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Nitrogen fixation and phosphatase activity in periphyton growing on nutrient diffusing substrata: evidence for differential nutrient limitation in stream periphyton

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Abstract. We explored N_2 fixation and alkaline phosphatase activity (APA) in periphyton from a N-limited stream ecosystem by coupling measurements of these processes with nutrient diffusion substrata (NDS) experiments. We measured periphyton biomass accumulation (as ash-free dry mass [AFDM] and chlorophyll *a* [CHLA]), N_2 fixation, and APA to evaluate the relative importance of N_2 fixation as an N source to the periphyton community and APA as an indicator of P deficiency in a seemingly N-limited system. We used fritted-glass-disc NDS and estimated AFDM, CHLA, N_2 fixation, and APA on days 6, 18, and 29 after deployment. Periphyton AFDM steadily increased on NDS over time, but was not influenced by nutrients. CHLA was elevated in the N treatment on days 18 and 29, indicating autotrophic N limitation. Consistent with N limitation, N_2 fixation was high but not different in the control and P treatments and was virtually undetectable on the N treatment. N_2 fixation in control and P treatments was detectable in both light and dark incubations, and dark rates were 4 to 73% of the light rates on days 18 and 29. The average contribution of total N_2 fixation to periphyton in control and P treatments was 0.93 mg N/m^2 on day 18 and 1.0 mg N/m^2 on day 29. APA was significantly elevated on the control and was highest in the N treatment despite no apparent P limitation of periphyton biomass accumulation. P enrichment always decreased APA. Measurable N_2 fixation and the change in CHLA suggest that autotrophs were primarily N limited. However, APA observed in controls demonstrated that some portion of the periphyton community was experiencing P deficiency. This result suggests that periphyton metabolism was related to both N and P availability, but that biomass accumulation might have been limited primarily by N. One explanation for these findings is that different organisms, perhaps occupying different trophic positions within the community, might have been limited by different elements.

Key words: algae, epilithon, NDS, nitrogenase activity, nitrogen fixation, phosphatase activity.

A common paradigm in aquatic ecology has been that N usually limits primary production in coastal marine environments, whereas P usually limits primary production in freshwater environments. However, this paradigm is not withstanding challenges, particularly those directed from meta-analyses on large data sets (Francoeur 2001, Elser et al. 2007). As a result, the efficacy by which freshwater systems were thought to overcome N limitation and come into equilibrium with P limitation has come into question. Schindler (1977) suggested that P generally would limit primary production in freshwater lakes over long time scales because N_2 fixation could supply sufficient N to counterbalance ecosystem N deficiency. This paradigm has been generally accepted by stream ecologists, but few studies have explored the role of instream N_2 fixation as a source of N to streams (Marcarelli et al. 2008), and the

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importance of N_2 fixation in alleviating N limitation to benthic microbial communities has been assessed only rarely (Scott et al. 2007). What is certain is that N limits primary production in streams over relatively short time scales as frequently as P does (Francoeur 2001) and that populations within the periphyton community might experience differential nutrient limitation (i.e., some limited by N, some limited by P).

Nitrogenase catalyzes the fixation of gaseous N_2 into biologically available NH_4^+ (Voet and Voet 2004). In aquatic environments, N_2 fixation is carried out by certain cyanobacteria, heterotrophic bacteria, and some diatoms with cyanobacterial endosymbionts (Howarth et al. 1988). Periphyton N_2 fixation and its relationship with dissolved inorganic N (DIN) availability has been studied extensively, but most of these studies have focused on lentic habitats (Doyle and Fisher 1994, Rejmánková and Komárková 2000, Inglett et al. 2004, Scott et al. 2007). Even modest levels of DIN will signal organisms to suspend nitrogenase synthesis and subsequently stop N_2 fixation because N_2 fixation is energetically expensive. However, N_2 fixation can provide a significant amount of N to the entire periphyton community when DIN concentrations are low. For instance, Grimm and Petrone (1997) demonstrated that fixed N_2 provided up to 85% of the net N flux into the benthos of a desert stream.

Marcarelli and Wurtsbaugh (2006) recently showed that P and temperature interacted to control N_2 fixation in periphyton from oligotrophic streams. P enrichment increased periphyton N_2 fixation rates by increasing the density of N_2-fixing organisms within the periphyton community. Higher temperature also increased N_2 fixation by increasing the density of N_2-fixing organisms and increasing the rate of cell-specific N_2 fixation. P enrichment also increased periphyton biomass (as chlorophyll *a* [CHLA]), but N enrichment had no effect on periphyton biomass. Therefore, the study by Marcarelli and Wurtsbaugh (2006) provided a good example of how N_2 fixation can supplement the N supply to periphyton communities that are strongly P limited. What remains unclear is how much N can be provided by N_2 fixation in streams experiencing stronger N limitation. Eutrophic streams might experience stronger and more frequent N limitation than oligotrophic streams because anthropogenic nutrient sources usually have a lower N:P ratio than do natural nutrient sources (Downing and McCauley 1992). However, N_2 fixation in eutrophic streams is an area of research that has been neglected (Marcarelli et al. 2008).

Enzyme activities are an important source of information on the physiological state of microbial communities. In particular, enzymes associated with nutrient cycling are informative indicators of nutrient status (Hill et al. 2006). For instance, phosphatases catalyze the hydrolysis of dissolved organic phosphate esters and are generated by a number of aquatic microbes, including bacteria, algae, and protozoans (Chróst 1991). Studies on natural systems and in laboratory cultures have demonstrated that microbial phosphatase activity increases when P limits growth (Healey and Hendzel 1980, Chróst and Overbeck 1987). Alkaline phosphatase activity (APA) has been widely studied in planktonic systems (Rose and Axler 1998), but has received less attention in benthic environments. Patterns of increasing periphyton APA are related to decreasing inorganic P availability and decreasing periphyton P content in lotic (Klotz 1992, Mulholland and Rosemond 1992) and lentic (Kahlert et al. 2002, Scott et al. 2007) benthic habitats. However, most periphyton APA measurements, particularly for streams, were done in studies with the goal of relating microbial enzyme activities to microbial biomass distributions (Sinsabaugh et al. 1991, Romaní and Sabater 2000, 2001). Scott et al. (2007) used APA as an indicator of P deficiency in N_2-fixing microbial communities in a freshwater wetland, but similar studies have not been done in streams.

