield was not affected by N availability. Instead, the biomass of the blooms in Lake Rayburn was statistically equivalent regardless of the N source. This finding supports the notion that  $\text{N}_2$  fixation can supply sufficient N, relative to P, for phytoplankton to achieve maximum biomass yield (Schindler 2012).  $\text{N}_2$  fixation was least efficient in fueling HABs (in both  $\text{RR}_{\text{TN}}$  and  $\text{RR}_{\text{PC}}$ ) in the least eutrophic reservoir (Lake Brittany) and vice versa. The more eutrophic reservoirs had greater total N concentrations and more phytoplankton biomass in the beginning of the experiments, which may have led to more rapid light-limitation of phytoplankton blooms in mesocosms than in the mesotrophic reservoir even though the nutrient additions were identical. Thus, the magnitude of stoichiometric imbalance, which in our study was controlled by the experimental nutrient additions and the initial conditions, probably exerts a strong control on  $\text{N}_2$ -fixation rates and efficiency during HAB events.

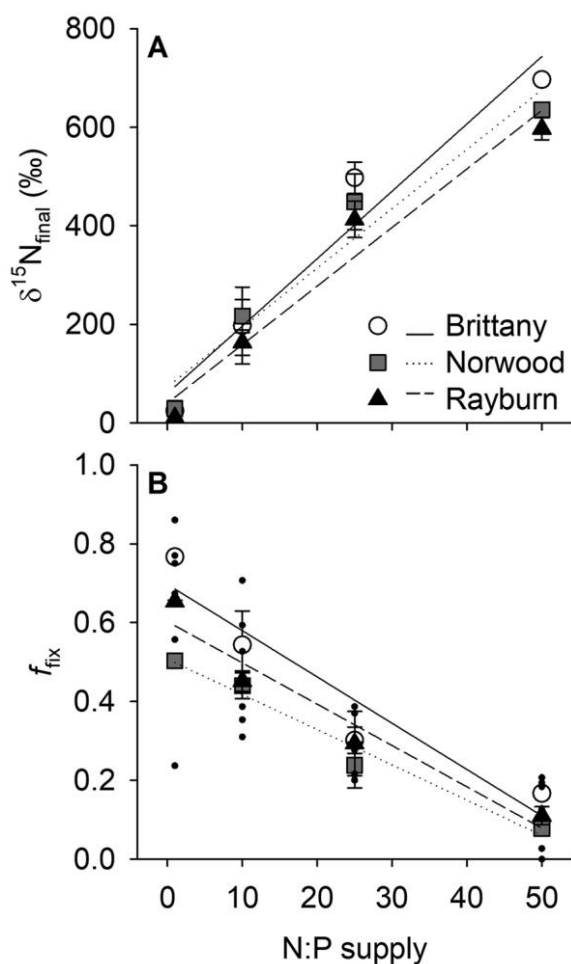


Figure 3. Ordinary least-squares (OLS) regressions for mean ( $\pm$ SD,  $n = 3$ ) stable-isotope ( $\delta^{15}\text{N}$ ) (A) and median fraction of total N composed of fixed N ( $f_{\text{fix}}$ ) data vs N : P supply by reservoir for samples collected at the end of the mesocosm experiment ( $n = 12/\text{reservoir}$ ). See Table 2 for results of regression analyses. In panel B, black dots represent the range of feasible values calculated using IsoSource and a multimixing stable-isotope model constrained by mass balance.

### Importance of N<sub>2</sub> fixation across the N:P supply gradient

N<sub>2</sub> fixation contributed to TN accumulation across all treatments in the simulated blooms in each reservoir despite the relatively high energetic costs (Turpin et al. 1985). However, the magnitude and importance of N<sub>2</sub> fixation was significantly affected by N : P supply. Fixed N made up a maximum of 77% of the TN pool at the end of the experiment when N : P supply was 1 and a minimum of 8% when N : P supply was 50, with a median of 37% across all treatments in all reservoirs (Fig. 3B). In a similar mesocosm experiment, Vrede et al. (2009) found that fixed N comprised nearly 50% of the PN pool at  $\sim 32$  N : P supply after 21 d. The average contribution of fixed N in our mesocosms enriched with 25 N : P in the current study was  $\sim 28\%$  less

than what was found at similar N : P by Vrede et al. (2009). However, the mesocosms in the 25 N : P supply treatment received  $>2\times$  as much  $\text{NO}_3^-$  as those in the experiment conducted by Vrede et al. (2009) because we chose larger dosing rates to simulate intense algal bloom events. Thus, lower  $f_{\text{fix}}$  values would be expected in our experiment, even after a longer period of time (37 vs 21 d).

