Phytoplankton N_2 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_2 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_2 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 ^{15}N tracer to simulate HABs growing along a large molar N:P gradient (1–50). N_2 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_2 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_2 fixation regardless of N-limitation (Howarth et al. 1988b, Paerl 1990, Paerl et al. 2001). Perhaps most important, N acquisition via N_2 fixation is a highly energy-intensive process compared to the direct uptake of NH_4^+ or NO_3^- 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_2 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_3^- or NH_4^+ 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 to 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_2-fixing *Microcystis aeruginosa* has been linked directly to N availability across multiple scales of investigation (Horst et al. 2014).

What is less obvious is whether N_2-fixing cyanobacterial blooms can become toxic. Beversdorf et al. (2013) demonstrated an indirect link whereby N_2-fixing cyanobacteria blooms were not toxic but were followed seasonally by toxic non-N_2-fixing cyanobacterial blooms after the N concentration of surface waters had increased. van de Waal (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_2 suggests the existence of stoichiometric or energetic tradeoffs between assimilated N aportioned for growth and assimilated N aportioned for toxin production. Thus, the efficiency of N_2 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_2-fixation efficiency may be a useful indicator of how much cyanobacteria rely on N_2 during bloom formation, but it can be defined in a number of ways. We define gross N_2 fixation as the amount of N accumulated from fixation and N_2 fixation efficiency as the amount of phytoplankton biomass achieved with active N_2 fixation relative to the amount of biomass accumulated from cyanobacteria growth on reactive N sources (i.e., NH_4^+ or NO_3^-). 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_{PC} = final PC/initial PC) would indicate the efficiency by which fixed N_2 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_{PC} vs N:P would indicate complete N_2-fixation efficiency.

We measured gross N_2 fixation and experimentally tested phytoplankton N_2-fixation efficiency across a wide range of N:P supply ratios in mesocosms simulating conditions favorable for HABs. We used a mass-balance-constrained ^{15}N stable-isotope-tracer method in mesocosms placed in 3 small, temperate reservoirs to test the degree to which N_2 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_{PC} (and RR_{TN}) 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_2-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_2 fixation because of a metabolic tradeoff between nitrogenase and toxin synthesis.

METHODS

Study sites

We conducted mesocosm experiments in 3 small (<0.2 km^2) 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

104 monitored the study reservoirs approximately weekly from May–October 2012. We used a 4-L
105 Van Dorn horizontal sampler to collect vertically integrated water samples from the euphotic
106 zone (typically 0–<5 m depth) at 5 locations equally spaced along the thalwegs of each reservoir.
107 The 5 samples were mixed to create a whole-reservoir composite sample and returned to the
108 laboratory on ice.

109 Within 24 h of collection, we filtered a subsample from each composite sample onto a
110 precombusted (4 h at 450°C) GF/F filter (Whatman, Maidstone, UK) for particulate C (PC) and
111 particulate N (PN) and onto an acid-washed GF/F filter for particulate P (PP). We froze the
112 filters for later analysis. Filtrate from filtered samples (GF/F) was retained, frozen, and later
113 analyzed for $NO_3^- + NO_2^-$-N (henceforth NO_3^--N), total dissolved N (TDN), and total dissolved
114 P (TDP). We preserved a subsample of the composite sample with M3 fixative ( ) for
115 phytoplankton enumeration with the aid of an inverted microscope method (Utermöhl 1958). We
116 identified phytoplankton based on the method outlined by Olrik et al. (1998). We allowed 5 mL
117 of sample to settle in a Utermöhl chamber overnight and identified and enumerated a minimum
118 of 200 cells at 100× magnification. We used taxon-specific geometric formulae recommended by
119 Rott (1981) and Olrik et al. (1998) to calculate phytoplankton biovolumes. Last, we filtered a
120 subsample of the composite sample from each reservoir onto GF/F filters, froze the filters, and
121 then analyzed them for microcystin on 26 July and 17 August 2012.
122
123 **Mesocosm experiment**
124 We conducted a mesocosm experiment from 31 July to 7 September 2012 in each of the 3
125 reservoirs to address how N:P supply ratios affected N_2 fixation, TN and biomass accumulation,
126 and microcystin concentrations, with respect to initial conditions. Mesocosms were white, 166-L

polyethylene containers (Brute®) 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 of 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_2PO_4 and, depending on the N:P treatment, either 50, 500, 1250, or 2500 µg N/L as KNO_3 enriched with ^{15}N ($\delta^{15}N$ = 962‰) so that we could distinguish it from N supplied by N_2 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 $\delta^{15}N$ analysis. In addition, we combined water samples from each

