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ORIGINAL PAPER

The role of N_2 fixation in alleviating N limitation in wetland metaphyton: enzymatic, isotopic, and elemental evidence

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Abstract The role of nitrogen (N_2) fixation in balancing N supply to wetland metaphyton was assessed by comparing primary production with enzymatic, isotopic, and elemental correlates. Primary production, N_2 fixation (acetylene reduction, AR), phosphatase activity, C:N:P ratio, and N isotopic composition of metaphyton were measured along a nutrient gradient in a freshwater marsh during May through September 2004. N_2 fixation and phosphatase activity in metaphyton were negatively correlated with inorganic N and P concentrations, respectively. Although metaphyton N_2 fixation demonstrated a clear spatial pattern along the nutrient gradient, N_2 fixation rates varied monthly and decreased sharply in September. However, the percent contribution of N_2 fixation to N uptake by metaphyton consistently decreased throughout the summer. Furthermore, the decreased contribution of N_2 fixation to N uptake corresponded with an increase in metaphyton N content during the growing season. Nitrogen isotopic data suggested the sustained importance of an atmospheric N_2 source through September at the most downstream (nutrient poor) site even though the percent contribution of N_2 fixition to N uptake was lowest in that month. This suggests that metaphyton were efficiently accumulating and recycling fixed N_2 in support of primary production. Over the course of the summer, metaphyton primary production showed a weak inverse correlation with metaphyton phosphatase activity (r^2 = 0.58). The largest residuals in this regression corresponded to the largest vartiation in metaphyton N content. When metaphyton primary production was normalized to metaphyton N content, production rates for the entire growing season were more strongly inversely correlated with metaphyton phosphatase activity (r^2 = 0.78). Results of the study suggest that N_2 fixation in N poor areas may adequately supplement community N requirements in metaphyton, thereby causing limitation by other elemental resources such as P.

Keywords Proximate limiting nutrient · Nitrogenase · Phosphatase · CNP stoichiometry · $\delta^{15}N$

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Introduction

Primary production in freshwater aquatic ecosystems will proceed until limited by resource availability. In general, photoautotrophs become limited by either the availability of light energy or mineral resources (*e.g.* nitrogen or phosphorus). When mineral resources are sufficient, photoautotrophs will grow until their own biomass decreases the amount of available light energy or until nutrients have been exhausted. Schindler (1977) suggested that N resources of lakes could only be exhausted temporarily and therefore P would limit primary production in these systems over extended time scales. This hypothesis was based on the ability of planktonic heterocystous cyanobacteria to fix atmospheric N (N_2) at a rate sufficient to supplement the N supply to an ecosystem. Cyanobacterial N_2 fixation rates, and conditions controlling the establishment of cyanobacteria and initiation of N_2 fixation, have since been widely described for a variety of aquatic habitats (Horne et al. 1979; Reuter et al. 1986; Doyle and Fisher 1994) with the greatest attention given to planktonic systems (see reviews by Howarth et al. 1988a, b; Vitousek 2002).

In wetland and shallow lake environments (lentic ecosystems), periphyton communities can dominate microbial primary production, particularly in nutrient poor systems (Vadeboncoeur et al. 2002). In particular, floating microbial mats or ''metaphyton'' (Stevenson 1996), which form by fragmentation of epipelic, epilithic, or epiphytic communities, can be highly productive and radically alter ecosystem nutrient cycling (Wetzel 1996). These communities often efficiently retain and recycle sequestered nutrient stocks (Borchardt 1996). However, nutrient limitation to primary production by metaphyton, and periphyton in general, has been widely demonstrated in enrichment experiments utilizing both whole-system (McDougal et al. 1997; Havens et al. 1999; McCormick et al. 2001; Rejmánková and Komárková 2005) and diffusion substrate approaches (Fairchild et al. 1985; Scott et al. 2005). Many of these studies demonstrated periods of N and/or P limitation, and/or periods of N + P co-limitation. Co-limitation describes a scenario whereby only combined enrichments of N and P (and possibly other micronutrients as well) resulted in growth stimulation. When N or both N and P are in low supply and cyanobacteria are present, N_2 fixation should commence when the energetic costs of N limitation (*i.e.* unrealized primary production) exceed the energetic cost of N_2 fixation (for example see terrestrial model by Rastetter et al. 2001). When N stocks have been replenished, N_2 fixation will cease as its energetic cost begins to exceed the potential production achievable solely with DIN (Vitousek and Howarth 1991, Tyrrell 1999). Although these models have been well developed for planktonic (Schindler 1977) and terrestrial communities (Rastetter 2001), N_2 fixation dynamics have not been extensively studied in periphyton communities.

Some recent studies have suggested the importance of N_2 fixation to metaphyton in subtropical and tropical marshes (Rejmánková and Komárková 2000; Rejmánková et al. 2004; Inglett et al. 2004). These studies used traditional methods for assessing N_2 fixation, such as acetylene reduction (Flett et al. 1976), as well as more novel approaches such as the natural N isotopic composition of N_2 fixing communities (Gu and Alexander 1993; France et al. 1998). In a recent study, Scott et al. (2005) found that periphyton were increasingly N-limited and exhibited increasing N_2 fixation potential along a nutrient gradient in a created wetland. However, this and other studies have not attempted to quantify the role of N_2 fixation and N accumulation in alleviating N limitation to primary production by the periphyton community.