The N<sub>2</sub>-fixation rates measured in our study ranged from 0.61 to 4.31  $\mu\text{g N L}^{-1} \text{h}^{-1}$ , which were comparable to those observed in various other studies. N<sub>2</sub>-fixation rates in several Texas reservoirs ranged from 0 to 11.7  $\mu\text{g N L}^{-1} \text{h}^{-1}$  (Forbes et al. 2008, Scott et al. 2008, 2009) and similar ranges were observed in Arkansas reservoirs nearby our study locations (Scott and Grantz 2013, Grantz et al. 2014). Vrede et al. (2009) observed an average rate of 3.62  $\mu\text{g N L}^{-1} \text{h}^{-1}$  (assuming a 13-h photoperiod each day) across a 21-d period in mesocosms treated with P only. Beversdorf et al. (2013) measured rates ranging from 0 to 4.65  $\mu\text{g N L}^{-1} \text{h}^{-1}$  in Lake Mendota during summer 2010 and 2011. As expected, N<sub>2</sub> fixation was significantly stimulated as N : P decreased in mesocosms across all 3 of our study reservoirs (Fig. 4), and the response of N<sub>2</sub> fixation to N : P was similar regardless of initial conditions. The occurrence of N<sub>2</sub> fixation at 50 N : P was not unexpected because our mesocosms were a closed system where even excessive dissolved nutrients were exhausted.

### N<sub>2</sub>-fixation efficiency, initial conditions, and ecosystem dynamics

Despite substantially increased N<sub>2</sub>-fixation rates at low N : P,  $\text{RR}_{\text{TN}}$  and  $\text{RR}_{\text{PC}}$  increased with increasing inorganic N availability (Fig. 5A, B), indicating that phytoplankton blooms relying heavily on N<sub>2</sub> fixation accumulated less N and produced less biomass than those relying on reactive N from the water column. These results are contradictory to those found in the study by Vrede et al. (2009), in which N<sub>2</sub> fixation effectively alleviated N deficiency over a 21-d period in a temperate, eutrophic lake in Sweden. However, phytoplankton biomass (as PC) did not increase in Lake Rayburn even though TN accumulation increased as N : P increased (Table 2). Thus, the phytoplankton species dominating assemblages at low N : P treatments in Lake Rayburn were evidently more efficient than those dominating assemblages at higher N : P when generating biomass per unit N supplied via N<sub>2</sub> fixation. This finding indicated that conditions at the onset of bloom formation can affect N<sub>2</sub>-fixation efficiency.

The increase in  $\text{RR}_{\text{TN}}$  and  $\text{RR}_{\text{PC}}$  across the N : P supply gradient was significantly greater in Lake Brittany than in Lakes Norwood and Rayburn. Lake Brittany had the lowest initial TN and PC concentrations, so  $\text{RR}_{\text{TN}}$  and  $\text{RR}_{\text{PC}}$  values were expected to exceed those in the other reservoirs. The slopes of the  $\text{RR}_{\text{TN}}$  or  $\text{RR}_{\text{PC}}$  vs N : P supply regressions were not necessarily expected to be greater in Lake Brit-



Table 3. Slope comparisons (N : P × reservoir interaction term) from 2-way analyses of variance (ANOVA) and analysis of covariance (ANCOVA) in cases where slopes were not different among reservoirs. Slopes and intercepts were considered significantly different among reservoirs when  $p < 0.05$ . See Table 2 for abbreviations.

Variable	N : P		Reservoir		N : P × reservoir	
	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
Variable vs experimental N : P (2-way ANOVA with interaction)						
$\delta^{15}\text{N}$ (‰)	454	<0.001	0.331	0.721	0.937	0.403
$f_{\text{fix}}$	281	<0.001	9.67	0.001	1.70	0.200
$N_{\text{fix}}$	68.0	<0.001	5.72	0.008	0.869	0.429
$\text{RR}_{\text{TN}}$	35.0	<0.001	6.62	0.004	7.422	0.002
$\text{RR}_{\text{PC}}$	37.5	<0.001	21.2	<0.001	22.3	<0.001
Variable vs. experimental N : P (2-way ANOVA without interaction where applicable)						
$\delta^{15}\text{N}$ (‰)	455	<0.001	2.46	<0.001	–	–
$f_{\text{fix}}$	269	<0.001	10.1	<0.001	–	–
$N_{\text{fix}}$	68.6	<0.001	9.08	<0.001	–	–

tany because as the initial TP concentration was lower and TN : TP was higher than in Lakes Norwood and Rayburn (Table 1) and indicated P-limited conditions (Guildford and Hecky 2000). However, initial biomass N : P (29) was considerably less than initial TN : TP (95) in Lake Brittany, a result suggesting that potentially recalcitrant dissolved fractions in Lake Brittany had a high initial N : P that skewed the