150 mesocosm for each treatment and filtered a subsample onto a GF/F filter. We froze filters for
151 later analysis for microcystin. We retained the filtrate, froze it, and later analyzed it for NO_3^--N,
152 TDN, and TDP.
153
154 **Laboratory analyses**
155 We thawed and dried frozen filters for 24 h (50°C) and analyzed them for PC and PN on
156 a Thermo Flash 2000 Organic Elemental Analyzer (Thermo Fisher Scientific, , Netherlands). We
157 digested PP filters in a persulfate solution and analyzed them colorimetrically based on the
158 ascorbic acid method (APHA 2005). We analyzes filtrate for TDN and TDP simultaneously on a
159 Skalar San Plus ( ) following a persulfate digestion (APHA 2005). We analyzed filtrate
160 subsamples from routine monitoring for NO_3^--N colorimetrically using the Cd-reduction method
161 (APHA 2005). We conducted PP and NO_3^--N colorimetric analyses on a Trilogy Lab
162 Fluorometer (Turner Designs, ,) with a spectrophotometer adaptor containing 800- and 600-nm
163 filters, respectively. We quantified microcystin in particulate matter with enzyme-linked
164 immunosorbent assay (ELISA; An and Carmichael 1994) after extraction from filters with
165 acidified 75% aqueous methanol. All ^{15}N filters were freeze-dried and then analyzed at the
166 University of Arkansas Stable Isotope Laboratory using a Finnigan Delta Plus mass spectrometer
167 ( ) following combustion in a Carlo Erba NC2500 elemental analyzer (connected via a Finnigan
168 Conflo II interface;). The $^{15}NL^{14}N$ ratio was expressed in δ notation relative to air (Peterson and
169 Fry 1987).
170
171 **Calculations and statistical analyses**
172 We used an approach similar to that of Vrede et al. (2009) to calculate N_2 fixation in the

mesocosms based on the ^{15}N stable isotope. The $\delta^{15}N$ of the final sample ($\delta^{15}N_{final}$) was defined by a multisource mixing model as:

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

where $\delta^{15}N_{initial}$, $\delta^{15}N_{added}$, and $\delta^{15}N_{fix}$ are the isotopic signatures of seston at the beginning of the experiment, ^{15}N-enriched fertilizer (962‰), and N_2 gas from the atmosphere (0‰), respectively. The variable $f_{initial}$, f_{added}, and f_{fix} are the fractional contributions of initial seston N, N fertilizer, and fixed N to the final N concentration in each mesocosm. The sum of $f_{initial}$, f_{added}, and f_{fix} was assumed to equal 1. The isotopic composition of PN and TDN were assumed to be 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_{fix} 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 $\delta^{15}N$ 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 $\delta^{15}N$ has to be to the actual mixture signature. We used a source increment of 1% and a mass-balance tolerance 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:

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

where TN_{final} is the mass of TN of the final sample and $TN_{initial}$ is the mass of TN present at the

196 beginning of the experiment. For each treatment in each reservoir, we used the mean and SD of
197 the TN_{final} values to estimate a 95% confidence interval (CI) by generating 1000 random values
198 (normal distribution; SigmaPlot 11;). We calculated the TN_{final}:$TN_{initial}$ ratio for each of the
199 randomly generated TN_{final} values based on the $TN_{initial}$ measured for each reservoir. The upper
200 and lower limits of the range of TN_{final}:$TN_{initial}$ values were defined as the 97.5th and 2.5th
201 percentiles of the distribution. We used these upper and lower limits to constrain the solutions
202 generated using IsoSource after calculating the *F* ratio (right-hand term) in Eq. 2. The minimum,
203 median, and maximum amount of total fixed N (TN_{fix}) was calculated as the product of the 5th,
204 50th, and 95th percentiles of f_{fix} and the final TN concentrations of each mesocosm.
205 We derived N_{fix} from TN_{fix} values calculated from the median value of f_{fix} and expressed
206 as µg N L^{-1} h^{-1}. Each N_{fix} value was considered to represent the average N_2 fixation rate for that
207 mesocosm over the course of the experiment (assuming a photoperiod of 13 h for each day of the
208 experiment). We conducted ordinary least squares (OLS) regression analyses in SAS (version
209 9.1; SAS Institute, Cary, North Carolina) to estimate the effect of N:P supply on $\delta^{15}N$, f_{fix}
210 (median), N_{fix}, and RR_{TN} and RR_{PC}. RRs were the final concentration divided by the initial
211 concentration in each mesocosm (accounting for volume loss throughout the course of the
212 experiment). We used RRs to estimate gross N_2 fixation (RR_{TN}) and the efficiency of N_2 fixation
213 (RR_{PC}) across N:P supply in each reservoir because a slope > 0 indicates that added reactive N
214 yielded more biomass than N_2 fixation. Therefore, any slopes in OLS regressions for RR_{PC} that
215 were significant ($p < 0.05$) were considered to indicate that N_2 fixation was not efficient in terms
216 of biomass accumulation, respectively, for that reservoir.
217 To test for effects of initial conditions, we compared the slopes between each variable
218 ($\delta^{15}N$, f_{fix}, N_{fix}, RR_{TN}, and RR_{PC}) vs N:P supply among reservoirs and subsequently tested for

