ABSTRACT

Effects of Nutrient Enrichment on Alkaline Phosphatase Activity
and Nitrogen Fixation Potential in Stream Periphyton

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In an initial experiment, I evaluated methodological considerations of using a novel nutrient-diffusing substrata (NDS) design to estimate the effect of nitrogen (N) and phosphorus (P) enrichment on alkaline phosphatase activity (APA) and N_2 fixation. Diffusion rates were effectively estimated, and suggested that this NDS design may also be useful for long-term studies. Levels of APA and N_2 fixation potential were substantial in controls, indicative of differential nutrient limitation in the periphyton. In a second experiment, I investigated the effect of ambient N and P concentrations on periphyton accrual, APA, and N_2 fixation using a P gradient of selected stream sites, which incidentally served also as an N gradient. Presence of both APA and N_2 fixation on controls, which decreased up the P gradient, suggested that periphyton communities were differentially limited by both N and P. This was further corroborated by responses to NDS enrichments of N and P.

Effects of Nutrient Enrichment on Alkaline Phosphatase Activity
and Nitrogen Fixation Potential in Stream Periphyton

by

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A Thesis

Approved by the Department of Biology

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Robert D. Doyle, Ph.D., Chairperson

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LIST OF ABBREVIATIONS AND ACRONYMS

AFDM	ash-free dry mass
APA	alkaline phosphatase activity
Chl.*a*	chlorophyll *a*
FGD	fritted glass disc
GPP	gross primary productivity
LEON-01	Leon River near Hamilton, Texas (stream site in Ch.3 experiment)
LEON-02	Leon River in Gatesville, Texas (stream site in Ch.3 experiment)
LTA	log-transformed, anchored values
N	nitrogen
N_2	dinitrogen
NBOS-04	North Bosque River in Meridian, Texas (stream site in Ch.3 experiment)
NDS	nutrient-diffusing substrata
P	phosphorus
PALU-01	Paluxy River near Glen Rose, Texas (stream site in Ch.3 experiment)
S.E.	standard error
TN	total nitrogen
TP	total phosphorus

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CHAPTER ONE

Introduction

A common cause of degraded water quality in the USA is nutrient pollution. Although required for aquatic life, excessive inputs of nutrients, such as nitrogen (N) and phosphorus (P), trigger a cascade of ecological events resulting in an increasingly eutrophic state. Eutrophication can result in algal blooms and hypoxia, which can suffocate many forms of aquatic life in freshwater ecosystems. In addition, many algae and other microbes produce harmful and/or bad-tasting compounds, thus further degrading water quality.

The concentration and ratio of available N and P are important factors regulating growth and production of algae and other microbes. Both nutrients are essential components of amino acids, nucleic acids, and other biomolecules necessary for life. Phosphorus has often been considered to be the limiting nutrient of primary producers because atmospheric nitrogen can be fixed by cyanobacteria to compensate for lack of N (Redfield 1958, Schindler 1977). However, different species are likely to contain different assortments, amounts, and ratios of these biomolecules. Indeed, different algal species have different nutrient requirements and contents (Rhee and Gotham 1980, Tilman et al. 1982, Borchardt 1996). Still, a meta-analysis of lotic nutrient amendment experiments by Francoeur (2001) revealed that the results of many nutrient amendment experiments were inconsistent with the theoretical prediction that multiple nutrients independently limit algal community biomass.

In addition to measures of growth and production, N_2 fixation rates and alkaline phosphatase activity (APA) can be used as indicators of N and/or P limitation. N_2 fixation can be performed by cyanobacteria and diatoms with cyanobacterial endosymbionts, typical of the *Epithemia* and *Rhopalodia* genera. Under conditions of N deficiency, these taxa will begin fixing atmospheric dinitrogen (N_2) gas. This process is energetically expensive, however, and will not occur if other sources of available N are in the environment (Howarth et al. 1988).