In this paper we explore the importance of N_2 fixation as a source of N to the metaphyton community in the created wetland studied by Scott et al. (2005). We used metaphyton enzymatic activities (nitrogenase and phosphatase), isotopic composition ($\delta^{15}N$), and elemental content (C:N:P ratios) to determine if N_2 fixation and N accumulation might alleviate N limitation of metaphyton primary production along the nutrient gradient during a growing season. Specifically, we sought to answer the following questions: (1) Does N_2 fixation contribute substantially to N uptake in N-limited metaphyton? (2) Does N accumulate in N-limited metaphyton, thereby decreasing the need for N_2 fixation? (3) Is primary production by N_2 fixing metaphyton correlated with proxies of P limitation over the entire growing season? Overall, the objective of the study was to form a more detailed understanding of the community processes that initiate and ultimately

inhibit N_2 fixation in what are perceived to be N-limited benthic microbial communities.

Materials and methods

Site description

The study was conducted at the Lake Waco Wetland (LWW) complex, near Waco, Texas, USA (Fig. 1). The complex is an 80-ha off-channel constructed marsh that receives water pumped from the North Bosque River. Water meanders through five wetland cells before flowing back into the North Bosque River. The average depth of the wetland is approximately 0.5 m and the hydraulic residence time ranges between 10 to 30 days. During the course of this study, the mean pumped inflow rate was 6.0 million gallons per day (range = 0–11 MGD) and the average hydraulic residence was 17.5 days. A floating metaphyton community generally occurs in the relatively deep ($\geq$ 0.5m), open-water areas of this wetland.

Metaphyton samples were collected from areas 1A, 1B, and 2B of the LWW (Fig. 1) in May, July, and September 2004. These sampling areas were positioned along the nutrient gradient which corresponds to the flow path of water (Fig. 1; see Scott et al. 2005). Approximately 400 cm^2 of metaphyton were harvested, placed into a plastic container with water collected at the site, and transported to the laboratory. Eight replicate samples were collected from each area on all dates. In the laboratory, metaphyton samples were subsampled for measurements of primary production, N_2 fixation via acetylene reduction, phosphatase activity, C, N, and P content, and $\delta^{15}N$ composition. In addition to metaphyton samples, four liters of water were collected from all sites on each sampling date to use as incubation water in laboratory bioassays for primary production and N_2 fixation. Water samples were also collected in each sampling area on a biweekly basis during the course of the study for water chemistry analysis.

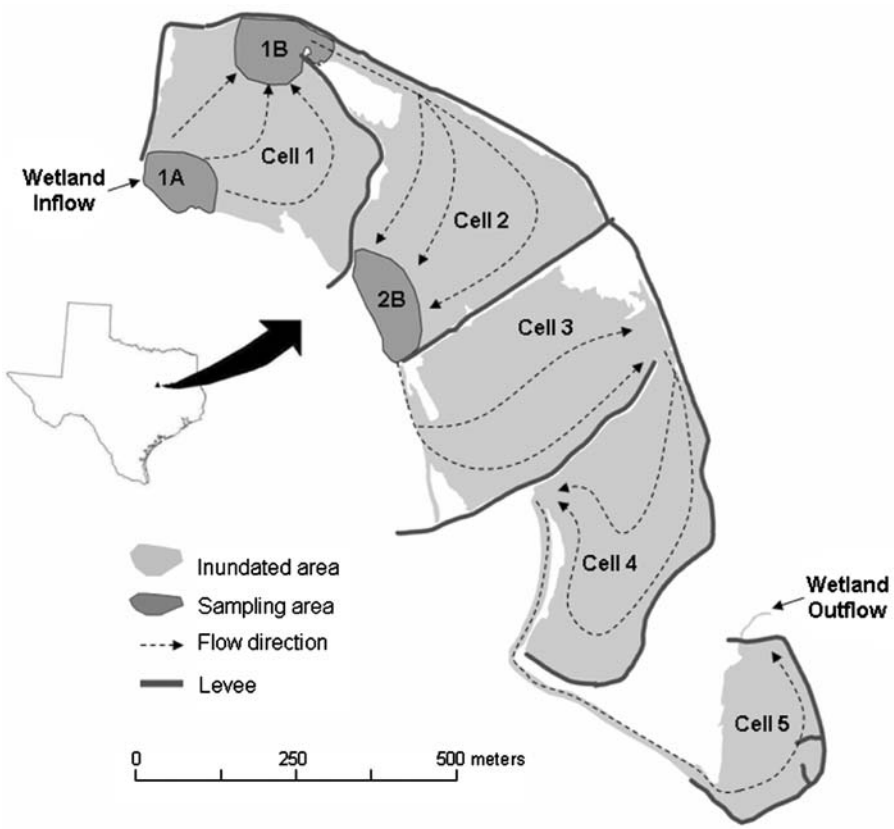

Fig. 1 Lake Waco wetland, near Waco, Texas, USA. Metaphyton samples were collected in May, July, and September 2004 within areas 1A, 1B, and 2B. Water chemistry was measured biweekly from May–September 2004 in each sampling area