219 differences in *y*-intercepts with analysis of covariance (ANCOVA) in cases where slopes were
220 equal among reservoirs. We used generalized linear models (GLMs) in Systat (version 13.1;) to
221 compare the slopes of the N:P and each variable among reservoirs by including the N:P ×
222 reservoir interaction term. In cases where the slopes were not statistically different among
223 reservoirs ($p > 0.05$ for N:P × reservoir for a given variable), we reran the GLM model without
224 the interaction term to generate the ANCOVA.
225
226 **RESULTS**
227 **Reservoir conditions**
228 Lakes Brittany, Norwood, and Rayburn are mesotrophic to slightly eutrophic reservoirs
229 with average TN and TP ranging from 435 to 699 µg N/L and 15 to 35 µg P/L, respectively (Fig.
230 1A, B) and do not typically experience HABs. Norwood and Rayburn had similar nutrient
231 concentrations, which were generally greater than in Brittany. However, molar TN:TP was
232 similar across reservoirs (average across reservoirs: 44.7 to 65.1; Fig. 1C). A clear trend in NO_3^--
233 N drawdown was observed in all 3 reservoirs as summer thermal stratification progressed. The
234 date on which NO_3^--N was reduced below the detection limit (16.5 µg N/L) differed
235 substantially among reservoirs. Norwood experienced drawdown ~1 mo after Brittany and
236 Rayburn (Fig. 1D). An apparent distinction in PC (used here as a proxy for phytoplankton
237 biomass) concentrations between Brittany and the other reservoirs indicated that Brittany was
238 less productive and possibly more nutrient limited than Norwood or Rayburn (Fig. 1E).
239 Both Rayburn and Brittany had measurable concentrations of microcystin on the 2 dates
240 it was analyzed (before and in the middle of the mesocosm experiments; Fig. 1F). The
241 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 Rayburn (0.70 µg/L) than in Brittany (0.01 µg/L) just before the mesocosm experiments began in late July (Fig. 1F). Each reservoir experienced an increase in N_2-fixing and non-N_2-fixing cyanobacteria from late May to late June following NO_3^--N drawdown (Fig. 2A–C). These taxa subsequently decreased in Brittany and Rayburn, and another relative increase in N_2-fixing cyanobacteria biomass was observed in Brittany in September. Norwood experienced cyanobacteria dominance (with N_2-fixers accounting for nearly 70% of total phytoplankton biomass) sustained late into the growing period following NO_3^--N drawdown (Fig. 2B).

Mesocosm experiment

The $\delta^{15}N_{initial}$ of seston varied across reservoirs, ranging from 0.33‰ in Norwood to 9.66‰ in Brittany (Table 1). A strong, positive linear trend between $\delta^{15}N_{final}$ (seston $\delta^{15}N$ 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_{fix} values generated by IsoSource ranged from 0.08 to 0.77 across all treatments and reservoirs (Fig. 3B). A significant trend of decreasing f_{fix} as N:P supply increased was observed in each reservoir (Table 2). The slope of f_{fix} 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_{final} at low N:P supply in Brittany than in Norwood and Rayburn (Fig. 3B). Furthermore, N_{fix} ranged from 0.61 to 4.31 µg N L^{-1} h^{-1} throughout the experiment, with the highest rates occurring in the lowest N:P supply treatment in Brittany and Rayburn (Fig. 4). Slopes of N_{fix} vs N:P supply regressions did not differ among reservoirs. However, the intercept for the N_{fix} regression was significantly lower in Norwood than in

Brittany and Rayburn, which were not different from each another (Table 3). Thus, N_{fix} were lower in Norwood than in Brittany and Rayburn, especially across low N:P (Fig. 4).

RR_{TN} 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_{TN} increase/unit N:P supply was significantly greater in Brittany than in Norwood and Rayburn, which were not different from each other (Table 3). Likewise, RR_{PC} increased significantly with N:P supply, but the increase was statistically significant only in Brittany and Norwood (Table 2). RR_{PC} increased with N:P supply significantly more in Brittany than in Norwood and Rayburn (Table 3). Mean RR_{PC} increased ~66% from the 1 to 50 N:P supply in Norwood, and an increase of 127% on average was observed in 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 µg/L occurred only in Rayburn at the highest N:P supply treatment, where they exceeded the guideline by nearly 3× (Fig. 6). Microcystin concentrations in the highest N:P supply treatment in Rayburn were 4 to 6× greater than ambient reservoir concentrations measured just before the start of the mesocosm experiment in late July and in mid-August, respectively.

DISCUSSION

N_2 fixation rates were strongly related to the experimental N:P conditions across the 37-d mesocosm experiments in all 3 reservoirs, but N_2 fixation was not fully efficient. RR_{TN} was positively correlated with experimental N:P in all reservoirs, a result indicating that gross 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 N_2 fixation alone may have prohibited N_2 fixation from meeting potential N demands. RR_{PC} also was positively correlated with experimental N:P Lakes Brittany and Norwood. These findings support the idea that 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, RR_{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 were statistically equivalent regardless of the N source. This finding supports the notion that N_2 fixation can supply sufficient N, relative to P, for phytoplankton to achieve maximum biomass yield (Schindler 2012). N_2 fixation was least efficient in fueling HABs (in both RR_{TN} and RR_{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 N_2 fixation rates and efficiency during HAB events.

Importance of N_2 fixation across the N:P supply gradient

N_2 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_2 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 composed nearly 50% of the PN pool at ~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 ~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× as much 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_{fix} values would be expected in our experiment, even after a longer period of time (37 vs 21 d).

