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Estimating Nitrogen Fixation Rates, Importance, and Short-Term Efficiency in Small, Temperate Reservoirs Using Delta15N Techniques

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ABSTRACT

Nitrogen (N_2) fixation can give certain species of cyanobacteria a competitive advantage in lake and reservoir phytoplankton. These species of cyanobacteria, along with others that cannot fix N_2, can form toxic compounds that impair water quality when present in high concentrations. N_2 fixation rates may be substantial in small (< 1.0 km^2), temperate reservoirs since these systems experience thermal stratification and often nitrogen (N) limitation throughout a substantial proportion of the year. However, the effects of N_2 fixation on N cycling, alleviation of short-term N limitation, and water quality are not well-understood. A mesocosm experiment and ecosystem-scale observational study were conducted to 1) determine the efficiency of N_2 fixation under varying N relative to phosphorus (P) supply, 2) examine the effects of N_2 fixation on autotrophic biomass accumulation and microcystin production, and 3) measure N_2 fixation rates and importance to autotrophic N demand and zooplankton N assimilation. Results of the mesocosm experiment indicated that N_2 fixation was increased at low N:P supply under high P. However, N_2 fixation was inefficient at alleviating N limitation when fixed N was the primary source of N. Additionally, microcystin production occurred only at high N:P supply when N_2 fixation was low, indicating that reducing external N inputs may have a positive effect on water quality. Results of whole-reservoir determination of N_2 fixation using seston $\delta^{15}N$ natural abundances indicated that N_2 fixation rates throughout the warm season were substantial and influenced by water temperature. Annual N_2 fixation rates ranged from 2.2 – 6.6 g N m^{-2} yr^{-1}, and contributed up to 19% of the annual autotrophic N demand. Zooplankton were assimilating fixed N in most of the study reservoirs, representing a possible mechanism of ecosystem fixed N retention. Collectively, these results suggest that N_2 fixation plays a substantial role in N cycling in small, temperate reservoirs, but likely cannot alleviate short-term N limitation.

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1. INTRODUCTION

Some species of cyanobacteria are capable of fixing dinitrogen gas (N_2) into bioavailable forms of N such as NH_3 and NH_4^+ via the nitrogenase enzyme (N_2 fixation). N_2 fixation gives these unique cyanobacteria a competitive advantage over other autotrophs in N-limited lakes and reservoirs. Much of the research on cyanobacteria and N_2 fixation in lakes and reservoirs has been focused toward identifying the conditions under which both N_2-fixing and non-N_2-fixing cyanobacteria are likely to dominate phytoplankton assemblages. Smith (1983) determined that lacustrine cyanobacteria tended to proliferate when water column total N:P (TN:TP; all N:P ratios in this thesis will be presented as molar ratios) was less than 64, with low cyanobacterial incidence above this value. Another study concluded that both N_2-fixing and non-N_2-fixing species of cyanobacteria can be dominant at TN:TP of up to 80 in large shallow lakes (Noges et al. 2008). Other studies have found that both N_2-fixing and non-N_2-fixing cyanobacteria can be abundant even in lakes with high TN:TP (Paerl et al. 2011; Vanni et al. 2011). However, Downing et al. (2001) suggest that the concentrations of TN or TP are better predictors for cyanobacterial biomass than TN:TP and Scott et al. (2008) found that NO_3^- concentrations were negatively correlated with biomass-specific N_2 fixation. Additionally, factors such as temperature (Scott et al. 2008), watershed area relative to lake area (Forbes et al. 2008), light, turbulence, depth, water residence time, dissolved organic carbon (DOC), micronutrients (e.g. Fe or Mo which are cofactors of nitrogenase) and biological interactions (Howarth et al. 1988a; Paerl et al. 2001) can affect cyanobacterial dominance in lacustrine environments.

Questions still remain for limnologists regarding when and why N_2-fixing cyanobacteria become abundant in lakes and reservoirs and what factors affect N_2 fixation rates and efficiencies. Factors determining the contribution of N_2 fixation to ecosystem N fluxes and

autotrophic N demand are not well understood in lacustrine systems. In a review of several early studies on N_2 fixation in freshwater ecosystems, Howarth et al. (1988b) illustrated that N_2 fixation may be relatively unimportant in oligotrophic and mesotrophic lakes when considered as a percentage of total N input. However, N_2 fixation rates tended to be considerably greater in eutrophic lakes, where total N input from N_2 fixation ranged from 5 – 80%. Moreover, N_2 fixation was relatively unimportant when considered as a percentage of the N needs for net primary production, which ranged from 0.03 – 8.9% across lakes of all trophic states (Howarth et al. 1988b). Unfortunately, the review by Howarth et al. lacked data for oligotrophic or mesotrophic systems, and only one reservoir was examined. Reservoir N_2 fixation data are especially rare compared to natural lake data, and even more so when reservoirs differing in trophic state are considered (but see Forbes et al. 2008, Scott et al. 2008, Scott et al. 2009, and Scott and Grantz 2013 for examples of reservoir N_2 fixation studies).

In addition to the lack of N_2 fixation data in various lacustrine systems, existing N_2 fixation data have been mostly derived from use of the acetylene reduction technique (Stewart et al. 1967). A major drawback of this popular method is that the theoretical conversion factor used to estimate N_2 fixation (3 moles of ethylene produced is equivalent to 1 mole of N_2 fixed) may not be entirely valid for mixed (i.e. natural) cyanobacteria communities. Several studies have observed deviations from a 3:1 conversion factor in various freshwater systems (Graham et al. 1980; Paerl 1982). Furthermore, the acetylene-reduction technique requires precise and labor-intensive incubation procedures immediately after sample collection, only yields information regarding the instantaneous potential for N_2 fixation, and likely under- or overestimates N_2 fixation rates when *in situ* conditions are altered (Flett et al. 1975, 1976). Therefore, even though the relative trends of N_2 fixation observed in various systems have likely been

reasonable, N_2 fixation rates and subsequently the contribution of N_2 fixation to ecosystem N inputs may be inaccurate.

Other methods for measuring N_2 fixation rates have also been developed and used to varying degrees. N_2 fixation was quantified in early studies using $^{15}N_2$ tracer experiments (see Neess et al. 1962) before being replaced by the less-expensive acetylene reduction technique. However, $^{15}N_2$-uptake remains a useful tool for calibrating acetylene reduction assays (e.g. Seitzinger and Garber 1987) and is still used in some studies to directly measure N_2 fixation (Mulholland et al. 2004; Mulholland et al. 2006). Natural abundance of ^{15}N in particles has also been used in both marine (Montoya et al. 2002; Wannicke et al. 2010) and freshwater (Patoine et al. 2006; Jankowski et al. 2012) studies to quantify the amount of fixed N present in biomass. Additionally, concurrent application of dissolved ^{15}N tracers and examination of biomass ^{15}N have been used to determine the relative importance of N_2 fixation to phytoplankton in freshwater and marine mesocosm experiments (Vrede et al. 2009; Wannicke et al. 2010).

Natural abundance of $\delta^{15}N$ (the ratio of ^{15}N : ^{14}N relative to a known standard) in suspended particles may be a particularly useful method for estimating N_2 fixation in lacustrine systems. Some of the first freshwater studies that utilized $\delta^{15}N$ natural abundance noted that periods of decreased particulate organic matter (POM) $\delta^{15}N$ were possibly due to increases in N_2 fixation (Yoshioka and Wada 1994; Gu et al. 1994). Indeed, the flux of fixed N derived from isotopically "light" atmospheric N ($\delta^{15}N \approx 0‰$) into phytoplankton results in a predictable decrease in biomass $\delta^{15}N$. Influences of N_2 fixation on particulate $\delta^{15}N$ is especially prominent if the phytoplankton were enriched with isotopically "heavy" N derived from human or watershed sources such as treated wastewater effluent, animal waste runoff, and some fertilizers (Kendall 1998).

The $\delta^{15}N$ natural abundance method has been used to directly quantify N_2 fixation in lakes and reservoirs rather than just observe apparent temporal patterns in ^{15}N. Through the use of $\delta^{15}N$ of phytoplankton biomass and stable isotope mixing models, the amount of fixed N can be estimated even after N_2 fixation has occurred. For example, Patoine et al. (2006) estimated the mass of N supplied by N_2 fixation as well as whether fixed N was being incorporated into zooplankton (primary consumers), based on phytoplankton and zooplankton $\delta^{15}N$ values collected for 10 years in six Canadian lakes and reservoirs. However, that study was spatially limited within individual lakes and reservoirs, as $\delta^{15}N$ measurements were only made at one location in each system. N_2 fixation has been shown to be spatially variable within lacustrine systems, especially reservoirs (Scott et al. 2008; Scott et al. 2009). Considering the size of the lakes (and two reservoirs) studied by Patoine et al. (lake area as large as 500 km^2), N_2 fixation estimates were likely not representative of whole-ecosystem rates.

A more recent study by Jankowski et al. (2012) also used a $\delta^{15}N$ natural abundance approach to estimate the relative proportions of fixed N, human derived N (from wastewater effluent), and natural watershed N to total N inputs. That $\delta^{15}N$ model developed by the authors was used with a single sampling event of several lakes near Seattle, Washington rather than with multiple measurements of the same systems. Unlike the model used by Patoine et al. (2006), the Jankowski model incorporated stoichiometric constraints to the estimation of N_2 fixation (fixed N was only estimated in the model when lake N:P ratios fell below a certain threshold). The ^{15}N mixing model was also reformatted to account for trophic enrichment of $\delta^{15}N$ to determine the proportion of fixed N in zooplankton. However, there are some drawbacks to the approach used by Jankowski et al. (2012). Although the authors collected samples from several lakes, they only used a single sample event to infer the importance of N_2 fixation within each ecosystem. Several

studies have demonstrated that N_2 fixation in lakes and reservoirs is temporally variable (Scott et al. 2008; Beversdorf et al. 2013; Scott and Grantz 2013). Moreover, the single sampling event occurred at only one site in each lake. As with the study conducted by Patoine et al. (2006), the lack of multiple samples from different locations within each lake coupled with the known spatial variability of N_2 fixation (see above) further increases the probability that the importance of N_2 fixation was over- or underestimated in each lake. Thus, while studies like those conducted by Patoine et al. (2006) and Jankowski et al. (2012) have advanced the use of $\delta^{15}N$ to estimate N_2 fixation in lakes and reservoirs, more can be done to refine this method and apply it to more understudied systems like small reservoirs.

A unique benefit of using $\delta^{15}N$ to track N_2 fixation is the ability to examine assimilation of fixed N into primary consumers using zooplankton $\delta^{15}N$. Aside from the two studies described above, relatively few studies have examined the assimilation of atmospherically derived N into the food web via zooplankton on an ecosystem scale in lakes and reservoirs. There is reason to believe that fixed N may not be incorporated into the food web so easily given that cyanobacteria are known to be a particularly low quality food for grazers such as cladocerans and copepods. Low palatability is primarily due cyanobacterial characteristics such as: large size (DeMott et al. 2001; Ghadouani et al. 2003), low levels of highly unsaturated fatty acids (DeMott and Müller-Navarra 1997), and toxin production (e.g. microcystin), which may harm sensitive species of zooplankton (Lampert et al. 1987; but see Wilson et al. 2006). However, some studies have demonstrated that certain zooplankton selectively graze unicellular, short filament, or small colonial cyanobacteria, although some of these organisms pass through the gut with relatively little digestion (Starkweather and Kellar 1983; Haney 1987; Fulton and Paerl 1988). Nonetheless, few studies have determined the extent that fixed N is assimilated into

zooplankton under natural conditions (Woodland et al. 2013). Aside from directly ingesting N_2-fixing cyanobacteria, assimilation of fixed N into the food web may occur when zooplankton graze on other phytoplankton that have taken up fixed N recently released by cyanobacteria (MacGregor et al. 2001). Regardless, the transfer of fixed N into the food web is important in regards to lacustrine N fluxes, as it may be a mechanism for ecosystem fixed N retention.

The ^{15}N stable isotope can be a valuable tool for characterizing N_2 fixation in small reservoirs, especially considering that more data are needed to determine rates, importance, and biogeochemical correlates or predictors of N_2 fixation in these relatively understudied systems. Therefore, my thesis was comprised of two studies that utilized $\delta^{15}N$ techniques. The first study was a mesocosm experiment conducted in three small reservoirs, aimed at determining the effects of N:P supply ratios on: 1) N_2 fixation rates, 2) N_2 fixation efficiency, and 3) microcystin production. The second part of my thesis was an observational study conducted in six small reservoirs representing a natural watershed land use and trophic state gradient to determine the following: 1) N_2 fixation rates throughout the warm season, 2) correlates and predictors of N_2 fixation specific to reservoir watershed land use or trophic state, and 3) fixed N contribution to zooplankton biomass. N_2 fixation rates were hypothesized to be substantial and contribute to autotrophic N demand and zooplankton N assimilation in small, temperate reservoirs during the warm season due to long periods of thermal stratification and N limitation. However, N_2 fixation was hypothesized to be less efficient at alleviating N limitation than NO_3^--N uptake, since N_2 fixation has been shown to be energetically more expensive even under controlled laboratory conditions (Turpin et al. 1985). Additionally, microcystin concentrations were also predicted to be less when N_2 fixation was the primary source of N due to a lower capacity for N_2-fixing cyanobacteria to allocate energy to microcystin production under N-limiting conditions.

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2. NITROGEN FIXATION EFFICIENCY ACROSS A EUTROPHIC N:P SUPPLY GRADIENT

2.1 Introduction

The ability of some species of cyanobacteria to fix atmospheric dinitrogen gas (N_2) into bioavailable forms (e.g. ammonium or NH_4^+) gives them a competitive advantage over other phytoplankton in N-limited lacustrine systems (Horne and Goldman 1972; Howarth et al. 1988a; Paerl et al. 2001). The process of N_2 fixation and its role in lacustrine N cycling has gained renewed research interest in recent years, as N limitation has become increasingly identified in various aquatic systems (Elser et al. 2007; Lewis and Wurtsbaugh 2008). Many of the N_2 fixation studies in lakes and reservoirs have been aimed at quantifying rates of N_2 fixation and its importance to total N loading, as well as the spatial and temporal variation of N_2 fixation (Scott et al. 2008a; Scott et al. 2009; Scott and Grantz 2013). However, the ability of N_2 fixation to alleviate N limitation and offset N deficiency at short timescales (within a single warm season) is still not well understood. Some studies have demonstrated that N_2 fixation can alleviate N limitation in a single warm season (Scott et al. 2008b), while other studies have shown that compensated N supply relative to P cannot be achieved at such a timescale (Havens et al. 2003). The debate over what timescales N_2 fixation can increase internal N loading enough to drive perpetually N-limited systems to P limitation (Schindler et al. 2008; Scott and McCarthy 2010; Scott and Grantz 2013) has elicited the need for more N_2 fixation data in lakes and reservoirs.

Many factors constrain N_2 fixation such as: nutrient availability, water column turbulence, temperature, light, and micronutrients (Howarth et al. 1988b; Paerl 1990; Paerl et al. 2001). N_2 fixation rates have been shown to be relatively high in eutrophic freshwater systems, and can contribute substantially to total N loads (Howarth et al. 1988a). Furthermore, TN:TP has

been shown to influence the dominance of cyanobacteria in natural phytoplankton assemblages and has continued to be used as a metric when discussing N_2 fixation (Smith 1983; Havens et al. 2003; Noges et al. 2008). Under naturally low N but high P supply (low N:P supply), N_2-fixing cyanobacterial dominance would be expected (Smith 1983; Paerl et al. 2001), although how efficient these organisms would be at relieving N limitation to other phytoplankton and the whole lake or reservoir in general remains unknown. On a seasonal timescale, N_2 fixation efficiency depends on the amount of N that can be fixed relative to the demand for N by phytoplankton. No studies to date have experimentally assessed natural community N_2 fixation efficiency across varying N:P supply under eutrophic (high P) conditions.

Determining how efficient N_2 fixation is in natural phytoplankton communities when primary factors such as turbulence, P availability, and light are non-limiting is critical for understanding the ability of N_2-fixers to alleviate N limitation over short timescales (e.g. within a single growing season). Assimilation of N via the reduction of N_2 is a highly energy-intensive process compared to direct uptake of ammonia (NH_3) or NH_4^+ due to the energy costs of the enzyme systems necessary for N_2 fixation (Turpin et al. 1985; Herrero et al. 2004). And when compared to nitrate (NO_3^-) assimilation, N_2 fixation is also unfavorable as a result of large energy costs associated with differentiating and maintaining specialized cells, called heterocytes, in which the nitrogenase enzyme can function (Turpin et al. 1985). Therefore, N_2 fixation as the sole source of N may not fuel as much biomass accumulation as NO_3^- or NH_4^+ assimilation would under prime growth conditions.