Primary production

Metaphyton primary production was determined by measuring the rate of O_2 production in high-light, low-light, and dark incubations (Wetzel and Likens 2000). Three small subsamples ($\leq$ 1 cm^2) were cut away from each metaphyton sample. Two portions were transferred into transparent BOD bottles with 300 ml water from the corresponding site and the third portion was transferred into an opaque BOD bottle with 300 ml water from the site. The concentration of dissolved O_2 was determined to a precision of 0.1 ppm before incubation with a YSI 5000 dissolved O_2 meter. Prior to addition to BOD bottles, dissolved O_2 concentration of incubation water was reduced to $\sim$ 3.0 ppm by bubbling with N_2 gas amended with 350 ppm CO_2. All BOD bottles were placed in a water bath incubation set to in-situ temperature conditions. One transparent bottle was incubated under high-light intensity ($\sim$ 390–460 µmol m^{-2} s^{-1}) and the other under low light intensity ($\sim$ 45–55 µmol m^{-2} s^{-1}). Samples were incubated until dissolved O_2 concentrations increased by $\sim$ 1 to 1.5 ppm in low light incubations ($\sim$ 1–4 h). Final dissolved O_2 concentration, incubation time, and photon flux density were recorded at the end of incubation. The photon flux density encountered by each incubation bottle was determined by measuring irradiance at the location of each bottle in the water bath with a LI-COR LI-250 light meter equipped with a spherical sensor. In addition to metaphyton samples, samples containing only water from each sampling location were incubated in duplicate at each light level to account for photosynthesis and respiration by plankton.

Following incubation, the samples were filtered onto a pre-washed, -dried, and -weighed glass fiber filter then oven-dried at 60°C overnight. The dry weight of sample was calculated as the final weight of the sample and filter minus the original filter weight. Gross photosynthesis for samples at each light level was calculated as:

$$GP = \frac{[(O_{2e} - O_{2r}) \times 0.375 \times 0.3]}{(PQ) \times (t) \times (DW)}$$

where GP is the rate of gross photosynthesis (mg C g DW^{-1} h^{-1}), O_{2e} is the change in dissolved O_2 concentration (mg O_2 l^{-1}) in the transparent bottle over time t, O_{2r} is the change in dissolved O_2 concentration (mg O_2 l^{-1}) in the opaque bottle over time t, PQ is the photosynthetic quotient (dimensionless constant = 1.2; see Wetzel and Likens 2000), and DW is the dry weight of the sample in grams. The constant 0.375 represents the ratio of carbon fixed to oxygen generated during photosynthesis, and the constant 0.3 was the incubation volume in liters. Low-light photosynthetic rates were standardized to the average low-light condition by dividing the photosynthetic rate by the measured incubation light intensity and multiplying by the average low-light incubation condition (40 µmol m^{-2} s^{-1}). Standardized low-light gross photosynthesis was multiplied by 8 h, light-saturated (determined from high-light incubations) gross photosynthesis was multiplied by 4 h, and these rates were summed to derive an estimate of daily metaphyton primary production expressed as mg C g DW^{-1} day^{-1}.

N_2 fixation

Acetylene reduction was used to estimate the rate of N_2 fixation in metaphyton samples. In this assay, acetylene is reduced to ethylene by the nitrogenase enzyme at a rate proportional to the reduction of N_2 to NH_4^+ (Flett et al. 1976). In the laboratory, three small subsamples ($\leq$ 1 cm^2) were cut away from each metaphyton sample and placed into Popper Micromate syringes with 30 ml site water. One syringe was wrapped in foil for dark incubation while the other two were used for high-light and low-light incubations. Five milliliters of acetylene gas were injected into each syringe which was then gently mixed to allow rapid dissolution of acetylene. Syringes were incubated as described above for primary production. At the end of incubation, 15 ml of air were drawn into each syringe which was then vigorously agitated to establish equilibrium conditions of gases between aqueous and vapor phases. Water and vapor volumes were recorded to account for partitioning between phases and ethylene concentration of the vapor was determined using a Carle AGC Series gas chromatograph (GC). The GC was equipped with a flame-ionization detector and a 1.8 m column packed with 80% Porapack N and 20% Porapack Q (80/100 mesh). The column temperature was 70°C, helium was used as the carrier gas, and 10 ppm ethylene standards were used to calibrate the instrument daily. For each

light level (dark, low-light, high-light), the hourly ethylene production rate was converted to an hourly N_2 fixation rate assuming that the production of 3 μmol ethylene was equivalent to the fixation of 1 μmol N_2 (Flett et al. 1976). Low-light N_2 fixation rates were standardized to average low-light conditions and used with light-saturated N_2 fixation rates to derive estimates of daily metaphyton N_2 fixation (μg N g DW^{-1} day^{-1}) following the same method described above for primary production. In addition to the absolute rates of N_2 fixation, we estimated the percent contribution of N_2 fixation to N uptake by dividing the metaphyton N_2 fixation rate the by rate of gross N uptake by metaphyton (μg N g DW^{-1} day^{-1}). Gross nitrogen uptake was estimated by dividing the rate of metaphyton primary production by the metaphyton C:N ratio.