The N_2 fixation rates measured in our study ranged from 0.61 to 4.31 µg N L^{-1} h^{-1}, which were comparable to those observed in various other studies. N_2 fixation rates in several Texas reservoirs ranged from 0 to 11.7 µg N L^{-1} 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 µg N L^{-1} 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 µg N L^{-1} h^{-1} in Lake Mendota during summer 2010 and 2011. As expected, N_2 fixation was significantly stimulated as N:P decreased in mesocosms across all 3 of our study reservoirs (Fig. 4), and the response of N_2 fixation to N:P was similar regardless of initial conditions. The occurrence of N_2 fixation at 50 N:P was not unexpected because our mesocosms were a closed system where even excessive dissolved nutrients were exhausted.

N_2 fixation efficiency, initial conditions, and ecosystem dynamics

Despite substantially increased N_2 fixation rates at low N:P, RR_{TN}, and RR_{PC} increased with increasing inorganic N availability (Fig. 5A, B), indicating that phytoplankton blooms relying heavily on N_2 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_2 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 with increased as N:P inreased (Table 2). Thus, the phytoplankton species dominating assemblages at low N:P treatments in Rayburn were evidently more efficient than those dominating assemblages at higher N:P when generating biomass per unit N supplied via N_2 fixation. This finding indicated that conditions at the onset of bloom formation can affect N_2-fixation efficiency.

The increase in RR_{TN} and RR_{PC} across the N:P supply gradient was significantly greater in Brittany than in Norwood and Rayburn. Lake Brittany had the lowest initial TN and PC concentrations, so RR_{TN} and RR_{PC} values were expected to exceed those in the other reservoirs. The slopes of the RR_{TN} or RR_{PC} vs N:P supply regressions were not necessarily expected to be greater in Brittany because as the initial TP concentration was lower and TN:TP was higher than in 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 Brittany, a result suggesting that potentially recalcitrant dissolved fractions in Lake Brittany had a high initial N:P that skewed the magnitude of stoichiometric imbalance. As a result, N may have been limiting growth in Brittany initially because of a relatively refractory initial TDN pool and low DIN (NO_3^--N < 16.5 µg/L). Inaccessibility of TDN as a source of N was corroborated by phytoplankton identification and enumeration data in Brittany, which indicated that 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 ratios were 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).

N_2 fixation and microcystin production during blooms

Microcystin concentrations greater than the WHO impairment standard (1 µg/L) were detectable only in the 50 N:P treatment in Rayburn (Fig. 6). Lake Rayburn had the highest ambient concentration of microcystin near the beginning of the experiment (0.701 µg/L; Fig. 1F), indicating significant microcystin production in Rayburn at the beginning of the mesocosm experiment. Thus, initial conditions of the experiment may have excluded potential microcystin producers from the mesocosms in Brittany and Norwood (Sarnelle 2007). The relative increase in microcystin concentrations after nutrient enrichment with high N (and P) only in Rayburn highlights the potential importance of 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_2-fixers and non-N_2-fixers during bloom development and senescence may lead to differential toxicity among blooms (Beversdorf et al. 2013).

380

381 **Study implications**

382 Our results indicate that N_2 fixation may not alleviate N limitation effectively in HAB
383 events driven by high P concentrations. N_2 fixation contributed large amounts of N to
384 mesocosms with high P supply and an imbalance of N in 3 small, temperate, man-made
385 reservoirs, but phytoplankton accumulated more N and produced more biomass when more
386 reactive N was available in the water column. This response was reservoir-dependent, with
387 phytoplankton biomass seemingly decoupled from N in the Lake Rayburn experiment. These
388 contrasting results highlight the importance of biological and chemical conditions unique to
389 individual reservoirs, which in the case of our study, strongly influenced the initial conditions of
390 the mesocosm experiments. An ongoing debate exists within the scientific literature regarding
391 whether N plays a significant role in controlling primary productivity in lakes over long time
392 scales (Elser et al. 2007, Lewis and Wurtsbaugh 2008). This debate has been extended to
393 whether N, in combination with P, should be managed to reduce the harmful effects of
394 accelerated eutrophication (Schindler et al. 2008, Conley et al. 2009). N can degrade water
395 quality via accelerated eutrophication in some lakes (Finlay et al. 2010). However, the evidence
396 supporting the N mitigation as a tool to reduce the harmful effects of eutrophication in lakes is
397 limited, but increasing (Paerl et al. 2016). Our results show that cyanobacterial biomass yield
398 increases in blooms where inorganic N is more readily available than P in the water column.
399 Thus, from the perspective of short-term bloom management, lower N concentrations may
400 reduce the frequency and magnitude of toxic bloom formation.

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FIGURE CAPTIONS

Fig. 1. Total N (TN) (A), total P (TP) (B), molar TN:TP (C), 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 on both sampling dates in Norwood.

Fig. 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.

Fig. 3. Ordinary least squares regressions for mean (±SD, $n = 3$) stable-isotope ($\delta^{15}N$) (A) and median fraction of total N composed of fixed N (f_{fix}) data vs N:P supply by reservoir for samples collected at the end of the mesocosm experiment ($n = 12$/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.