Additionally, the proliferation of N_2-fixing and/or non-N_2-fixing cyanobacteria is particularly undesirable in lakes and reservoirs due to their tendency to produce aesthetically displeasing surface scums, odors, and potentially harmful toxins such as microcystin (Paerl et al.

2001). Cyanobacteria capable of producing toxins have been shown to be present under both low and high TN:TP conditions (Beversdorf et al. 2013; Chislock et al. 2013). Scott et al. (2013) found that microcystin concentrations in Canadian lakes were greater when TN:TP was optimal for biomass production (approximately 26 – 50). Increased N availability relative to P may be of key importance for microcystin production (Horst et al. 2014). Nonetheless, more microcystin data, especially under low N:P conditions, would be beneficial for understanding when and why microcystin production occurs in lakes and reservoirs.

Therefore, the objectives of this study were to experimentally test the efficiency of N_2 fixation and microcystin production across a wide range of N:P supply ratios under high P (eutrophic conditions). A mass balance-constrained ^{15}N stable isotope tracer method was used in mesocosms placed in three small, temperate reservoirs to specifically test the hypothesis that N_2 fixation would not effectively alleviate N limitation at low N:P supply under eutrophic conditions due to limitation by the high energetic costs of N_2 fixation. N_2 fixation efficiency was also hypothesized to vary between reservoirs due to different initial conditions. Additionally, microcystin was measured across the same N:P supply gradient to test the hypothesis that toxin production is elevated at greater N:P supply because of increased biomass or N availability (Scott et al. 2013; Horst et al. 2014).

2.2 Methods

2.2.1 *Study Sites*

Mesocosm experiments were conducted in three small (< 0.2 km^2), monomictic reservoirs in the Springfield Plateau region of northwest Arkansas. Lake Brittany (36°28’08”N, 94°12’04”W), Lake Norwood (36°28’45”N, 94°14’44”W), and Lake Rayburn (36°27’43”N,

94°14’21”W) are all steeply-sloped reservoirs with mean depths of 7.6 – 8.8 m and maximum depths of 21.0 – 23.3 m. All three reservoirs have similar watershed land use characteristics, with primarily forested (64 – 78%) and urban (14 – 25%) watersheds. These reservoirs are designated for recreational use, especially sport fishing. The study reservoirs were monitored approximately weekly from May – October 2012. Vertically-integrated water samples were collected from the euphotic zone at 5 locations on each lake using a 4 L Van Dorn horizontal sampler. The five samples were mixed, creating a whole-reservoir composite sample, and returned to the laboratory on ice.

Within 24 h of collection, a subsample from each composite sample was filtered onto a pre-combusted (4 h at 450 °C) GF/F filter (Whatman) for particulate carbon (PC) and particulate N (PN) and onto an acid-washed GF/F filter for particulate P (PP). The filters were then frozen for later analysis. Filtrate from filtered samples (GF/F) was retained, frozen, and later analyzed for nitrate + nitrite-N (henceforth NO_3^--N), total dissolved N (TDN), and total dissolved P (TDP). A subsample of the composite water was preserved with M3 fixative for phytoplankton enumeration. Phytoplankton were enumerated using an inverted microscope method (Utermöhl 1958) and identified using the method outlined by Olrik et al. (1998). Taxa-specific geometric formulas recommended by Rott (1981) and Olrik et al. (1998) were used to calculate phytoplankton biovolumes. Additionally, a subsample of whole-reservoir composite water from each reservoir was filtered onto GF/F filters (Whatman), frozen, and then analyzed for microcystin on 26 July and 17 August 2012.

2.2.2 *Mesocosm experiment*

A mesocosm experiment was conducted from 31 July to 7 September 2012 in each of the three reservoirs to address how N:P supply ratios affected N_2 fixation, TN and biomass accumulation, and microcystin concentrations, with respect to initial conditions. Mesocosms were white, 166 L polyethylene containers (Brute®) closed at the bottom and open to the atmosphere. All mesocosms were submerged in each reservoir for 4 weeks to allow for leaching of any potentially harmful chemicals and then cleaned prior to the experiment. Twelve mesocosms were attached to a floating PVC frame on 31 July in each of the three reservoirs (36 mesocosms total). Mesocosms in each reservoir were filled with 130 L of vertically integrated euphotic zone water. Each mesocosm was randomly assigned an N:P (molar) treatment of 1, 10, 25, or 50 (3 mesocosms per treatment per reservoir). The same amount of P was added to all of the mesocosms, and N was manipulated according to the N:P treatment. Nutrients were added approximately biweekly, and by the end of the experiment all mesocosms had received a total of 3.6 µmol L^{-1} P as NaH_2PO_4 and depending on the N:P treatment, either 3.6, 36, 90, or 180 µmol L^{-1} N as KNO_3 enriched with ^{15}N ($\delta^{15}N$ = 962‰) in order to distinguish it from N supplied by N_2 fixation which is isotopically "light" (approximately 0‰) . Upon addition of nutrients, mesocosms were stirred thoroughly to allow equal distribution of N and P throughout the water column.

Seston samples were collected just before the first nutrient addition (day 0) and on the last day of the experiment (day 37) for biological and chemical analyses. Initial samples were collected from mesocosm source water for each reservoir. A final sample was collected from each mesocosm after homogenizing the contents of the container, including any periphyton that may have grown on the sides during the experiment. Samples were collected into acid-washed

dark bottles, stored on ice, taken back to the laboratory, and filtered within 48 h. Upon returning to the lab, 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. Additionally, water samples from each mesocosm for each treatment were combined and a subsample was filtered onto a GF/F filter to be analyzed for microcystin. Filters were then frozen for later analysis. Filtrate from filtered samples (GF/F) was retained, frozen, and later analyzed for NO_3^--N, TDN and TDP.

2.2.3 *Laboratory Analyses*

Frozen PC and PN filters were re-dried for 24 h (50 °C) and analyzed using a Thermo Flash 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Inc., Netherlands). Particulate P filters were digested in a persulfate solution and analyzed colorimetrically using the ascorbic acid method (APHA 2005). Filtrate was analyzed simultaneously for TDN and TDP on a Skalar San Plus following a persulfate digestion (APHA 2005). Filtrate subsamples from routine monitoring were also analyzed for NO_3^--N colorimetrically using the cadmium reduction method. Both the PP and NO_3^--N colorimetric analyses were conducted on a Turner Designs Trilogy Lab Fluorometer with a spectrophotometer adaptor containing an 800 nm and 600 nm filter, respectively. Microcystin in particulate matter was quantified using enzyme-linked immunosorbent assay (ELISA; An and Carmichael 1994) after extraction from filters with 75% aqueous methanol. All ^{15}N filters were freeze-dried and then analyzed at the University of Arkansas Stable Isotope Laboratory using a Finnigan Delta Plus mass spectrometer following combustion in a Carlo Erba NC2500 elemental analyzer (connected via a Finnigan Conflo II

interface). The ratio of ^{15}N to ^{14}N was expressed in conventional delta notation, relative to air (Peterson and Fry 1987).

2.2.4 *Calculations and Statistical Analyses*

A similar approach to Vrede et al. (2009) was employed to calculate N_2 fixation in the mesocosms using the ^{15}N stable isotope. The $\delta^{15}N$ of the final sample ($\delta^{15}N_{final}$) was defined by a multi-source mixing model as:

$$\delta^{15}N_{final} = (\delta^{15}N_{intial} \cdot f_{initial}) + (\delta^{15}N_{added} \cdot f_{added}) + (\delta^{15}N_{fix} \cdot f_{fix}) \quad (2.1)$$

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 three-source mixing model (Equation 2.1) cannot have a single solution because it has three unknown variables that cannot be solved with simultaneous equations. Therefore a range of solutions for f_{fix} was calculated using IsoSource version 1.3 (U.S. EPA, Western Ecology Division, Corvallis, OR, U.S.A.). IsoSource was utilized following the procedure developed by Phillips and Gregg (2003). Briefly, IsoSource is a software package used to calculate a range of feasible source combinations that satisfy a mixing model with more than one 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. For this study, a source increment of 1% and a mass balance tolerance equal to the average within-treatment standard deviation were used for IsoSource computations. TN data were used to constrain the range of values calculated using IsoSource to only those solutions that satisfied the mass balance equation described as:

$$TN_{final} / TN_{initial} = (f_{initial} + f_{added} + f_{fix}) / f_{initial} \quad (2.2)$$

where TN_{final} is the mass of TN of the final sample and $TN_{initial}$ is the mass of TN present at the beginning of the experiment. For each treatment in each reservoir, the average and standard deviation of the TN_{final} values were used to estimate a 95% confidence interval by generating 1000 random values (normal distribution; SigmaPlot 11). The TN_{final} $TN_{initial}^{-1}$ ratio was then calculated for each of the randomly generated TN_{final} values using the $TN_{initial}$ measured for each reservoir. The upper and lower limits of the range of TN_{final} $TN_{initial}^{-1}$ values were defined as the 97.5% and 2.5% percentiles of the distribution. These upper and lower limits were used to constrain the solutions generated using IsoSource after calculating the *f* ratio (right-hand term) in Equation 2.2. The minimum, median, and maximum amount of total fixed N (TN_{fix}) was calculated as the product of the 5%, 50%, and 95% percentiles of f_{fix} and the final TN concentrations of each mesocosm.

N_2 fixation rates (N_{fix}) were derived from TN_{fix} values calculated from the 50^{th} percentile (median hereafter) of f_{fix} and expressed as µg N L^{-1} h^{-1}. Each N_{fix} value was considered to represent the average N_2 fixation rate for that mesocosm over the course of the experiment

(assuming a photoperiod of 13 h for each day of the experiment). Ordinary least squares (OLS) regression analyses were conducted using SAS 9.1 (SAS Institute Inc., Cary, NC, USA) to estimate the effect of N:P supply on $\delta^{15}N$, f_{fix} (median), N_{fix}, and the response ratios (RR) derived for TN (RR_{TN}) and PC (RR_{PC}). Response ratios were the final concentration divided by the initial concentration in each mesocosm (accounting for volume loss throughout the course of the experiment). Response ratios were used to determine the efficiency of N_2 fixation across N:P supply in each reservoir because any slope greater than zero indicates that added reactive N yielded more biomass than N_2 fixation. Therefore, any slopes in OLS regressions for RR_{TN} and RR_{PC} that were significant ($P < 0.05$) were considered to indicate that N_2 fixation was inefficient in terms of TN or biomass accumulation for that reservoir.

In order to test for effects of initial conditions, the slopes and intercepts of the OLS regressions developed for each parameter versus N:P supply were compared by reservoir. Analysis of covariance (ANCOVA) was used (SAS 9.1) to compare slopes and intercepts, with $P < 0.05$ indicating a significant difference between reservoirs for each test (i.e. slope and intercept).

2.3 Results

2.3.1 *Reservoir Conditions*

Lakes Brittany, Norwood, and Rayburn are mesotrophic to slightly eutrophic reservoirs with average TN and TP ranging from 435 – 699 μg L^{-1} and 15.3 – 35 μg L^{-1}, respectively (Fig. 2.1). Norwood and Rayburn had similar nutrient concentrations, which were generally greater than in Brittany. However, TN:TP (by moles) was similar across reservoirs, ranging from 44.7 – 65.1. A clear trend in NO_3^--N drawdown was observed in all three reservoirs as summer and

consequently thermal stratification progressed. Interestingly, the date that NO_3^--N was drawn down below the detection limit (16.5 µg L^{-1}) differed substantially across reservoirs, as Norwood experienced drawdown approximately one month after Brittany and Rayburn (Fig. 2.1D). Furthermore, an apparent distinction in PC (used here as a proxy for phytoplankton biomass) concentrations between Brittany and the other reservoirs indicated that Brittany was less productive and possibly more nutrient limited than Norwood or Rayburn (Fig. 2.1E).

Rayburn and Brittany were the only reservoirs to have measurable concentrations of microcystin on the two dates it was analyzed (Fig. 2.1F). However, the concentrations on both dates in each reservoir were below the World Health Organization's (WHO) provisional guideline value of 1 µg L^{-1}, although microcystin was relatively higher in Rayburn (0.70 µg L^{-1}) than in Brittany (0.01 µg L^{-1}) just before the mesocosm experiments began in late July (Fig. 2.1F). 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 (Figure 2.2). A subsequent decrease in these taxa was observed in Brittany and Rayburn, with another relative increase in N_2-fixing cyanobacteria biomass observed in September in Brittany. Interestingly, 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. 2.2).

2.3.2 *Mesocosm Experiment*

The $\delta^{15}N_{initial}$ varied across reservoirs, ranging from 0.33‰ in Norwood to 9.66‰ in Brittany (Table 2.1). A strong, positive linear trend between $\delta^{15}N_{final}$ and N:P supply was observed in each reservoir (Fig. 2.3A), although this relationship was not significantly different between reservoirs (Table 2.3). The median f_{fix} values generated using IsoSource ranged from

0.08 – 0.77 across all treatments and reservoirs (Fig. 2.3B). A significant trend of decreasing f_{fix} as N:P supply increased was also observed in each reservoir (Table 2.4). However, ANCOVA results revealed that f_{fix} regression slopes were not significantly different between reservoirs, but that the intercepts were significantly different (Table 2.3), indicating that fixed N in Brittany comprised more of TN_{final} at low N:P supply compared to Norwood and Rayburn (Fig. 2.3B). Furthermore, N_2 fixation rates ranged from 0.61 – 4.31 μg N L^{-1} h^{-1} throughout the entire experiment, with the highest rates occurring in the lowest N:P supply treatment in Brittany and Rayburn (Fig. 2.4). Slopes of N_{fix} versus N:P supply regressions were not different between reservoirs. However, the intercept for the N_{fix} regression in Norwood was significantly lower than in Brittany and Rayburn (which were not different from each other; Table 2.3). Thus, N_2 fixation rates in Norwood were particularly less than in Brittany and Rayburn across the entire N:P supply gradient (Fig. 2.4).

RR_{TN} increased with N:P supply in all reservoirs (Fig. 2.5A), and the slope of this regression was significantly greater than zero in each reservoir (Table 2.2). Furthermore, the RR_{TN} increase per unit N:P supply was significantly greater in Brittany than in Norwood and Rayburn (which were not significantly different from each other; Table 2.3). Likewise, RR_{PC} significantly increased with N:P supply, but only significantly in Brittany and Norwood (Table 2.2). RR_{PC} increased with N:P supply significantly more in Brittany than in Norwood and Rayburn (Table 2.3). Mean RR_{PC} increased approximately 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. 2.5B).

Microcystin was detected in each treatment across all reservoirs (Fig. 2.6). However, only the highest N:P supply treatment in Rayburn had concentrations greater than the WHO's

provisional guideline value of 1 µg L^{-1} by nearly a factor of three (Fig. 2.6). Microcystin concentrations in the highest N:P supply treatment in Rayburn were greater than ambient reservoir concentrations measured just before the start of the mesocosm experiment and in August by a factor of approximately 4 and 6, respectively.

2.4 Discussion

The results of this study indicate that despite warm conditions and virtually no water column turbulence, N_2 fixation was not entirely efficient over the 37 day mesocosm experiment. The magnitude of TN and biomass accumulation was lower when N_2 fixation was the primary source of N compared to dissolved NO_3^-. The inefficiency of N_2 fixation may have been a result of the limiting energetic cost of differentiating heterocytes and the formation of complex enzymatic systems needed for N_2 reduction to occur (Turpin et al. 1985; Hererro et al. 2004). Cyanobacteria have been shown to grow optimally when water temperature is > 20 °C (Paerl et al. 2001), and N_2 fixation is known to be disrupted by filament breakage caused by excessive turbulence (Howarth et al. 1988b; Paerl et al. 2001). Thus, the mesocosm experiment described here represented ideal conditions for N_2-fixing cyanobacteria. N_2 fixation would likely be even less efficient on the same timescale if other factors known to influence N_2 fixation were introduced.

Importance of N_2 fixation across the N:P supply gradient

N_2 fixation contributed to TN accumulation across all treatments in each reservoir despite its 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 comprised up to 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 (Fig. 2.3B). In a similar mesocosm experiment, Vrede et al. (2009) found that fixed N comprised nearly 50% of the PN pool at approximately 32 N:P supply after 21 days. The average contribution of fixed N in the mesocosms enriched with 25 N:P in the current study was approximately 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 over twice as much NO_3^- than those in the experiment conducted by Vrede et al. (2009). Thus, lower f_{fix} values in this experiment would be expected, even after a longer period of time (37 days compared to 21 days).