Nutrient-diffusing substrata (NDS) are useful tools for identifying nutrient limitation in periphyton communities over short time scales (15–60 d). NDS have been used in lakes (Fairchild and Lowe 1984, Pringle and Bowers 1984, Fairchild et al. 1985), wetlands (Scott et al. 2005), streams (Lowe et al. 1986, Winterbourn and Fegley 1989, Tate 1990, Marcarelli and Wurtsbaugh 2006, Tank et al. 2006), and large rivers (Corkum 1996, Scrimgeour and Chambers 1997). Periphyton accumulates on diffusion surfaces when NDS are deployed in natural waters, and differences in accumulation rates between nutrient treatments and controls are interpreted as evidence of periphyton nutrient limitation. Periphyton accumulation is most often measured as ash-free dry mass (AFDM) or CHLA. AFDM represents the combined organic matter of algae and heterotrophs (e.g., bacteria, fungi) in the periphyton, whereas CHLA represents the biomass of algae only. Many studies also have used taxonomic assessments to explore changes in algal community composition after nutrient enrichment with NDS. However, few studies have coupled NDS experiments with more-complex measurements of periphyton community function such as N_2 fixation and APA.

We measured periphyton N_2 fixation, APA, and biomass accumulation (as AFDM and CHLA) to understand the importance of N_2 fixation in alleviating N limitation to stream periphyton. We worked in a stream-like outflow of a constructed freshwater marsh. N_2 fixation and APA must be measured in enclosed containers. Therefore, we used the micro-NDS design

developed by Gibeau and Miller (1989) and modified by Marcarelli and Wurtsbaugh (2006), wherein fritted glass discs (FGDs) are used as diffusion surfaces at the end of plastic tubes containing enrichment media. FGDs provide a solid surface that can be removed from NDS and placed inside closed syringes for N_2 fixation and APA assays. We estimated nutrient diffusion rates from NDS by measuring the rate of nutrient depletion from enriched agar because our NDS design differed slightly from those previously described in the literature.

Based on ambient nutrient levels and previous periphyton work in our study area (Scott et al. 2005), we expected that surface-water N would be sufficiently low and P would be sufficiently high to induce N limitation in periphyton. Thus, we hypothesized that biomass accumulation rates would be enhanced by N enrichment but not by P enrichment. We also hypothesized that N_2 fixation would be elevated on unenriched control NDS and highest on P-enriched NDS, where the N:P ratio of nutrient supply was lowest. We also hypothesized that N_2 fixation would be deactivated by N enrichment, whereas APA would be elevated on the N treatment only if P became limiting in response to N enrichment. Last, we hypothesized that N_2 fixation and APA would be more sensitive than AFDM and CHLA to changes in nutrient availability. Our goals were to evaluate N_2 fixation as a source of N to the periphyton community and to assess the importance of P deficiency in a seemingly N-limited system.

Methods

Study site

We deployed NDS in the stream-like outflow of the Lake Waco Wetland (LWW), near Waco, Texas, USA, from 23 May 2006 to 21 June 2006. Previous research on metaphyton in the study area indicated that N limits periphyton production primarily in the downstream areas of this created wetland (Scott et al. 2005, 2007). The outflowing stream connecting LWW with Lake Waco is ~100 m long, has an average width of 3 m, and has primarily sandy substrate with *Typha latifolia* growth along its margins. Thalweg depth at the deployment location was 0.4 m. Velocity and discharge during the study were ~0.15 m/s and ~0.1 m^3/s, respectively. The deployment location was an open site with low marginal *Typha* growth and lacked canopy cover.

NDS and experimental design

We constructed NDS based on the designs of Gibeau and Miller (1989) and Marcarelli and Wurtsbaugh (2006). Both of these designs used a FGD as a growth substrate that enabled nutrients to diffuse from an agar medium through the porous surface of the FGD. Our design differed from previous designs in that larger vessels (50-mL centrifuge tubes) were used to store agar in an effort to minimize the ratio of FGD surface area to agar volume. We cut 22-mm-diameter holes in the caps of 50-mL centrifuge tubes (Carolina Biological Supply, Burlington, North Carolina), and inserted FGDs (25-mm diameter, 4–5.5-μm porosity; Wilmad LabGlass, Buena, New Jersey) inside the cap, exposing most of the FGD surface through the top of the cap. We inserted a 3-mm-wide rubber gasket inside the cap to seal the bottom of the FGD to the top rim of the centrifuge tube.

We prepared NDS with 20 g/L agar and either no nutrient enrichment (control), N enrichment (0.51 M N from $NaNO_3$), or P enrichment (0.065 M P from $Na_3PO_4{\cdot}12H_2O$). We randomly placed NDS in a rack (60 × 90 cm) made from wire netting framed with plastic-coated plant stakes. We used a 2nd set of stakes to anchor the rack in the substrate at its deployment location in the wetland outflow. We deployed 51 NDS of each treatment (control, N, and P) to determine rates of biomass accumulation (AFDM and CHLA), N_2 fixation, APA, and N and P diffusion from NDS for a 29-d study period. We retrieved 3 randomly chosen NDS from each treatment on days 1, 2, 3, 12, and 24 to determine agar N and P concentrations and periphyton AFDM. We retrieved 12 NDS from each treatment on days 6, 18, and 29 to determine agar N and P concentrations and periphyton AFDM, CHLA, N_2 fixation rates, and APA.

