Defining the balance between cyanobacterial N_2 fixation and denitrification in Falls of the Neuse Reservoir, NC

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Executive Summary:
The balance between N_2 fixation by cyanobacteria and N removal via denitrification is a critical driver of phytoplankton nutrient limitation in lakes and reservoirs. Denitrification in shallow, highly productive lakes and reservoirs can remove significant quantities of N that in combination with efficient trapping of P, can lead to strong N limitation. N limitation has the potential to favor heterocystous cyanobacteria that are capable of N_2 fixation. Falls Lake's phytoplankton community contains high densities of heterocystous cyanobacteria. However, no data existed on microbial N additions through N_2 fixation or removal by denitrification for Falls Lake. This project addressed this knowledge gap of nutrient dynamics in Falls Lake by tackling three research questions:

1) Do microbial processes cause a net production (N_2 fixation) or removal (denitrification) of biologically available N from Falls Lake?
2) Is N_2 fixation quantitatively important relative to stream loads and atmospheric deposition, and therefore, worth including in water quality models?
3) What factors stimulate or constrain N_2 fixation in Falls Lake?

Rates of microbial N_2 fixation were measured from spring through fall during 2019 to 2022 at six main channel and ten creek arm stations using the acetylene reduction technique. Concentrations of bioavailable N and P, light availability, physical conditions, and the biomass and composition of the phytoplankton community were also measured to understand factors that relate to N_2 fixation. Measured rates were scaled up to annual lake-wide estimates. The measured rates were also compared to the biomass of heterocystous cyanobacteria during each measurement to produce a biomass-specific N_2 fixation rate. The biomass-specific rate was used to estimate a time series of N_2 fixation based on historical records of heterocystous cyanobacteria biomass measured by the NC Dept. of Environmental Quality (NCDEQ). Lakewide N_2 fixation estimates were used to constrain the N budget and calculate lake-wide N losses via denitrification using a mass balance approach. Denitrification rates calculated by the mass balance complemented direct measurements of denitrification made on sediment cores by Dr. Piehler's lab as part of the Collaboratory's Falls Lake Nutrient Study and denitrification estimates produced by a sediment diagenesis model calibrated with sediment porewater concentration data from Falls Lake. A series of nutrient addition experiments were also conducted to determine the limiting nutrient for phytoplankton growth in Falls Lake.

Direct measurements and biomass-based estimates of N_2 fixation indicated that N_2 fixation contributes less than 1% and about 6% of total N inputs to Falls Lake, respectively. Such a small fraction of total N inputs justifies omitting the process in eutrophication models for Falls Lake. Based on the mass balance and direct core measurements of denitrification it appears that denitrification exceeds N_2 fixation and that the balance of these microbial processes result in a net loss of N from Falls Lake. Net loss of N could help maintain N limited phytoplankton which is consistent with N limited growth observed in nutrient addition experiments conducted in spring and summer 2021. Most of the N and P within Falls Lake are bound up in plankton biomass. P is not available in great excess and appeared to be an important constraint on N_2 fixation. This situation of N limitation but with the potential for stimulation of N_2 fixation by P suggests that dual management of N and P is warranted for preventing undesirable levels of phytoplankton biomass in Falls Lake.

Introduction

The balance between N_2 fixation by cyanobacteria and N removal via denitrification is a critical driver of phytoplankton nutrient limitation in lakes and reservoirs (Scott and McCarthy 2010). Denitrification in shallow, highly productive lakes and reservoirs can remove significant quantities of N that in combination with efficient trapping of P can lead to strong N limitation (Grantz et al. 2014). N limitation has the potential to favor cyanobacteria groups capable of N_2 fixation. Examination of DWR's phytoplankton community composition data indicated that heterocystous cyanobacteria capable of N_2 fixation regularly comprise 25% or more of the phytoplankton biomass during the summer, but water quality models for Falls Lake including NCDEQ's model (Lin) and current modeling efforts by the Upper Neuse River Basin Association (UNRBA) do not contain a N_2 fixing cyanobacteria group. Omission of a N_2 fixing cyanobacteria group precludes the ability to simulate these N inputs to the reservoir and could create severe errors in estimation of phytoplankton biomass responses if N_2 fixation is an important process or could become quantitatively important if N inputs are reduced (Schindler et al. 2008).

Constraining N inputs by N_2 fixation significantly enhances our understanding of phytoplankton nutrient responses in Falls Lake and fills a significant data gap in the N mass balance for Falls Lake. Filling this gap of N inputs to Falls Lake allows calculation of lake-wide N losses via denitrification using a mass balance approach (Molot and Dillon 1993). Denitrification rates calculated by the mass balance complement direct measurements of denitrification made on sediment cores by Dr. Piehler's lab as part of the Collaboratory's Falls Lake Nutrient Study and denitrification estimates produced by a sediment diagenesis model calibrated with sediment porewater concentration data from Falls Lake (Alpering 2018). Collectively, these efforts provide significant information on water column and sediment N cycling within Falls Lake that will aid understanding responses of Falls Lake water quality to a rapidly changing watershed and climate and will inform future modeling efforts.

This project sought to answer three research questions:

4) Do microbial processes cause a net production (N_2 fixation) or removal (denitrification) of biologically available N from Falls Lake?
5) Is N_2 fixation quantitatively important relative to stream loads and atmospheric deposition, and therefore, worth including in water quality models?
6) What factors stimulate or constrain N_2 fixation in Falls Lake?

METHODS

Sampling: Between July 2019 and August 2022, a series of sampling campaigns were conducted along a transect of 6 main channel stations and at 10 creek arm sites to measure N_2 fixation and the biological, physical, and chemical characteristics at each site. Details of the sampling campaigns are provided in the Supplemental Information.

Measurement of water column N_2 fixation: All N_2 fixation measurements were conducted using the acetylene reduction assay and details are provided in the Supplemental Information. During fall 2019 and summer 2020, N_2 fixation measurements conducted at mid-channel locations were made at different light levels to understand how N_2 fixation responded to the strong vertical light gradient in Falls Lake. For creek arm samples collected during 2021 and the creek arm and mid-channel samples collected during summer 2022, N_2 fixation measurements were made at a single irradiance level (20% incident light). For all measurements, water temperature was maintained at the *in situ* temperature.

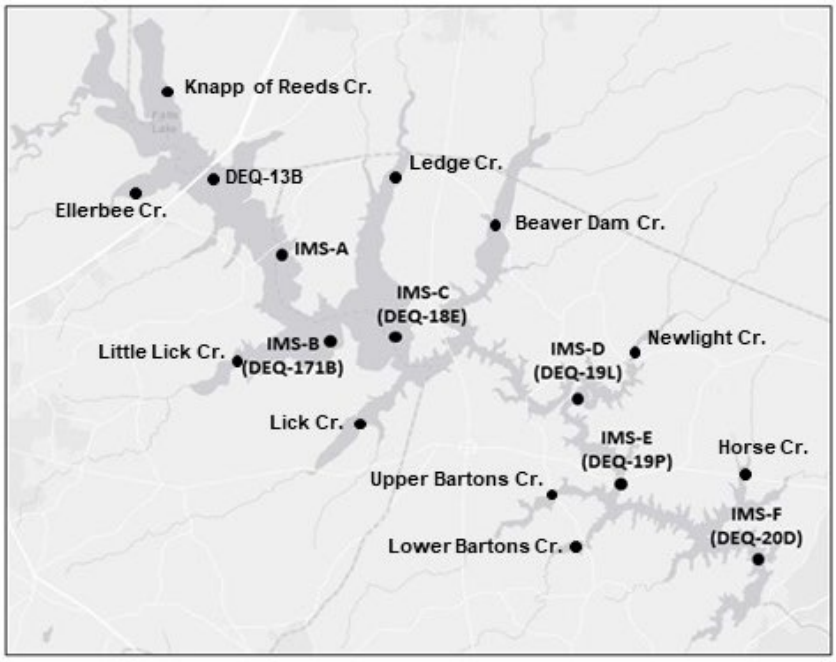

Figure 1. Map of main channel and creek sampling stations for measurements of N fixation rate. Five of the six main channel stations coincided with stations sampled monthly by NC Dept. of Environmental Quality

Measurement of nutrients, vertical structure of the water column, and phytoplankton biomass and community composition: Depth profiles of temperature, conductivity, dissolved oxygen, pH, and photosynthetically active radiation (PAR) were measured at each sampling event. The euphotic zone depth was calculated as the depth of 1% PAR penetration. For each sample, nutrient measurements included dissolved ammonium, nitrate+nitrite, phosphate, total dissolved nitrogen, and silicate, and particulate nitrogen. Phytoplankton biomass was estimated as chlorophyll *a* and accessory photopigments measured by HPLC were used to estimate the biomass of different phytoplankton classes including cyanobacteria. An aliquot of each sample was additionally preserved in Lugol's solution for species-level microscopic identification and enumeration of the phytoplankton community. Biomass of potentially N_2 fixing, heterocystous cyanobacteria within the order Nostocales were microscopically quantified based on methods in Hall and Paerl (2011). Details of the analytical procedures are provided in the Supplemental Information.

Relationships between N_2 fixation and bioavailable, inorganic N and P nutrient forms (nitrate, ammonium, and phosphate), and cyanobacteria biomass determined by accessory photopigments and by microscopy were explored using Spearman's rank correlations to improve understanding of the controls on N_2 fixation in Falls Lake. Details of data treatment including treatment of censored values are provided in the Supplemental Information.

Examination of the relationships between directly measured rates of N_2 fixation by microscopically determined biomass of heterocystous cyanobacteria biomass allowed an estimate of the biomass-specific rate of N_2 fixation and comparing our observed biomass-specific N_2 fixation rates against literature values allowed us to assess how active the N_2 fixing cells of Falls Lake are compared to that observed in other systems, and whether observed variation in rates is likely due to changes in biomass or changes in biomass-specific activity levels. The relationship developed between heterocystous cyanobacteria biomass and directly measured N_2 fixation was additionally used in conjunction with time series data of heterocystous cyanobacteria biomass collected by NCDEQ at four stations (Figure 1; NEU013B, NEU018E, NEU19P, NEU20D) to estimate an approximate monthly time series of N_2 fixation from 2011 to 2020.

Estimates of lake-wide, annual N input due to N_2 fixation were calculated in two ways. The first method involved scaling up the direct measurements of N_2 fixation made in this project to produce a lake-wide estimate. The second method involved scaling up and averaging the monthly time series of N_2 fixation estimated based on heterocystous cyanobacteria biomass measured by NCDEQ at the four NCDEQ stations. Details of the procedure used to scale up these measurements can be found in the Supplemental Information but both methods accounted for the photic volume of areas of the lake represented by the available N_2 fixation estimates and both methods assumed a 12 h per day photoperiod when N_2 fixation is possible. Interannual variability of the biomass-based (second method) annual N_2 fixation rates were compared against annual stream loads of N to investigate whether stream loads were related to N_2 fixation as would be expected if reduced N loads enhanced N limitation within the phytoplankton community.