The N_2 fixation rates measured in the current study ranged from 0.61 – 4.31 µg N L^{-1} h^{-1} which were comparable to those observed in various other studies. Natural N_2 fixation rates in several Texas reservoirs have been observed to range from 0 – 11.7 µg N L^{-1} h^{-1} (Forbes et al. 2008; Scott et al. 2008a; Scott et al. 2009). Vrede et al. (2009) observed an average rate of 3.62 µg N L^{-1} h^{-1} (assuming a 13 hour photoperiod each day) across a 21 day period in mesocosms treated with P only. Additionally, Beversdorf et al. (2013) measured natural rates ranging from 0 – 4.65 µg N L^{-1} h^{-1} in Lake Mendota during the summers of 2010 and 2011. As expected, N_2 fixation was significantly stimulated as N:P supply decreased across all reservoirs (Fig. 2.4), and the response of N_2 fixation to N:P supply was similar regardless of initial conditions. The occurrence of N_2 fixation at 50 N:P supply was not unexpected, as N or P can limit growth when TN:TP is 20 – 50 (Guildford and Hecky 2000). Regardless, decreased DIN (e.g. NO_3^-) under P-replete conditions resulted in increased importance of N_2 fixation as a source of N, regardless of initial nutrient conditions.

N_2 fixation efficiency

Despite substantially increased N_2 fixation rates at low N:P supply, RR_{TN} and RR_{PC} increased with increasing inorganic N availability (Fig. 2.5), indicating that N_2 fixation was not highly efficient even though conditions in the mesocosms were ideal. These results are contradictory to the study conducted by Vrede et al. (2009), in which N_2 fixation was found to effectively alleviate N deficiency over a 21 day period in a temperate, eutrophic lake in Sweden. However, while TN accumulation was greater with increased N:P supply in Rayburn, biomass (as PC) did not increase (Table 2.2). Thus, the phytoplankton species dominating assemblages in low N:P supply treatments in Rayburn were evidently more efficient at generating biomass per unit N supplied via N_2 fixation, indicating that initial conditions at the onset of nutrient perturbations can have an effect on N_2 fixation efficiency.

Interestingly, the increase in RR_{TN} and RR_{PC} across the N:P supply gradient was significantly greater in Brittany compared to Norwood and Rayburn. Brittany had the lowest initial TN and PC concentration, so RR_{TN} and RR_{PC} values were expected to exceed those in the other reservoirs. The slope of the RR_{TN} or RR_{PC} versus N:P supply regression was not necessarily expected to be greater in Brittany, as the initial TP concentration was lower and TN:TP was higher than in Norwood and Rayburn (Table 2.1), which indicated P-limited conditions (Guildford and Hecky 2000). However, initial biomass N:P was considerably less (29) than initial TN:TP (95) in Brittany, and relatively low compared to Norwood (33) and Rayburn (40). Therefore, N may have been limiting growth in Brittany initially, due to a relatively refractory initial TDN pool and low DIN (NO_3^--N < 16.5 µg L^{-1}). 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 comprised approximately 25% of the

natural phytoplankton assemblage near the beginning of the experiment (Fig. 2.2). These results suggest that initial TN:TP ratios were not entirely useful for predicting the response to N and P enrichment, since TDN was likely not an available source of N even though phytoplankton have been shown to incorporate labile dissolved organic N (DON) in other studies (see Bronk et al. 2007).

Microcystin production

Detrimental levels of microcystin were only measured at 50 N:P supply in Rayburn (Fig. 2.6). Interestingly, Rayburn had the highest ambient concentration of microcystin near the beginning of the experiment (0.701 µg L^{-1}; Fig. 2.1F), indicating that microcystin-producing cyanobacteria were present in Rayburn at the beginning of the mesocosm experiment. The relative increase in microcystin concentrations after nutrient enrichment with high N (and P) only in Rayburn may highlight the importance of initial conditions at the onset of nutrient enrichment. The occurrence of microcystin concentrations above the WHO's provisional guideline of 1 µg L^{-1} only at 50 N:P supply is consistent with Scott et al. (2013), who found that microcystin concentrations typically exceeded this guideline in Canadian lakes when TN:TP was approximately 26 – 50. The results of this experiment were also similar to those of another study which found that the cyanotoxin, saxitoxin, was enhanced in limnocorrals receiving 40 and 122 N:P treatments, but not in mesocosms receiving 7 N:P treatments in a eutrophic lake (Chislock et al. 2014). Since biomass did not significantly increase with N:P supply in Rayburn, the occurrence of detrimental levels of microcystin at high N:P may have been driven by N availability rather than biomass production. Indeed, the importance of N in microcystin production was demonstrated in a field experiment conducted by Horst et al. (2014), who found that microcystin cell quota was significantly decreased when P additions caused N deficiency.

2.5 Conclusions

N_2 fixation contributed large amounts of N to mesocosms experiencing high P supply and an imbalance of N in three small, temperate reservoirs. However, the reduction of N supply with no concomitant reduction in P supply significantly decreased RR_{TN} and RR_{PC} (PC was used as a proxy for autotrophic biomass), indicating that N_2 fixation could not effectively alleviate N limitation at high P and low N:P supply on a seasonal time scale. Initial nutrient conditions were important, however, as N_2 fixation evidently fueled efficient biomass accumulation at low N:P in the more initially eutrophic reservoir. Furthermore, microcystin concentrations above the WHO's provisional guideline of 1 $\mu g\ L^{-1}$ were measured only in the mesocosms receiving the highest N:P supply, which may have been influenced by initial microcystin conditions. A reduction in N supply relative to P may not always result in less biomass accumulation, but may be beneficial for limiting microcystin production. These results add to a growing collection of evidence that dual N and P reduction strategies deserve consideration in eutrophication management strategies (Conley et al. 2009; Paerl 2009).

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2.7 Tables

Table 2.1. Initial conditions measured in mesocosm source water in each reservoir.

	Mesocosm Experiment – Initial		
Parameter	Brittany	Norwood	Rayburn
$\delta^{15}N$ (‰)	9.66	0.33	1.81
TN ($\mu g\ L^{-1}$)	378	637	785
TP ($\mu g\ L^{-1}$)	8.8	24.6	20.2
TN:TP (by moles)	95.3	57.2	86.1
NO^{3-}-N ($\mu g\ L^{-1}$)	-	-	-
PC ($mg\ L^{-1}$)	0.65	1.95	2.13

Table 2.2. Results from OLS linear regression analyses run on various parameters vs. N:P supply for each reservoir (n = 12). Slopes (m) were considered significantly different from 0 when $P < 0.05$.

Variable	m	y_0	F	P	r^2
$\delta^{15}N$ (‰)					
Brittany	13.67	60.32	138.61	<0.0001	0.93
Norwood	12.05	73.70	144.58	<0.0001	0.93
Rayburn	11.88	40.62	185.24	<0.0001	0.95
f_{fix}					
Brittany	-0.012	0.70	69.60	<0.0001	0.87
Norwood	-0.009	0.51	182.28	<0.0001	0.95
Rayburn	-0.010	0.60	105.60	<0.0001	0.91
N_{fix} ($\mu g\ L^{-1}\ hr^{-1}$)					
Brittany	-0.048	3.88	13.21	0.0046	0.57
Norwood	-0.041	2.60	29.94	0.0003	0.75
Rayburn	-0.060	3.75	37.15	0.0001	0.79
RR_{TN}					
Brittany	0.108	4.82	18.12	0.0017	0.64
Norwood	0.033	2.78	10.11	0.0098	0.50
Rayburn	0.028	2.70	12.36	0.0056	0.55
RR_{PC}					
Brittany	0.776	28.95	29.12	0.0003	0.74
Norwood	0.096	8.75	8.789	0.0142	0.47
Rayburn	0.045	10.13	2.966	0.1157	0.23

Table 2.3. Results of analysis of covariance (ANCOVA) comparing the slopes of OLS linear regressions developed for various parameters versus N:P supply. Slopes and intercepts were considered significantly different between reservoirs when $P < 0.05$.

Parameter	Slope		Intercept	
	F	*P*	*F*	*P*
$\delta^{15}N$				
B vs. N	1.11	0.3041	0.56	0.4615
B vs. R	1.51	0.2331	4.54	0.0452
N vs. R	0.02	0.9005	2.32	0.1423
f_{fix}				
B vs. N	3.23	0.0874	18.24	0.0003
B vs. R	0.53	0.4771	4.49	0.0463
N vs. R	1.60	0.2211	7.37	0.0130
N_{fix}				
B vs. N	0.20	0.6632	17.29	0.0004
B vs. R	0.58	0.4567	1.76	0.1993
N vs. R	2.39	0.1381	9.81	0.0050
RR_{TN}				
B vs. N	7.54	0.0125	39.69	<0.0001
B vs. R	9.05	0.0069	43.86	<0.0001
N vs. R	0.13	0.7172	0.56	0.4635
RR_{PC}				
B vs. N	21.32	0.0002	82.52	<0.0001
B vs. R	25.00	<0.0001	75.68	<0.0001
N vs. R	1.47	0.2388	0.15	0.7023

2.8 Figure Legends

Figure 2.1. Parameters measured May – October 2012 (33 sample dates) in Brittany (open circles), Norwood (shaded squares), and Rayburn (filled triangles) including: TN (A), TP (B), TN:TP (C), NO_3^--N (D), PC (E), and microcystin (F). Note that TN:TP is by moles, and PC is in mg L^{-1}. Vertical dashed line represents the beginning of the mesocosm experiment on 31 July 2012. Microcystin was below detection on both sample dates in Norwood (F).

Figure 2.2. Relative phytoplankton biomass in Brittany (top), Norwood (middle), and Rayburn (bottom) on six sample dates from May – September 2012. Taxanomic groups were identified based on Olrik et al. (1998), although cyanobacteria (cy.) were split into two categories: N_2-fixing and non-N_2-fixing cyanobacteria based on identified species' capability for carrying out N_2 fixation. Flagellates, diatoms, green algae, non-N_2-fixing cyanobacteria, and N_2-fixing cyanobacteria are represented from top to bottom in descending shade, respectively. Vertical dashed line represents the beginning of the mesocosm experiment on 31 July 2012.

Figure 2.3. Stable isotope and N_2 fixation data vs. N:P supply (from samples collected at the end of the mesocosm experiment) including: $\delta^{15}N$ in‰ (A) and median f_{fix} or the fraction of TN comprised of fixed N (B). OLS regression analyses were conducted for each parameter by reservoir (n = 12) with $P < 0.05$ indicating a slope significantly different than 0. See Table 2.2 for results of regression analyses. Shown are means (n = 3) ± standard deviations (error bars). For panel B, black dots represent the range of feasible values calculated using IsoSource and a multi-mixing stable isotope model constrained by mass balance.

Figure 2.4. N_2 fixation rates representing average rates throughout the experiment in each reservoir, accounting for volume loss and assuming a 13 h photoperiod each day. OLS regression analyses were conducted for each parameter by reservoir (n = 12) with $P < 0.05$ indicating a slope significantly different than 0. See Table 2.2 for results of regression analyses. Shown are treatment means (± standard deviation as error bars) by reservoir. Black dots represent mean N_2 fixation rate minima and maxima calculated from the range of f_{fix} values generated using IsoSource.

Figure 2.5. Mean response ratios, or the ratio of final concentration to initial concentration, for A) TN (RR_{TN}) and B) PC (RR_{PC}) in each reservoir. Error bars represent standard deviations within treatments. OLS regression analyses were conducted for each RR by reservoir, with $P <$ 0.05 indicating a slope significantly different than 0. See Table 2.2 for results of regression analyses.

Figure 2.6. Microcystin measured from a subsample collected from a treatment composite sample (samples from all three mesocosms from that treatment were combined) in each reservoir. Horizontal dashed line represents the WHO's provisional guideline of 1 μg L^{-1}, above which concentrations are considered unsafe for human health.

2.9 Figures

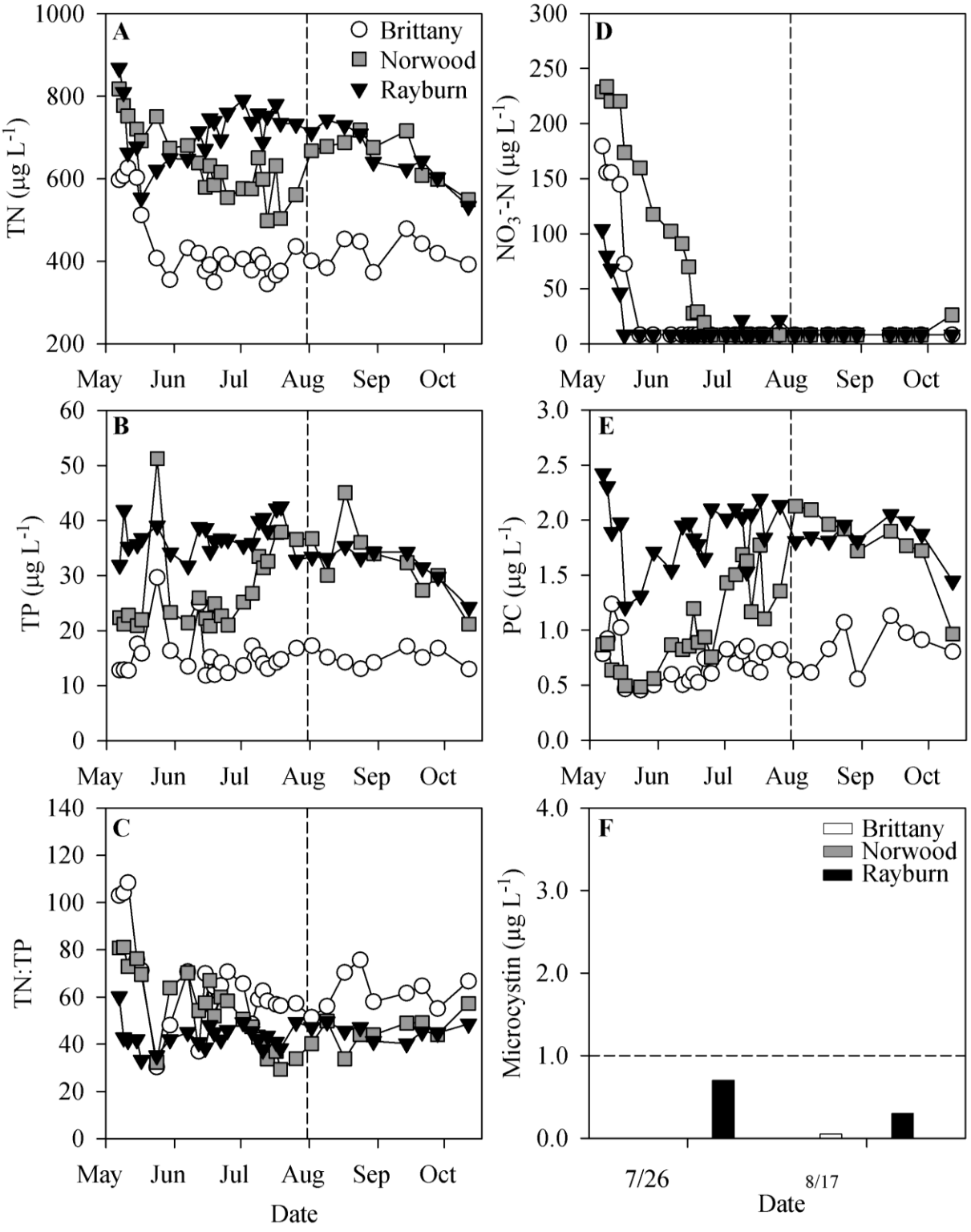

Figure 2.1

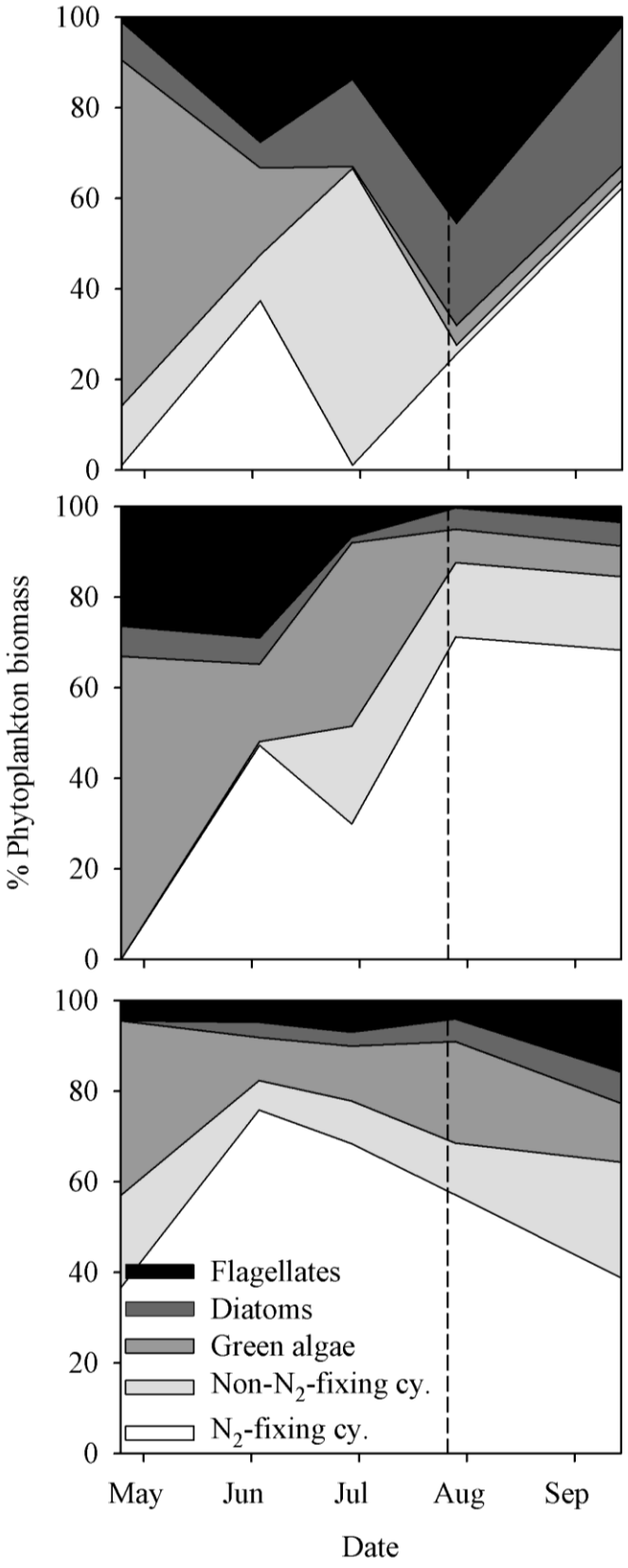
100
80
60
40
20
0
% Phytoplankton biomass
Flagellates
Diatoms
Green algae
Non-N2-fixing cy.
N2-fixing cy.
May
Jun
Jul
Aug
Sep
Date