Fig. 4. Ordinary least-squares regression for mean (±SD) treatment N_2-fixation rates (N_{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 N_2-fixation rate minima and maxima calculated from the range of f_{fix} values generated using IsoSource.

547 Fig. 5. Ordinary least-squares regression for mean (±SD) treatment response ratios (RR = the
548 ratio of final to initial concentration) as a function of molar total N (TN:total P (TP) for
549 TN (RR_{TN}) (A) and particulate C (RR_{PC}) (B) in each reservoir. OLS regression analyses
550 were conducted for each RR by reservoir, with $p < 0.05$ indicating a slope significantly
551 different than 0. See Table 2 for results of regression analyses.
552 Fig. 6. Microcystin measured from a subsample collected from a treatment composite sample
553 (samples from all 3 mesocosms from that treatment combined) in each reservoir.
554 Horizontal dashed line represents the World Health Organization's provisional guideline
555 of 1 µg/L, above which concentrations are considered unsafe for human health.

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. $\delta^{15}N$ = isotopic composition of seston, PC is particulate C, and Chl *a* is chlorophyll *a*. Initial values were.

Reservoir/condition	$\delta^{15}N$ (‰)	TN (mg/L)	TP (mg/L)	TN:TP	PC (mg/L)	Chl *a* (µg/L)
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
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
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

561 Table 2. Results from ordinary least squares linear regression analyses for median fraction of
562 total N composed of fixed N (f_{fix}), the N_2-fixation rate (N_{fix}), the response ratio of total N (RR_{TN}),
563 and the response ratio of particulate C (RR_{PC}) vs N:P supply for each reservoir (n = 12). Slopes
564 (m) were considered significantly different from 0 when $p < 0.05$.

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

565

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

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

570

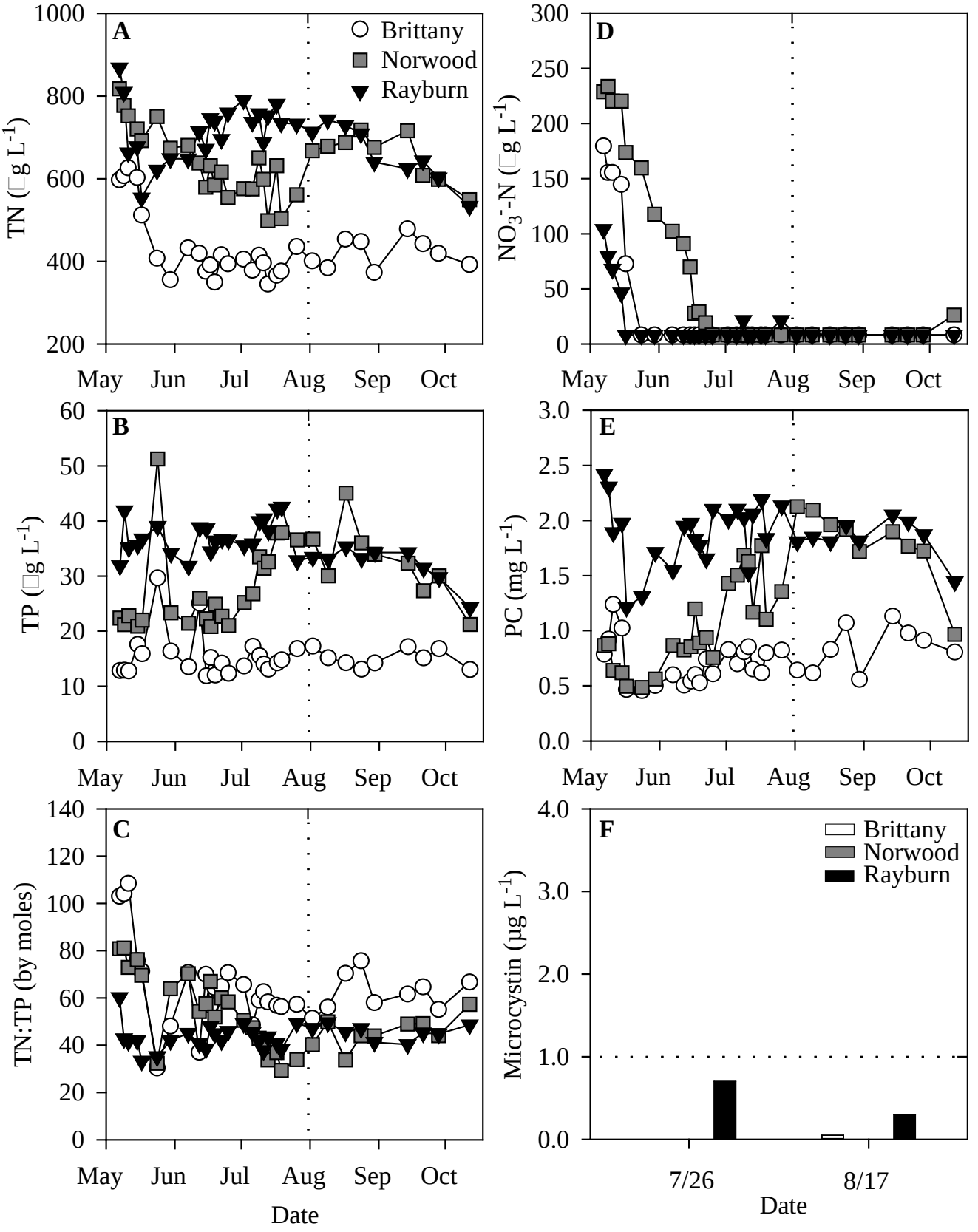
A
Brittany
Norwood
Rayburn
TN (□g L-1)
1000
800
600
400
200
May
Jun
Jul
Aug
Sep
Oct
D
NO3--N (□g L-1)
300
250
200
150
100
50
0
B
TP (□g L-1)
60
50
40
30
20
10
0
E
PC (mg L-1)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C
TN:TP (by moles)
140
120
100
80
60
40
20
0
Date
F
Microcystin (µg L-1)
4.0
3.0
2.0
1.0
0.0
7/26
8/17
Date