Water chemistry and temperature

We collected 50-mL surface-water grab samples upstream and immediately downstream of the NDS rack on days 3, 6, 12, 18, and 24 after deployment. We estimated total N (TN), total P (TP), $NO_2 + NO_3$-N (NO_x-N), and PO_4-P levels using colorimetric methods (APHA 1992) on a Lachat® Quik-Chem 8500 flow-injection autoanalyzer (Hach, Loveland, Colorado). We analyzed TN and TP following alkaline and acid persulfate digestions, respectively. We monitored water temperature throughout the study period with a HOBO® Water Temp Pro temperature logger (Onset Computer Corporation, Bourne, Massachusetts) attached to the NDS rack.

N and P diffusion rates

We estimated nutrient diffusion rates by monitoring how rapidly nutrients disappeared from NDS agar. We sampled 3 randomly chosen NDS from each treatment

(control, N, and P) on days 1, 2, 3, 6, 12, 18, 24, and 29 after deployment. We extracted agar from the centrifuge tubes and melted it in a beaker with ~900 mL deionized water. We cooled the solution to room temperature, diluted it to 1 L, and collected 7-mL duplicate subsamples that we held frozen for <28 d until analysis for dissolved NO_x-N and PO_4-P concentrations.

Estimation of N_2 fixation, APA, AFDM, and CHLA

We kept periphyton intact on FGDs from NDS for measurement of N_2 fixation and APA before removal for biomass estimation (AFDM, CHLA). We measured N_2 fixation on 5 FGDs and APA on another 5 FGDs from each treatment × sampling day combination. We used the 2 remaining NDS as dark incubation controls in N_2-fixation assays. We conducted N_2 fixation and APA assays in 50-mL Popper micromate syringes with 3-way stopcocks (Popper and Sons, New Hyde Park, New York). We transferred periphyton on FGDs into syringes with either 30 mL of stream water for N_2 fixation assays or 30 mL of 1.2% Tris buffer for APA assays.

N_2 fixation.—We estimated N_2 fixation using acetylene reduction (Flett et al. 1976). We injected 5 mL of acetylene gas, generated from the dissolution of calcium carbide, into syringes containing samples in 30 mL stream water. We gently agitated samples to dissolve the acetylene and then incubated them under artificial lighting (250 μmol m^{-2} s^{-1}) for 2 to 4 h. We incubated 2 samples from each treatment in the dark to estimate the importance of N_2 fixation by heterotrophs. In addition, we incubated streamwater samples under lights to account for any background N_2 fixation in seston. We removed samples from incubation and analyzed them one at a time and recorded the length of incubation to the nearest minute. We drew 15 mL of air into each syringe and agitated the samples to establish equilibrium between water and vapor phases. We withdrew 1 μL of air from each syringe and injected it into a Carle AGC series gas chromatograph (Hach Carle, Loveland, Colorado) equipped with a flame ionization detector and a 1.8-m column packed with 80% Porapack N and 20% Porapack Q. The column temperature was 70°C. We used 10-ppm ethylene standards to calibrate the instrument daily. To estimate N_2 fixation, we assumed that the production of 3 μmol ethylene was equivalent to the fixation of 1 μmol N_2 (Flett et al. 1976).

APA.—We estimated APA using the rate by which phosphate was cleaved from 4-methylumbelliferyl phosphate (MUFP) resulting in the production of methylumbelliferone (MUF), which fluoresces (emission wavelength 455 nm) when irradiated at 365 nm wavelength (Healey and Hendzel 1979). We injected 4 mL of 0.2-mM MUFP into syringes containing samples in 1.2% Tris buffer. We gently agitated syringes, incubated samples at room temperature, and measured fluorescence after 5, 20, and 45 min. We removed subsamples from incubation and analyzed samples one at a time and recorded the length of incubation to the nearest minute. We measured fluorescence on a Turner 10 AU fluorometer (Turner Designs, Sunnyvale, California) calibrated with 50-, 100-, 250-, 500-, and 1000-μg/L MUF standards. We used the slope of a linear regression of time (min) vs MUF concentration to calculate APA activity. Instances in which the MUFP concentration was not saturating were rare. However, we derived APA activity from the exponential increase in MUF concentration vs time using regression when we observed this phenomenon.

Periphyton biomass.—After N_2 fixation and APA assays, we removed FGDs from syringes and scraped and rinsed periphyton to form a 50-mL slurry. We collected two 25-mL aliquots onto separate glass fiber filters (GFFs) for AFDM and CHLA analyses, respectively. We estimated AFDM on prewashed, precombusted, and preweighed GFFs. We dried AFDM samples at 100°C for 1 h, weighed them to estimate dry mass, combusted them for 1 h at 500°C, and weighed them again to estimate ash content. We determined phaeophytin-corrected CHLA using the method described by Biggs and Kilroy (2000). We extracted CHLA overnight in 90% ethanol (heated) and measured pigments by absorbance on a Beckman DU series spectrophotometer (Beckman Coulter, Fullerton, California).

Data analysis

We used paired *t*-tests (SAS 9.1.3; SAS Institute, Cary, North Carolina) to compare water-column TN, TP, NO_x-N, and PO_4-P concentrations upstream and downstream of the NDS rack. We used nonlinear regression models of nutrient diffusion vs day of retrieval (SigmaPlot 9.0; SYSTAT, Point Richmond, California) to estimate nutrient diffusion rates from agar.

We used 2-factor analysis of variance (ANOVA; SAS) with day of retrieval (date) and nutrient treatment (nutrient) as main effects and a date × nutrient interaction term on AFDM, CHLA, N_2 fixation, and APA. We used standard 2-way factorial ANOVA rather than repeated-measures ANOVA because we did not take repeated measurements from the same samples over time, but rather sampled a unique set of NDS during each collection; thus,

TABLE 1. Mean (±1 SE) surface-water N and P concentrations upstream and downstream of the nutrient diffusing substrate rack. TN = total N, TP = total P, NO_x-N = NO_2 + NO_3-N. $n = 5$.