Assessing nutrient limitation and effects of nutrient availability on N_2 fixation: Nutrient addition bioassay experiments were conducted at three creek stations during spring and summer 2021 to determine the limiting nutrient in the creek arms and to determine the extent to which N_2 fixation is impacted by P availability. For each experiment, triplicate Cubitainers were amended with the following treatments: a control with no added nutrients, nitrate addition, phosphate addition, and nitrate plus phosphate (see Paerl et al., 2014 for details). Phytoplankton growth and N_2 fixation were assessed after a three-day incubation period. The control and P addition treatment were additionally reassessed after one week to determine the degree to which enhanced P availability can stimulate shifts toward N_2 fixing cyanobacteria taxa. This information is useful for determining the potential for P inputs to stimulate N_2 fixation and can provide a useful upper constraint for modeled N_2 fixation rates (Del Guidice and Obenour 2021). Details of the set up and assessment methods for these experiments are provided in the Supplemental Information.

Characterizing the N mass balance: Annual tributary loads of total N and total P for tributaries to Falls Lake and atmospheric deposition of N over the period 2006 to 2019 were taken from NCDEQ's 2021 Status Report of the Falls Lake Nutrient Strategy (NCDEQ 2021). Annual fluxes of total N and total P out of Falls Lake were calculated using the weighted regressions on time, discharge, and season (WRTDS) model (Hirsch et al. 2010) on USGS gaged discharge (USGS gage 02087183) and monthly concentration data collected by NC DEQ's Ambient Monitoring System (station J1890000). Annual N inputs were calculated as the sum of tributary loads,

atmospheric deposition, and N_2 fixation. Net retention of N (TN_{ret}, units kg N/y) and P (TP_{ret}, units kg P/y) was determined as the difference between annual inputs and outputs through river flux. Under an assumption that net retention of P is due solely to sedimentation, the whole lake denitrification rate (DNF) can be estimated based on the ratio of N:P retention (N/P_{ret}) and the average N:P mass ratio of the lake's surface sediments (N/P_{sed}) which was estimated as 3.67 (Equation 1; Molot and Dillon 1993). Details on tributary loads and sediment N:P ratio are provided in the Supplemental Information.

Equation 1. $$DNF = TN_{ret}(N/P_{ret} - N/P_{sed})/(N/P_{ret})$$

RESULTS

Patterns of N_2 fixation in the main channel and creek arms: Rates of N_2 fixation in the main channel generally ranged from 0 to 4 nmol N/L/h (Figure 2) with an average of 1.3. There was no clear downstream spatial pattern along the main channel (Figure 2). The highest observed rate was 11.6 nmol N/L/h in a sample collected in May 2020 from station A and incubated just below the surface at 0.25 m depth (SI Figure 2).

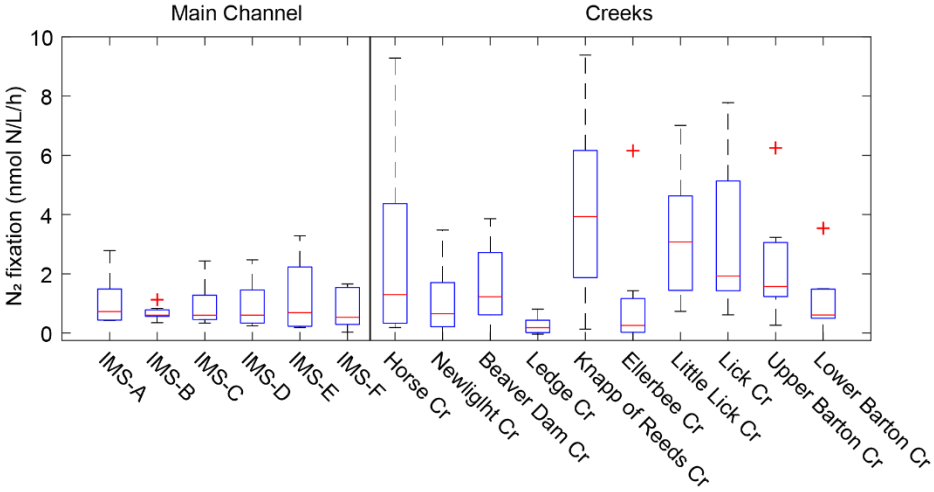

Figure 2. Boxplots of N_2 fixation at main channel and creek arm locations. Boxes represent interquartile range. Red lines indicate median values. Whiskers extend to 1.5 times the interquartile range and outliers beyond the whiskers are red plus symbols.

For the main channel measurements conducted in 2019 and 2020, rates were generally highest for sample aliquots incubated at higher levels of irradiance (Fig. SI-2). This indicates that light availability is likely a constraint on N_2 fixation in Falls Lake.

Rates of N_2 fixation in the creek arms ranged from 0 to ~10 nmol N/L/h (Figure 2) and averaged 3.2 nmol N/L/h, about three time higher than the mid channel sites. Among the creeks, it appears that creeks on the eastern side of the lake including Horse, Newlight, Beaver Dam and Ledge Creeks tend to have lower rates of N fixation than creeks on the western side of the reservoir (Figure 2). Within the spring through fall sampling conducted by this project, seasonality of N_2 fixation was weak but rates were generally highest during the heat of July and August (Figure SI-3).

Relationship of N_2 fixation to in situ nutrient concentrations: Highly-bioavailable, dissolved inorganic forms of N and P were generally found in very low concentrations at both the main channel and creek arm sites (Figure SI-4). Median nitrate values were below detection at all sites except for the upper main channel site, IMS-A, which had a low median nitrate of 4 µg/L. Main channel nitrate never exceeded 30 µg/L but high concentrations of 120, 260, and 1100 µg/L were

observed on single occasions at Beaverdam, Ledge, and Ellerbee Creeks (Figure SI-4). Ammonium ranged from 5 to 90 µg/L but median values were around 10 µg/L for both main channel and creek sites (Figure SI-4). Phosphate concentrations were generally between 2-4 µg/L (Figure SI-4) but ranged as high as 12 in the main channel sites and up to 115 and 50 µg/L on single occasions at New Light and Ellerbee Creeks. These occasional spikes in bioavailable nitrate and phosphate at the creek sites likely reflect recent nutrient pulses from the watershed with insufficient time lapsed for significant drawdown by phytoplankton.

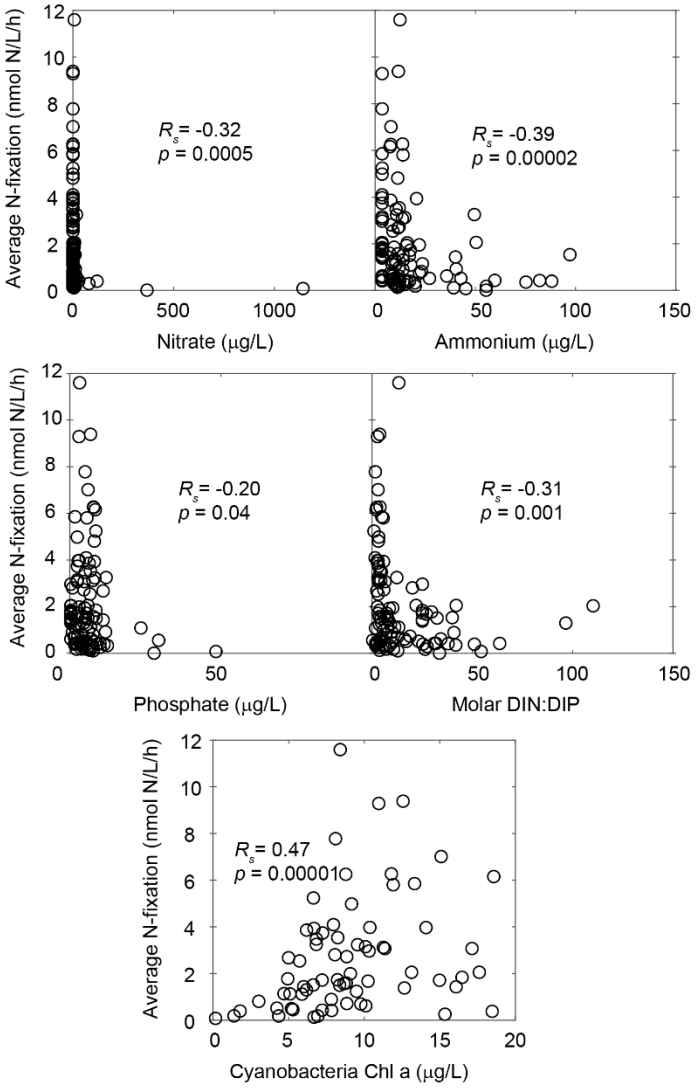

Figure 3. Scatter plots of N_2 fixation versus nutrients and cyanobacterial biomass estimated by HPLC pigment analyses. Rs and p values are results from Spearman's rank correlations.

N_2 fixation exhibited a significant negative relationship with nitrate, ammonium, phosphate, and the molar ratio of dissolved inorganic nitrogen to phosphate (Figure 3). Negative relationships of N_2 fixation with ammonium and nitrate might be expected because elevated ammonium is known to inhibit synthesis of the nitrogenase enzyme complex required for N_2 fixation (Agawin et al. 2007) and high nitrate availability often favors the growth of eukaryotes that can outcompete N_2 fixers (Paerl et al. 2014). The negative relationships with phosphate, however, were unexpected but may have resulted from the impacts of pulse flow events that increase phosphate concentrations while simultaneously diminishing the biomass of N_2 fixing cyanobacteria due to rapid flushing. When creek arm stations were removed from these analyses, N_2 fixation at the the main channel sites exhibited a weak but statistically significant positive relationship with phosphate. In general, these observed relationships between bioavailable nutrients and N_2 fixation are broadly consistent with the current paradigm that N_2 fixation is promoted under conditions of low N and high P availability (Andersen et al. 2019). However, the confounding influence of

flow on delivery of these nutrients and simultaneous flushing of N_2 fixing biomass cannot be eliminated as a potential cause of the observed relationships.

Nutrient limitation of phytoplankton growth: Nutrient addition bioassays were conducted from water collected in June and August 2021 from Ledge, Knapp of Reeds, and Upper Barton Creeks. These experiments revealed an N limited phytoplankton community. Addition of P, however, did significantly stimulate the cyanobacterial fraction of the phytoplankton in the August experiments and increased rates of N_2 fixation by 400-1000% compared to the control. The relatively low levels of phosphate concentrations and in situ N_2 fixation, and high degree of stimulation of N_2 fixation by P additions during these August experiments provides evidence that availability of bioavailable P is likely a key constraint on N_2 fixation in Falls Lake. Greater detail of these experimental results is provided in the Supplemental Information.