Figure 2.2

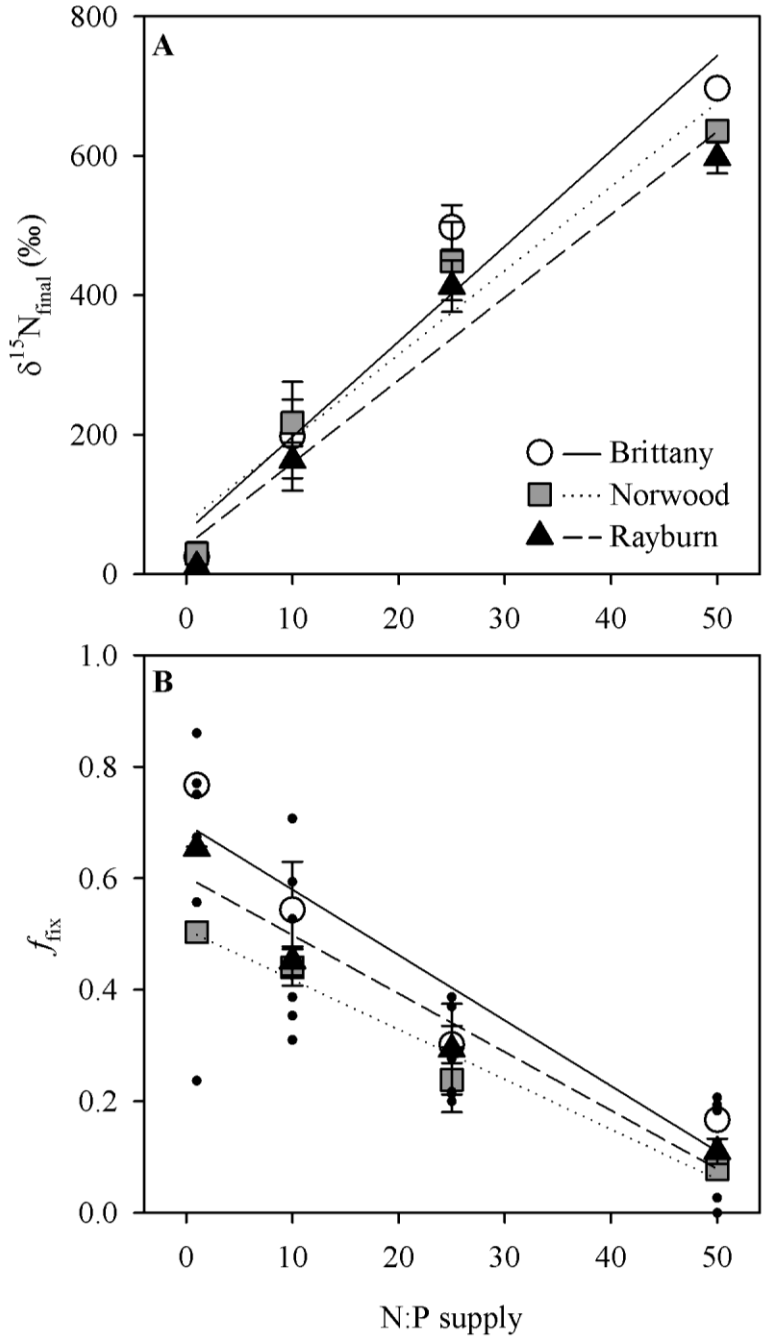
A
B
800
600
400
200
0
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
δ15Nfinal (‰)
ffix
N:P supply
Brittany
Norwood
Rayburn

Figure 2.3

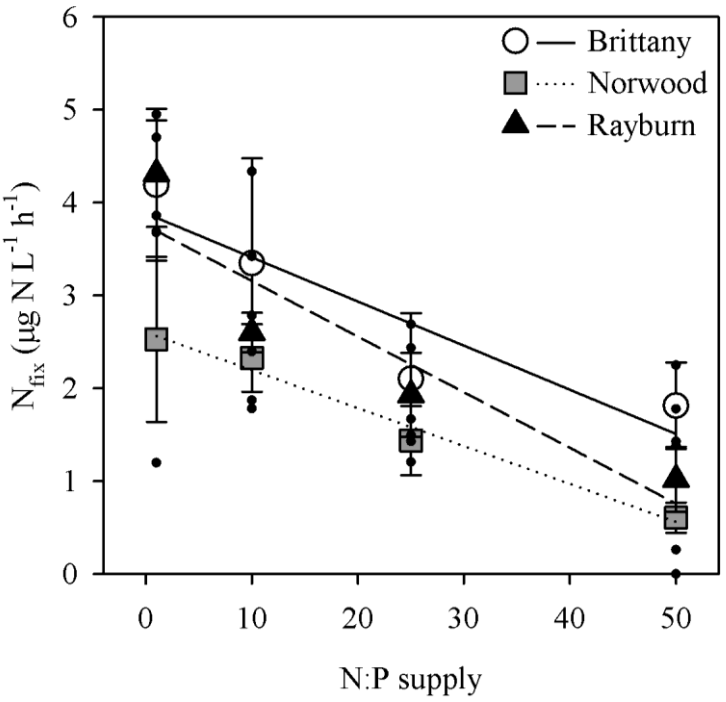
Brittany
Norwood
Rayburn
N_{fix} (µg N L^{-1} h^{-1})
N:P supply
0
1
2
3
4
5
6
0
10
20
30
40
50

Figure 2.4

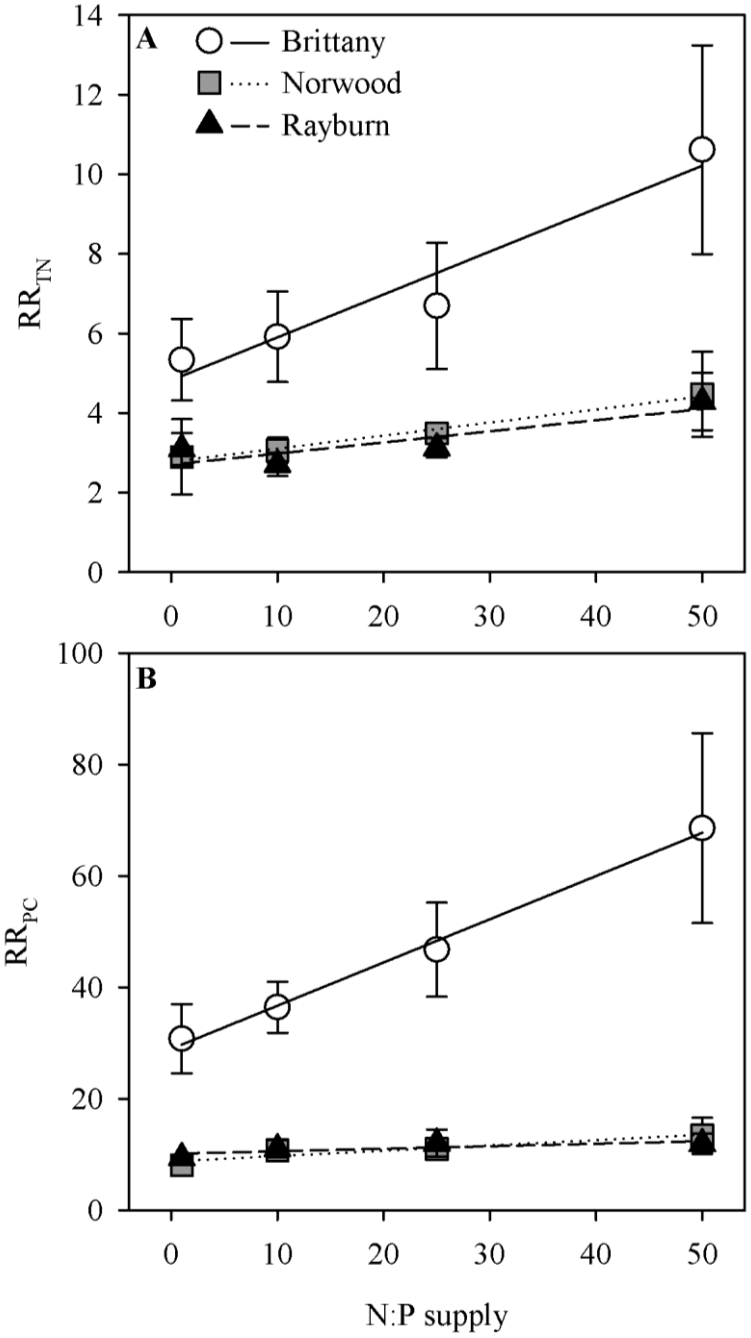

Figure 2.5

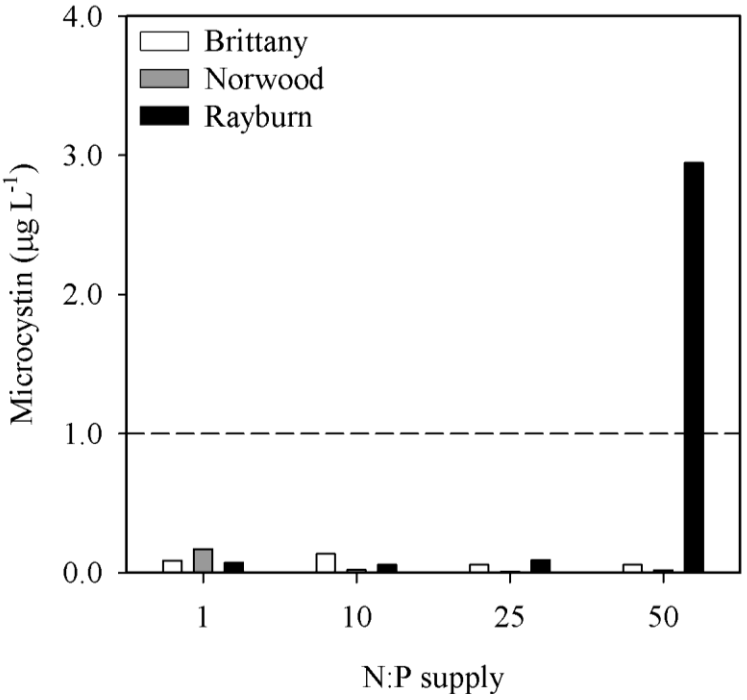
Brittany
Norwood
Rayburn
Microcystin (µg L-1)
4.0
3.0
2.0
1.0
0.0
1
10
25
50
N:P supply

Figure 2.6

3. NITROGEN FIXATION RATES AND IMPORTANCE ACROSS A NATURAL TROPHIC GRADIENT IN SIX SMALL, TEMPERATE RESERVOIRS

3.1 Introduction

The reduction of dinitrogen gas (N_2) to bioavailable N (N_2 fixation) by various species of cyanobacteria may be of relative importance to N cycling in lakes and reservoirs. Inputs of N from N_2 fixation can be substantial relative to external (Howarth et al. 1988a; Patoine et al. 2006) and internal (Scott and Grantz 2013) N loading in both lakes and reservoirs. However, patterns and importance (e.g. relative amount of N supplied to autotrophs) of N_2 fixation in reservoirs are still not well-understood, especially at the ecosystem scale. Some studies have attempted to determine N_2 fixation rates within and between reservoirs (Scott et al. 2008; Scott et al. 2009; Forbes et al. 2008), although few have examined small (< 1.0 km^2), temperate reservoirs (Scott and Grantz 2013). Furthermore, most freshwater N_2 fixation studies to date utilized the acetylene reduction technique (Stewart et al. 1967) to quantify instantaneous N_2 fixation rates or potential rates. Acetylene reduction methods may not be appropriate for estimating ambient N_2 fixation due to cyanobacteria community differences in the ethylene:N_2 conversion factor (Paerl 1982; Graham et al. 1980). Additionally, lacustrine N_2 fixation studies that employ the acetylene reduction technique tend to be spatially and temporally limited as the method requires labor-intensive incubated bioassays that are prone to error to be carried out immediately after sample collection (Flett et al. 1975, 1976).

A more recently-developed technique that utilizes the natural abundance ^{15}N stable isotope signature of phytoplankton has been employed by several researchers in marine systems (e.g. Montoya et al. 2002 and Wannicke et al. 2010) and is gaining traction for freshwater systems (Patoine et al. 2006; Jankowski et al. 2012). The contribution of N produced via N_2

fixation (fixed N) to phytoplankton N that has already occurred (rather than can potentially occur) can be estimated using ^{15}N signatures of sestonic biomass (consisting primarily of phytoplankton) collected easily from lakes and reservoirs. The N derived from N_2 fixation tends to be isotopically different from other sources such as N in wastewater or agriculture runoff (Kendall 1998; Fry 2006). The ^{15}N technique could allow for N_2 fixation to become a water quality parameter measured as easily as chlorophyll *a* or particulate N (PN). Furthermore, examining the ^{15}N content of zooplankton allows for the unique ability to determine if fixed N is incorporated into zooplankton (Patoine et al. 2006; Sommer et al. 2006). Some zooplankton taxa have been shown to directly graze on cyanobacteria (Starkweather and Kellar 1983; Haney 1987; Fulton and Paerl 1988) and assimilate fixed N (Patoine et al. 2006; Sommer et al. 2006). However, assimilation of fixed N by zooplankton is rarely studied in freshwater systems. Determination of the transfer of fixed N to zooplankton could be important for understanding different mechanisms of interannual fixed N retention in lakes and reservoirs, such as incorporation into the upper, more temporally stable portions of the food web.

Use of the ^{15}N natural abundance technique represents a potentially straightforward and important tool for further understanding the role of N_2 fixation in small reservoirs. Regardless of the method used, relatively few N_2 fixation studies have been conducted in small reservoirs compared to natural lakes and large impoundments. While generally understudied compared to lakes (Kennedy et al. 2003), reservoirs have been shown to play a globally significant role in carbon (C) burial (Dean and Gorham 1998) and landscape N retention and/or removal (Harrison et al. 2009). A heightened understanding of N_2 fixation's contribution to N cycling and the variables that influence when and why N_2 fixation occurs in reservoirs could be considerably

useful for predicting how these systems will continue to respond to nutrient perturbations in a dynamic landscape.