	Concentration (μg/L)			
Analyte	Upstream	Downstream	Paired t	1-tailed p
TN	556.2 ± 27.3	592.8 ± 35.2	1.58	0.09
TP	105.5 ± 5.3	104.3 ± 6.1	–0.64	0.28
NO_x-N	49.6 ± 3.2	62.8 ± 4.5	2.46	0.03
PO_4-P	24.5 ± 0.4	29.9 ± 3.1	1.70	0.08

adjustments for serial autocorrelation were inappropriate. Post hoc comparisons of significant main effects or interaction terms were done with LSMEANS with Tukey adjustments (SAS). The significance level for all tests was $p < 0.05$.

We applied $\log_{10}(x)$ transformations to most data sets before analyses because group variances tended to increase with mean response magnitude. Before transformation, we anchored each data set by setting the minimum data value to 1.0 by adding or subtracting a constant to the values in the data set. This technique standardizes the effect of transformations (Sokal and Rohlf 1995, Osborne 2002) and keeps the resultant log-transformed values positive.

Results

Water chemistry and N and P diffusion rates from NDS

Mean NO_x-N concentrations were higher downstream than upstream of the NDS rack during the 29-d study period ($t = 2.46$, $p = 0.03$). TN, TP, and PO_4-P concentrations did not differ between upstream and downstream samples (Table 1). Mean water temperature was 25.5°C (range: 23–28°C) during the study period.

Mean agar concentrations of NO_x-N and PO_4-P declined exponentially during the study period. Day-29 NO_x-N and PO_4-P remained within ~40% and 75% of day-0 concentrations, respectively. NO_x-N concentrations of the control and P treatment were ~0. PO_4-P concentrations of the control and N treatment also were low, but the PO_4-P concentrations tended to be slightly higher in the N treatment than in the control. The 1st derivatives of exponential-loss equations predicted the release rates for NO_x-N and PO_4-P of the N and P treatments, respectively (Fig. 1A, B). The predicted release rate of NO_x-N from the N treatment decreased by >50% by day 2, but the release rate decreased more gradually after day 3 (Fig. 1A). NDS released 2.9 mg NO_x-N cm^{-2} d^{-1} on day 6, 1.2 mg NO_x-N cm^{-2} d^{-1} on day 18, and 0.57 mg NO_x-N cm^{-2} d^{-1} on day 29. The release rate of PO_4-P from the P treatment did not exhibit a rapid initial decline (Fig. 1B). NDS released 0.41, 0.26, and 0.18 mg PO_4-P cm^{-2} d^{-1} on days 6, 18, and 29, respectively.

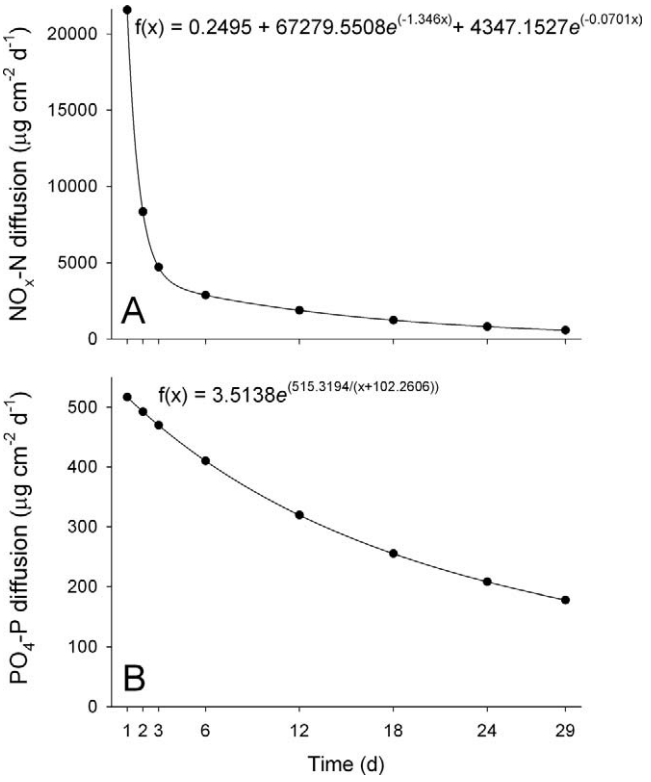

FIG. 1. N (A) and P (B) release rates from nutrient diffusing substrates.

Effects of N and P enrichment on periphyton accrual

Periphyton AFDM increased significantly during the study period ($F_{7,129} = 92.88$, $p < 0.0001$; Fig. 2). However, area-normalized AFDM did not differ significantly among nutrient treatments ($F_{2,129} = 1.30$, $p = 0.2751$), nor was the nutrient × date interaction significant ($F_{14,129} = 0.54$, $p = 0.9056$). AFDM accrual appeared linear and was similar among nutrient treatments between days 1 and 6. AFDM accrual rate was 0.1197 mg cm^{-2} d^{-1} between days 1 and 6 and was 0.2197 mg cm^{-2} d^{-1} between days 24 and 29. Area-normalized CHLA varied significantly among nutrient treatments over the study period ($F_{4,97} = 5.12$, $p = 0.0009$; Fig. 3). CHLA was significantly greater in the N treatment than in control or P treatments on days 18 and 29.

N_2 fixation and APA following nutrient enrichment

N_2 fixation varied significantly among nutrient treatments during the study period, regardless of

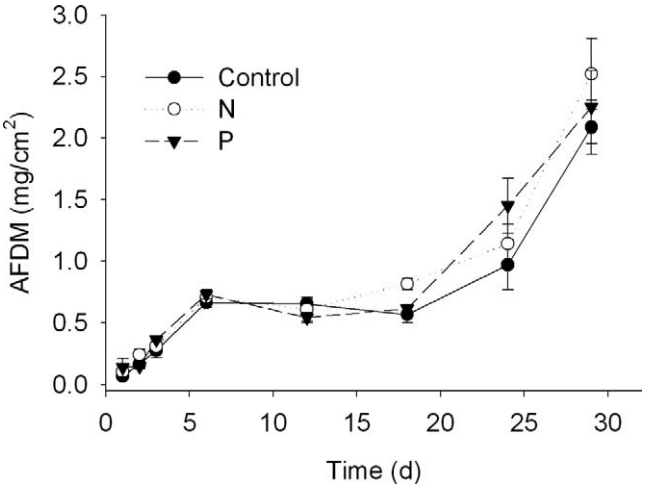

FIG. 2. Mean (±1 SE) periphyton biomass measured as area-normalized ash-free dry mass (AFDM) on control, N, and P nutrient diffusing substrate treatments over 29 d.