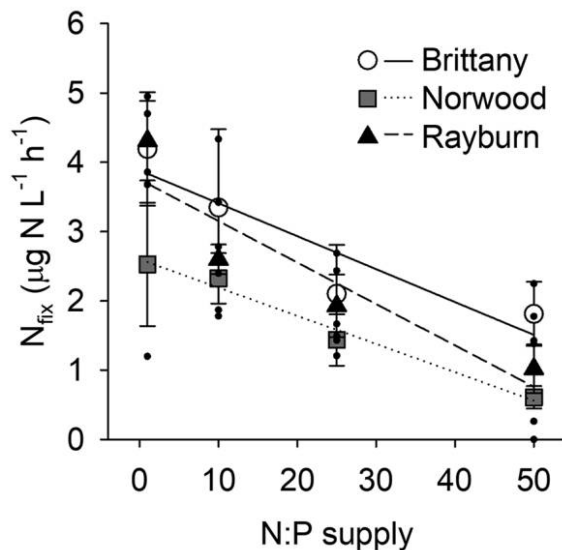


Figure 4. Ordinary least-squares regression for mean ( $\pm$ SD) treatment  $\text{N}_2$ -fixation rates ( $N_{\text{fix}}$ ) as a function of molar total N (TN) : total P (TP) supply accounting for volume loss and assuming a 13-h photoperiod each day during the experiment in each reservoir ( $n = 12$ ). See Table 2 for results of regression analyses. Black dots represent mean  $\text{N}_2$ -fixation rate minima and maxima calculated from the range of  $f_{\text{fix}}$  values generated using IsoSource.

magnitude of stoichiometric imbalance. As a result, N may have been limiting growth in Lake Brittany initially because of a relatively refractory initial TDN pool and low dissolved inorganic N (DIN) ( $\text{NO}_3^-$ -N < 16.5  $\mu\text{g/L}$ ). Inaccessibility of TDN as a source of N was corroborated by phytoplankton identification and enumeration data in Lake Brittany, which indicated that  $\text{N}_2$ -fixing cyanobacteria made up ~25% of the natural phytoplankton assemblage near the beginning of the experiment (Fig. 2A). These results suggest that initial TN : TP was not entirely useful for predicting the response to N and P enrichment because TDN probably was not immediately available to phytoplankton (Bronk et al. 2007).

#### $\text{N}_2$ fixation and microcystin production during blooms

Microcystin concentrations greater than the WHO impairment standard (1  $\mu\text{g/L}$ ) were detectable only in the 50 N : P treatment in Lake Rayburn (Fig. 6). Lake Rayburn had the highest ambient concentration of microcystin near the beginning of the experiment (0.701  $\mu\text{g/L}$ ; Fig. 1F), indicating significant microcystin production in Lake Rayburn at the beginning of the mesocosm experiment. Thus, initial conditions of the experiment may have excluded potential microcystin producers from the mesocosms in Lakes Brittany and Norwood (Sarnelle 2007). The relative increase in microcystin concentrations after nutrient enrichment with high N (and P) only in Lake Rayburn highlights the potential importance of  $\text{N}_2$  fixation as a tradeoff to toxin production, which is consistent with analyses of large-scale databases (Scott et al. 2013, Yuan et al. 2014). Our microcystin results were generated with enzyme-linked immunosorbent assay and should be interpreted with caution, but the findings are consistent with those reported in recent literature. Other investigators have shown that saxitoxins

were greatest in field experiments with high, but not low, N : P (Chislock et al. 2014) and that microcystin production decreased with increasing P inputs that decreased N : P (Horst et al. 2014). These patterns are interesting, but more research is needed to understand how species interactions between N<sub>2</sub>-fixers and non-N<sub>2</sub>-fixers during bloom development and senescence may lead to differential toxicity among blooms (Beversdorf et al. 2013).