The objectives of this study were to: 1) determine rates of N_2 fixation throughout the warm season in six small reservoirs using the ^{15}N stable isotope, 2) develop regression models for ^{15}N-derived N_2 fixation estimates for comparison with other models and 3) determine the likelihood of zooplankton assimilation of fixed N. Nutrient concentrations, temperature, and seston and zooplankton ^{15}N were monitored in the study reservoirs from April – October 2013. N_2 fixation was hypothesized to be predictably related to variations in seston $\delta^{15}N$ due to the effect of fixed atmospheric N (low $\delta^{15}N$) on natural phytoplankton communities enriched with ^{15}N from inorganic N uptake early in the growing season when external N inputs are generally higher. Therefore, multi-source ^{15}N mixing models were used to determine the fractional contribution of fixed N to sestonic N and subsequently N_2 fixation rates (which were also scaled to obtain annual rates). Multiple linear regression analyses were used to determine predictive models for these estimated biweekly rates of N_2 fixation. Additionally, zooplankton were hypothesized to show signs of assimilation of fixed N based on studies that have found some zooplankton taxa capable of directly grazing cyanobacteria (Starkweather and Kellar 1983; Haney 1987; Fulton and Paerl 1988). Simple linear regressions were used to determine if fixed N was being incorporated into the food web via zooplankton.

3.2 Methods

3.2.1 *Study Sites*

Lakes Brittany (36°28’08”N, 94°12’04”W), Elmdale (36º11’45”N, 94º12’50”W), Fayetteville (36º08’11”N, 94º07’46”W), Norwood (36°28’45”N, 94°14’44”W), Rayburn

(36°27’43”N, 94°14’21”W), and Wedington (36º05’27”N, 94º22’02”W) are small (< 1.0 km^2) flood control and multi-use recreational impoundments located in and around Northwest Arkansas (Table 3.1). These reservoirs are warm and monomictic, experiencing strong thermal stratification during the late spring through mid-fall. The reservoirs in this study represent a trophic gradient ranging from oligotrophic/slightly mesotrophic Lake Brittany to highly eutrophic Lake Fayetteville due to a watershed land use gradient that exists across these reservoirs (Table 3.1).

3.2.2. *Field Sampling*

Weekly sampling of each reservoir took place approximately biweekly during the warm season from April – October, 2013. Each reservoir was sampled within 24 h of each other (i.e. some sampling events were split into two sampling days). On each sampling date, a depth profile of temperature and light extinction were collected at a site overlying the deepest point in each reservoir (always near the dam). Temperature was measured at approximately 0.5 – 1.0 m intervals from the surface to the hypolimnion (or bottom in the shallower reservoirs) using a multiparameter datasonde (600 XLM YSI, Yellow Springs, OH, USA) and light extinction was measured from the surface to the euphotic zone depth (Z_{eu}) with a Licor quantum sensor (LI-250A Light Meter/Photometer, LI-193 bulb, LI-COR®, Lincoln, NE, USA). The Z_{eu} was described as the depth at which light intensity was 1% of photosynthetically active radiation (PAR) measured at the surface. Surface water temperature was determined at a depth of 0.5 m. Vertically-integrated water samples were collected from the euphotic zone at 5 sites approximately equidistant from the dam to the inflow on each reservoir using a 4 L Van Dorn horizontal sampler. Additionally, a zooplankton sample was collected at each site using a

Wisconsin tow-net (80 µm mesh; Wildco, Yulee, FL) towed from the Z_{eu} to the surface. The five water samples were mixed, creating a whole-reservoir composite sample for later seston and filtrate analysis, and the five zooplankton samples were similarly mixed to be later filtered and analyzed. All samples from each reservoir were kept in dark, acid-washed bottles on ice and taken back to the laboratory the same day. Wedington, which is managed by the U.S. Forest Service, was inaccessible on the final sampling date due to the October shutdown of the U.S. government.

Within 24 h of collection, a subsample from each composite water sample was filtered onto a pre-combusted (4 h at 450 °C) GF/F filter (Whatman) for particulate carbon (PC) and PN analysis, a GF/F filter for chl *a* extraction, and onto an acid-washed GF/F filter for particulate P (PP) analysis. The filters were then frozen for later analysis. Filtrate from filtered samples (GF/F) was retained, frozen, and later analyzed for soluble reactive P (SRP), nitrate + nitrate-N (henceforth NO_3^--N), ammonium-N (NH_4^+-N), total dissolved N (TDN), and total dissolved P (TDP). Additionally, subsamples from each composite water and zooplankton sample were filtered onto heat-treated tissue quartz filters (Pall) and frozen for later ^{15}N analysis.

3.2.3 *Laboratory Analyses*

Frozen PC and PN filters were re-dried for 24 h (50 °C) and analyzed using a Thermo Flash 2000 Organic Elemental Analyzer (Thermo Fisher Scientific Inc., Netherlands). Particulate P filters were autoclave-digested in a persulfate solution and analyzed colorimetrically (as SRP) using the ascorbic acid method (APHA 2005). Filtrate subsamples were analyzed for SRP using the ascorbic acid method following a 1% persulfate digestion (APHA 2005). Filtrate subsamples were analyzed for TDN using a TOC-V_{CSH} and TNM-1

analyzer (Shimadzu Scientific Instruments, Columbia, MD; APHA 2005). Filtrate subsamples were also analyzed for NO_3^--N colorimetrically using the cadmium reduction method, and NH_4^+-N was analyzed colorimetrically using the Hach method (APHA 2005). Collectively, NO_3^--N and NH_4^+-N were considered to represent dissolved inorganic N (DIN). Total dissolved P was analyzed spectrophotometrically on a Cary 300 UV-Vis (Agilent Technologies, Foster City, CA) using the ascorbic acid method, following a 1% persulfate digestion (APHA 2005). The SRP (including digested PP), NO_3^--N, and NH_3-N analyses were all conducted on a Turner Designs Trilogy Lab Fluorometer with a spectrophotometer adapter containing an 800, 600, and 540 nm filter, respectively. For the ^{15}N analysis, filters were freeze-dried and then analyzed at the University of Arkansas Stable Isotope Laboratory using a Finnigan Delta Plus mass spectrometer after combustion in a Carlo Erba NC2500 elemental analyzer (connected via a Finnigan Conflo II interface). The ratio of ^{15}N to ^{14}N was expressed in conventional delta notation, relative to air (Peterson and Fry 1987).

3.2.4 *Seston $\delta^{15}N$ Mixing Models*

The sources of ^{15}N to sestonic N on a given sampling date were assumed to be three: N recycled from sestonic N measured on the previous sampling date ($\delta^{15}N_{prev}$), N from watershed sources ($\delta^{15}N_{ws}$), and N supplied via N_2 fixation ($\delta^{15}N_{fix}$). The $\delta^{15}N$ of filtered material from a whole-reservoir composite seston sample (henceforth $\delta^{15}N_{seston}$), was assumed to be equivalent to these sources based on:

$$\delta^{15}N_{seston} = (\delta^{15}N_{prev} \cdot f_{prev}) + (\delta^{15}N_{ws} \cdot f_{ws}) + (\delta^{15}N_{fix} \cdot f_{fix}) \quad (3.1)$$

where *f* values were the relative contribution to seston N, respectively for each source. All $\delta^{15}N$ values were known a priori, and all *f* values were unknown. The $\delta^{15}N_{ws}$ for each reservoir was assumed to be represented by the greatest $\delta^{15}N_{seston}$ measured throughout the study. The $\delta^{15}N_{prev}$ was simply defined as the $\delta^{15}N_{seston}$ from the previous sampling date. Fractionation was assumed to be negligible during N recycling between sampling dates, which has been previously demonstrated (Scott et al. 2007). The $\delta^{15}N_{fix}$ was held constant across all reservoirs and sampling dates at -1‰. While 0‰ is regularly used to describe $\delta^{15}N_{fix}$ (Patoine et al. 2006; Vrede et al 2009), -1‰ was used in this study as it is more in line with studies that have demonstrated $\delta^{15}N_{fix}$ to naturally range from -1.7 – -0.5‰ (Mingawa and Wada 1984; Rolff 2000; Fry 2006).

A unique solution could not be calculated for f_{fix} since both f_{ws} and f_{prev} in Equation 3.1 were also unknown. However, a range of possible f_{fix} values could be estimated using IsoSource version 1.3 (US EPA, Western Ecology Division, Corvallis, OR, USA). Briefly, IsoSource is a software package used to calculate a range of feasible source combinations that satisfy a mixing model with more than one 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. A source increment of 1‰ and a mass balance tolerance of 0.1‰ were used, following the procedure developed by Phillips and Gregg (2003). The minimum, median, and maximum f_{fix} values were calculated using IsoSource. There were some exceptions to the number of N sources considered when using IsoSource in Rayburn and Wedington. During a simultaneous whole-ecosystem study in Rayburn, the reservoir was fertilized twice with commercial-grade chemical fertilizer. Approximately 0.326 g N m^{-2} and 0.023 g P m^{-2} (N:P =

32) in the form of Sportmax® (15-42-4 [% N-P_2O_5-K_2O]) plus calcium nitrate (15.5-0-0) was applied to the reservoir on DOY 158, Rayburn was fertilized again on DOY 200 with 0.479 g N m^{-2} and 0.034 g P m^{-2} (N:P = 31) using the same fertilizer mixture. The measured $\delta^{15}N$ of the fertilizer mixture ($\delta^{15}N_{fert}$) used was 0.71‰, therefore introducing a fourth possible source of N to phytoplankton, with an isotopic signature similar to that of fixed N. Additionally, chemical-grade fertilizer was applied to Wedington once during the study. Approximately 0.128 g N m^{-2} and 0.086 g P m^{-2} (N:P = 3) in the form of liquid pond fertilizer (10-34-0; $\delta^{15}N_{fert}$ assumed to be 0.71‰) was applied to Wedington on DOY 127. Based on previous whole-reservoir fertilization events (Thompson 2013), fertilizer was only considered an active source of N to sestonic N for up to 21 days following fertilization. After this time, any N fertilizer was assumed to be fully incorporated and represented by $\delta^{15}N_{prev}$ and f_{prev}. Therefore, a range of possible f_{fix} values was generated for each sampling day within the two weeks following fertilization (in each reservoir) using IsoSource as stated previously, but with four sources (including the addition of $\delta^{15}N_{fert}$) rather than three.

3.2.5 *Reservoir N_2 Fixation Rates*

Minimum, median, and maximum volumetric N_2 fixation rates (mg N L^{-1} day^{-1}) were estimated by multiplying the minimum, median, and maximum f_{fix} by the volumetric autotrophic N demand on each sampling date. Volumetric autotrophic N demand on a given sampling date was assumed to equal the sestonic C:N (PC:PN) multiplied by the daily volumetric primary productivity (PPr). PPr was predicted for each sampling date from measured chl *a* concentrations using the linear model (r^2 = 0.826, P<0.0001) developed by Knoll et al. (2003) for 12 reservoirs that were similar in size, depth, and trophic state to the reservoirs in this study.

Volumetric N_2 fixation rates were multiplied by the volume of the euphotic zone for each sampling date and divided by the surface area of the lake to estimate daily areal N_2 fixation rates (mg N m^{-2} day^{-1}). The volume of the euphotic zone on a sampling date was derived from reservoir capacity curves, which were developed from bathymetric data for Brittany, Norwood, Rayburn, and Wedington (J.T. Scott, unpublished data) and estimated for Elmdale and Fayetteville based on bathymetric data for Wedington as discussed by Grantz et al. (2012).

Areal N_2 fixation rates calculated for each sampling date (N_{fix}) were assumed to represent the average rate for half the days between the previous and subsequent sampling dates. The last sample was collected in late October just before autumn mixing. A substantial NH_4^+-N flux from the hypolimnetic pool stored throughout the warm season occurs in these reservoirs at the onset of autumn mixing (Scott and Grantz 2013), which usually begins in late October. The $\delta^{15}N$ of hypolimnetic mineralized organic N (a large source of the NH_4^+-N) or NH_4^+-N supplied by dissimilatory nitrate reduction to ammonium (DNRA) is not well-known, especially for these systems. Consequently, f_{fix} values could not be estimated after autumn mixing due to several new N sources with unknown $\delta^{15}N$. However, N_{fix} was assumed to be zero after the last sampling date since this period is characterized by mixed conditions, high availability of inorganic N (both NH_4^+-N and NO_3^--N), and low water temperatures (Tõnno and Noges 2003; Scott et al. 2008, Scott and Grantz 2013). Likewise, N_{fix} was assumed to be zero before the first sampling date due to similar conditions not favorable for N_2 fixation to occur, which was found by Scott and Grantz (2013) in three of the study reservoirs during previous years.

A yearly N_2 fixation rate was calculated for each reservoir as the sum of rate values calculated for each sampling day and the estimates of N_2 fixation rates between sampling days. The yearly autotrophic N demand was used to determine what percentage of N demand was

satisfied by N_2 fixation throughout the entire year, as well as just during the study period (approximately the warm season). For the January – March and November – December periods, autotrophic N demand was calculated using historical chl *a* averages from the same months (data not shown) and average sestonic C:N data collected for each reservoir during the current study. Therefore, the yearly autotrophic N demand for each reservoir was calculated as the sum of all estimated rates of N demand for the study period and the estimated average rate of N demand representing the late autumn – spring period.

3.2.6 *Statistical Analyses*

Multiple linear regression was used to identify variables that could predict N_2 fixation and the relative strength of those predictors. In order to develop multiple linear regression models for N_{fix}, multicollinearity among independent variables (e.g. temperature, dissolved nutrients, and nutrient ratios) was investigated prior to multiple linear regression analysis by using Spearman's rank-order correlation. Spearman's rank-order correlation analyses were conducted using SAS 9.1 to determine correlations between all independent and dependent variables. A Spearman's correlation coefficient greater than 0.75 or less than -0.75 (and $P < 0.05$) between two independent variables was considered to be a significant and strong correlation that indicated collinearity existed between those two predictor variables. For the dependent variable N_{fix}, the independent variables chl *a*, PN, PC, and PC:PN were not used as possible predictor variables for N_{fix} since they were used to estimate PPr, autotrophic N demand, and consequently N_{fix}. Based on results of the Spearman's rank-order correlation analysis, the independent variables used in multiple linear regression analyses included surface water temperature, Z_{eu}, NO_3^--N, TN, TP, and TN:TP. Multiple linear regression has been used to

predict N_2 fixation from water temperature and total and dissolved nutrients in other studies (Scott et al. 2008; Forbes et al. 2008).

Stepwise multiple linear regression analyses were conducted to determine predictor variables of median N_{fix} (i.e. estimated from only the median f_{fix} values calculated using IsoSource) using SAS 9.1. A P value < 0.05 was required for independent variables to be added or removed from the model. All data were log-transformed before analysis in order to satisfy assumptions of normally distributed residuals. Variance inflation factors (VIF) were also used to determine collinearity between independent variables included in the model. The VIF is the inverse proportion of the variance for a predictor variable not explained by the other predictor variables present in the model. A VIF greater than 2.5 indicated that the other predictor variables were explaining a large proportion of the variance for the independent variable in question and was assumed to be negatively affecting the model (Allison 1999). Any predictor variables with a VIF value > 2.5 were removed and the analysis was run again. NO_3^--N was the only predictor variable that often fell below a detection limit. Several data below detection contributed to distribution skewness, therefore the values < 16.5 µg L^{-1}(detection limit) were assigned random numbers between 0 and 16.5 µg L^{-1} in order to smooth the distribution of the tail at the lower end. Additionally, linear regression (OLS) was used to compare annual N_2 fixation rates to the independent variables used in the multiple linear regression analyses described previously. Linear regression analyses were conducted (SAS 9.1) for annual N_2 fixation versus the means (DOY 95 – 294) of surface water temperature, Z_{eu}, NO_3^--N, TN, TP, and TN:TP. Regressions were considered significant when $P < 0.05$.

The likelihood of zooplankton assimilation of fixed N was determined using OLS linear regression. Regression analyses for zooplankton $\delta^{15}N$ ($\delta^{15}N_{zoop}$) versus $\delta^{15}N_{seston}$ were run using

SAS 9.1 for each reservoir and the entire dataset. Seston was assumed to represent the primary food source for zooplankton communities at any given time. Therefore, zooplankton $\delta^{15}N$ should theoretically reflect $\delta^{15}N_{seston}$ plus a known enrichment factor of approximately 3.4‰ (range 2.2 – 4‰; Minagawa and Wada 1984; Vander Zanden and Rasmussen 2001), unless selective feeding on individual taxa with $\delta^{15}N$ signatures differing from seston was occurring. Thus, slopes from regression analyses that were significantly greater than 0 ($P < 0.05$) indicated that zooplankton were incorporating fixed N into their biomass. However, this test did not identify whether fixed N assimilation was through direct feeding on N_2 fixers or on other phytoplankton comprised of recycled fixed N, as this was beyond the scope of the study.