normalization method ($p < 0.001$; Fig. 4A–C). N_2 fixation did not differ significantly among nutrient treatments on day 6. However, N_2 fixation was significantly greater in control and P than in N treatments on days 18 and 29. N_2 fixation did not differ significantly between control and P treatments (Fig. 4A–C). Biomass (CHLA)-normalized N_2 fixation in control and P treatments was significantly lower on day 29 than on day 18 (Fig. 4C). A similar pattern was apparent for biomass (AFDM)-normalized N_2 fixation, but the difference between days was not significant

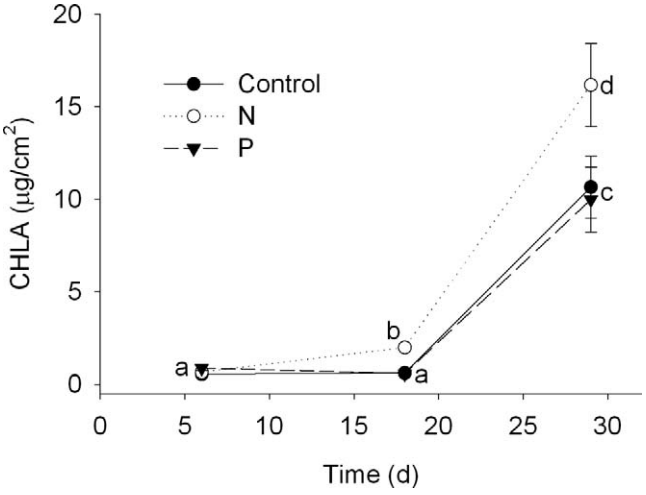

FIG. 3. Mean (±1 SE) periphyton biomass measured as chlorophyll *a* (CHLA) on control, N, and P nutrient diffusing substrate treatments over 29 d. Values with the same letter are not significantly different (Tukey–Kramer adjusted posthoc comparisons).

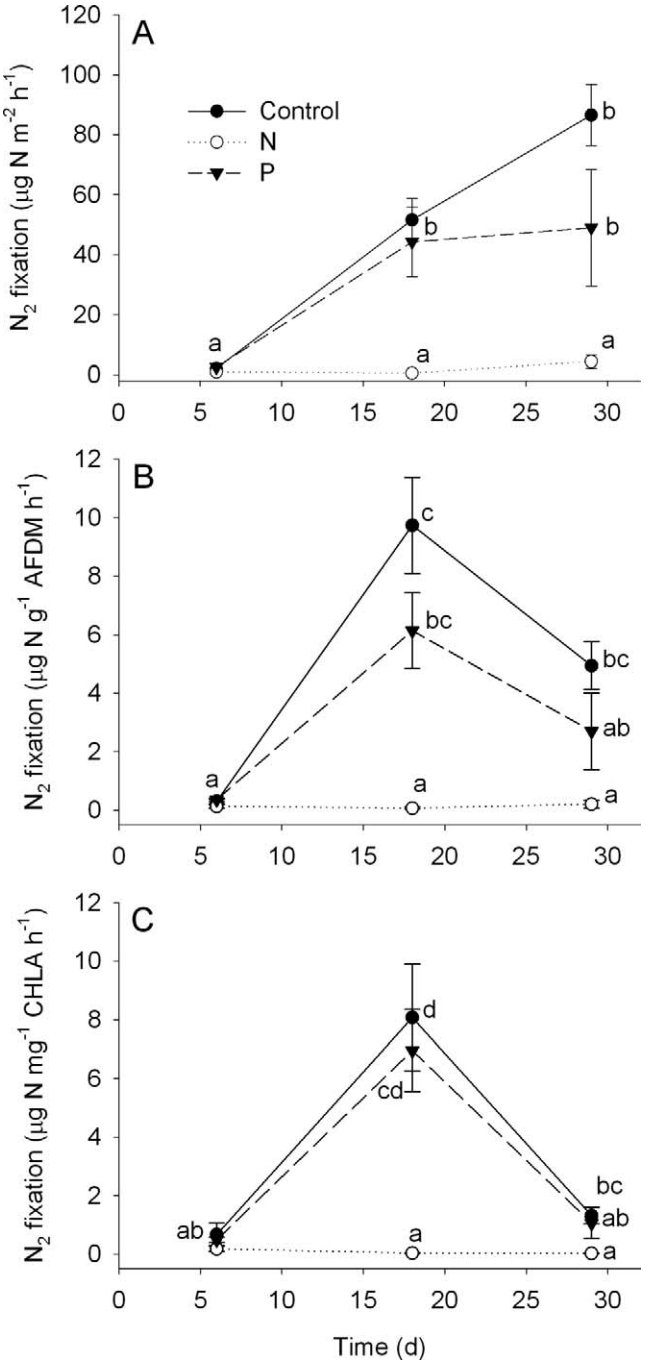

FIG. 4. Mean (±1 SE) N_2 fixation rates normalized by area (A), periphyton ash-free dry mass (AFDM) (B), and periphyton chlorophyll *a* (CHLA) (C) on control, N, and P nutrient diffusing substrate treatments over 29 d. Values with the same letter are not significantly different (Tukey–Kramer adjusted post hoc comparisons).

(Fig. 4B). N_2 fixation in dark incubations was consistently >0 in control and P treatments (Table 2). N_2 fixation in dark incubations accounted for 4.0 to 73.3% of N_2 fixation in light incubations, except in the

TABLE 2. Mean periphyton N_2 fixation rates (±1 SE) in dark incubations. $n = 2$.