Relationship between cyanobacteria biomass and N_2 fixation: A weak but statistically significant positive relationship was observed between cyanobacterial biomass determined by HPLC pigment analyses and N_2 fixation (Figure 3). Low cyanobacteria pigments were related to low rates of N_2 fixation. However, high cyanobacteria pigments were not always related to elevated N_2 fixation. This result was not surprising for two reasons. First, heterocystous cyanobacteria that are potentially capable of N_2 fixation may not conduct N_2 fixation due to P limitation or inhibition by excess N availability as was seen in the nutrient addition experiments. Second, many of the cyanobacteria known to dominate Falls Lake cyanobacteria assemblages are non-N_2 fixing members of the *Oscillatoriales* and *Chroococcales* (Touchette et al.2007) and it is impossible to distinguish between non-N_2 fixing and N_2 fixing cyanobacteria based on pigment composition.

The biomass of potentially N_2 fixing, heterocystous taxa in the order *Nostocales* was quantified microscopically for the main channel samples. Biovolume of potential N_2 fixing cyanobacteria ranged from 0 to ~2.8 10^9 μm^3/ L (Figure SI-8) and exhibited a much stronger ($R_S = 0.69$, $p < 0.00001$) relationship with N_2 fixation compared to the relationship with cyanobacterial chlorophyll *a* (Figure 3). The ratio of microscopically quantified N_2 fixing biomass to N_2 fixation was used to estimate a biomass-specific N_2 fixation activity for heterocystous cyanobacteria in Falls Lake of 8.5×10^{-9} nmol N/μm^3 N-fixer biovolume/h. Details on the derivation of this rate are provided in the Supplemental Information.

Estimates of N_2 fixation based on direct measurements: Based on the values of direct measurements of N_2 fixation from the main channel and creek arm sites and the assumptions used to scale our bottle incubation measurements up to lake-wide annual rates (see Supplemental Information), the annual N input due to N_2 fixation is ~3800 kg N/ y (Table 1). The estimated N_2 fixation of the creek arms 2000 kg N/y was just slightly higher than the estimate for the main channel, 1800 kg N/y. This relatively minor difference in annual estimate despite an average 2-fold higher measured N_2 fixation in the creek arms (Figure 2) was caused by the smaller total area of the creek arms (18 km^2 for the creek arms vs 26 km^2 for the main channel) but also by the shallower photic zone depth of many of the creek arms. Details on photic zone depths at the different sites are provided in the Supplemental Information.

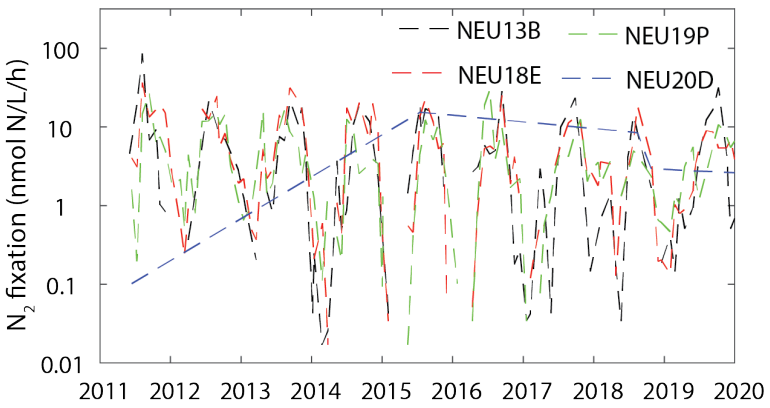

Figure 4. Time series of estimated N_2 fixation at four locations based on the product of heterocystous cyanobacterial biomass measured by NCDEQ and the median biomass-specific N_2 fixation rate measured in this study from main-channel sites.

Estimates of N_2 fixation based on heterocystous cyanobacteria biomass: The heterocystous cyanobacteria community composition in the long-term NCDEQ record was nearly identical to that observed during this study's N_2 fixation measurements (Figure SI-9). This provided confidence that substantial shifts in the cyanobacterial community that could impact N_2 fixation rates had not occurred over the long-term record. Estimates of N_2 fixation rates based on heterocystous cyanobacteria biomass measured by NCDEQ varied over nearly four orders of magnitude from ~0.01 to 100 nmol N/L/h. Much of the variability was due to pronounced seasonality with average N_2 fixation fluctuating from about 0.1 nmol N/L/h during winter to above 10 nmol N/L/h during summer (Figure 4).

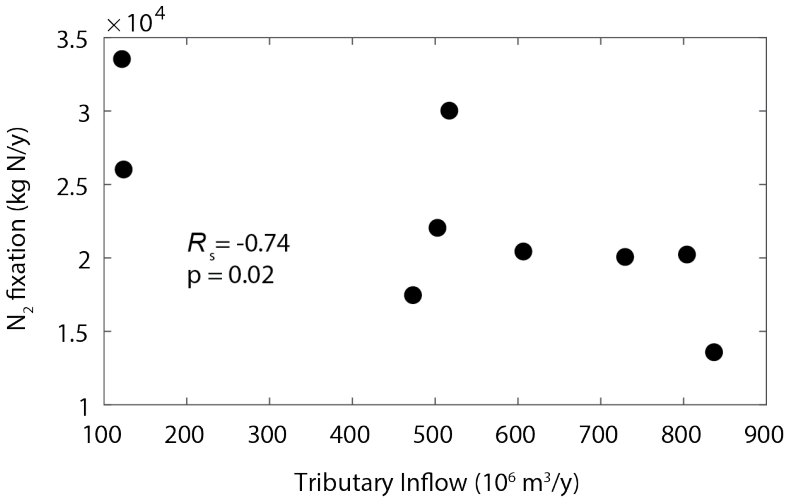

Figure 5. Annual N_2 fixation rate estimated from NCDEQ measurements of heterocystous cyanobacterial biomass versus annual tributary inflow to Falls Lake. R_s and p values are from a Spearman's rank correlation.

Estimates of lake-wide annual N_2 fixation rates estimated based on heterocystous cyanobacteria biomass ranged from 14,000 to 34,000 kg N/y and averaged 28,000 kg N/y, approximately sevenfold higher than the annual estimate based on scaling up direct measurements (3,800 kg N/y). Thus, depending on which method is used, N_2 fixation supplied an average of <1% of tributary N loads if the direct measurements are used (Table 1) versus an average of ~6% if the estimates based on cyanobacteria biomass are used (Table 2).

In dry years like 2011 and 2012 that had low tributary loads, estimates based on cyanobacteria biomass indicated that N_2 fixation may equal up to 20% of the tributary N load, roughly the same magnitude as atmospheric deposition (Table 2). The increased relative importance of N_2 fixation during dry years was not only caused by decreases in tributary loads. There also was a general trend of increasing

magnitude of estimated N_2 fixation under low flow conditions (Figure 5) which might indicate compensation of N deficits by N_2 fixation when tributary N loads are low.

N mass balance: The annual estimates of N_2 fixation derived using both methods were added to annual estimates of tributary N loads and atmospheric deposition to develop a mass balance for N for the years 2006 to 2019 (Tables 1 and 2). First, we describe the nutrient budget obtained using the scaled-up direct measurements to produce a constant annual average N_2 fixation rate (Table 1), and we subsequently contrast that nutrient budget with the budget derived from annually-variable N_2 fixation estimates based on heterocystous cyanobacterial biomass (Table 2).

Table 1. Mass balance of total N and total P and calculations of lake-wide annual denitrification for the period 2006-2019 using a constant annual N_2 fixation rate derived from direct measurements made by this study.

Year	Trib. N load (kg N/y)	Atm. dep. (kg N/y)	N_2 fix. (kg N/y)	N_2 fix. (% Trib. Load)	Total TN flux in (kg N/y)	River TN flux out (kg N/y)	TN retain (%)	Trib. P load (kg N/y)	River TP flux out (kg P/y)	TP retain (%)	Denit. (kg N/y)	Denit. (% Trib. Load)
2006	4.3×10^5	7.1×10^4	3.8×10^3	0.9	5.1×10^5	2.3×10^5	55	7.9×10^4	1.3×10^4	84	4.0×10^4	9
2007	3.1×10^5	5.7×10^4	3.8×10^3	1.2	3.7×10^5	2.6×10^5	32	4.1×10^4	1.6×10^4	61	2.5×10^4	8
2008	5.2×10^5	6.3×10^4	3.8×10^3	0.7	5.9×10^5	2.0×10^5	65	8.6×10^4	1.1×10^4	88	1.1×10^5	21
2009	6.7×10^5	5.3×10^4	3.8×10^3	0.6	7.2×10^5	4.6×10^5	37	7.7×10^4	2.6×10^4	67	7.6×10^4	11
2010	4.8×10^5	5.0×10^4	3.8×10^3	0.8	5.3×10^5	3.6×10^5	32	5.3×10^4	2.1×10^4	60	5.3×10^4	11
2011	2.0×10^5	5.9×10^4	3.8×10^3	1.9	2.7×10^5	8.3×10^4	69	1.6×10^4	3.9×10^3	75	1.4×10^5	69
2012	2.1×10^5	5.8×10^4	3.8×10^3	1.8	2.7×10^5	8.6×10^4	69	1.7×10^4	3.8×10^3	77	1.4×10^5	67
2013	4.7×10^5	5.7×10^4	3.8×10^3	0.8	5.3×10^5	3.8×10^5	29	4.5×10^4	2.0×10^4	57	5.9×10^4	13
2014	5.1×10^5	5.5×10^4	3.8×10^3	0.7	5.7×10^5	4.7×10^5	18	5.0×10^4	2.6×10^4	48	1.4×10^4	3
2015	5.3×10^5	6.0×10^4	3.8×10^3	0.7	6.0×10^5	4.0×10^5	34	5.5×10^4	2.1×10^4	62	7.5×10^4	14
2016	5.2×10^5	5.9×10^4	3.8×10^3	0.7	5.8×10^5	6.4×10^5	-10	5.9×10^4	3.5×10^4	41	-1.5×10^5	-28
2017	4.8×10^5	5.4×10^4	3.8×10^3	0.8	5.4×10^5	3.4×10^5	37	6.9×10^4	1.9×10^4	73	1.5×10^4	3
2018	8.2×10^5	5.7×10^4	3.8×10^3	0.5	8.8×10^5	6.4×10^5	27	1.1×10^5	3.3×10^4	70	-4.4×10^4	-5
2019	6.0×10^5	5.7×10^4	3.8×10^3	0.6	6.6×10^5	6.0×10^5	9	6.5×10^4	3.3×10^4	50	-5.9×10^4	-10
Avg	**4.8×10^5**	**5.8×10^4**	**3.8×10^3**	**0.8**	**5.4×10^5**	**3.7×10^5**	**36**	**5.9×10^4**	**2.0×10^4**	**65**	**3.8×10^4**	**13**

Using scaled-up direct measurements of N_2 fixation, total N inputs to Falls Lake including tributary inputs, atmospheric deposition, and N_2 fixation ranged from 2.7×10^5 in 2011 which was a very dry year to 8.8×10^5 kg N/y during 2018, a very wet year (Table 1). The percent of total N inputs removed by sedimentation and denitrification in Falls Lake varied from -10% in 2016 to 69% in 2011 and 2012, and averaged 35% (Table 1). As is often the case, the percentage of tributary loads of TP that were retained by Falls Lake was considerably higher than for TN, ranging from 41 to 88%, and averaging 65% (Table 1). The mass ratio of TN to TP inputs retained in Falls Lake varied from -2.3 in 2016 to 15.8 in 2015 and averaged 5.6 (data not shown). This average ratio of TN to TP retention was higher than the average mass ratio of TN:TP in the surficial sediments (3.7) which is indicative of N losses by denitrification (Molot and Dillon 1993). Lake-wide denitrification calculated via equation 1 ranged from -1.5×10^5 to 1.4×10^5 kg N/y and averaged 3.6×10^4 kg N/y (Table 1). In comparison to the total tributary loads of N, denitrification represented between -24 and 51%, and averaged a modest 13% of the total annual tributary loads of N (Table 1).