Phosphatase activity

Phosphatase activity in metaphyton was measured fluorometrically using methylumbelliferone phosphate (MUFP) as a substrate. In the presence of phosphatase enzymes the phosphate group on MUFP is hydrolyzed yielding methylumbelliferone (MUF). MUF fluoresces when irradiated at 365 nm wavelength. In samples saturated with MUFP, the rate of increasing fluorescence is proportional to the rate of MUF production, and subsequently, phosphatase activity (Healy and Hendzel 1979; Petterson 1980). Periphyton subsamples were transferred into 15 ml culture tubes with 9 ml 1.2 % TRIS buffer (pH 8.3). One milliliter of 10^{-4} mol l^{-1} MUFP was added to each tube and samples were mixed gently. Samples were incubated at room temperature under ambient indoor lighting. Fluorescence was measured after 5, 15, and 45 min on a Turner 10 AU fluorometer calibrated with 50, 100, 250, 500, and 1000 ppb MUF standards. Dry weight of all samples was determined as previously described and phosphatase activity expressed as nmol P_{ase} g DW^{-1} min^{-1}.

Metaphyton isotopic and elemental composition

A subsample of each metaphyton sample was oven-dried overnight at 60°C and ground to a fine powder for determination of C, N, and P content and N isotopic composition. C and N content were determined simultaneously using a Thermo Finnigan FlashEA 1112 elemental analyzer. Phosphorus content was determined colorimetrically on a Lachat Quickchem 8500 following a 3 h digestion in concentrated H_2SO_4 at 350°C (Clesceri et al. 1998). Nitrogen isotopic composition was measured using a continuous flow isotope ratio mass spectrometer connected to a Carlo Erba NA1500 elemental analyzer. Measured $^{15}N/^{14}N$ ratios are expressed in delta notation (δ):

$$\delta^{15}N_{sample} = \left[\left(\frac{R_{sample}}{R_{air}}\right) - 1\right] \times 1000$$

where $\delta^{15}N_{sample}$ is the isotopic composition of the sample expressed in units of per mil (‰), R_{sample} is $^{15}N/^{14}N$ ratio measured in the sample, and R_{air} is the $^{15}N/^{14}N$ ratio of air.

Water chemistry

Biweekly water chemistry samples were collected in acid-washed 1 l polyethylene bottles and returned to the laboratory for analysis of nitrite-nitrogen plus nitrate-nitrogen (NO_2–N + NO_3–N), ammonia–nitrogen (NH_4–N), and soluble reactive phosphorus (SRP). NO_2–N + NO_3–N was determined colorimetrically on a Beckman DU 650 spectrophotometer following cadmium reduction (Clesceri et al. 1998). NH_4–N and SRP were also determined colorimetrically using the phenate and molybdenum blue methods, respectively (Clesceri et al. 1998). Water temperature, specific conductance, and pH were measured during sample collection with a YSI 6600 multiparameter datasonde.

Results

Water chemistry

Results of water chemistry sampling are provided in Table 1. Nitrite-N + NO_3–N followed a general pattern of decreasing concentration between sites positioned along the flow path of water. A similar pattern was apparent for NH_4–N only in September. In May and July, SRP concentrations were generally similar amongst all sites. In September, average SRP concentration was highest at site 1B, followed by sites

Table 1 Mean water chemistry values at all sites for the period in which metaphyton sampling was conducted (mean ± SD; $n = 2$ for all events except SRP in July where $n = 1$)

Site	Water Temp(°C)	Spec Cond (µS cm^{-1})	pH	NO_2–N + NO_3–N (µmol l^{-1})	NH_4–N (µmol l^{-1})	DIN (µmol l^{-1})	SRP (µmol l^{-1})	DIN:SRP (molar)
May								
1A	25 ± 2	736 ± 59	7.8 ± 0.0	14 ± 0.6	1.5 ± 0.1	15 ± 0.6	0.14 ± 0.12	110 ± 98
1B	25 ± 3	715 ± 51	7.9 ± 0.2	3.1 ± 2.1	1.4 ± 0.5	4.5 ± 2.2	0.19 ± 0.12	23 ± 18
2B	26 ± 3	642 ± 65	7.8 ± 0.2	0.4 ± 0.1	1.0 ± 0.8	1.4 ± 0.8	0.15 ± 0.11	10 ± 10
July								
1A	30 ± 1	708 ± 344	8.0 ± 0.3	50 ± 57	3.0 ± 1.7	53 ± 57	0.35	150 ± 160
1B	27 ± 0	665 ± 344	8.0 ± 0.3	7.3 ± 6.6	2.9 ± 2.3	10 ± 7.0	0.17	60 ± 41
2B	29 ± 0	592 ± 399	8.0 ± 0.7	0.8 ± 0.4	1.4 ± 0.6	2.2 ± 0.7	0.18	12 ± 3.8
September								
1A	26 ± 0	490 ± 12	8.1 ± 0.3	15 ± 6.9	5.5 ± 1.2	21 ± 7.0	0.08 ± 0.00	250 ± 84
1B	23 ± 1	501 ± 83	7.8 ± 0.6	7.1 ± 8.0	1.1 ± 0.9	8.2 ± 8.0	0.25 ± 0.13	32 ± 36
2B	24 ± 1	507 ± 83	7.8 ± 1.1	0.4 ± 0.2	0.6 ± 0.5	1.1 ± 0.6	0.05 ± 0.01	24 ± 13

1A then 2B. However, all differences observed in SRP concentrations between sites were minor when compared to differences observed in dissolved inorganic nitrogen (DIN = NO_2–N + NO_3–N + NH_4–N). The ratio of DIN:SRP consistently decreased along the flow path of water during all months.