Alkaline phosphatase activity (APA) has also been utilized as an indicator of nutrient status in aquatic ecosystems in both classical studies (eg. Healey and Hendzel 1979, Fitzgerald and Nelson 1966, Kuenzler and Perras 1965) and more recent studies (Newman et al. 2003, Kahlert et al. 2002). In the aquatic environment, alkaline phosphatases catalyze the hydrolysis of dissolved organic phosphate esters (Kuenzler and Perras 1965). Although all microbes are capable of directly utilizing APA as a means of overcoming phosphorus deficiency, in periphyton mats, symbioses between bacteria and algae may exist in which cyanobacteria provide carbon to bacteria via photosynthesis and bacteria provide phosphorus via APA (Scott and Doyle 2006, Sharma et al. 2005). In periphyton, bacterial production and photosynthetic production can be positively correlated (Scott and Doyle 2006). However, algal production can increase more rapidly than bacterial production with increasing nutrients (Carr et al. 2005).

Many nutrient limitation studies have utilized nutrient-diffusing substrata (NDS) to test for effects of different nutrient enrichments. NDS have been used in investigations of nutrient limitation 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) and large rivers (Corkum 1996, Scrimgeour and Chambers 1997). For treatments, NDS media is typically enriched with N, P, N+P, or with no enrichment as a control. NDS designs vary. Many NDS use an agar media in containers such as Petri dishes (Corkum 1996), clay pots (Fairchild and Lowe 1984), and plastic vials sealed with a porous porcelain or fused silica crucible cover (Gibeau and Miller 1989). Another NDS design uses nutrient enriched aqueous media inside polyethylene bottles by capping the bottle with an open-top lid over a nylon membrane or glass-fiber filter (Matlock et al. 1998).

In this study, a new, modified form of NDS based on the design of Gibeau and Miller (1989) was evaluated. Before implementation at various streams, determination of nutrient diffusion rates from this NDS design was useful in deciding the length of time NDS may be deployed in the field. Periphyton responses to nutrient enrichments were also monitored over time to elucidate the deployment period with maximum separation of APA and N_2 fixation rates between treatments for clearest results in future studies. Since the APA estimation method utilizes both a pH buffer and a large organic compound, it was performed following measure of N_2 fixation potential and performed alone on other samples. Thus, it was also imperative to determine whether or not measuring N_2 fixation potential using the acetylene-reduction technique jeopardizes APA readings. Therefore, a method refinement study was performed using NDS in a stream-like downstream section of a nutrient-depletion gradient in a constructed freshwater marsh to evaluate these issues.

In streams, very few studies have utilized APA and N_2 fixation rates to study effects of nutrient ratios and concentrations on periphyton. Recently, Marcarelli and

Wurtsbaugh (2006) effectively demonstrated that P availability and temperature can interplay to control N_2 fixation and periphyton community composition in streams. In another recent study, APA was found to be inversely related to TP and soluble reactive phosphorus concentrations and levels of cellular P (Bowman et al. 2003). APA has also been used as an indicator of P limitation across streams of different watershed land use and geology (Klotz 1985), and can be directly related to the N:P ratios in ambient stream water (Klotz 1992). To our knowledge, the dual use of N_2 fixation rates and APA for indication of nutrient status is currently nonexistent in the literature. Therefore, the effects of ambient N and P concentrations on periphyton accrual, APA, and N_2 fixation were also investigated in a second study using a P gradient of selected stream sites (Chapter 3).

CHAPTER TWO

Evaluating a Novel Nutrient-Diffusing Substrate Design to Estimate Alkaline Phosphatase Activity and Nitrogen Fixation of Periphyton

Introduction

Nutrient pollution is a common cause of degraded water quality in the USA. Although required for aquatic life, excessive inputs of nutrients, such as nitrogen (N) and phosphorus (P), trigger a cascade of ecological events resulting in an increasingly eutrophic state. Eutrophication can result in algal blooms and hypoxia, which can suffocate many forms of aquatic life in freshwater ecosystems. In addition, many algae and other microbes produce harmful and/or bad-tasting compounds, thus further degrading water quality.