Treatment	Day	Dark N_2 fixation ($\mu g\ N\ m^{-2}\ h^{-1}$)	% of mean light N_2 fixation
Control	6	15.0 ± 9.17	727.0
	18	19.1 ± 3.28	37.0
	29	10.0 ± 6.14	11.6
N	6	0.00 ± 0.00	0.0
	18	0.00 ± 0.00	0.0
	29	0.00 ± 0.00	0.0
P	6	0.81 ± 0.81	33.3
	18	32.3 ± 16.18	73.3
	29	1.96 ± 0.67	4.0

control treatment on day 6 when N_2 fixation was 7× higher in dark than in light incubations (Table 2).

APA always was significantly lower in P than in control or N treatments, regardless of normalization method ($F_{2,36} = 57.17$–91.67, $p < 0.0001$; Fig. 5A–C). APA varied significantly among nutrient treatments over the study period ($F_{4,36} = 2.92$–5.19, $p = 0.0022$–0.0343; Fig. 5A–C). On day 18, area-normalized APA was greater in the N than in the control treatments (Fig. 5A). In the control treatment, area-normalized APA did not differ significantly between days 6 and 18, but was significantly greater on day 29 than on days 6 and 18. In the N treatment, area-normalized APA increased significantly between days 6 and 18, but did not change between days 18 and 29. In control and N treatments, biomass-normalized APA was significantly lower on day 29 than on day 18 (Fig. 5B, C).

Discussion

NDS diffusion rates and surface-water chemistry

Initial rapid loss of nutrients from NDS limits their usefulness in long-term studies (Corkum 1996, Rugenski et al. 2008). Most NDS studies are limited to the period of measurable nutrient diffusion from treatments. Deployment periods for NDS designs similar to ours range from 2 wk (Bernhardt and Likens 2004) to 2 mo (Von Schiller et al. 2007), but typically are 3 to 4 wk so that diffusion rates remain relatively constant (Tank and Dodds 2003). Bernhardt and Likens (2004) reported that day-14 N and P diffusion rates were only 10% and 25%, respectively, of day-2 rates. In our study, depletion of N was initially rapid and exponential, but resulted in only 60% loss during the 29-d study period. Depletion of P was more gradual, and resulted in only 25% loss during the study period. Our results also suggest that N and P diffusion remained high enough on day 29 (0.57 mg NO_x-N $cm^{-2}\ d^{-1}$, 0.18 mg PO_4-P $cm^{-2}\ d^{-1}$) to inhibit periphyton N_2 fixation and APA. Furthermore, N and P diffusing from NDS at the end of the study period was high relative to N and P requirements of periphyton growing on them. We

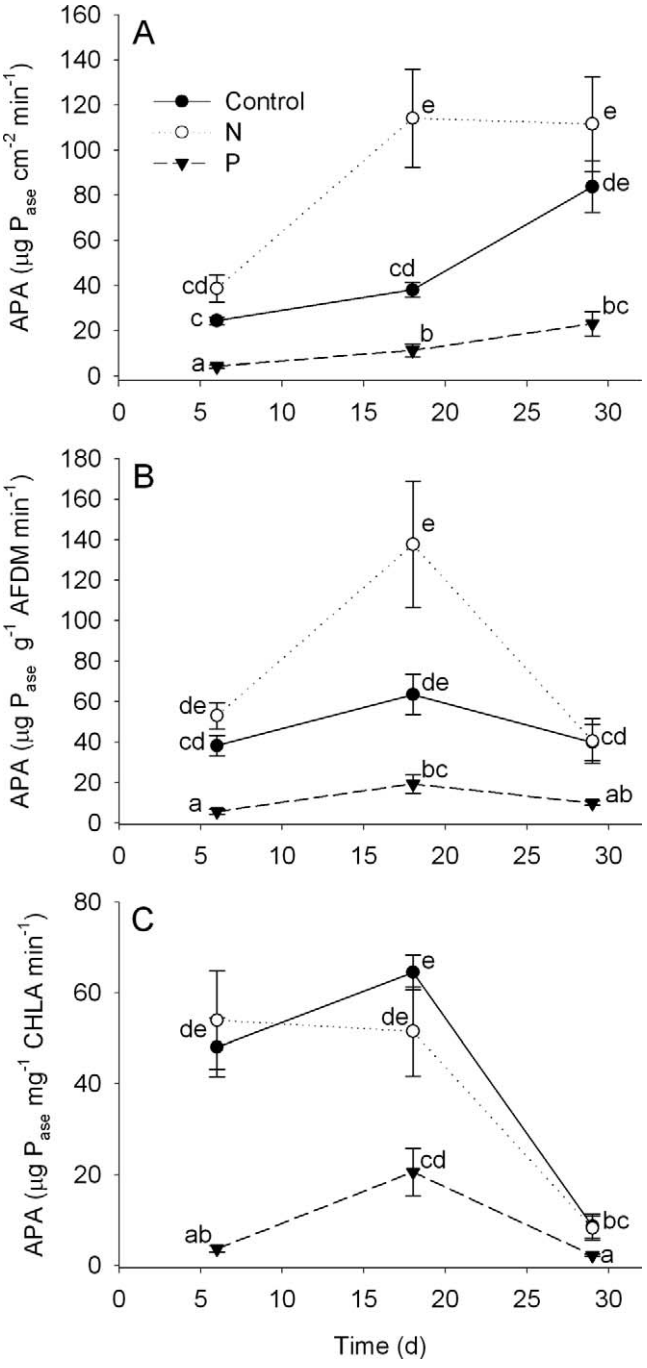

FIG. 5. Mean (±1 SE) alkaline phosphatase activity (APA), expressed as mass phosphatase (P_{ase}) per minute and normalized to area (A), periphyton ash-free dry mass (AFDM) (B), and periphyton chlorophyll *a* (CHLA) (C) on control, N, and P nutrient diffusing substrate treatments over 29 d. Values with the same letter are not significantly different (Tukey–Kramer adjusted posthoc comparisons).

did not measure periphyton N and P content, but N and P content of stream epilithon (Stelzer and Lamberti 2001) and metaphyton from the wetland that provides the water for our study stream (Scott et al. 2007) range from 1.0 to 2.0% N and 0.1 to 0.2% P. Mean periphyton dry mass on day 29 of our study was ~3 mg/cm^2. If we apply N and P contents from Stelzer and Lamberti (2001) and Scott et al. (2007) to periphyton in our system, periphyton N content probably ranged from 30 to 60 μg/cm^2 and periphyton P probably ranged from 3 to 6 μg/cm^2. Thus, the N and P diffusion rates were sufficient to replace periphyton N 10 to 20× and periphyton P 30 to 60× on day 29. Sufficient N and P were provided by NDS by the end of our study period to maintain the integrity of our nutrient-enrichment treatments.