For the nutrient budget that was derived using N_2 fixation estimates derived from heterocystous cyanobacterial biomass, the approximately six-fold higher of estimate N inputs from N_2 fixation and its negative relationship with tributary nutrient loading resulted in small increases in estimated rates of denitrification compared to the budget based on scaled-up direct measurements

of N_2 fixation, average of 4.6×10^4 (Table 2) versus 3.8×10^4 kg N/y (Table 1). However, higher estimates of N_2 fixation increased average denitrification as a percent of tributary loadings from 13 (Table 1) to 21% (Table 2).

The finding that there was more outflow than input of N during 2016 may be an artifact created by underestimation of tributary N inputs or overestimation of river fluxes of N out of Falls Lake. Outflowing TN flux estimated here using the WRTDS method was similar (5% lower) to the outflowing TN flux estimated by NC DEQ using the LOADEST model (NCDEQ 2019). So, it appears more likely that the negative retention of N during 2016 is caused by an underestimation of N inputs. During late 2015 and 2016, an unknown source contributed pulses of very high nutrient levels into Knapp of Reeds Creek (Draft UNRBA Falls of the Neuse Reservoir (Lake) Watershed Modeling Report 2022). Due to the episodic nature of these pulses, it is unlikely that their contribution to the annual N and P loading was accurately characterized in the NC DEQ's loading estimates for 2016. If the denitrification estimate from 2016 is removed from the data set, the long term average denitrification estimate would increase to 16% of tributary loading for the budget based on direct N_2 fixation measurements or 26% of tributary loading for the budget based on NCDEQ's estimates of heterocystous cyanobacteria biomass.

Table 2. Mass balance of total N and total P and calculations of lake-wide annual denitrification for the period 2006-2019 using annual N_2 fixation rates estimated based on biomass of potentially N_2 fixing cyanobacteria quantified by NCDEQ and biomass-specific rates of N_2 fixation measured in this study.

Year	Trib. N load (kg N/y)	Atm. dep. (kg N/y)	N_2 fix. (kg N/y)	N_2 fix. (% Trib. Load)	Total TN flux in (kg N/y)	River TN flux out (kg N/y)	TN retain (%)	Trib. P load (kg N/y)	River TP flux out (kg P/y)	TP retain (%)	Denit. (kg N/y)	Denit. (% Trib. Load)
2011	2.0×10^5	5.9×10^4	4.1×10^4	20	3.0×10^5	8.3×10^4	73	1.6×10^4	3.9×10^3	75	1.8×10^5	87
2012	2.1×10^5	5.8×10^4	3.2×10^4	15	3.0×10^5	8.6×10^4	72	1.7×10^4	3.8×10^3	77	1.7×10^5	80
2013	4.7×10^5	5.7×10^4	3.7×10^4	8	5.7×10^5	3.8×10^5	33	4.5×10^4	2.0×10^4	57	9.2×10^4	20
2014	5.1×10^5	5.5×10^4	2.5×10^4	5	5.9×10^5	4.7×10^5	21	5.0×10^4	2.6×10^4	48	3.6×10^4	7
2015	5.3×10^5	6.0×10^4	2.7×10^4	5	6.2×10^5	4.0×10^5	36	5.5×10^4	2.1×10^4	62	9.8×10^4	18
2016	5.2×10^5	5.9×10^4	2.5×10^4	5	6.0×10^5	6.4×10^5	-6	5.9×10^4	3.5×10^4	41	-1.2×10^5	-24
2017	4.8×10^5	5.4×10^4	2.2×10^4	4	5.6×10^5	3.4×10^5	39	6.9×10^4	1.9×10^4	73	3.2×10^4	7
2018	8.2×10^5	5.7×10^4	1.7×10^4	2	9.0×10^5	6.4×10^5	28	1.1×10^5	3.3×10^4	70	-3.1×10^4	-4
2019	6.0×10^5	5.7×10^4	2.5×10^4	3	6.8×10^5	6.0×10^5	12	6.5×10^4	3.3×10^4	50	-3.8×10^4	-6
Avg	**4.8×10^5**	**5.7×10^4**	**2.8×10^4**	6	**5.7×10^5**	**4.0×10^5**	**34**	**5.4×10^4**	**2.1×10^4**	**62**	**4.6×10^4**	**21**

Comparison of mass balance estimates vs. other estimates of lake-wide denitrification: Four campaigns that directly measured rates of Falls Lake denitrification from sediment cores were made by Dr. Michael Piehler's lab. These measurements were made during October 2019, May 2020, August 2020, and July 2021 in conjunction with this project's surface water sampling from the six main channel and ten creek arm sites to measure N_2 fixation. Denitrification measurements were made on the cores by quantifying the rate of N_2 gas evolution by the sediments with a membrane inlet mass spectrometer during a continuous flow incubation. Average values of denitrification for the first three campaigns have been scaled up to the surface area of Falls Lake and to annual rates (see Piehler 2023 final report). The scaled-up rates ranged from 50,000 to nearly 500,000 kg N/y. The average across the three campaigns was 237,000 kg N/y or approximately 47% of the average tributary load of N from 2006 to 2019 (Figure 6).

A second estimate of lake-wide denitrification was calculated by a sediment diagenesis model that was calibrated to vertical profiles of porewater ammonium and nitrate plus nitrite concentrations from 29 Falls Lake sediment cores collected throughout the main channel and

main creek arms of Falls Lake (Alperin 2018). Depth profiles of modeled denitrification were integrated over depth and then the average denitrification rate of the 29 cores (127 μg $N/m^2/d$) was scaled to the 50,000,000 m^2 sediment surface area of Falls Lake. Lake-wide denitrification estimated via the model was 2300 kg N/y or <1% of tributary inputs (Figure 6). Thus, the three methods of estimating a lake-wide denitrification rate provide estimates that range widely from removing < 1% to removing nearly half of the annual tributary N load. Resolving the large discrepancy between the diagenesis model results and the denitrification estimates made by the mass balance and direct measurements on cores methods would be a fruitful avenue for future research.

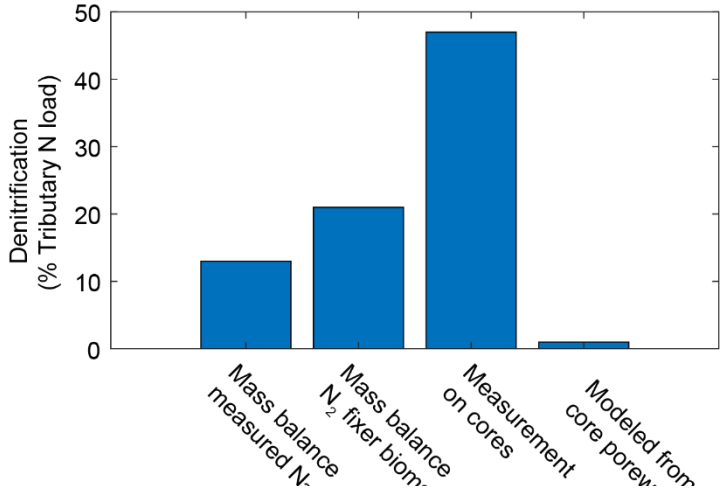

Figure 6. Lake-wide estimates of denitrification based on the N mass balance with N2 fixation calculated using direct measurements or estimated based on heterocystous cyanobacterial biomass, direct measurements of denitrification on sediment cores, and modeled denitrification based on sediment pore water N concentrations.

Key takeaways/ Management implications

1) Direct measurements of N_2 fixation indicate that N_2 fixation contributes less than 1% of total N inputs to Falls Lake. Estimated N_2 fixation based on the biomass of cyanobacteria capable of N_2 fixation is about 6% of tributary inputs. Both methods agree that N_2 fixation is a small percentage of total N_2 inputs which provides a justification for omitting the process in eutrophication models for Falls Lake.

2) Based on the mass balance and direct core measurements of denitrification it appears that denitrification is greater than N_2 fixation and that the balance of these microbial processes result in a net loss of N from Falls Lake. Net loss of N could help maintain N limited phytoplankton which is consistent with N limited growth observed in nutrient addition experiments conducted in spring and summer 2021.

3) Most of the N and P within Falls Lake are bound up in plankton biomass. Neither N or P is available in great excess and small additions of N commonly led to P limitation. P availability also appeared to be an important constraint on N_2 fixation. This situation of weak N limitation and the potential for stimulation of N_2 fixation by P suggests that dual management of N and P is warranted for preventing undesirable phytoplankton biomass in Falls Lake.

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Supplemental Information

Details of sampling campaigns: From July 2019 to July 2020, a series of five N_2 fixation assessment campaigns were conducted along a transect of 6 main-channel stations (Figure 1). Between May and October 2021, a series of five N_2 fixation assessments were conducted at ten stations located with major creek arms. During July and August 2022, two N_2 fixation assessment campaigns were conducted at the 6 main-channel and 10 creek arm sites. During mid-channel sampling, integrated surface water from the surface to twice Secchi depth was collected from each of the six stations, IMS-A to IMS-F (Figure 1). Creek arms stations were sampled from ~0.2 m deep by holding an open bottle just below the surface. Sample bottles were maintained at in situ temperature until after N_2 fixation assays were initiated within 3 hours of sample collection. After N_2 fixation assays were initiated, samples were placed on ice for transport to the lab at UNC Chapel Hill's Institute of Marine Sciences.

Details of N_2 fixation measurement methods: During fall 2019 and summer 2020, N_2 fixation measurements were made at mid-channel locations using acetylene reduction assays along the vertical light gradient according to Grantz et al. (2014) as follows. For each station, 50 mL aliquots of the photic zone composite sample were added to six, 70 mL clear-glass (borosilicate) serum vials and a seventh vial that was made opaque by wrapping in black tape. Once each vial was stoppered using a rubber septum, four mL of head space was extracted from each vial and four mL of acetylene gas was injected. The six clear vials were suspended at depths from 0.25 m, 0.75 m, 1.25 m, 1.75 m, 2.25 m and 2.75 m. The seventh dark bottle from each station was incubated at 3 m depth. These in situ incubations maintained in situ temperature conditions, and spanned the light gradient from and bottles were suspended at several depths corresponding to ~50% to 0% of incident PAR. At each depth an additional bottle of deionized water was also amended with acetylene and identically incubated as a control to account for non-biological reduction of acetylene gas. At each depth the rate of non-biological acetylene reduction was subtracted off the measured acetylene reduction from all the stations. Incubations were conducted for three to four hours during midday.