The concentration and ratio of available N and P are important factors regulating growth and production of algae and other microbes. Both nutrients are essential components of amino acids, nucleic acids, and other biomolecules necessary for life. Phosphorus has often been considered to be the limiting nutrient of primary producers because atmospheric nitrogen can be fixed by cyanobacteria to compensate for lack of N (Redfield 1958, Schindler 1977). However, different species are likely to contain different assortments, amounts, and ratios of these biomolecules. Indeed, different algal species have different nutrient requirements and contents (Rhee and Gotham 1980, Tilman et al. 1982, Borchardt 1996). Still, a meta-analysis of lotic nutrient amendment experiments by Francoeur (2001) revealed that the results of many nutrient amendment

experiments were inconsistent with the theoretical prediction that multiple nutrients independently limit algal community biomass.

In addition to measures of growth and production, N_2 fixation rates and alkaline phosphatase activity (APA) and N_2 fixation rates can be used as indicators of N and/or P limitation. N_2 fixation can be performed by cyanobacteria and diatoms with cyanobacterial endosymbionts, typical of the *Epithemia* and *Rhopalodia* genera. Under conditions of N deficiency, these taxa will begin fixing atmospheric dinitrogen (N_2) gas. This process is energetically expensive, however, and will not occur if other sources of available N are in the environment (Howarth et al. 1988).

APA has also been utilized as an indicator of nutrient status in aquatic ecosystems in both classical studies (e.g. Healey and Hendzel 1979, Fitzgerald and Nelson 1966, Kuenzler and Perras 1965) and more recent studies (Newman et al. 2003, Kahlert et al. 2002). In the aquatic environment, alkaline phosphatases catalyze the hydrolysis of dissolved organic phosphate esters (Kuenzler and Perras 1965). Although all microbes are capable of directly utilizing APA as a means of overcoming phosphorus deficiency, in periphyton mats, symbioses between bacteria and algae may exist in which cyanobacteria provide carbon to bacteria via photosynthesis and bacteria provide phosphorus via APA (Scott and Doyle 2006, Sharma et al. 2005). Moreover, bacterial production and photosynthetic production can be positively correlated in periphyton (Scott and Doyle 2006). However, algal production may increase more rapidly than bacterial production with increasing nutrients (Carr et al. 2005).

N_2 fixation and APA are dynamic response variables that can be affected by other factors. In a lake inlet/outlet stream reciprocal transplant experiment, both warm outlet

(mean 18°C) and cold inlet (mean 7.1°C) communities showed greatest N_2 fixation when P enrichment was given and incubation was in the warm outlet (Marcarelli and Wurtsbaugh 2006). Algal community APA can also be higher in warmer temperatures (Hernández et al. 1996). In benthic algae tested by Hernández et al. (1996), optimum temperature for APA ranged from 25 to > 30°C. Since these studies were performed on algal communities of a respective region, however, this does not necessarily mean that N_2 fixation and APA become decreasingly important with increasing latitude. Although grazers have been shown to alter benthic algal nutrient composition (Evans-White and Lamberti 2005) and availability (McCormick and Stevenson 1991) and community structure (McCormick and Stevenson 1989), to our knowledge, no studies have quantified the dual effect of grazers and nutrient enrichment on N_2 fixation potential and APA. Micronutrients, such as iron and molybdenum in particular, can also interplay to limit N_2 fixation (Howarth et al. 1988, Wurtsbaugh and Horne 1983) and growth (Winterbourn 1990, Wuhrmann and Eichenberger 1975). The trace metal, zinc, is an important structural component of the alkaline phosphatase enzyme, and although not usually in short supply, copper toxicity can cause a displacement of zinc, thus resulting in reduced APA (Reuter 1983, Foy et al. 1978, Vallee 1959). Carbon is rarely limiting to algae, but has been found to be at least growth-limiting in soft-water lakes (Barnese and Schelske 1994, Fairchild and Sherman 1993, Fairchild et al. 1989, Hein 1997).

Of the nutrients required by benthic algae, it is most often N and P that are in shortest supply to benthic algal communities (Borchardt 1996), thus N_2 fixation and APA can still be valuable indicators of nutrient status. Trends in lakes have shown that shifts to N limitation usually favor nitrogen fixing organisms (Levine and Schindler 1999).

This trend has also been observed in a constructed freshwater marsh, and N_2 fixation rates there were greatest at the outflow where the highest surface-water N:P ratio was observed (Scott et al. 2005). In the Everglades, surface-area-specific APA was found to be a sensitive indicator of P enrichment (Newman et al. 2003).