3.3 Results

3.3.1 *Reservoir Water Quality Characteristics*

Means of DOY 95 – 294 data for various parameters and estimates from each reservoir are presented in Table 3.2. Mean chl *a* and PC were generally greater in Elmdale and Fayetteville compared to the other study reservoirs. Mean Z_{eu}, chl *a*, PC, and N demand were generally least in Brittany and greatest in Fayetteville (Table 3.2), which represented the lower and upper limit of the trophic gradient, respectively. Drawdown of NO_3^--N to below detection (< 16.5 µg L^{-1}) was observed in all reservoirs (Fig. 3.1). However, the length of time needed for NO_3^--N drawdown ranged from 39 – 152 days after the study began on DOY 95 (in Wedington and Brittany, respectively). Moreover, mean PN:PP (approximately biomass N:P) was generally less than TN:TP and similar across all reservoirs, which suggests that nutrient limitation may have also been similar across reservoirs. Seston $\delta^{15}N$ ranged from -0.30 – 7.79‰ across all reservoirs throughout the study period. (Fig. 3.1). Mean $\delta^{15}N_{seston}$ for the entire study was 2.76 ±

1.90‰. Additionally, the maximum $\delta^{15}N_{seston}$ observed in each reservoir was used as the $\delta^{15}N_{ws}$ for Equation 3.2. Reservoir-specific $\delta^{15}N_{ws}$ ranged from 2.18 – 7.79‰ (Table 3.2). The mixing model results also indicated that 30 – 43% and 29 – 43% of the N demand was supplied from the previous sample date (i.e. retained and recycled in the epilimnion between sample dates) and the watershed, respectively, on average during the warm season across all reservoirs (Table 3.2).

Evidence indicated that autumn mixing was occurring or had just occurred on the last sampling date (DOY 294) in Elmdale and Fayetteville. The only observations of measurable NH_4^+-N (> 37.5 µg L^{-1}) were made in Elmdale and Fayetteville on DOY 294. Additionally, NO_3^--N was also greater than 100 µg L^{-1} in Elmdale and Fayetteville on the last sampling date, after being undetectable since June in both reservoirs (Fig. 3.1). Moreover, temperature profiles collected on this sampling date indicated that thermal stratification was negligible. Therefore, f_{fix} and consequently N_{fix} were not calculated for the last sampling date in Elmdale and Fayetteville, since new sources of N with unknown $\delta^{15}N$ were available for uptake in the euphotic zone. However, due to conditions unsuitable for N_2 fixation after autumn mixing, N_{fix} was assumed to be zero for this sampling date in Fayetteville and Elmdale as discussed in Section 3.2.5.

3.3.2 *Reservoir N_2 Fixation Rates and Contribution to N Demand*

The median fractional contribution of N_2 fixation to autotrophic N demand on a given sampling date (f_{fix}) ranged from 0 – 0.72, and the median f_{fix} value calculated for each sampling date using IsoSource was 0.31 ± 0.19 on average across all reservoirs. Reservoir N_2 fixation rates (calculated using median f_{fix} values) estimated on each sampling date ranged from 0 – 92 mg N m^{-2} d^{-1} across all reservoirs (Fig. 3.2). Mean N_{fix} was generally greater in Fayetteville (33 mg N m^{-2} d^{-1}) and least in Brittany (11 mg N m^{-2} d^{-1}). However, N_{fix} variability was substantial

across all reservoirs (standard deviation ranged from 11 – 30 mg N m^{-2} d^{-1}). N_{fix} ranged from 0 – 124 mg N m^{-2} d^{-1} across all reservoirs when f_{fix} maxima were used and from 0 – 86 mg N m^{-2} d^{-1} when f_{fix} minima were used. Interestingly, an increase in N_{fix} was observed in Wedington following whole-reservoir chemical fertilization with a relatively unbalanced N:P supply of approximately 3 (Fig. 3.2). Increased N_{fix} was not readily apparent in Rayburn following either of two events of chemical fertilization with approximately 30 N:P supply, which was considered to be relatively balanced according to the range (20 – 50 N:P) suggested by Guildford and Hecky (2000). Results of multiple linear regression analyses for the log-transformed N_{fix} (calculated using only the median f_{fix} values) are presented in Table 3.3. Log-transformed surface temperature was the strongest predictor of log-transformed N_{fix} (partial $r^2 = 0.22$) followed by TN, and NO_3^--N in the model developed using data from all reservoirs ($r^2_{adj} = 0.37$, $P < 0.0001$).

Annual whole-reservoir N_2 fixation rates were substantial in all reservoirs, especially relative to estimated autotrophic N demand (Table 3.4). These N_2 fixation rates were considered yearly rates since N_2 fixation was assumed to be zero before the study began and after it ended (see section 3.2.5). Yearly N_2 fixation rates calculated using median f_{fix} derived N_{fix} values ranged from 2.2 – 6.6 g N m^{-2} yr^{-1}. However, these rates ranged from 0.8 – 10.6 when the minimum and maximum f_{fix} values (of the range calculated using IsoSource) were considered. The contribution of N_2 fixation to autotrophic N demand during the study period ranged from 18 – 34% when median N_{fix} values were considered and from 5 – 52% when N_{fix} values derived from minimum and maximum f_{fix} were used. The yearly N_2 fixation rate was apparently greatest in Fayetteville and least in Brittany. However, the relative contribution of fixed N to N demand was apparently greatest in Norwood and least in Elmdale. Additionally, results of OLS linear regression analyses indicated that despite being identified as predictor variables from multiple

linear regression analysis, surface temperature, TN, and NO_3^--N were not significant predictors of annual N_2 fixation (Fig. 3.3). Annual N_2 fixation was negatively correlated with mean Z_{eu} (Fig. 3.3), which was considered an indicator of trophic status (i.e. lower mean Z_{eu} is associated with higher trophic state). Linear regression analyses were also conducted for N_2 fixation versus TP and TN:TP; however, those regressions were not significant ($P > 0.05$) and are not presented.

3.3.3 *Zooplankton Assimilation of Fixed N*

Zooplankton $\delta^{15}N$ ranged from 1.04 – 9.86‰ across all reservoirs and was generally greater in Elmdale and Fayetteville compared to the other study reservoirs (Table 3.2). Results of OLS regression analyses indicated that fixed N may have been consistently assimilated into zooplankton in Brittany ($r^2 = 0.55$, $P = 0.0025$) and Fayetteville ($r^2 = 0.51$, $P = 0.0063$). Additionally, significant relationships between $\delta^{15}N_{seston}$ and $\delta^{15}N_{zoop}$ were observed in Norwood and Elmdale, but these correlations were relatively weak (Table 3.5). $\delta^{15}N_{zoop}$ was not significantly correlated with $\delta^{15}N_{seston}$ in Rayburn and Wedington (Table 3.5), indicating that zooplankton assimilation of fixed was likely not occurring. However, $\delta^{15}N_{seston}$ was a strong predictor of $\delta^{15}N_{zoop}$ ($r^2 = 0.71$) across all reservoirs (Fig. 3.4).

3.4 Discussion

The results of this study indicate that N_2 fixation was substantial and temporally variable throughout the warm season in six small, temperate reservoirs. N_2 fixation rates estimated using biweekly variations in $\delta^{15}N_{seston}$ were comparable to or greater than rates reported in other lakes and reservoirs. Additionally, biweekly N_2 fixation rates were found to be predicted by the combination of variables such as surface water temperature, TN, and NO_3^--N. However, these

variables were less useful for predicting annual N_2 fixation rates between reservoirs, which were influenced more by reservoir trophic state. Fixed N was also found to not only be an important source of N for autotrophic N demand during the warm season, but incorporated into zooplankton biomass as well.

Seston $\delta^{15}N$ and biweekly N_2 fixation rates

Stable isotopes of seston N have been used in other studies to determine interannual N_2 fixation (Patoine et al. 2006) as well as spatial patterns of the instantaneous proportion of fixed N in seston (Jankowski et al. 2012). Seston and zooplankton $\delta^{15}N$ values observed in this study were comparable to those found in other N stable isotope studies (Patoine et al. 2006; Jankowski et al. 2012). The $\delta^{15}N_{seston}$ maxima (i.e. $\delta^{15}N_{ws}$) were generally greater in the reservoirs with dominant proportions of urban and agricultural development in their watersheds (Table 3.2). However, no wastewater treatment plant is operated in either the Fayetteville or Elmdale watersheds, indicating that the magnitude of maximum $\delta^{15}N_{seston}$ values in these reservoirs was likely more influenced by animal waste runoff from pasture which is the primary form of agriculture in both watersheds. Indeed, the $\delta^{15}N$ of NO_3^--N derived from oxidation of animal waste N is typically higher (10 – 20‰) than other sources such as inorganic fertilizer (-4 – 4‰; Kendall 1998). The lower maximum $\delta^{15}N_{seston}$ values observed in the forested watersheds are more consistent with ^{15}N-deplete forest soil, although still higher than would be expected (Natelhoffer and Fry 1988). However, due to the prominence of karst features in the Ozark Plateau (Petersen et al. 1999) these reservoirs likely have groundwater inputs, which are often characterized by ^{15}N-enriched NO_3^--N (4 – 10‰; Kendall 1998). Groundwater inputs of NO_3^--N with $\delta^{15}N$ values in the above range might explain the relatively high $\delta^{15}N_{seston}$ maxima observed. Moreover, the maximum $\delta^{15}N_{seston}$ in each reservoir was always within the first four sampling

days, a period characterized by detectable reservoir NO_3^--N that was usually greater than 100 µg L^{-1} (Fig. 3.1). The subsequent changes in $\delta^{15}N_{seston}$ throughout the growing season were therefore presumably due to the variations in inputs of atmospheric N from N_2 fixation and watershed N from external inputs as hypothesized.

The N_2 fixation rates estimated from $\delta^{15}N_{seston}$ and modeled primary productivity on individual sampling days were often substantial and similar to those measured in three of the study reservoirs by Scott and Grantz (2013). In that study, pelagic N_2 fixation rates measured in three of the current study's reservoirs (Elmdale, Fayetteville, and Wedington) using the acetylene reduction technique (Flett et al. 1976) were as high as approximately 225 mg N m^{-2} d^{-1}. The biweekly estimates of N_2 fixation in this study ranged up to 92 mg N m^{-2} d^{-1}, which was within the range reported by Scott and Grantz (2013). Additionally, N_2 fixation throughout the warm season was considerably variable (Fig. 3.2), which was also observed by Scott and Grantz (2013). Temporal variation of lacustrine N_2 fixation within a single growing season has been shown in other studies as well (Scott et al. 2008; Beversdorf et al. 2013). The relative variability in N_2 fixation throughout the study period was likely a result of variations in external nutrient loading or internal N regeneration. Additionally, many factors are known to influence N_2 fixation and N_2-fixing cyanobacterial dominance, such as water column turbulence and micronutrient (Fe and Mo) availability (Howarth et al. 1988b; Paerl 1990) which were not quantified in this study.

Rayburn and Wedington were both enriched at the whole-reservoir scale with chemical fertilizer during this study as part of separate efforts to increase fish production. Interestingly, the N:P of the fertilizer used in Rayburn was greater than the N:P of the fertilizer used in Wedington by a factor of 10 (Fig. 3.2). While no specific statistical analyses were conducted to

discern differences in N_2 fixation after fertilization, N_{fix} seemingly increased in Wedington and decreased in Rayburn after fertilization (Fig. 3.2). These apparent responses to whole-reservoir fertilization are likely related to the rates and N:P ratios of the chemical fertilizers used in each reservoir. Wedington was fertilized with high P (0.086 g m^{-2}) at low N:P (approximately 3) and N_{fix} increased an average of 200% in the three weeks following fertilization (Fig. 3.2). However, Rayburn was fertilized twice with lower P (0.023 and 0.034 g m^{-2}) at balanced N:P (approximately 31 for both events) and N_{fix} decreased on average 46% and 56% in the three weeks following both fertilization events. Increased N_2 fixation at low N:P supply, especially at high P, would be expected based on results of various field experiments (Levine and Schindler 1999; Vrede et al. 2009; Baker et al. *in preparation*). Moreover, the fertilization rates described above would have resulted in a TN:TP of approximately 31 (assuming efficient uptake of nutrients) in Wedington and 60 – 82 in Rayburn. N would therefore be expected to limit productivity to a greater extent in Wedington than in Rayburn, considering that N limitation has been shown to occur at TN:TP < 50 (Guildford and Hecky 2000). Thus, these results suggest that low N:P supply at the whole-reservoir scale may indeed induce N deficiency and increase N_2 fixation.

Predictors of N_2 fixation during the warm season

Surface water temperature (depth = 0.5 m) was found to be an important predictor variable in the multiple linear regression model developed for the biweekly N_{fix} dataset. Specifically, increased surface water temperature in tandem with increased TN and decreased NO_3^--N produced a significant model for whole-reservoir areal N_2 fixation (Table 3.3). However these other predictor variables were less important than surface temperature (partial r^2 ranged from 0.06 – 0.09). Many cyanobacteria have been shown to grow optimally in water

temperatures greater than 20 °C (Paerl et al. 2001). Scott et al. (2008) found that water temperature was a strong predictor of volumetric N_2 fixation in a large temperate reservoir, and N_2 fixation has also been shown to be favored by warmer temperatures in other studies (Howarth et al. 1988b; Noges et al. 2008). Furthermore, an analysis of a large lake dataset by Beaulieu et al. (2013) demonstrated that cyanobacterial biomass was greater at increased water temperature. While N_2 fixation rates do not represent total cyanobacterial biomass, increased rates are likely associated with increased N_2-fixing cyanobacteria biomass.

Annual N_2 fixation and importance to autotrophic N demand

Considered on an annual basis, the whole-reservoir N_2 fixation rates estimated in this study are among the highest reported in freshwater lakes and reservoirs. Moreover, these annual rates were comparable to those measured by Scott and Grantz (2013). Annual N_2 fixation was apparently greatest in Fayetteville and least in Brittany when median values of f_{fix} (from IsoSource ranges) were used to calculate rates (Table 3.4). Scott and Grantz (2013) also found that annual N_2 fixation in Fayetteville generally exceeded Elmdale and Wedington. Interestingly, the predictor variables of N_2 fixation identified using multiple linear regression were not as useful for predicting annual N_2 fixation across reservoirs. Annual N_2 fixation was only significantly correlated with Z_{eu}, used here as an indicator of trophic status (Fig. 3.3). Annual N_2 fixation was negatively correlated with mean Z_{eu}, indicating that the magnitude of N_2 fixation is influenced by reservoir trophic state (i.e. greater in more eutrophic systems). N_2 fixation rates have been found to be relatively high in eutrophic lakes (Howarth et al. 1988a; Beversdorf et al. 2013). Additionally, Scott et al. (2009) observed that the magnitude of N_2 fixation increased with reservoir trophic status.

N_2 fixation was a substantial source of N to autotrophic N demand in all of the study reservoirs. Some studies have observed that N_2 fixation contributes a relatively small proportion of N to autotrophic N demand. For example, Howarth et al. (1988a) estimated that N_2 fixation supplied up to 8.9% of the annual autotrophic N demand in a review of several N_2 fixation studies in various lakes varying in size, location, and trophic state. Gettel (2006) estimated that up to 7% of autotrophic N demand was met by N_2 fixation in some oligotrophic arctic lakes. The results of this study suggest that N_2 fixation may have contributed as much as 34% of the autotrophic demand during the warm season, and 19% annually (Table 3.4). Similarly, Patoine and Leavitt (2008) found that N_2 fixation also supplied a substantial amount of N to phytoplankton, with up to nearly 40% of autotrophic N demand attributed to fixed N. Additionally, N_2 fixation supplied up to 29% of the autotrophic N demand in a large natural lake within a single year (Horváth et al. 2013); however, total warm season N demand and N_2 fixation were not estimated. Furthermore, the importance of N_2 fixation relative to photosynthetic needs in reservoirs has not been addressed to the same extent as in natural lakes. Collectively these results suggest that N_2 fixation can be an important source of N in small temperate reservoirs that experience several months of thermal stratification, warm water temperatures, and low concentrations of DIN. However, further understanding of the importance and temporal variation of fixed N recycling within reservoirs may help to explain why N_2 fixation plays an apparently larger role in autotrophic N supply in these systems compared to natural lakes and even oceans (Howarth et al. 1988a).

Zooplankton assimilation of fixed N

The incorporation of fixed N into the upper trophic levels, which are more stable components of the food web, is one possible mode of ecosystem fixed N retention. Zooplankton

$\delta^{15}N$ was shown to significantly decrease with decreasing $\delta^{15}N_{seston}$ (assumed to be the primary food source for zooplankton) in four of the six reservoirs studied (Table 3.5). These results are comparable to what was found by Patoine et al. (2006) in several Canadian lakes and by Sommer et al. (2006) in the Baltic Sea. Interestingly, the strength of this relationship was greatest in the two reservoirs that represented opposing trophic states and N_2 fixation rates (Brittany and Fayetteville), suggesting that phytoplankton or zooplankton community composition may play a more important role in fixed N assimilation rather than nutrient conditions and N_2 fixation itself. These results indicate that assimilation of fixed N into consumers was likely occurring in several of the study reservoirs, regardless of N_2 fixation rates. However, more studies in lakes and reservoirs across trophic gradients are needed to determine how community composition of phytoplankton and zooplankton is affected by N_2 fixation in order to better understand the role of fixed N accumulation in upper levels of the food web.