Figure 1

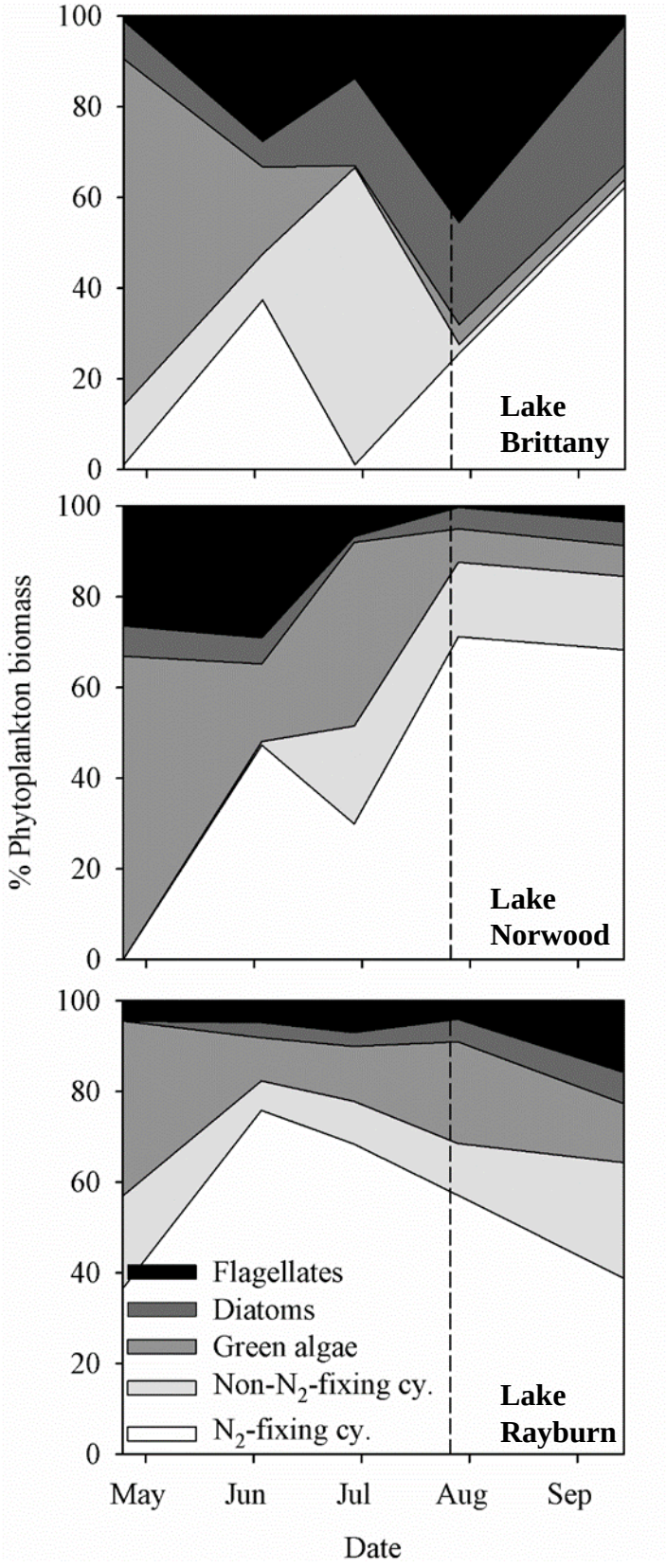
% Phytoplankton biomass
100
80
60
40
20
0
Lake Brittany
Lake Norwood
Lake Rayburn
Flagellates
Diatoms
Green algae
Non-N2-fixing cy.
N2-fixing cy.
May
Jun
Jul
Aug
Sep
Date