In streams, relatively few studies have utilized APA and N_2 fixation rates to study effects of nutrient ratios and concentrations on periphyton. In one study, APA was used as an indicator of P limitation across streams of different watershed land use and geology (Klotz 1985). As previously mentioned, Marcarelli and Wurtsbaugh (2006) effectively demonstrated that P availability and temperature interplay to control N_2 fixation and periphyton community composition in oligotrophic streams. In addition, other studies have typically utilized only one of these two enzymatic nutrient limitation indicators.

Many nutrient limitation studies have utilized nutrient-diffusing substrata (NDS) to test for effects of different nutrient enrichments. NDS have been used in investigations of nutrient limitation 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) and large rivers (Corkum 1996, Scrigmouer and Chambers 1997). For treatments, NDS media is typically enriched with N, P, N + P, or with no enrichment as a control. NDS designs vary. Many NDS use an agar media in containers such as Petri dishes (Corkum 1996), clay pots (Fairchild and Lowe 1984), and plastic vials sealed with a porous porcelain or fused silica crucible cover (Gibeau and Miller 1989). Another NDS design uses nutrient enriched aqueous media inside polyethylene bottles by capping the bottle with an open-top lid over a nylon membrane or glass-fiber filter (Matlock et al. 1998).

We evaluated a new, modified form of NDS based on the design of Gibeau and Miller (1989). Nutrient diffusion rates of NO_3-N and PO_4-P from nutrient treatments of this NDS design were also estimated, which may be useful in deciding the length of time NDS should be deployed in the field. Periphyton response to nutrient enrichments were also monitored over time to elucidate the deployment period with maximum separation of APA and N_2 fixation rates between treatments for clearest results in future studies. These rates were normalized to surface area, ash free dry mass (AFDM), and chlorophyll *a* (Chl.*a*) for comparison. Additionally, to evaluate whether APA could be effectively measured on same-sample NDS as those used to measure N_2 fixation potential, it was imperative to determine whether or not measuring N_2 fixation potential using the acetylene-reduction technique jeopardizes APA readings. Since the APA method utilizes both a pH buffer and a large organic compound, it was performed following measure of N_2 fixation potential and performed alone on other samples.

Using NDS in a stream-like outflow of a constructed freshwater marsh, I quantified 1) diffusion rates of nutrients from an agar media NDS design, 2) APA of samples used for estimate of N_2 fixation potential for comparison with samples used to measure APA alone, 3) accumulation of periphyton over time, and 4) N_2 fixation potential and APA of periphyton over time. I hypothesized that (i) N_2 fixation will be highest in the P treatment and APA would be highest in the N treatment, (ii) APA readings taken after the acetylene-reduction procedure would not differ from APA readings measured alone, and (iii) the overall limiting nutrient would be revealed as the single nutrient treatment (N or P) yielding greater growth than the control.

Methods

Study Site

The Lake Waco Wetland Complex (LWW) is located near Waco, Texas, USA. The complex is a 80-ha constructed marsh with dense stands of *Typha latifolia* and *Schoenoplectus californicus*. The LWW consists of 5 wetland cells. Cell 1 receives pumped inflow from the North Bosque River. Research by Scott et al. (2005) has suggested that N is removed more efficiently than P from the wetland. A nonlinear flow path is followed from cell 1 through cells 2, 3, 4, and 5 respectively before returning to the river. Currently, the North Bosque River and its receiving waters, Lake Waco, are receiving nutrient pollution from point and non-point anthropogenic sources in the watershed. The LWW was constructed as habitat mitigation for a loss of fringe wetlands of Lake Waco's littoral zone that occurred during a 2 meter conservation pool rise of Lake Waco. Flooding of the LWW began in stages in 2001, and flooding of all cells was completed in November 2003.

The experiment was performed in the stream-like outflow of cell 4. It is primarily composed of sandy substrate with *Typha latifolia* growth along its margins. The outflow is approximately 100 meters long with an average width of 3 meters. Thalweg depth at the deployment location was 0.38 meters. Velocity and discharge at deployment location was ~0.15 m/s and ~0.1 m^3/s. This deployment location was an open site of comparatively low marginal *Typha* growth, and completely lacking in canopy cover.