Metaphyton primary production and enzyme activity

Metaphyton primary production did not follow a consistent pattern among sites and dates (Table 2). In May, average primary production was highest at site 1A, lower at site 1B, and lowest at site 2B. However, this trend disappeared in July when highest rates were observed at site 1B, followed by 1A then 2B. Site 1B remained the most productive site in September, but site 2B displayed higher primary production than site 1A during this month.

Metaphyton N_2 fixation was not detected at site 1A on any sampling event, but was always measurable at sites 1B and 2B (Table 2). N_2 fixation was always light-dependent (data not shown). Furthermore, the N_2 fixation rate was negatively correlated with average DIN concentration in the water column (Fig. 2A) which resulted in a consistent spatial pattern of increasing metaphyton N_2 fixation along the flow path of water (*i.e.* between sites; Table 2 and Fig. 3). N_2 fixation comprised 0–33% of total N uptake depending on site and month (Table 2). Metaphyton N uptake at site 2B had the greatest contribution from N_2 fixation in each month, followed by site 1B. However, the percent contribution of N_2 fixation to total N uptake decreased throughout the summer at these sites.

Phosphatase activity was always greatest at site 2B followed by site 1A then site 1B, except in September when phosphatase activity at site 1A exceeded that observed at site 2B (Table 2). In general, phosphatase activity was negatively correlated with SRP concentration in the water column (Fig. 2B).

Metaphyton elemental and isotopic composition

The elemental composition of metaphyton exhibited both spatial and temporal heterogeneity. Nitrogen content increased at sites 1B and 2B between May and September (Fig. 3). Although the differences in N content observed at these sites between May and July, and July and September were not statistically significant, trends suggest N content was increasing at these sites throughout the summer. Mean N content at site 1A however, increased slightly from May to July before falling again in September, but these trends were not statistically significant. The carbon to nitrogen ratio (C:N) of metaphyton was highest at sites 1A and 1B in May but relatively consistent among all other site-date combinations (Table 2). Insufficient sample was collected for the determination of P content in the May samples. The ratio of carbon to phosphorus (C:P) and nitrogen to phosphorus (N:P) in July and September tended to exhibit

Table 2 Primary production, enzyme activities, and C:N:P ratios of metaphyton at each site on each sampling date

Month site	Primary production (mg C g DW^{-1} day^{-1})	N_2 Fixation (μg N g DW^{-1} day^{-1})	% N uptake from N_2 fixation	Phosphatase activity (nmol P_{ase} g W^{-1} min^{-1})	Metaphyton C:N (molar)	Metaphyton C:P (molar)	Metaphyton N:P (molar)
May							
1A	39.0 ± 12.0 (7)	BDL[a]	0.0 ± 0.0	60.5 ± 16.6	20.8 ± 2.66	No data	No data
1B	22.0 ± 11.7	42.6 ± 33.6	7.6 ± 10	41.6 ± 21.2 (7)	24.3 ± 3.65	No data	No data
2B	10.6 ± 3.2	222 ± 38.1	33 ± 5.8	104.4 ± 24.3 (4)	17.5 ± 1.87	No data	No data
July							
1A	65.0 ± 23.8	BDL[a]	0.0 ± 0.0	23.9 ± 12.6 (7)	14.9 ± 1.36	263 ± 98.5	17.9 ± 7.06
1B	82.3 ± 36.2 (7)	67.8 ± 106 (7)	1.6 ± 2.8	14.2 ± 5.1 (7)	16.7 ± 3.73	264 ± 72.0	15.8 ± 1.40
2B	41.4 ± 16.3	385 ± 285	12 ± 9.1	76.4 ± 33.8	15.8 ± 1.03	383 ± 100	24.3 ± 6.39
September							
1A	27.1 ± 10.5 (7)	BDL[a]	0.0 ± 0.0	95.7 ± 50.8	15.6 ± 1.0	549 ± 122	35.3 ± 7.34
1B	107 ± 46.5	6.10 ± 6.30	0.1 ± 0.1	31.9 ± 15.1	14.5 ± 1.8	347 ± 54.3	24.0 ± 1.91
2B	42.2 ± 6.7	60.7 ± 32.6	2.5 ± 1.9	77.2 ± 25.0	18.9 ± 2.9	510 ± 55.7	27.4 ± 3.34

All values are mean ± SD. For all values, $n = 8$ except where indicated parenthetically

[a] BDL = below detection limit (1ppm ethylene)

more heterogeneity than that observed in C:N. In July, C:P and N:P were similar at sites 1A and 1B but higher at site 2B (Table 2). This difference was due to diminished metaphyton P content at site 2B in July. In September, C:P and N:P were greatest at sites 1A and 2B and lower at site 1B (Table 2). Again, these differences were primarily the result of the relatively large difference in metaphyton P content observed between sites. In addition to these spatial trends, metaphyton C:P at site 1A increased from July to September and metaphyton N:P at sites 1A and 1B increased from July to September. The N:P ratio in metaphyton was not strongly related to the ratio of DIN:SRP in July but appeared to be more positively correlated in September (Tables 1 and 2).

Nitrogen isotopic composition followed a distinct spatial pattern throughout the summer where $\delta^{15}N$ was greatest at site 1A, lower at site 1B, and lowest at site 2B (Fig. 3). September was the only month where $\delta^{15}N$ at site 1B was not statistically less than $\delta^{15}N$ at site 1A. $\delta^{15}N$ at site 2B in September did remain lower than sites 1A and 1B.