### Study implications

Our results indicate that N<sub>2</sub> fixation may not alleviate N limitation effectively in HAB events driven by high P concentrations. N<sub>2</sub> fixation contributed large amounts of N to mesocosms with high P supply and an imbalance of N in 3 small, temperate, man-made reservoirs, but phytoplankton accumulated more N and produced more biomass

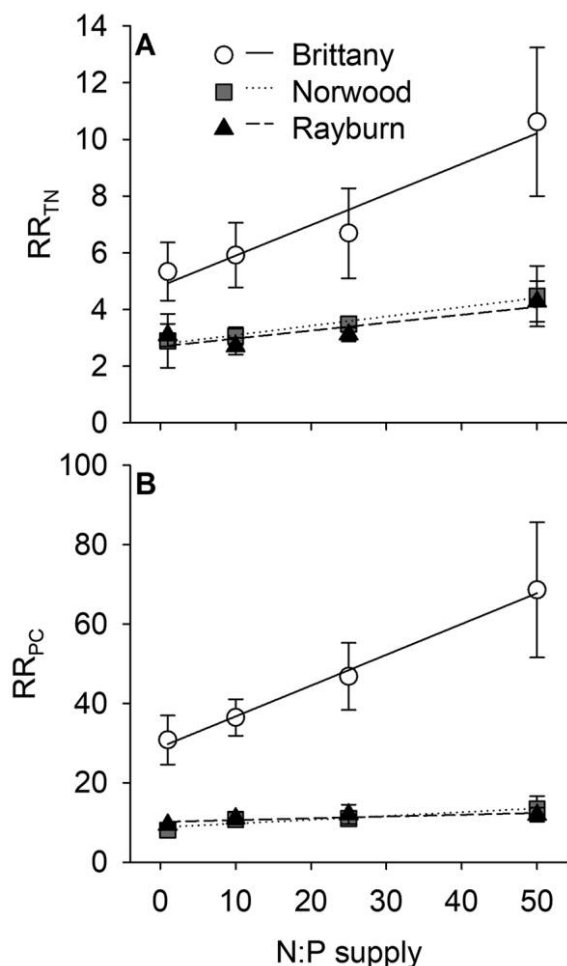


Figure 5. Ordinary least-squares regression for mean ( $\pm$ SD) treatment response ratios (RR = the ratio of final to initial concentration) as a function of molar total N (TN) : total P (TP) for TN (RR<sub>TN</sub>) (A) and particulate C (RR<sub>PC</sub>) (B) in each reservoir. OLS regression analyses were conducted for each RR by reservoir, with  $p < 0.05$  indicating a slope significantly different than 0. See Table 2 for results of regression analyses.

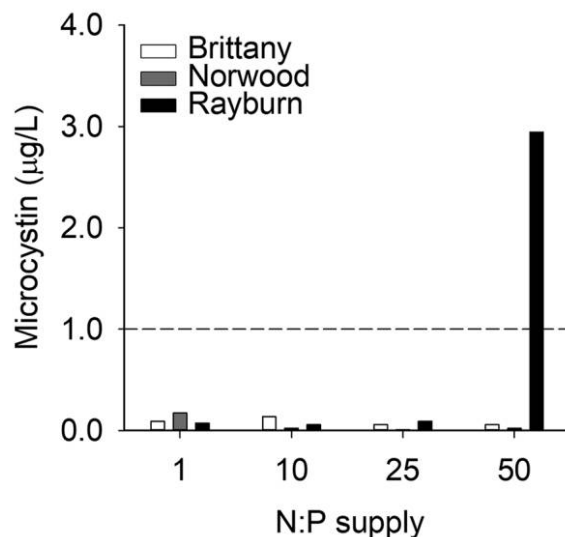


Figure 6. Microcystin measured from a subsample collected from a treatment composite sample (samples from all 3 mesocosms from that treatment combined) in each reservoir. Horizontal dashed line represents the World Health Organization's provisional guideline of 1  $\mu\text{g/L}$ , above which concentrations are considered unsafe for human health.

when more reactive N was available in the water column. This response was reservoir-dependent, with phytoplankton biomass seemingly decoupled from N in the Lake Rayburn experiment. These contrasting results highlight the importance of biological and chemical conditions unique to individual reservoirs, which in the case of our study, strongly influenced the initial conditions of the mesocosm experiments. An ongoing debate exists within the scientific literature regarding whether N plays a significant role in controlling primary productivity in lakes over long time scales (Elser et al. 2007, Lewis and Wurtsbaugh 2008). This debate has been extended to whether N, in combination with P, should be managed to reduce the harmful effects of accelerated eutrophication (Schindler et al. 2008, Conley et al. 2009). N can degrade water quality via accelerated eutrophication in some lakes (Finlay et al. 2010). However, the evidence supporting N mitigation as a tool to reduce the harmful effects of eutrophication in lakes is limited, but increasing (Paerl et al. 2016). Our results show that cyanobacterial biomass yield increases in blooms where inorganic N is more readily available than P in the water column. Thus, from the perspective of short-term bloom management, lower N concentrations may reduce the frequency and magnitude of toxic bloom formation.

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Author contributions: BCB, AEW, and JTS developed the research questions. BCB conducted fieldwork and laboratory analyses sans microcystin analyses conducted by AEW. BCB wrote the original draft that was edited by AEW and JTS.

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