For creek arm samples collected during 2021 and the creek arm and mid-channel samples collected during summer 2022, N_2 fixation measurements were also made using the acetylene reduction technique but only at a single irradiance level (20% incident light). Surface waters from each station were collected in the morning. Aliquots of water from each sample were then incubated in triplicate clear borosilicate serum bottles paired with triplicate dark bottles in a temperature-regulated water bath fitted with two layers of neutral density screening to reduce incident light to 20% of full-strength sunlight. Water temperature during the incubation was maintained at the *in situ* temperature observed at the last station sampled and deviated by less than 0.3 °C over the course of an approximate 4 hour incubation. As with the 2019-2020 main channel measurements, acetylene reduction within triplicated deionized water controls was subtracted from all rate measurements to remove any non-biological acetylene reduction.

Methods for quantifying biological, physical, and chemical conditions: At each sampling station, profiles of temperature, conductivity, dissolved oxygen, pH, and photosynthetically active radiation (PAR) were measured at the time of sampling using a YSI Exo or YSI6600

multiparameter data sonde, and a LiCor PAR sensor. The PAR attenuation coefficient was determined by linear regression of the natural log transformed PAR data on depth. Regressions with greater than 2 data points and R^2 values greater than 0.8 were used to calculate euphotic zone depths based on the depth of 1% PAR penetration.

Aliquots of each photic zone composite sample were filtered through Whatman GF/F filters within a few hours of collection under gentle vacuum (< 7 in Hg) using a manual pump. The liquid filtrate was saved for dissolved nutrient analysis (ammonium, nitrate+nitrite, phosphate, total dissolved nitrogen, silicate) using a Lachat Quikchem 8000 autoanalyzer, while filters were retained for analysis of chlorophyll a by fluorometry, taxa specific accessory pigments by HPLC, and particulate carbon and nitrogen by elemental analysis (Peierls et al. 2012; Hall et al. 2013). Biomass of the four dominant phytoplankton classes in units of chlorophyll *a* were calculated from accessory photopigment concentrations using the matrix factorization program ChemTax (Mackey et al. 1996) following protocols from Paerl et al. (2014). Potentially N_2 fixing, heterocystous cyanobacteria within the order Nostocales were microscopically enumerated using inverted microscopy (Hall and Paerl 2011), and trichome biovolumes of each observed taxa were estimated based on measured lengths and widths measured using a calibrated, ocular Whipple grid and an assumed cylindrical morphology (Olenina et al. 2006). From each sample, total biovolume of potential N_2 fixing cyanobacteria was computed as the sum of the biovolumes of all heterocystous cyanobacteria species present.

For the main channel and creek arm stations, relationships between N_2 fixation and bioavailable, inorganic N and P nutrient forms (nitrate, ammonium, and phosphate), and cyanobacteria biomass determined by HPLC/Chemtax and by microscopy were explored using Spearman's rank correlations to improve understanding of the controls on N_2 fixation in Falls Lake. For the rank correlation analyses, nutrient values that were below the limit of detection were set to half the detection limit. Detection limits for nitrate, ammonium, and phosphate were 0.7, 6.99, 0.65, and μg/L, respectively. For the main channel stations, maximum N_2 fixation rate within each profile (i.e. the N_2 fixation rate at an optimal irradiance) was used as a dependent variable rather than a depth-averaged rate to help separate the potential influence of light limitation of N_2 fixation from other factors. The average of triplicate values was used for the creek arm stations.

Methods for scaling up estimates of N_2 fixation to lake-wide, annual N_2 fixation: Estimates of lake-wide, annual N input due to N_2 fixation were calculated in two ways. The first method relied solely on the direct measurements of N_2 fixation. For main channel and creek arm stations, the depth average of each N_2 fixation profile and average of triplicates incubated at 20% incident irradiance were respectively used to represent N_2 fixation at that station. Each main channel and creek arm site was associated with a polygon whose area was measured using Google Earth (Figure SI-1, Table SI-1) and whose photic zone was calculated as the product of photic zone depth and area. First, we scaled each station measurement up to the total polygon photic volume and then, for each sampling date, we summed N_2 fixation of all creeks or all main channel

stations measured on that date. We then averaged separately the total creek and total channel N_2 fixation data across dates. Finally, we summed the average creek and average main channel data to construct a lake wide average N_2 fixation value that accounts for the spatial heterogeneity of N_2 fixation and differences in photic volume of the main channel and creek arm areas.

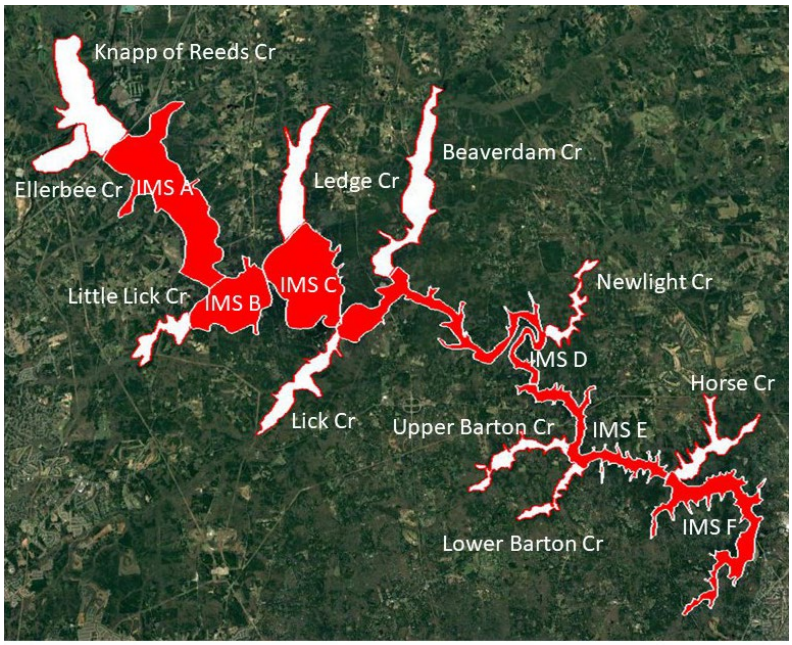

Figure SI-1. Map of lake segments used for scaling up N_2 fixation measurements to lake-wide rates of N_2 fixation.

The second method of estimating annual, lake-wide N_2 fixation rates was conducted by scaling up and averaging the monthly time series of N_2 fixation estimated based on heterocystous cyanobacteria biomass measured at the four NCDEQ stations. Monthly measurements of heterocystous cyanobacteria biomass were multiplied by the median biomass-specific N_2 fixation rate observed from the main channel stations in this study to obtain monthly estimates of N_2 fixation at the four NCDEQ stations. These monthly estimates were then averaged to estimate a monthly lake-wide average N_2 fixation rate. Monthly lake-wide N_2 fixation estimates were then averaged to estimate a lake-wide annual average N_2 fixation rate in units nmol N/L/h. This value was then scaled up to the photic volume of the lake using the average total photic volume for the lake (8.4×10^{10} L) that was calculated above for N_2 fixation estimates using direct measurements, and scaled up to the annual period by multiplying by a 12 hour photoperiod per day, and 365 days per year. This time series of estimated N_2 fixation rates is based on the assumption that biomass-specific rates observed in our study were the same throughout the 2011-2020 period. We have no data to validate this assumption, but also have no reason to suspect that it is not valid. Interannual variability of these biomass-based

Table SI-1. Area of polygons used to scale-up N_2 fixation measurements to a lake-wide N_2 fixation rate.

Polygon	Area (km^2)
IMS 1	7.85
IMS 2	3.37
IMS 3	5.46
IMS 4	4.41
IMS 5	2.21
IMS 6	3.13
Horse Creek	1.27
Newlight Creek	0.84
Beaver Dam Creek	3.34
Ledge Creek	2.66
Knapp of Reeds Creek	4.3
Ellerbee Creek	1.41
Little Lick Creek	1
Lick Creek	1.6
Upper Barton Creek	0.96
Lower Barton Creek	0.55

annual estimated N_2 fixation rates were compared against annual stream loads of N to investigate whether stream loads were related to N_2 fixation as would be expected if reduced N loads enhanced N limitation within the phytoplankton community.

Experimental assessment of nutrient impacts on phytoplankton growth and N_2 fixation: Additional (additional to samples used for N_2 fixation, nutrient and phytoplankton biomass/composition measurements) 60-liter water samples from Ledge, Knapp of Reeds, and Upper Barton Creeks were collected on 8 June and 23 August 2021 and 3 liters were dispensed into triplicated 4 liter Cubitainers. The triplicate Cubitainers were amended with the following treatments: a control with no added nutrients, nitrate addition, phosphate addition, and nitrate plus phosphate (see Paerl et al., 2014 for details). Growth in each treatment was assessed after 3 days and compared to initial measurements of phytoplankton biomass and community composition determined by HPLC pigments and microscopy. N_2 fixation rates were measured after 3 days incubation by acetylene reduction in light and dark bottles under two layers of neutral density screening in an outdoor water bath. Measuring N_2 fixation responses after 3 days primarily captures responses that are driven by changes in the physiology of the *in situ* cyanobacterial community versus responses that are increasingly likely to occur due to changes in the abundance of N_2 fixing taxa. To determine the degree to which P availability can regulate changes in N fixation, control and phosphate addition treatments were continued through a full week (7 days), and biomass, community composition, and N_2 fixation were measured again.

Estimates of tributary loads and sediment N:P ratios: The tributary load estimates were based on LOADEST models of load from the major gaged tributaries, and estimates of atmospheric deposition to the lake surface were based on interpolations of total inorganic nitrogen deposition within the US EPA's Clean Air Status and Trends (CASTNET) monitoring network (NCDEQ 2021). The lake-wide sediment N:P mass ratio was calculated as 3.67 (+/-1.25 S.D.) based on the average N:P ratio of the upper, 0-3 cm, sediment layer within 30 cores collected by Dr. Alperin. These 30 cores spanned the entire length of the reservoir and included both shallow and deeper channel locations. Although the TN:TP ratio of the surface sediments varied from 1.0 to 5.7, there were no consistent patterns of the ratio in relation to distance downstream or depth of overlying water (Alperin 2018).