Study limitations

Some assumptions are required for these N_2 fixation rates to be considered valid. The three-source mixing model used in this study is comprised if $\delta^{15}N_{ws}$ varies throughout the warm season in each reservoir. This model variable was assumed to remain constant throughout the study; however, changes in the relative contribution of groundwater, agricultural, and urban-influenced inputs to watershed N sources during the warm season may have occurred. For example, fertilizer application to fields in a watershed at different times during the study could have increased or decreased the $\delta^{15}N$ of N in runoff or even groundwater that eventually reach the reservoir. Variable $\delta^{15}N_{ws}$ may explain the unusually high N_{fix} values estimated early in the study when NO_3^--N was still available in Fayetteville and Norwood (Fig. 3.2). Additionally, while N supplied from the stored pool of NH_4^+ in the hypolimnion occurred in two of the

reservoirs at the end of the study (see section 3.3.1), Scott and Grantz (2013) found that internal N loading does variably occur in three of the study reservoirs (Elmdale, Fayetteville, and Wedington) during the warm season. The $\delta^{15}N$ of N from internal sources such as the hypolimnion is unknown, and may influence the $\delta^{15}N$ of N available to phytoplankton.

3.5 Conclusion

The results of this study demonstrate that rates of N_2 fixation are high and can make up a substantial contribution to autotrophic N demand in small reservoirs that experience warm water temperatures, thermal stratification, and DIN depletion throughout part of the year. The best predictor variable for N_2 fixation multiple linear regression models was surface water temperature. The results of the multiple linear regression analyses may indicate that N_2 fixation patterns within a single season are similar in small, temperate reservoirs regardless of trophic state. However, the magnitude of annual N_2 fixation rates generally increased with trophic state and was not influenced by the growing season means of the predictor variables identified using multiple linear regression. Additionally, N_2 fixation contributed up to 34% of the autotrophic N demand during the warm season and up to 19% for the entire year. Regardless of temporal scale, the magnitude of N_2 fixation's contribution of N to autotrophic N demand was greater than what has been observed in other freshwater lacustrine studies. While more data are needed to determine how fixed N might accumulate interannually in reservoirs, the results of the current study also suggested that zooplankton were assimilating fixed N in most of the reservoirs. The assimilation of fixed N into primary consumers represents a possible mechanism of reservoir fixed N retention through incorporation into higher trophic levels, and should be further quantified in future studies. Collectively, these results demonstrate that N_2 fixation is a

significantly more important process for N cycling in small, temperate reservoirs than has been observed for other freshwater ecosystems.

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3.7 Tables

Table 3.1. Reservoir and watershed characteristics for six reservoirs in the Northwest Arkansas area, USA.

Reservoir	Reservoir Area (km^2)	Watershed Area (km^2)	WA:RA[a]	Max. Depth (m)	Volume (m^3)	Watershed %Forested	Watershed %Agricultural	Watershed %Urban
Brittany	0.142	3.3	23.0	24	1.5×10^6	74.1	3.0	22.9
Elmdale	0.327	10.3	31.4	9	2.5×10^6	5.2	27.0	61.0
Fayetteville	0.604	24.0	39.7	10	3.0×10^6	9.8	37.0	41.0
Norwood	0.130	2.2	16.6	23	1.2×10^6	85.7	0.3	14.0
Rayburn	0.182	3.1	17.0	24	1.6×10^6	74.8	0.1	25.1
Wedington	0.499	19.3	38.7	10	1.7×10^6	80.0	13.0	1.7

Table 3.2. Whole-reservoir means ± standard deviation (n = 13 in Wedington and 14 in all other reservoirs) from DOY 95 – 294, 2013 for selected parameters and estimates. All f means are from median values of IsoSource-generated f ranges.

Parameter	Brittany	Elmdale	Fayetteville	Norwood	Rayburn	Wedington
Water temperature (°C)	23.2 ± 5.2	24.0 ± 5.0	23.2 ± 5.3	23.0 ± 5.4	23.5 ± 5.4	24.9 ± 5.2
Z_{eu} (m)	6.3 ± 0.9	4.2 ± 1.9	2.6 ± 0.9	4.7 ± 1.3	4.6 ± 1.0	5.3 ± 1.0
Chl *a* (μg L^{-1})	8 ± 4	24 ± 13	48 ± 31	15 ± 10	15 ± 5	12 ± 5
PC (μg L^{-1})	990 ± 188	2440 ± 1147	4198 ± 2007	1476 ± 640	1721 ± 452	1658 ± 646
PN (μg L^{-1})	158 ± 25	387 ± 156	636 ± 290	259 ± 107	278 ± 69	244 ± 74
PP (μg L^{-1})	12 ± 4	31 ± 13	55 ± 22	18 ± 5	21 ± 4	18 ± 4
TDN (μg L^{-1})	482 ± 149	438 ± 126	589 ± 248	421 ± 130	496 ± 193	358 ± 68
TDP (μg L^{-1})	6.8 ± 2.5	8.3 ± 3.7	13.7 ± 6.5	7.6 ± 2.1	7.7 ± 2.8	9.3 ± 8.2
TN (μg L^{-1})	639 ± 142	825 ± 98	1225 ± 243	680 ± 187	774 ± 178	603 ± 89
TP (μg L^{-1})	19 ± 5	39 ± 13	69 ± 24	25 ± 6	28 ± 6	27 ± 9
TDN:TDP	166 ± 47	136 ± 62	121 ± 113	129 ± 48	146 ± 42	116 ± 44
TN:TP	75 ± 12	51 ± 15	42 ± 9	60 ± 13	62 ± 15	52 ± 12
PN:PP	30 ± 7	30 ± 14	26 ± 10	32 ± 7	30 ± 9	30 ± 5
N demand (mg N m^{-2} d^{-1})	56 ± 49	104 ± 59	138 ± 79	82 ± 49	82 ± 37	58 ± 38
$\delta^{15}N_{seston}$ (‰)	2.59 ± 0.74	4.42 ± 1.72	4.74 ± 1.74	2.02 ± 1.39	2.05 ± 0.91	0.62 ± 0.69
$\delta^{15}N_{zoop}$ (‰)	4.71 ± 1.14	7.71 ± 1.05	7.28 ± 1.61	4.57 ± 1.08	4.80 ± 0.87	2.21 ± 0.84
$\delta^{15}N_{ws}$ (‰)	4.24	7.05	7.79	5.82	4.12	2.18
f_{fix}	0.20 ± 0.12	0.19 ± 0.13	0.21 ± 0.13	0.35 ± 0.16	0.21 ± 0.11	0.34 ± 0.20
f_{ws}	0.35 ± 0.21	0.43 ± 0.21	0.41 ± 0.20	0.29 ± 0.20	0.36 ± 0.18	0.34 ± 0.21
f_{prev}	0.38 ± 0.15	0.35 ± 0.14	0.43 ± 0.22	0.36 ± 0.13	0.34 ± 0.12	0.30 ± 0.14

Table 3.3. Results of multiple linear regression analysis for the dependent variable N_{fix}. All data were log-transformed, and a constant of 0.1 was added to N_{fix} prior to log transformation due to the occurrence of 0 values. Temperature was measured at 0.5 m depth. Concentrations of TN and NO_3^--N were measured from whole-reservoir composite, depth integrated euphotic zone samples.

Variable	Estimate	*F*	*P*	df	r^2 (adj)
Intercept	-5.101				
Temperature (°C)	1.710	22.23	< 0.0001		0.22
TN ($\mu g\ L^{-1}$)	1.481	9.97	0.0023		0.09
NO_3^--N ($\mu g\ L^{-1}$)	-0.229	7.64	0.0072		0.06
	Model Summary	15.10	< 0.0001	4, 76	0.37 (0.35)

Table 3.4. Yearly N_2 fixation rate estimates. Median values of the range of possible f_{fix} generated with IsoSource were used to calculate daily rates throughout the study period. The daily estimates were summed to obtain total N_2 fixation rates (assumed to represent yearly rates). See methods for details on how autotrophic N demand was estimated.

Reservoir	N_2 fixation (g N m^{-2})	% Contribution to autotrophic N demand	
		Warm season	Annual
Brittany	2.2 (1.2 - 3.4)	21 (11 - 31)	13 (7 - 20)
Norwood	5.8 (3.0 - 8.5)	34 (18 - 50)	19 (10 - 21)
Rayburn	3.3 (0.8 - 6.7)	20 (5 - 41)	11 (3 - 21)
Wedington			
2008[a]	6.6	—	—
2009[a]	4.4	—	—
2013	4.0 (1.8 - 6.1)	33 (15 - 52)	19 (8 - 29)
Elmdale			
2008[a]	12.0	—	—
2009[a]	7.1	—	—
2013	3.9 (1.5 - 6.4)	18 (7 - 30)	11 (4 - 18)
Fayetteville			
2008[a]	16.0	—	—
2009[a]	3.4	—	—
2013	6.6 (2.8 - 10.6)	24 (10 - 38)	13 (6 - 20)

[a] Estimates made by Scott and Grantz (2013).

Table 3.5. Results of OLS linear regression analyses for $\delta^{15}N_{zoop}$ versus $\delta^{15}N_{seston}$ in individual reservoirs. Sample size (n) = 14 for all reservoirs except Elmdale and Fayetteville (n = 13) due to the uncertainty of $\delta^{15}N_{seston}$ measured on DOY 294 (see section 3.3.1).

Reservoir	m	y_0	F	P	r^2
Brittany	1.14	1.76	14.55	0.0025	0.55
Norwood	0.429	3.71	5.29	0.0403	0.31
Rayburn	0.448	3.88	0.64	0.0881	0.22
Wedington	0.069	2.17	0.03	0.8544	0.00
Elmdale	0.463	5.51	6.20	0.0320	0.38
Fayetteville	0.744	3.56	11.33	0.0063	0.51

3.8 Figure Legends

Figure 3.1. $\delta^{15}N_{seston}$ (filled circles) and NO_3^--N (open circles) throughout the study period in each reservoir.

Figure 3.2. Estimates of N_2 fixation rates (N_{fix}) calculated on each sampling date using the median values of the range of possible f_{fix} generated with IsoSource. Error bars represent minimum and maximum N_{fix} values based on IsoSource f_{fix} estimates.

Figure 3.3. Selected variable means (DOY 95 – 294) versus annual N_2 fixation rates calculated using N_{fix} estimates derived from the median values of f_{fix} ranges generated using IsoSource. Solid lines represent significant OLS linear regressions ($P < 0.05$) and dashed lines represent regressions that were not statistically significant ($P > 0.05$).

Figure 3.4. Results of OLS linear regression analyse conducted between seston $\delta^{15}N$ ($\delta^{15}N_{seston}$) and zooplankton $\delta^{15}N$ ($\delta^{15}N_{zoop}$) measured throughout the study period across all reservoirs.

3.9 Figures

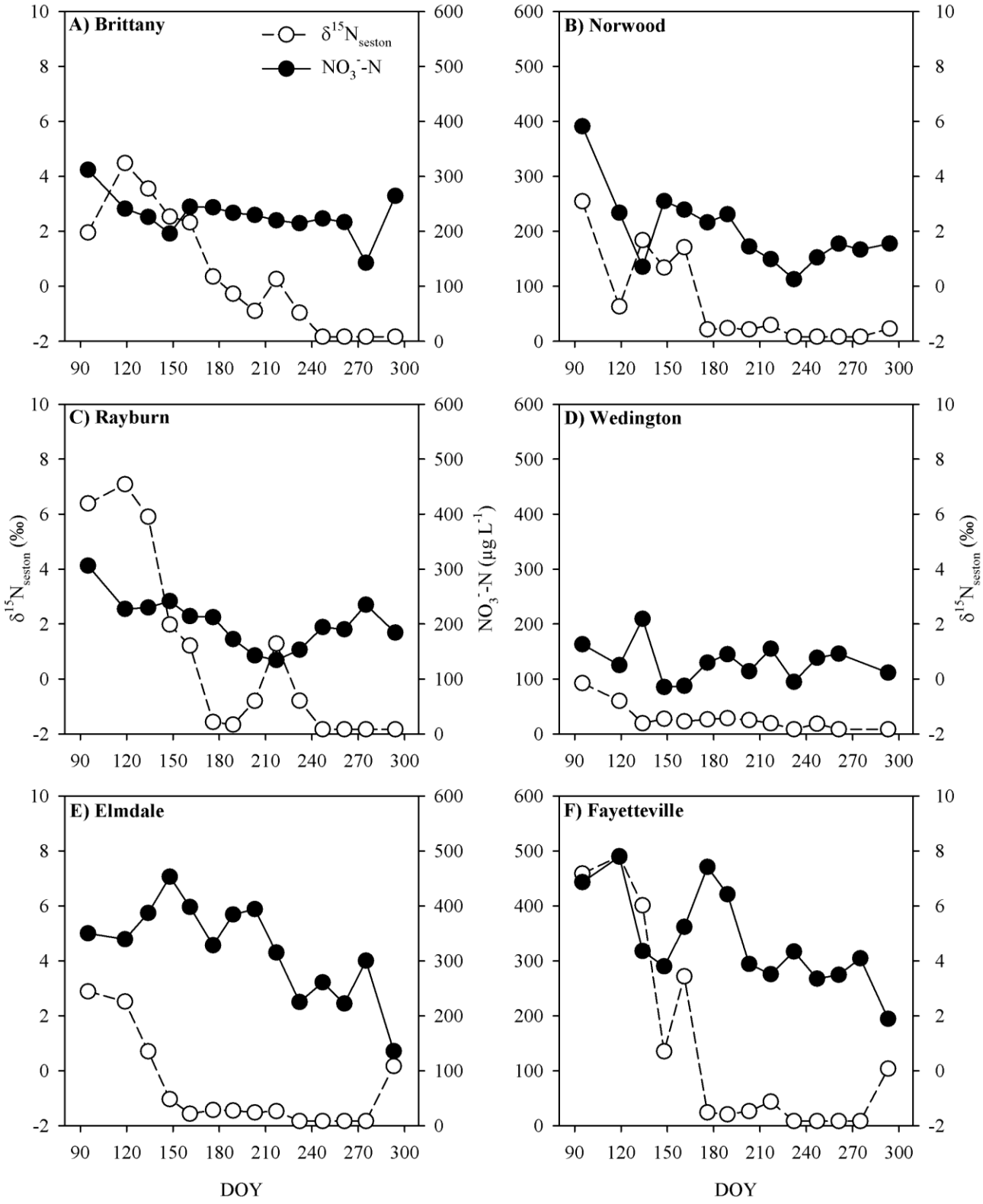

Figure 3.1

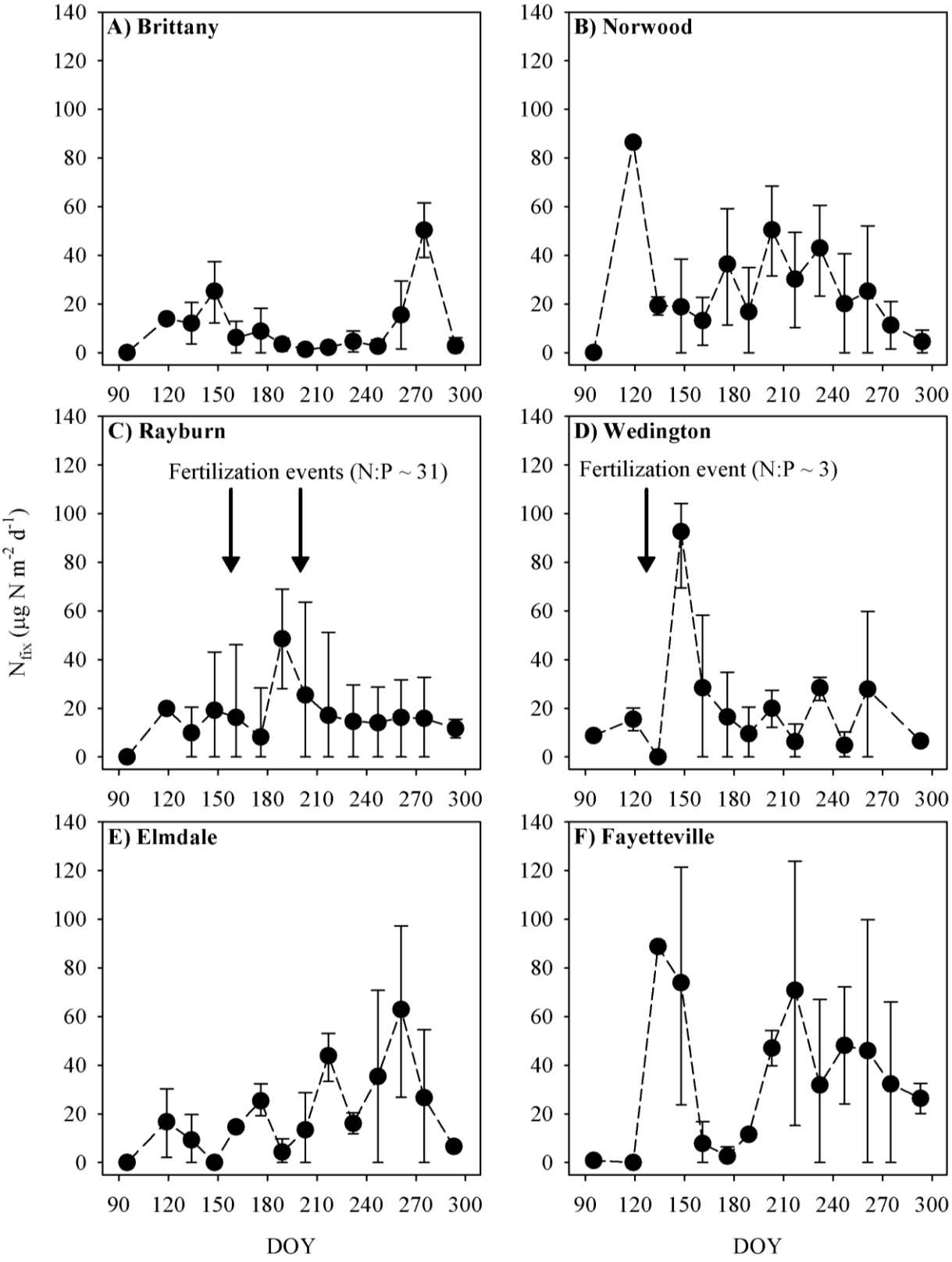
A) Brittany
B) Norwood
C) Rayburn
Fertilization events (N:P ~ 31)
D) Wedington
Fertilization event (N:P ~ 3)
E) Elmdale
F) Fayetteville
N_{fix} (µg N m^{-2} d^{-1})
DOY
DOY