Relationship between metaphyton N_2 fixation, $\delta^{15}N$, and N content

Metaphyton $\delta^{15}N$ decreased with increasing N_2 fixation during each sampling event (Fig. 3). However, correlation between these variables did not appear robust among months. Interestingly, statistically significant decreases in $\delta^{15}N$ between sites were always associated with statistically significant increases in N_2 fixation between sites with the exception of site 1B in July. In that month, metaphyton $\delta^{15}N$ at site 1B was statistically lower than $\delta^{15}N$ at site 1A, but N_2 fixation could not be statistically separated from zero at site 1B due to a relatively large degree of variability.

Although these clear spatial patterns existed in metaphyton N_2 fixation and $\delta^{15}N$, similar spatial patterns were not apparent in metaphyton N content or C:N ratios (Fig. 3 and Table 2). As previously mentioned however, a trend of increasing metaphyton N content through time was apparent, particularly at sites 1B and 2B (Fig. 3). Although this trend did not appear related to the absolute rates of N_2 fixation at these sites through time (Fig. 3), the decreasing contribution of N_2 fixation to N uptake through the summer was related to an overall temporal increase in metaphyton N content (Fig. 4).

Correlates of metaphyton primary production

Metaphyton primary production was seldomly correlated with water column nutrient chemistry or metaphyton stoichiometry through space or time (Tables 1 and 2). Two exceptions were the pattern of primary production between sites in May and

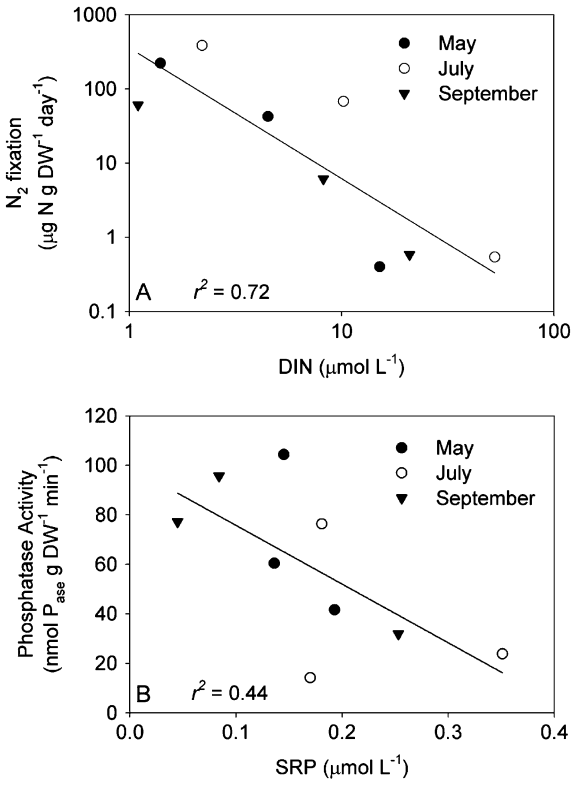

Fig. 2 Enzymatic activity versus nutrient concentrations for each sampling event. (**A**) Daily N_2 fixation, measured by acetylene reduction, versus mean DIN concentration in the water column. (**B**) Phosphatase activity versus mean SRP concentration in the water column

September, which appeared weakly correlated with water column DIN (Tables 1 and 2) and with metaphyton C:P (Table 2), respectively. Metaphyton phosphatase activity showed a negative correlation with metaphyton primary production over the entire summer when averaged by site (Fig. 5A). Interestingly, the largest residuals in this regression corresponed to the largest variations in metaphyton N content. Normalizing metaphyton primary production to metaphyton N content substantially strengthened this inverse correlation (Fig. 5B).

Discussion

Did N_2 fixation contribute substantially to N uptake in N-limited metaphyton?

In a previous study, Scott et al. (2005) found that N enrichment increased periphyton production and that periphyton N_2 fixation potential was high in the downstream portions of the Lake Waco Wetlands (LWW). Results of our study support those general conclusions. Metaphyton N_2 fixation in the LWW increased in response to decreasing DIN concentrations (Fig. 2A). This corresponded to increased metaphyton N_2 fixation along the flow path of water (*i.e.* between sites) throughout the summer of 2004 (Fig. 3). More importantly, the relative importance of N_2 fixation to N uptake by metaphyton also always increased between sites positioned along the flow path of water (Table 2). In the areas identified by Scott et al. (2005) as the most strongly N limited (*i.e.* sites 1B and 2B), N_2 fixation comprised 0.1–33% of metaphyton N uptake in the summer of 2004. This range is lower than that reported for eplithon in Lake Tahoe (72% N uptake from N_2 fixation; Reuter et al. 1986) but is similar to the range reported for epiphyton in an Amazon River floodplain lake (11% N uptake from N_2 fixation; Doyle and Fisher 1994).

Did N accumulate in N-limited metaphyton, thereby decreasing the need for N_2 fixation?