Nutrient-Diffusing Substrata and Rack Design

Nutrient-diffusing substrata (NDS) were constructed based on the design of Gibeau and Miller (1989) and were similar to that used by Marcarelli and Wurtsbaugh (2006). Both of these previous designs used a fritted glass disc (FGD) as a diffusion substrate, which passively allows nutrients to diffuse from an agar medium through the porous surface of the FGD. My design differed slightly in that I used a standard 50-ml centrifuge tube to store agar rather than smaller containers with more FGD surface area to agar volume. I hypothesized that the tall, narrow centrifuge tubes would reduce the surface-area-to-volume-ratio of the FGD relative to the nutrient stock solution and may result in a more protracted period of nutrient enrichment on the FGD.

The cap of a 50 ml centrifuge tube (Carolina Biological Supply, http://www.carolina.com) was drilled in the center to produce a 22 mm diameter hole. A Danco #42 Cap Thread Gasket and a 25 mm fritted glass disc (FGD) of fine porosity (4 - 5.5 microns) (Wilmad LabGlass, http://www.wilmad.com) was rested on top of the rim of the centrifuge tube.. The gasket helped secure and seal the FGD inside the centrifuge tube cap, thus limiting nutrient diffusion through FGD pores rather than allowing it leak around the margins of the FGD. Concentrated nutrients dissolved in agar and stored within each NDS allow for passive diffusion to occur, and the FGD provide substrate for the attachment of periphyton.

In this study, NDS were filled past the 50 ml mark of the tube to the rim with 20 g L^{-1} agar treated with either no nutrient enrichment (control), nitrogen enrichment (N), or phosphorus enrichment (P) prior to being capped. N treatments were made to a 0.5 molar solution of N using $NaNO_3$ in agar. P treatments will be made to a 0.05 molar solution of

P using $Na_3PO_4 \cdot 12H_2O$ in agar. Because the centrifuge tubes hold more than 50 ml volumes when filled to the rim, final room temperature volumes of each NDS were 56.75 ml.

A rack (60 cm x 90 cm) was constructed to hold NDS using wire netting framed with plastic-coated plant stakes for stability. Stakes were also used to anchor the rack into its deployment location. Each corner of the rack was attached by rope to a respective stake ~30 cm away that had been driven into the sandy bottom. Every other hexagonal hole in the wire netting was assigned a slot number. NDS were then randomly assigned a slot number by use of a random number generator.

Water Chemistry and Temperature

Surface-water samples were collected immediately upstream of the NDS rack to determine study period ambient nutrient concentrations in the stream-like outflow of the wetland. Total nitrogen (TN) and total phosphorus (TP), NO_3-N, and PO_4-P levels were estimated from surface-water samples on days 3, 6, 12, 18, and 24 following deployment. Water samples were also collected <0.5 meters downstream from the NDS rack on these same days to compare dissolved NO_3-N and PO_4-P levels with ambient concentrations. A Lachat® Quik-Chem 8500 flow-injection autoanalyzer was used to estimate these surface-water nutrients using standard methods. Study period water temperature was estimated using a HOBO® Water Temp Pro temperature logger attached to the rack.

Field Diffusion Rates of Nitrogen and Phosphorus

To determine field diffusion rates of nutrients from the agar, 3 NDS of each treatment (C, N, and P) were picked at random for determination of dissolved nitrogen

and phosphorus concentrations upon each retrieval date. The retrievals for this analysis took place on days 1, 2, 3, 6, 12, 18, 24, and 29 following deployment. Each NDS agar was added to an individual beaker with approximately 940 ml of deionized water, and melted at 100^{o}C in a hot water bath. The solution was then allowed to cool to room temperature. Finally, it was diluted further with deionized water to yield a 1 L solution. Duplicate 7 ml subsamples were taken from this solution and frozen for up to 28 days until analyzed of dissolved NO_3-N and PO_4-P concentrations. A regression curve was fit for the N and P treatments for the NO_3-N and PO_4-P concentrations respectively against the number of days following deployment. Using these regressions it was then possible to determine the release rates of each analyte from their respective nutrient treatment over time by use of the first derivative of these equations.