Figure 3.2

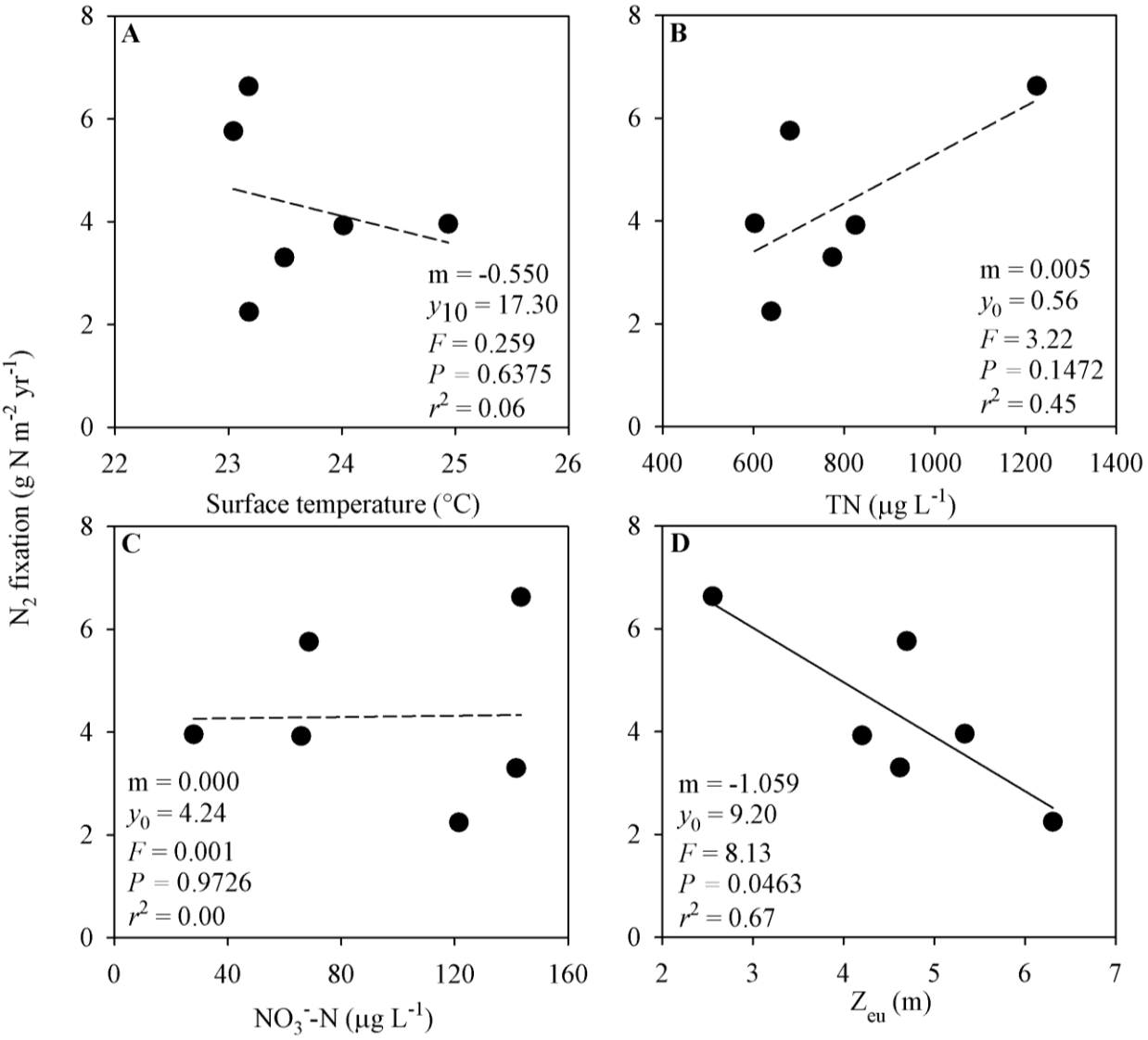
A
m = -0.550
y_{10} = 17.30
F = 0.259
P = 0.6375
r^2 = 0.06
Surface temperature (°C)
B
m = 0.005
y_0 = 0.56
F = 3.22
P = 0.1472
r^2 = 0.45
TN (µg L^{-1})
C
m = 0.000
y_0 = 4.24
F = 0.001
P = 0.9726
r^2 = 0.00
NO_3^--N (µg L^{-1})
D
m = -1.059
y_0 = 9.20
F = 8.13
P = 0.0463
r^2 = 0.67
Z_{eu} (m)
N_2 fixation (g N m^{-2} yr^{-1})

Figure 3.3

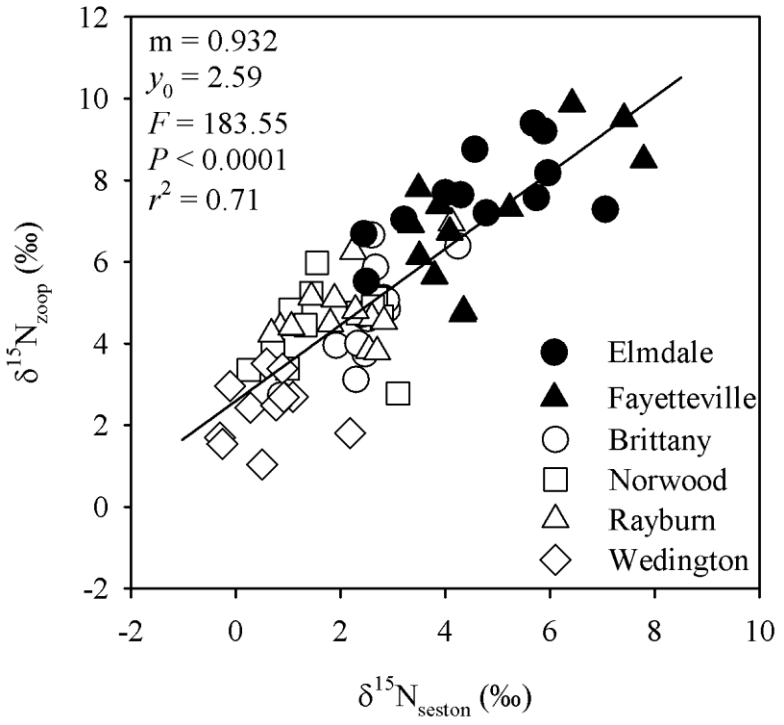
m = 0.932
y_0 = 2.59
F = 183.55
P < 0.0001
r^2 = 0.71
Elmdale
Fayetteville
Brittany
Norwood
Rayburn
Wedington
$\delta^{15}N_{zoop}$ (‰)
$\delta^{15}N_{seston}$ (‰)
12
10
8
6
4
2
0
-2
-2
0
2
4
6
8
10

Figure 3.4

4. CONCLUSION

The primary objectives of this study were to determine N_2 fixation's short-term efficiency, effects on water quality, and ecosystem-scale importance in reservoirs using $\delta^{15}N$ tracer and natural abundance techniques. Reservoirs have been increasingly recognized for their significant role in C burial (Dean and Gorham 1998) and landscape N retention and/or removal (Harrison et al. 2009; Grantz et al. 2012), and represent important ecosystems that have been generally understudied compared to natural lakes (Kennedy et al. 2003). Few studies have quantified N_2 fixation at any scale in small (< 1.0 km^2) reservoirs (Scott and Grantz 2013), which may represent a substantial proportion of impoundments globally (Downing et al. 2006). Additionally, few freshwater lacustrine N_2 fixation studies have utilized the $\delta^{15}N$ method (Patoine et al. 2006; Jankowski et al. 2012), and those studies were spatially and temporally limited.

The results of this study provided further evidence that natural community N_2 fixation may increase with low N:P supply at high P (Levine and Schindler 1999; Vrede et al. 2009). N_2 fixation was greater at low TN:TP exposure ($TN:TP_{exp}$), or the sum of initial TN and fertilizer N relative to the sum of initial TP and fertilizer P, in both the mesocosm experiment and at the whole-reservoir scale in this study. The N_2 fixation rates measured following N and P enrichment in this study (ranging from 3.2 – 56.0 µg N L^{-1} d^{-1}) were comparable to those measured by Vrede et al. (2009) in P-enriched mesocosms in a eutrophic lake, as shown in Fig. 4.1. Additionally, these volumetric N_2 fixation rates were similar to rates measured by Scott et al. (2009) in Waco Reservoir (up to approximately 23.4 µg N L^{-1} d^{-1}, assuming a 13 h photoperiod) and by Forbes et al. (2008) in several Texas reservoirs (approximately 22.2 µg N L^{-1} d^{-1} on average, assuming a 13 h photoperiod). Despite substantial rates in low N:P supply

treatments, N_2 fixation was found to be inefficient in regards to N and biomass accumulation on a short timescale (37 days) under considerably ideal conditions, which has not been explicitly field-tested. The inefficiency of N_2 fixation observed in this study may be related to the energetic costs of carrying out N_2 reduction in well-oxygenated water. N_2 fixation has been demonstrated in laboratory experiments to be inefficient compared to NH_4^+ or NO_3^- assimilation due to the energetic costs of differentiating heterocytes and constructing nitrogenase (Turpin et al. 1985; Herrero et al. 2004). Furthermore, N_2 fixation was less efficient in reservoirs with lower initial nutrient concentrations at the onset of unbalanced, N-manipulated N:P supply. Microcystin production was also observed only at high N:P supply. Thus, reductions in both N and P supply may result in decreased biomass and cyanotoxin production.

Whole-reservoir, seston δ^{15}N-derived N_2 fixation was substantial and temporally variable in all six study reservoirs, with rates comparable to those found in another study in three of the study reservoirs using the acetylene reduction technique (Scott and Grantz 2013). Results of multiple linear regression analysis indicated that surface water temperature, TN, and NO_3^--N were significant predictors of biweekly N_2 fixation throughout the warm season. Other studies have also demonstrated greater N_2 fixation when water temperature is increased and NO_3^--N availability is low (Howarth et al. 1988a; Scott et al. 2008). However, the magnitude of annual N_2 fixation was not influenced by these predictor variables, but was related to reservoir trophic state (indicated by mean Z_{eu} throughout the warm season). Thus, patterns and predictors of N_2 fixation within a single warm season may be similar across small, temperate reservoirs, but the amount of N supplied by N_2 fixation may be increased in more eutrophic systems.

The contribution of fixed N to autotrophic N demand was comparable to what has been observed in some lakes and reservoirs (Patoine and Leavitt 2008; Horváth et al. 2013), although

greater than in other various lacustrine systems (Howarth et al. 1988b). Further refinement of fixed N recycling dynamics in these reservoirs may help to identify the extent of N_2 fixation's role in phytoplankton N supply. Additionally, the results of this study suggest that despite the apparent low palatability of zooplankton to freshwater zooplankton (Ghadouani et al. 2003), fixed N was being assimilated into primary consumers either directly or indirectly in some of the reservoirs. The assimilation of fixed N into the food web may represent a mechanism for reservoir retention of fixed N and future studies should be aimed at further quantifying this.

Collectively, these results suggest that N_2 fixation plays an important role in N cycling in small, temperate reservoirs. N_2 fixation patterns within a single season are likely similar across small reservoirs, but reservoir trophic state may significantly affect the magnitude of fixed N inputs. However, N_2 fixation may not be entirely efficient at alleviating short-term N limitation, as inefficiency was demonstrated in this study to occur even under ideal conditions. Future studies should be directed at examining multiyear N_2 fixation rates, contribution to autotrophic N demand, and assimilation into higher trophic levels to further assess the role and limitations of N_2 fixation in small reservoirs.

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4.1 Figure Legends

Figure 4.1. N_2 fixation rates (N_{fix}) versus TN:TP_{exp}. The TN:TP_{exp} is defined as the sum of initial TN and fertilizer N relative to the sum of initial TP and fertilizer P. Regression analysis was conducted in SAS 9.1 (SAS Institute Inc., Cary, NC, USA) for combined data from the mesocosm experiment (filled circles) and whole-reservoir fertilization events (open circles) from this study, as well as the mean rate reported by Vrede et al. (2009) in P-enriched mesocosms (open triangle). An exponential decay (two parameter) model provided the best fit for the combined data.

4.2 Figures

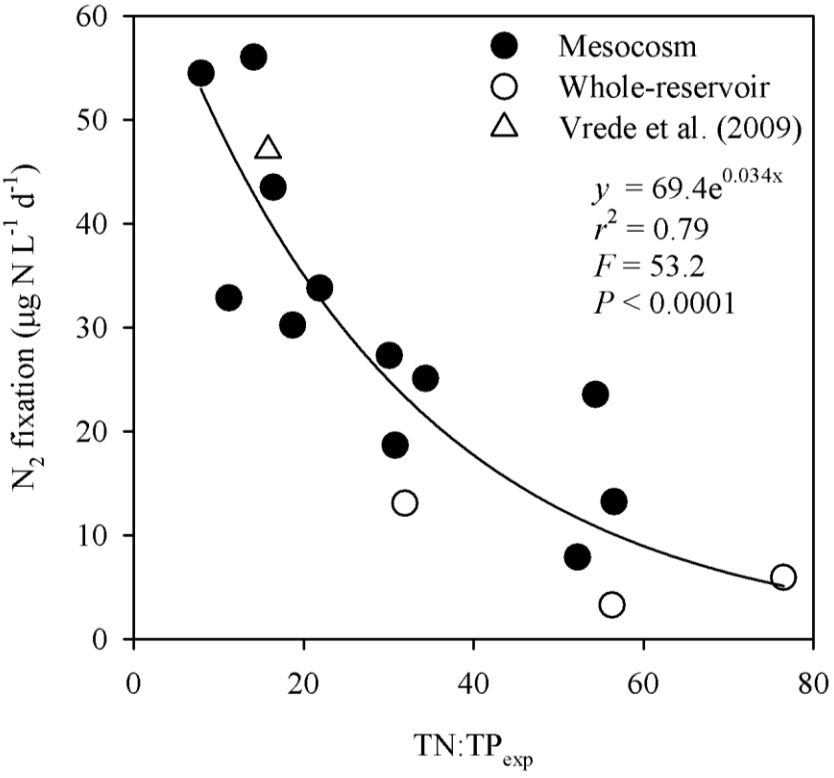

Figure 4.1