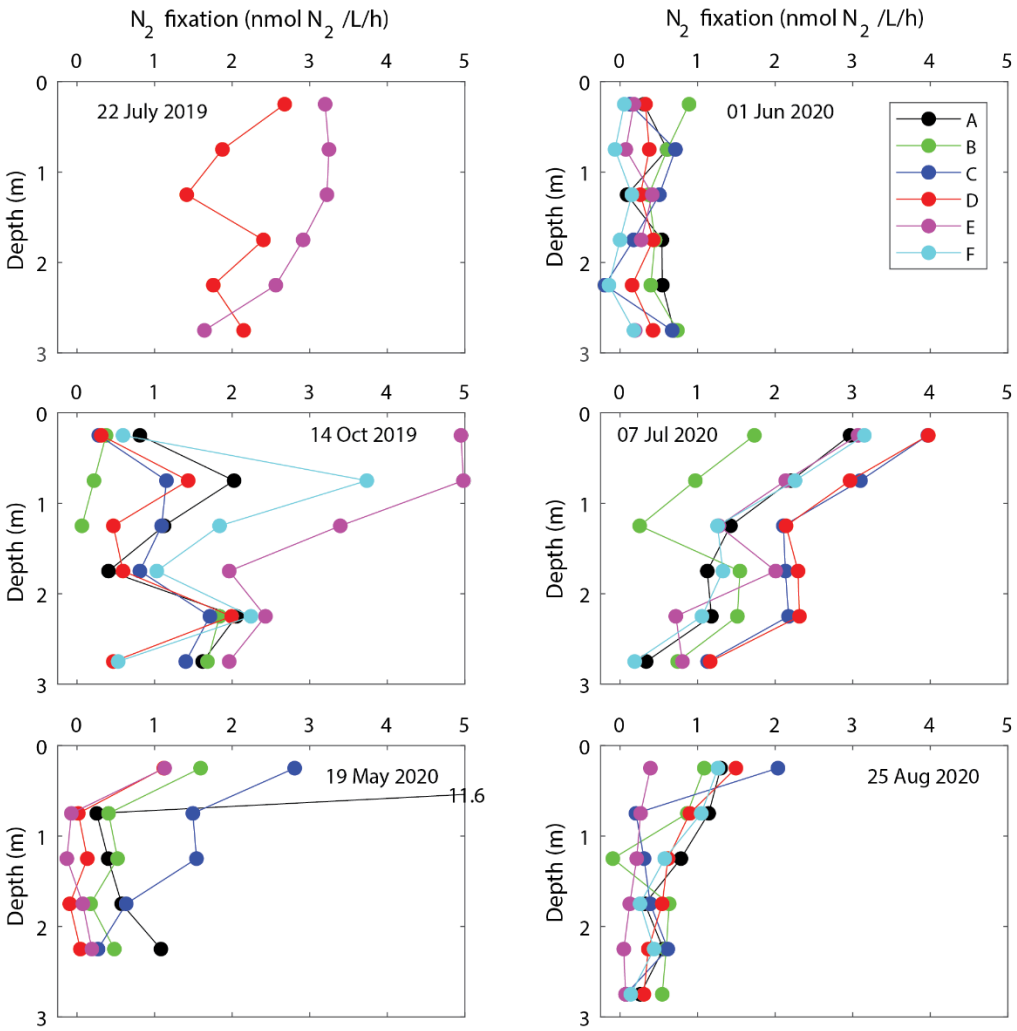

Figure SI-2. Depth profiles of N2 fixation measured at six main channel stations (IMS A-F) in Falls Lake during 2019 and 2020.

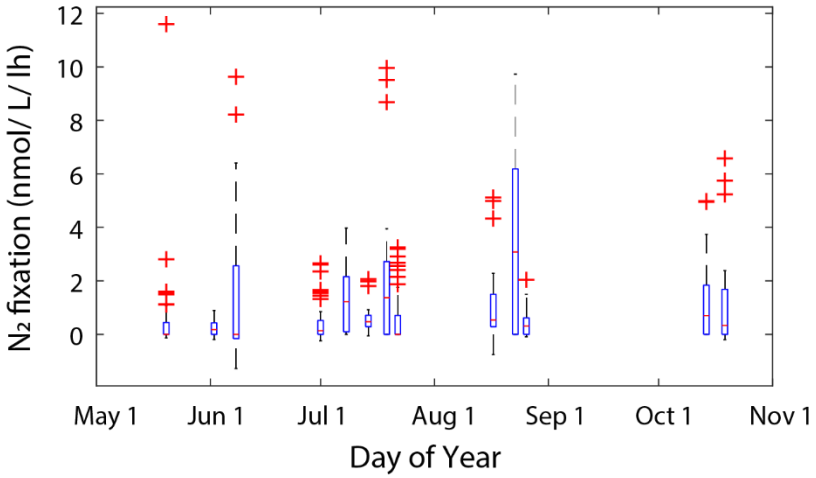

Figure SI-3. Boxplots of N2 fixation at different days of the year from both mid-channel and creek arm measurements made from 2019 to 2022. Boxes represent interquartile range. Red lines indicate median values. Whiskers extend to 1.5 times the interquartile range and outliers beyond the whiskers are red plus symbols.

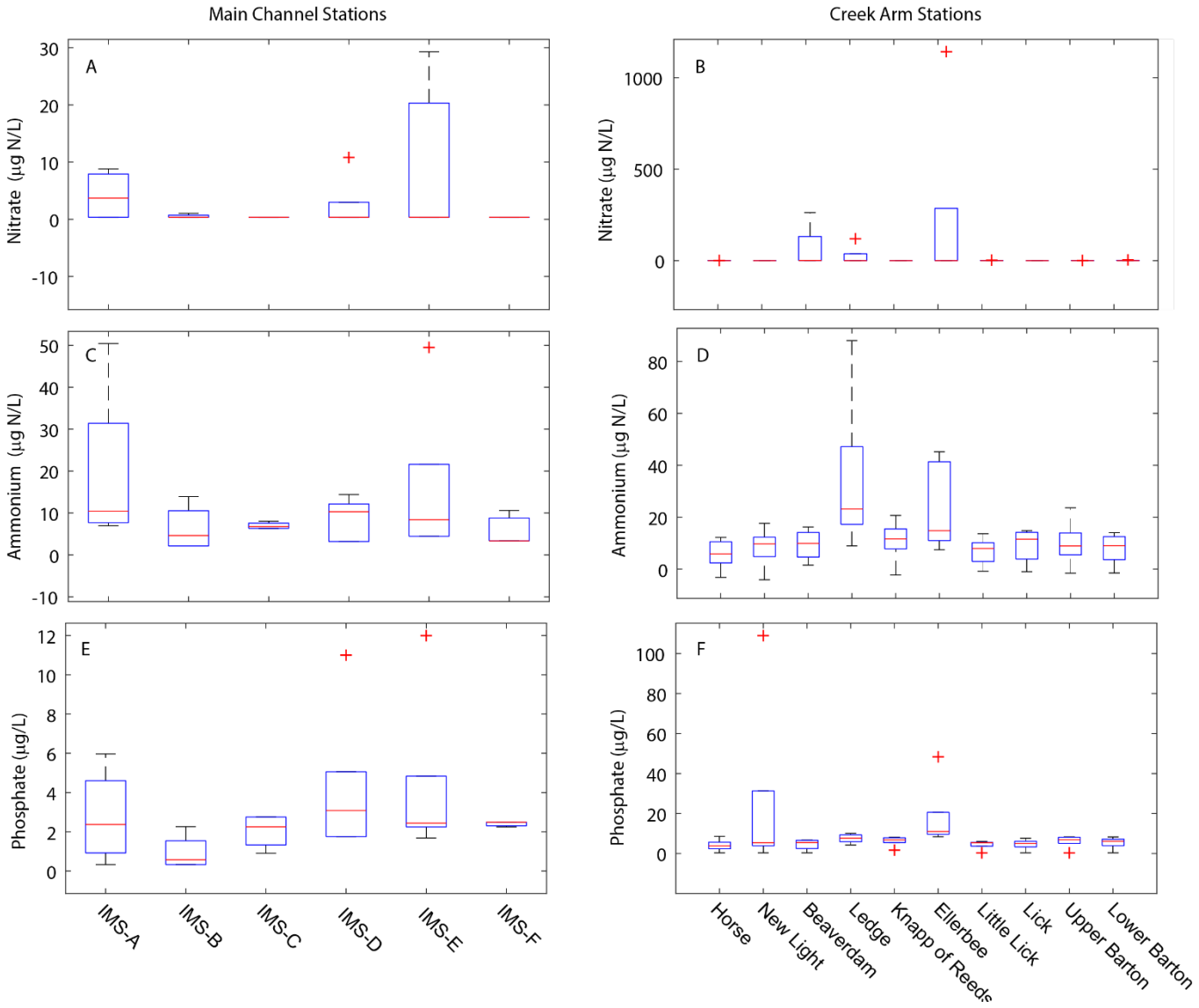

Figure SI-4. Boxplots of dissolved inorganic nutrients at main channel and creek arm stations from samples collected from 2019 to 2022. Boxplot configuration is identical to Figure SI-3.

Nutrient limitation of phytoplankton growth: In the June 2021, nutrient addition experiment, nitrogen was the primary limiting nutrient for phytoplankton assemblages collected from Ledge, Knapp of Reeds, and Upper Barton Creeks (Figure SI-5). After 3 days incubation, levels of total phytoplankton chlorophyll *a* in the control had decreased by 10-50% since the time of collection (Figure SI-5 A,C,E). Nitrogen additions prevented part of the loss experienced in the controls and increased chlorophyll *a* by 10-70% above the control by day 3 (Figure SI-5 A,C,E). Phosphorus additions resulted in no change compared to the control for Ledge and Knapp of Reeds Cr. but led to a significant decrease in chlorophyll *a* for Upper Bartons Creek. This situation is believed to arise when P additions stimulate heterotrophic bacteria that then compete with phytoplankton for growth limiting levels of bioavailable nitrogen (Andersen et al. 2005; Moisander et al. 2003). For all three creeks, addition of N and P together stimulated growth of total phytoplankton 20-25% above that of N addition alone. This indicates that the balance of N and P supply to phytoplankton is only marginally N deficient compared to P (Andersen et al. 2005). The control and P addition treatments were incubated for a full week to determine the degree to which addition of P would stimulate N_2 fixing cyanobacteria. P addition resulted in no change, a 15% increase, and 55% decrease compared to the control in total chlorophyll *a* after a full week's incubation of Ledge, Knapp of Reeds, and Upper Barton Creek water, respectively (Figure SI-5 A,C,E).

At the beginning of the June experiment, cyanobacterial chlorophyll *a* constituted about 20-40% of the total chlorophyll *a* (Figure SI-5 A-E). The pattern of stimulation for cyanobacteria was essentially the same as for total phytoplankton showing primary N limitation with additional stimulation with combined N and P additions (Figure SI-5 B,D,E). P additions had no effect on cyanobacteria biomass at Ledge and Knapp or Reeds Creek but caused a significant decrease at

Upper Bartons Creek.