We attempted to minimize the effect of confounding experimental factors by randomizing NDS positions in the stream. Thus, concentrations of dissolved N might have been slightly higher at downstream than at upstream NDS. NO_x-N concentrations were slightly higher in water collected 0.5 m downstream than in water collected immediately upstream of the rack. The slightly higher NO_x-N concentrations supplied to downstream NDS might have dampened periphyton response to nutrient treatments in downstream samples, but the concentrations of NO_x-N in downstream water were certainly lower than the exposure concentrations of NO_x-N on the NDS. Moreover, we did not detect any spatial pattern in biomass or enzyme activity as a function of the position of samples in the rack. Thus, we conclude that upstream–downstream differences in streamwater nutrients had minimal influence on our results, but the relative position of treatments should be considered carefully in the design of future NDS studies (Tate 1990).

N_2 fixation, APA, and biomass on NDS

N_2 fixation.—Periphyton N_2 fixation ranged from 44 to 86 μg N m^{-2} h^{-1} in control and P treatments on days 18 and 29. This range is 4 to 8× higher than the median of 22 values reported by Marcarelli et al. (2008). However, mean rates in some of the studies reviewed by Marcarelli et al. (2008) were as high as 410 to 8400 μg N m^{-2} h^{-1} (Buckley and Triska 1978, Howard-Williams et al. 1989, Grimm and Petrone 1997, Marcarelli and Wurtsbaugh 2006). The highest rates were reported from Sycamore Creek (Grimm and Petrone 1997), a strongly N-limited stream in the Sonoran Desert (Grimm and Fisher 1986). Furthermore, the highest rates occurred during mid-summer in all studies, consistent with summer N_2-fixation maxima in lentic systems (Howarth et al. 1988). Our study was done in late May through mid-June, and rates might be substantially higher during July and August.

We hypothesized that N_2 fixation would be highest in control and P treatments and that N enrichment would suppress N_2 fixation by periphyton. N_2 fixation rates were lower in N than in control treatments, but P enrichment did not stimulate N_2 fixation in our system. Mean N_2 fixation was always lower on P treatments than on controls, although this difference was never statistically significant. That P enrichment did not stimulate N_2 fixation was unexpected. We see no obvious explanation for this result, but offer the following possibilities. 1) The composition of the microbial community might have changed in the P treatment (sensu Fairchild et al. 1985), with the consequence that a different community of algae and bacteria had slightly lower N_2 fixation potential. However, we lack taxonomic data to support this hypothesis. Marcarelli and Wurtsbaugh (2006) found that P enrichment increased the number of N_2-fixing taxa in an oligotrophic stream, but other studies have reported that P enrichment decreased the rate of N_2 fixation in some stream periphyton communities (Marcarelli and Wurtsbaugh 2007). 2) Increased N remineralization might have occurred in response to P enrichment. Several studies have demonstrated the importance of N-mineralizing extracellular enzymes in periphyton (Francoeur and Wetzel 2003, Francoeur et al. 2006, Rier et al. 2007), but to our knowledge, no studies have explored how P enrichment affects N remineralization within the periphyton matrix. 3) Heterotrophic N_2 fixation might have decreased in response to P enrichment. Sundareshwar et al. (2003) reported reduced heterotrophic N_2 fixation in estuarine sediments after P enrichment, but found that glucose alone and glucose + P additions stimulated N_2 fixation. We did not replicate dark N_2 fixation measurements sufficiently for statistical comparisons, but N_2 fixation in the dark was 4 to 73% of N_2 fixation in the light on days 18 and 29. Thus, heterotrophic N_2 fixation could have influenced our results and might have been affected by availability of dissolved organic C in the periphyton matrix. More work is needed to elucidate these mechanisms.

Daily N_2 fixation was 0.93 mg N m^{-2} d^{-1} on day 18 and 1.0 mg N m^{-2} d^{-1} on day 29 (mean of control and P treatment N_2 fixation rates integrated over a 14:10 h day:night cycle). The rates of N diffusion in the N treatment suppressed N_2 fixation on days 18 (12.0 g N m^{-2} d^{-1}) and 29 (5.7 g N m^{-2} d^{-1}) and supplied 6×10^3 to 12×10^3 times more N to the N-enriched periphyton than was supplied by N_2 fixation to periphyton in control and P treatments. We cannot say what minimum rate of N supply from NDS might have

inhibited N_2 fixation. However, agar N concentrations can be modified to manipulate diffusion rates (Rugenski et al. 2008), and future studies might be able to clarify the relationships between diffusion rates of NO_3^- or NH_4^+ from NDS and up- or down-regulation of N_2 fixation by periphyton.

N_2 fixation rates in streams generally are much lower than instream denitrification and stream DIN uptake rates (Marcarelli et al. 2008). This conclusion suggests that N_2 fixation might seldom be an important source of N in stream ecosystems. However, the rates used in these comparisons were derived for the whole-reach scale and were expressed on an areal basis. Therefore, they do not consider N standing stocks within stream reaches. A more appropriate rate to compare might be the N-specific uptake rate (or N turnover rate of the periphyton N pool) expressed in units of time^{-1}. If we assume that periphyton N in our study probably ranged from 30 to 60 μg N/cm^2 on NDS, then N-specific uptake from N_2 fixation on control and P treatments in our study was 1.5×10^{-3}/d to 3.1×10^{-3}/d on day 18 and 1.7×10^{-3}/d to 3.4×10^{-3}/d on day 29. We did not measure total N uptake, but Dodds et al. (2004) reported that N-specific total N uptake by primary producers across several streams in North America ranged from 10^{-4}/d to 10^{-1}/d, a range that brackets our N_2 fixation estimates. Therefore, the importance of N_2 fixation as a source of N to primary producers and, ultimately, to consumers within the food web might be more obvious when considered in terms of stream biomass N.