Figure 2

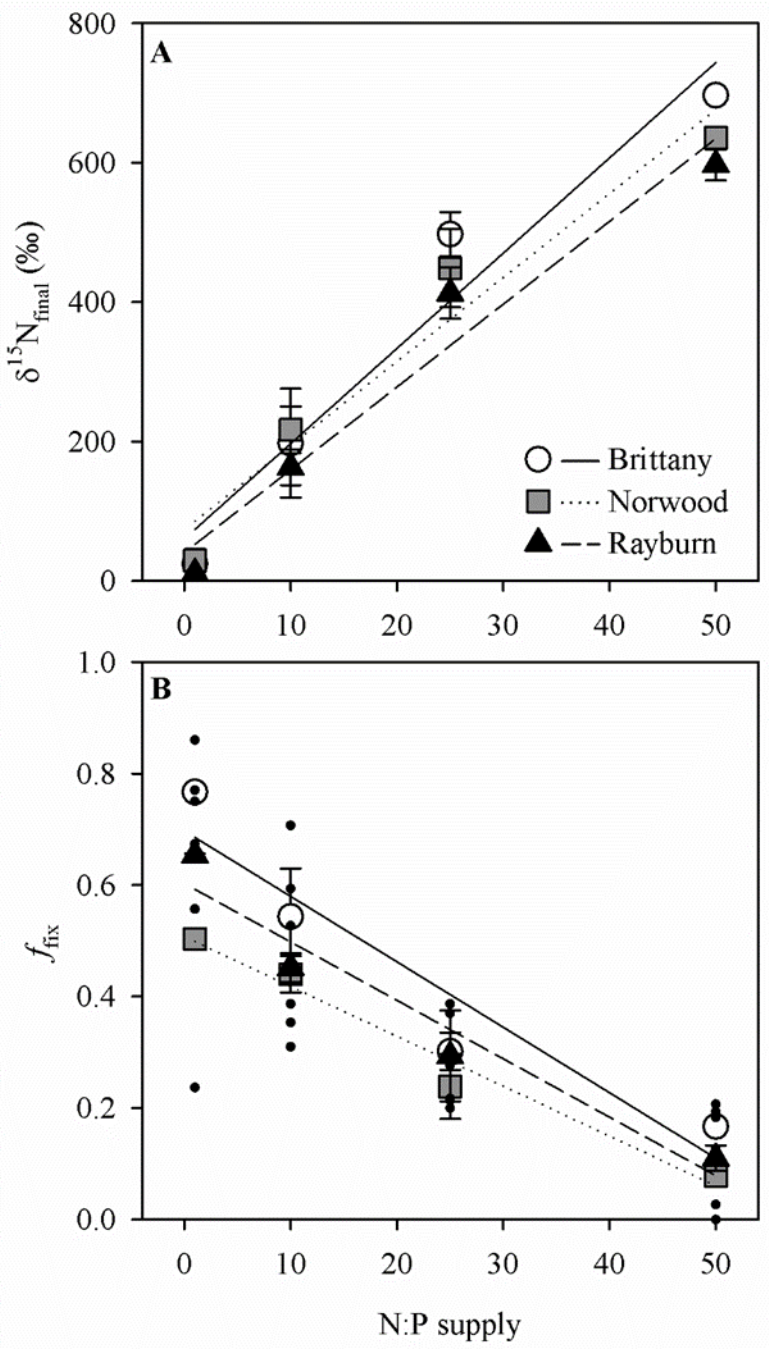
A
B
800
600
400
200
0
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
δ15Nfinal (‰)
ffix
N:P supply
Brittany
Norwood
Rayburn

Figure 3

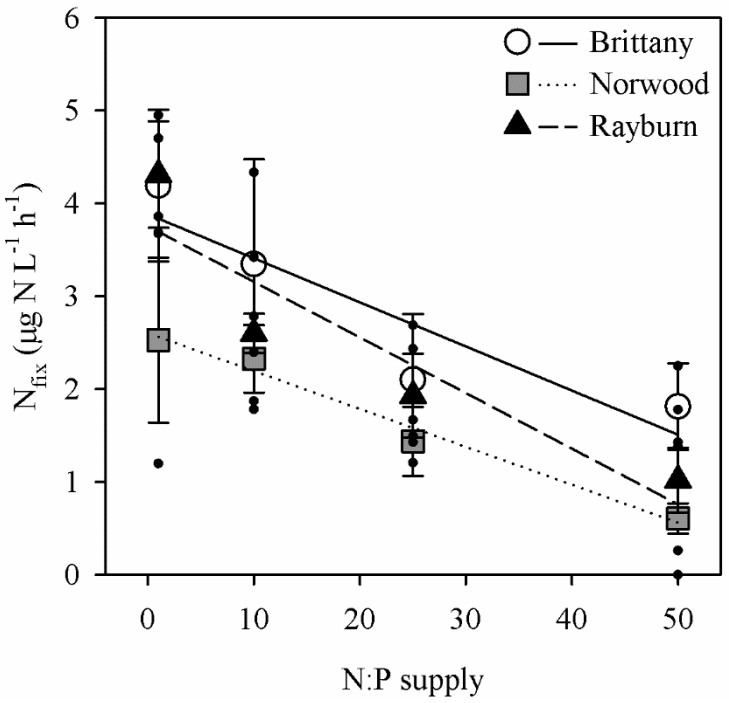
Brittany
Norwood
Rayburn
N_{fix} (µg N L^{-1} h^{-1})
0
1
2
3
4
5
6
0
10
20
30
40
50
N:P supply