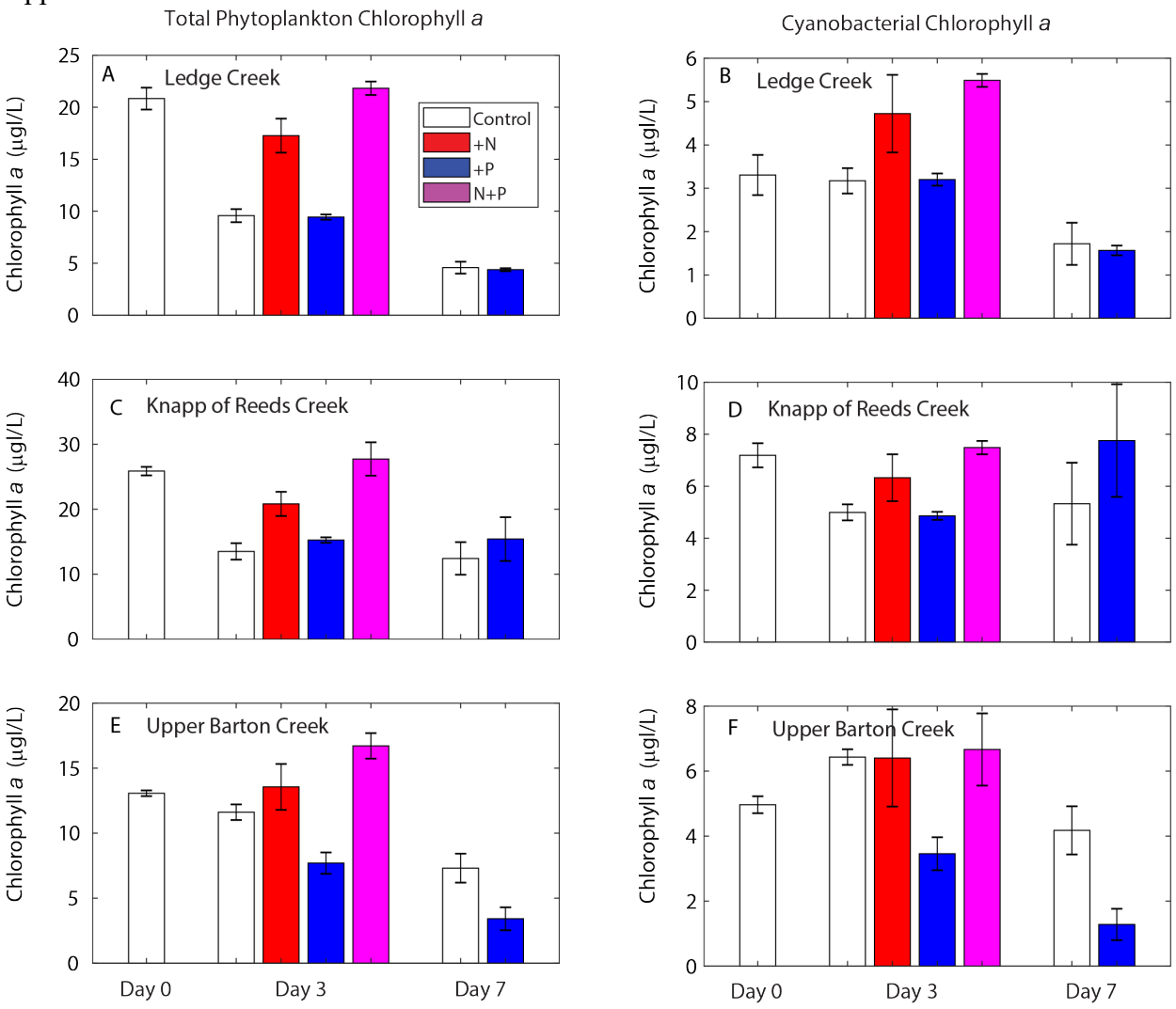

Figure SI-5. Effects of nutrient (N and P) additions on total phytoplankton biomass and cyanobacterial biomass (both as chlorophyll a) during the June 2021 experiment on three creek arms of Falls Lake. Bars (whiskers) represent the mean (STD) of triplicate values.

Results from the August nutrient addition experiment showed an approximate 25% increase above the control for total phytoplankton chlorophyll *a* from Ledge and Knapp of Reeds Creeks but no stimulation by N or P at Upper Barton Creeks after 3 days incubation (Figure SI-6 A,C,E). The combination of N and P resulted in greater than 100% stimulation of phytoplankton biomass at all 3 creeks after 3 days incubation. These results are indicative of weak N limitation that is very close to co-limitation by both N and P at Ledge and Knapp of Reeds Creeks, and co-limitation by both N and P at Upper Barton Creek (Andersen et al. 2005). Unlike in the June

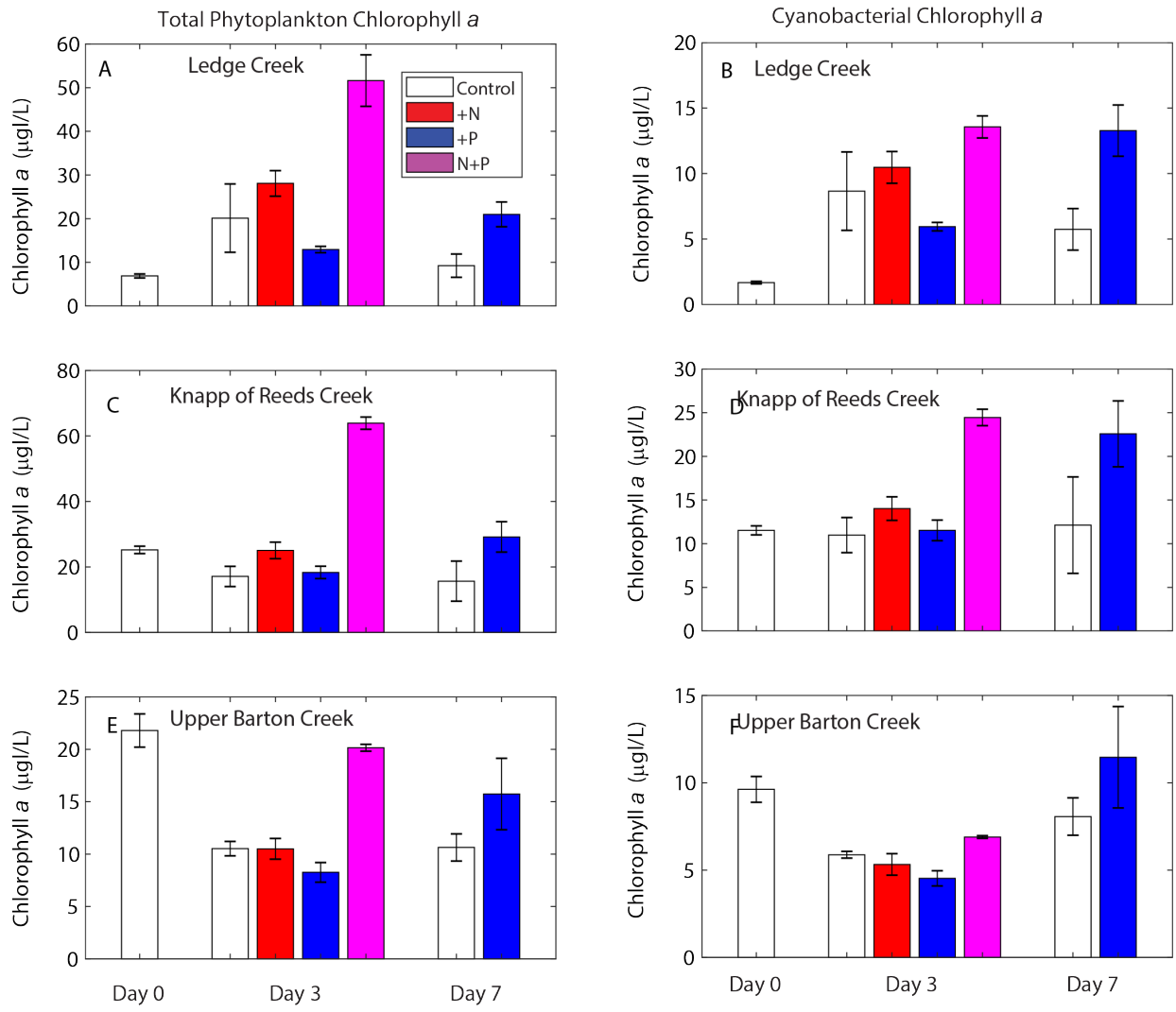

Figure SI-6. Effects of nutrient (N and P) additions on total phytoplankton biomass and cyanobacterial biomass (both as chlorophyll a) during the June 2021 experiment on three creek arms of Falls Lake. Bars (whiskers) represent the mean (STD) of triplicate values.

experiment, P additions did result in significant (40-120%) stimulation of biomass after a full week's incubation.

Cyanobacteria comprised about half of the phytoplankton biomass at the beginning of the August experiment (Figure SI-6 B,D,F). The response of cyanobacteria to nutrient additions closely mirrored that of total phytoplankton chlorophyll *a* but with a somewhat muted response to combined N and P additions. Most (~75%) of the increase in chlorophyll *a* that was observed in the P additions after a full week incubation was due to increases in cyanobacteria biomass as would be expected if P additions stimulated N_2 fixing cyanobacteria. We show below that this was indeed the case.

Effects of nutrient additions on N_2 fixation: In the June 2021 experiment, nutrient additions generally had little effect on N_2 fixation after 3 days incubation (Figure SI-7 A,C,E). A strange

exception to this trend was an ~80% suppression in N_2 fixation compared to the control in the P addition treatment for water from Upper Barton Creek after 3 days incubation (Figure SI-7 E). As discussed above, P additions can occasionally lead to lower phytoplankton biomass due to heightened competition for N by bacteria stimulated by P. Heightened N limitation should promote N_2 fixation, not depress it, and we cannot explain this strange result. After a full week's incubation of waters from Ledge and Knapp of Reeds Creek, P addition stimulated N_2 fixation