APA.—We hypothesized that P enrichment would inactivate APA and that N enrichment would stimulate APA. As in other studies (e.g., Klotz 1992), P enrichment always decreased APA. N enrichment stimulated area-normalized APA on day 18 but not on day 29. N enrichment stimulates APA in phytoplankton (e.g., Rose and Axler 1998) and in stream epilithon (Klotz 1992). Stimulation of APA by N enrichment suggests that organisms originally limited by N become P limited after N enrichment or that N enrichment enables P-limited organisms to up-regulate APA. Thus, P deficiency appears to be an important limitation on periphyton biomass production and metabolism. The concentration of inorganic P in our study stream (24.4–29.9 μg P/L) approached concentrations that saturate periphyton biomass accumulation (Bothwell 1989). However, mass transfer of nutrients from stream water into the periphyton matrix is the primary rate-limiting step for nutrient uptake by periphytic algal cells (Larned et al. 2004). Thus, internal nutrient regeneration within the periphyton matrix could be important in both oligotrophic and eutrophic streams because the rate at which nutrients enter the biofilm matrix might not keep up with demand by cells. This idea is supported by our data, which suggest that P deficiency existed in control and N treatments even though streamwater inorganic P concentration was consistently high.

Periphyton APA values approached 0 only on P-enriched NDS. We cannot say what minimum rate of P supply from NDS was necessary to inhibit periphyton APA. However, as with N, agar P concentrations can be modified to manipulate diffusion rates (Rugenski et al. 2008), and future studies might be able to clarify the relationships between diffusion rates of P from NDS up- or down-regulation of APA by periphyton.

Biomass.—We hypothesized that N enrichment, but not P enrichment, would increase periphyton biomass. CHLA, a measure of autotrophic biomass (Steinman et al. 2006), was higher in N than in control or P treatments on days 18 and 29, but N enrichment had no effect on AFDM. P enrichment had no effect on CHLA or AFDM. However, both N_2 fixation and APA were greatly influenced by periphyton biomass. AFDM and CHLA increased substantially between days 18 and 29. Area-normalized N_2 fixation and APA were highest (or equal to the highest observed value) on day 29 when CHLA and AFDM were highest, but AFDM- and CHLA-normalized N_2 fixation and APA were highest on day 18 when CHLA and AFDM were relatively low. This pattern suggests that periphyton was more metabolically active (with regard to N_2 fixation and APA) on day 18 than on day 29 and that the higher area-normalized rates on day 29 were driven by high periphyton biomass.

We hypothesized that N_2 fixation and APA would be more sensitive than periphyton biomass to differences in nutrient enrichment. N_2 fixation and APA were strongly influenced by nutrient treatments. CHLA accrual was stimulated by N enrichment, but not P enrichment, on days 18 and 29, but AFDM accrual was not sensitive to N or P enrichment. These results indicate that autrotrophs must have been limited by N availability throughout the study. However, the high rates of APA on control and N treatments suggest that some part of the periphyton community (autotrophs, heterotrophs, or both) was P limited. This conclusion is supported by the lower APA in P than in control or N treatments. Thus, our results suggest the potential for differential nutrient limitation among individual autotrophic and, perhaps, heterotrophic taxa in this periphyton community.

Differential nutrient limitation in periphyton

Our study design did not enable us to distinguish whether APA was derived from autotrophs or hetero-

trophs. We do not know whether only a subset of autotrophs, mostly heterotrophs, or a combination of both were experiencing P deficiency. Different autotrophic taxa within the same community can be limited by different elements (Dodds 1991, Armitage et al. 2006), as can autotrophs and heterotrophs within the same community (Sundareshwar et al. 2003, Zohary et al. 2005). Differential nutrient limitation of autotrophs and heterotrophs might maximize resource use within an ecosystem if groups facilitate resource delivery to each other, rather than compete for the same resource (Sundareshwar et al. 2003). The strength of algal–bacterial coupling in periphyton decreases as nutrient availability increases (Scott and Doyle 2006, Scott et al. 2008). This result suggests facilitation of N and P exchange between trophic levels. Thus, periphyton communities experiencing differential nutrient limitation between trophic levels might be more productive than predicted under a single-limiting-nutrient scenario. Exchange of resources between trophic levels in microbial benthos experiencing differential nutrient limitation could explain how benthic communities increase ecosystem production efficiency in nutrient-poor systems and could provide a mechanism to explain why benthic production is of primary importance in oligotrophic ecosystems (Vadeboncoeur et al. 2002).

Results of our study suggest that N_2 fixation can provide an important source of N to periphyton communities, but that P deficiency might still play a critical role in periphyton community metabolism and production. Both N_2 fixation and APA are sensitive indicators of microbial nutrient limitation in periphyton. N enrichment increased autotrophic biomass in our periphyton community. Moreover, although changes in total periphyton biomass did not generally reflect a response to added nutrients, N enrichment inhibited N_2 fixation and stimulated APA, and P enrichment inhibited APA. Therefore, microbial metabolism was related to both N and P availability. Furthermore, the simultaneous activity of both N_2 fixation and APA and their respective responses to nutrient enrichment suggests the importance of differential nutrient limitation and supports the need for a multielement approach to understanding nutrient constraints on ecological communities and the systems in which they live (sensu Sterner and Elser 2002). However, more work is needed to determine if differential nutrient limitation is a general characteristic of aquatic microbial communities and if patterns observed in nature might be explained by differences in elemental constraints among microorganisms of the same or of different trophic positions.

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