Figure 4

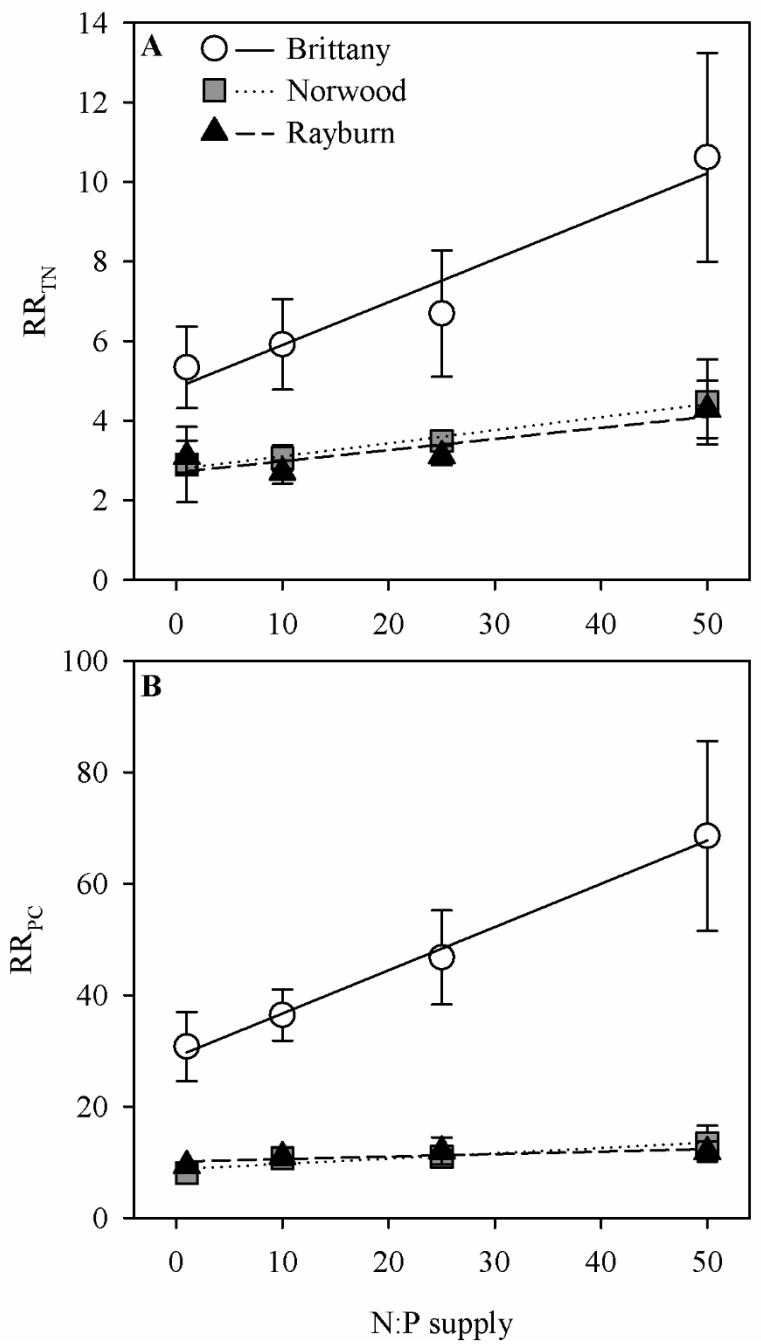

Figure 5

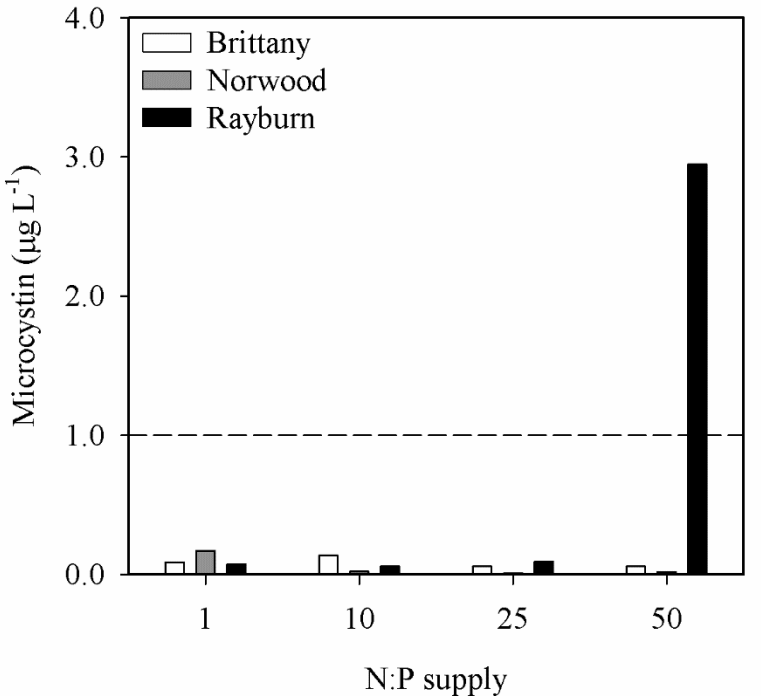
4.0
3.0
2.0
1.0
0.0
Microcystin (µg L-1)
Brittany
Norwood
Rayburn
1
10
25
50
N:P supply

Figure 6