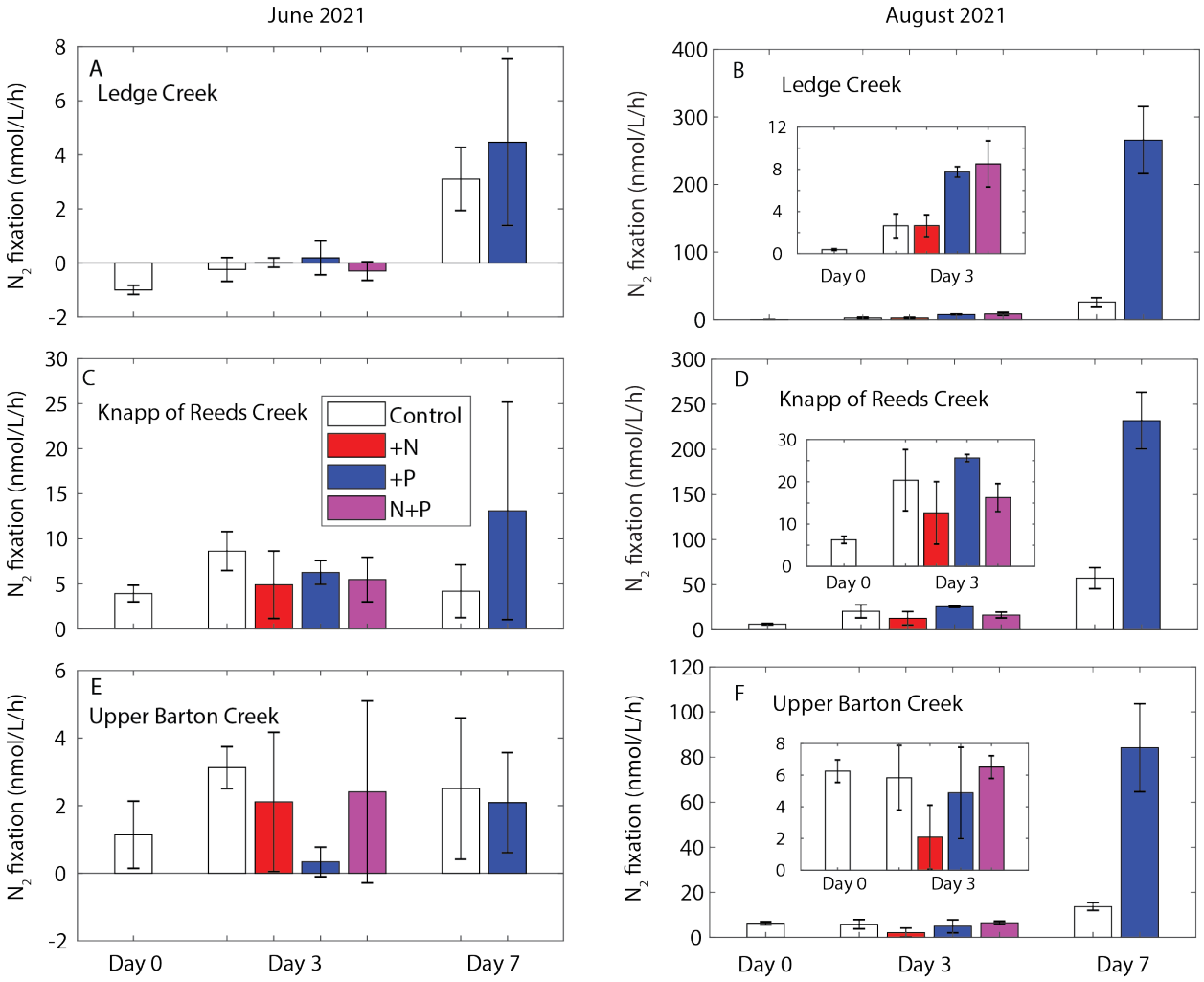

Figure SI-7. Response of N2 fixation to N and P additions in experiments conducted from Falls Lake creek arm waters in June and August 2021. Bars and whiskers represent means (STD) of triplicate values. Due to the large range of values, insets of results from Day 0 and Day 3 were added to figure panels for the August 2021 experiment.

by 30-200% of the control, though with a high degree of within-treatment variability.

The August 2021 experiment showed a much greater degree of stimulation by P additions. After 3 days incubation of Ledge Creek water, both treatments with added P resulted in more than a doubling of N_2 fixation (Figure SI-7 B,D,F). For Knapp of Reeds Creek, P addition increased N2 fixation by ~25% but treatments with added N caused suppression of N_2 fixation after 3 days. For Barton Creek, N addition alone caused a 70% reduction of N_2 fixation after 3 days but P addition did not cause significant stimulation above the control. After a full week incubation, N_2

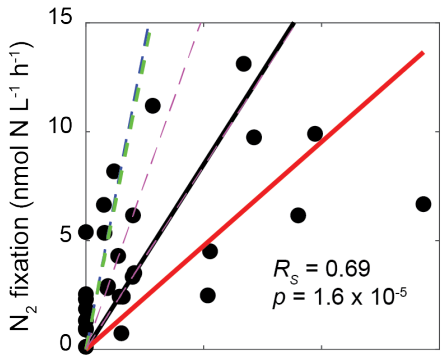

Figure SI-8. Measured rates of N_2 fixation versus biomass of heterocystous cyanobacteria at main channel sites on Falls Lake (filled black circles). Solid red line represents a zero-intercept, least squares regression. Solid black line represents the median ratio of N_2 fixation: N_2 fixer biovolume after excluding samples with zero N_2 fixer biovolume. Dashed blue, green, and the two magenta lines represent ratios respectively observed by Klawonn et al. (2016) from the Baltic Sea, Willis et al. (2016) from a culture of Cylindrospermopsis raciborskii, and Miosander et al. (2012) from C. raciborskii dominated assemblages from Lake George and the St. Johns River Florida.

fixation increased by 200-1000% compared to the day of collection in the control and P addition resulted in an additional 400-1000% stimulation compared to the control. The relatively low levels of phosphate concentrations and in situ N_2 fixation, and high degree of stimulation of N_2 fixation by P additions provides evidence that availability of bioavailable P is likely a key constraint on N_2 fixation in Falls Lake.

Derivation of a biomass-specific N_2 fixation rate for heterocystous cyanobacteria in Falls Lake: A zero-intercept linear regression of Nostocalean cyanobacteria biovolume versus rate of N_2 fixation was statistically significant (p = 0.001) but only explained 31 % of the variability in the rate of N_2 fixation. The slope of the zero-intercept regression of heterocystous cyanobacteria biomass on N_2 fixation (red line in Figure SI-8) indicated a lower N_2 fixation rate: biomass ratio than observed in other studies. The least square regression line appeared highly influenced by the highest biomass data point which only had modest N_2 fixation. The median ratio of N_2 fixation to N_2 fixer biovolume was more similar to literature values, especially the *Cylindrospermopsis raciborskii*- dominated natural assemblages (magenta lines in Figure SI-8) measured by Moisander et al. (2012). The N_2 fixer assemblage of Falls Lake was also dominated by *C. raciborskii* (Figure SI-9) and moderate biomass-specific N_2 fixation rates are likely typical of this species (Moisander et al. 2012; Willis et al. 2016). Based on agreement with previous literature values and the high influence of a few data points on the least square regression, estimates of N_2 fixation rates based on heterocystous cyanobacteria biomass that are described below were calculated using the median ratio of observed N_2 fixation vs biomass, 8.5×10^{-9} nmol N/μm^3 N-fixer biovolume/h.

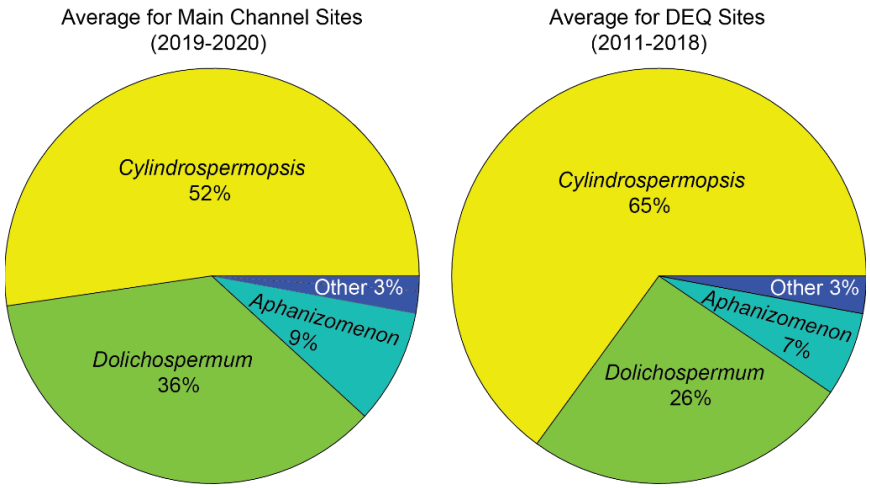

Figure SI-9. Average composition of heterocystous, potential N_2 fixing cyanobacteria taxa for the main channel sites in this study and from NC DEQ's stations NEU013B, NEU018E, NEU019P and NEU020D.

Photic zone depths used to scale N_2 fixation measurements to lake-wide estimates: The large upstream creek areas of Ledge, Knapp of Reeds, and Ellerbee Creeks had median photic zone depths of only about 1 m (Figure SI-10), and these shallow photic depths are estimated to strongly restrict N_2 fixation in these areas. There was a strong upstream to downstream increase in photic zone depth from about 1.5 m at IMS-A near Fish Dam Rd. to 2.75 m depth at IMS-F near the dam. The other creek areas had photic depths that were similar to the main channel areas that they flowed into.

One of the dangers of inferring rates from biomass of a large group species that likely have different levels of N_2 fixation activity (Moisander et al. 2012) is that community composition may vary between when rate measurements are made and the time period over which the biomass-specific rates are applied to estimate the time series of N_2 fixation. The heterocystous cyanobacteria community of the main channel samples collected by this study during 2019 and 2020 is very similar to that observed by NC DEQ over the period 2011 to 2018 (Figure SI-9). *Cylindrospermopsis raciborskii* is dominant followed by *Dolichospermum sp.*, *Aphanizomenon sp.* and then a small fraction composed by other members of the order Nostocales. This lessens the probability that significant changes in community composition of the heterocystous cyanobacteria community could lead to significant error or bias in the N_2 fixation estimates derived from biomass of heterocystous cyanobacteria.

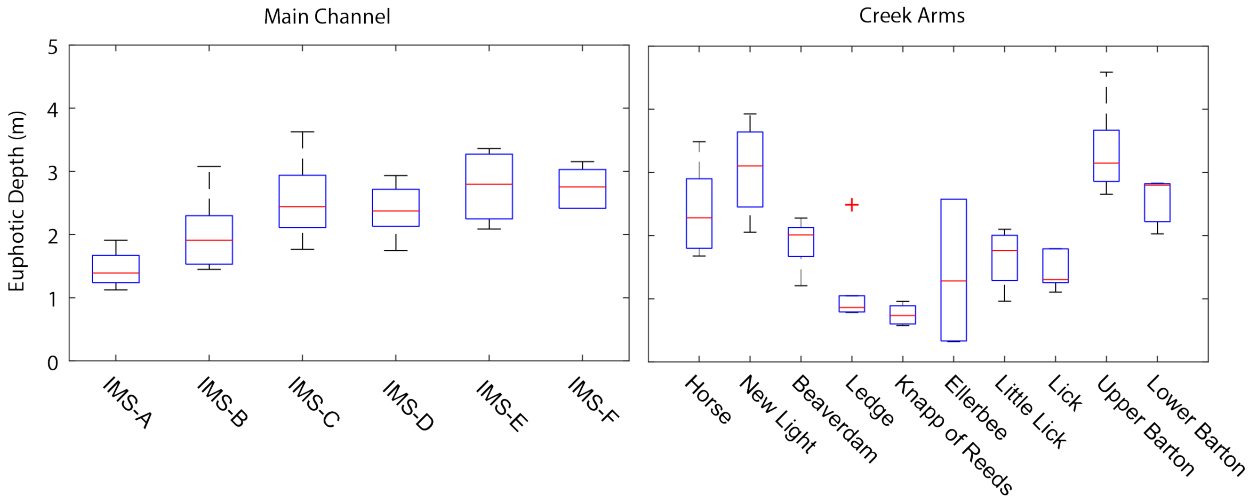

Figure SI-10. Boxplots of the euphotic zone depth at main channel and creek arm sites in Falls Lake. Boxplot configuration is identical to Figure SI-3.