Nitrogen content of metaphyton increased throughout the summer of 2004, particularly at sites 1B and 2B (Fig. 3). Metaphyton N content was lowest in May and relatively equal amongst sites (Fig. 3). Nevertheless, DIN concentrations showed a marked decrease along the flow path of water (*i.e.* between sites; Table 1) resulting in the increased percent contribution of N_2 fixation to metaphyton N uptake between sites positioned along the flow path of water (Table 2). The spatial pattern of increased N_2 fixation at downstream sites continued throughout the summer with highest N_2 fixation rates observed in July (Fig. 3). However, metaphyton primary production was also highest in July (Table 2) and the relative importance of N_2 fixation to N uptake actually decreased with time as metaphyton N content increased (Fig. 4). These results are in general agreement with trends observed in phytoplankton (Schindler 1977; Tyrrell 1999) and are supported by similar evidence in other metaphyton communities. In a Florida Everglades metaphyton community, Inglett et al. (2004) found that N accumulated with increasing N_2 fixation throughout the summers of

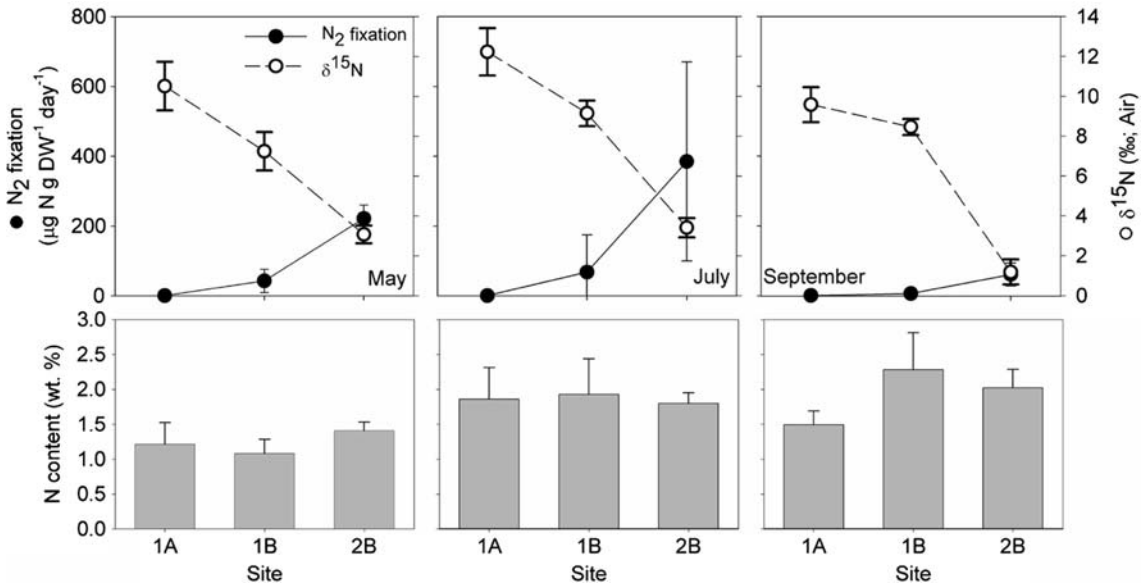

Fig. 3 Nitrogen fixation, nitrogen isotopic signature, and nitrogen content of metaphyton for each sampling event. Upper panels show the spatial trends of N_2 fixation and $\delta^{15}N$ along the flow path of water (sites 1A to 2B) for each sampling event. Lower panels show spatial trends of metaphyton N content for each respective sampling event. Error bars represent standard deviation (SD)

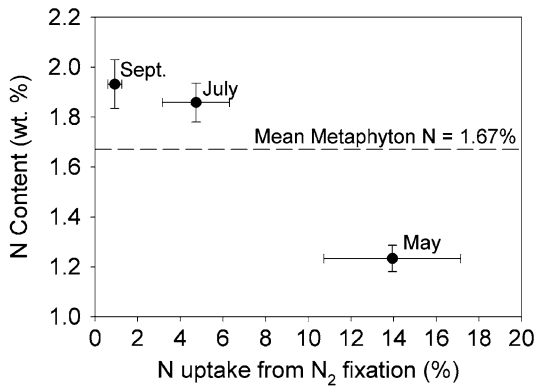

Fig. 4 Relationship between mean percent of N uptake from N_2 fixation in each month and mean metaphyton N content in each month. Horizontal dotted line represents mean metaphyton N content over the entire summer. Error bars represent standard error of the mean (SE)

1998 and 1999 and that metaphyton N content was always greatest in September when N_2 fixation rates began to decline. Although these results suggest that N_2 fixation may have been sufficient to balance metaphyton N content within one summer season, this study did not include measurements of metaphyton primary production over any time scale.

Interestingly, the relationship between the diminishing importance of N_2 fixation to N uptake and increasing metaphyton N content in our study was usually, though not always, supported by the N isotopic compostion of metaphyton. Because the N isotopic composition of autotrophs will usually reflect the isotopic compostion of their inorganic N source (Lajtha and Marshall 1994), it is often possible to estimate the relative contribution of DIN and atmospheric N_2 to communities that can utilize either source by measuring the isotopic composition of the community itself (Gu and Alexander 1993; France et al. 1998). In all months, metaphyton $\delta^{15}N$ at site 1A ranged from 9 to 12 ‰ (Fig. 3), which corresponds to the approximate value for NO_3-N in inflowing waters of this system (Dworkin 2003). In May and July, metaphyton $\delta^{15}N$ was systematically lower at sites 1B and 2B (Fig. 3), which suggests a consistent increase in the importance of fixed N_2 ($\delta^{15}N$ of N_2 = 0 ‰) at these locations (see similar analyses by Inglett et al. 2004; Rejmánková et al. 2004). By September, the percent contribution of N_2 fixation to N uptake at site 1B was low (Table 2) and metaphyton $\delta^{15}N$ reflected DIN as the primary source (~10 ‰; Fig. 3). However, the percent contribution

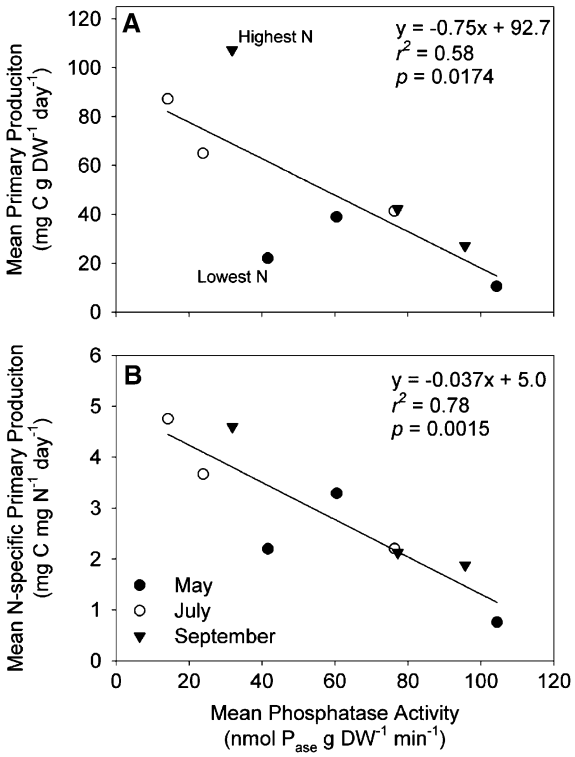

Fig. 5 Relationship between mean phosphatase activity and metaphyton primary production. (**A**) Mean phosphatase activity versus mean primary production; samples with lowest and highest N content had the largest residual values in the regression. (**B**) mean phosphatase activity versus mean nitrogen-specific primary production

of N_2 fixation to N uptake at site 2B was also low in September (Table 2) but metaphyton $\delta^{15}N$ reflected N_2 as the primary source ($\sim$1 ‰; Fig. 3). This suggests that metaphyton at site 2B were efficiently recycling N that was originally derived from an atmospheric source. Although not explicitly measured in this study, periphyton communities are known to retain and recycle nutrients with great efficiency (Borchardt 1996). It is also interesting that metaphyton $\delta^{15}N$ at site 2B was highest in May and decreased throughout the summer but that $\delta^{15}N$ at site 1B was lowest in May and increased throughout the summer eventhough N_2 fixation was always measurable at both sites (Table 2). This suggests that the 5 to 25-fold difference in the contribution of N_2 fixation to N uptake between sites 1B and 2B (Table 2) was enough to drive metaphyton at site 2B toward the atmospheric N_2 isotopic signature (0 ‰) but favor the DIN isotopic signature (10 ‰) in metaphyton at site 1B (see September panel in Fig. 3).

Was metaphyton primary production correlated with proxies of P limitation over the entire growing season?

Mean metaphyton primary production among all sites through the summer was correlated with mean metaphyton phosphatase activities (Fig. 5A). The largest residuals in this regression were associated with the hjghest and lowest metaphyton N content observed in the study. Normalizing metaphyton primary production to metaphyton N content resulted in a strengthened relationship between metaphyton primary production and phosphatase activity (Fig. 5B). When considered with the information from N_2 fixation above, these results suggest that N_2 fixation may have been sufficient to balance the N needs to metaphyton and result in P control of metaphyton production when considered over the entire summer. These findings are similar to the models proposed by Schindler (1977) and Vitousek and Howarth (1991), and more recently supported in a model of marine phytoplankton production (Tyrrell 1999).

Results of this study may also provide insight into the importance of N_2-fixing metaphyton communities in tropical marshes, which are generally considered to be strongly P-limited. Rejmánková and Komárková (2000) found that P enrichment increased metaphyton primary production but that N enrichment did not change metaphyton primary production in three marshes of northern Belize. In two of the three marshes where P enrichment increased primary production, a simultaneous increase in N_2 fixation rates and decrease alkaline phosphatase activity (APA) were observed. This suggests that P availability may have limited primary production but that P enrichment may have resulted in an N deficiency. Additionally, metaphyton N content was generally higher in the third marsh where N_2 fixation and APA did not change when enriched with P, than it was in the two marshes where N_2 fixation and APA responded to P enrichment. This further suggests that N rich metaphyton did not waste energy on N_2 fixation to bring N supply into balance, which is in general agreement with trends observed in our study.

Conclusions

Results of this study suggest that the seasonal accumulation of N, derived from both the atmosphere and/or water column, can alleviate periods of N limitation in metaphyton. As metaphyton N content increases in response to atmospheric and DIN inputs through the growing season, the relative contribution of N_2 fixation to N uptake declines. Nitrogen isotopic data collected in this study was particularly useful for identifying the importance of recycled N originally obtained by metaphyton via N_2 fixation. Overall, this study confirms the importance of fixed N_2 as a N source to metaphyton primary production and provides an example of proximate N limitation versus ultimate P limitation in a benthic microbial community.

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