Periphyton Sampling

Fifty-one NDS of each treatment (control, N, and P) were deployed. Multiple analyses were performed on periphyton of each NDS. Ash-free dry mass (AFDM) of periphyton scraped from FGD surfaces was estimated from triplicate samples on days 1, 2, 3, 6, 12, 18, 24 and 29. An additional 9 NDS (12 total per treatment) were retrieved on day 6, 18, and 29 retrievals. On these dates, N_2 fixation potential, APA, and Chl.*a* were estimated in addition to AFDM. Five of the replicates were used to measure N_2 fixation potential and APA on the same NDS to evaluate whether APA is significantly altered by the acetylene reduction method. Another five replicates were used to measure APA without the measure of N_2 fixation potential beforehand. Two NDS were used to measure N_2 fixation potential after light incubation.

Following these analyses, periphyton were scraped off FGD into its respective assay solution and diluted to 50 ml using deionized water. This 50 ml solution was homogenized using a vortex until well-mixed. Whenever filamentous algae presence resisted homogenization, it was cut into pieces and again placed on vortex. Before settling could occur in each NDS, 13 ml and 25 ml aliquots were removed for Chl.*a* and AFDM estimations respectively.

Ash-Free Dry Mass

The following steps were taken to determine AFDM:

(1) The desired number of 0.45 micron glass fiber filters (GFF) to be used for the study were rinsed in a vacuum filtration apparatus with deionized water to remove any possible residual particulate material. These GFF were combusted in a muffle furnace at 500^{o}C for 1 hour and stored in a desiccator until needed during NDS retrieval processing.

(2) All material on the FGD surface were scraped off and filtered onto an ashed GFF. The mass of the ashed GFF used for a sample was measured and noted beforehand.

(3) GFF were desiccated in a drying oven set at 100^{o}C for 1 hour. At this point, the dry mass (DM) of sample on the GFF was measured as the mass of this dried GFF with sample subtracted by the noted mass of the ashed GFF.

(4) Dried sample and GFF was then placed into a muffle furnace for 1 hour set at 500^{o}C. Mass was then measured. By subtracting this mass from the DM, AFDM is thus calculated.

Note that the AFDM values measured using this method on days 6, 18, and 29 were doubled to achieve an estimate of total AFDM for a sample since only half of the homogenized sample was used to estimate AFDM on these days.

Chlorophyll a

Alcohol solvents, such as ethanol in particular, have proven to be more efficient solvents than acetone at extracting pigments from algae (Holm-Hansen and Reimann 1978, Riemann and Ernst 1982, Sartory 1982). Although grinding can assist with extractions in acetone (Marker 1972), it may not compensate for the poor extraction efficiency of acetone (Biggs and Kilroy 2000). Additionally, grinding is an unnecessary additional step not required for extraction in ethanol (Sartory and Grobbelaar 1984). Therefore, the ethanol extraction method, as described in detail by Biggs and Kilroy (2000), was followed for extraction and determination of Chl.*a* content.

% Chlorophyll a

An autotrophic index (AI) is sometimes applied to datasets that contain measures of AFDM and Chl.*a* of samples, and is simply the value of AFDM (mg cm^{-2}) divided by Chl.*a* (mg cm^{-2}) (APHA et al 1992). A low AI would be indicative of a higher autotrophic composition in a sample with high viability (APHA et al 1992). Since Chl.*a* amount per AFDM is of interest in this study, we opted to utilize %Chl.*a* as a more intuitive approach. %Chl.a is simply (mg Chl.*a* cm^{-2} / mg AFDM cm^{-2}), or the inverse of AI, multiplied by 100%.

Alkaline Phosphatase Activity: Fluorometric Technique

A classic method utilized for determination of APA in algal communities involves measure of fluorescence (Healey and Hendzel 1979), caused by phosphate cleavage of a compound which yields a highly fluorescent molecule (Figure 2.1). The alkaline phosphatase substrate used was 4-methylumbelliferyl phosphate (MUP). The

dephosphorylated product, 4-methylumbelliferone (MU), is highly stable and fluorescent (Fernley and Walker 1965). In this study, APA was determined using a method based on Healey and Hendzel (1979).

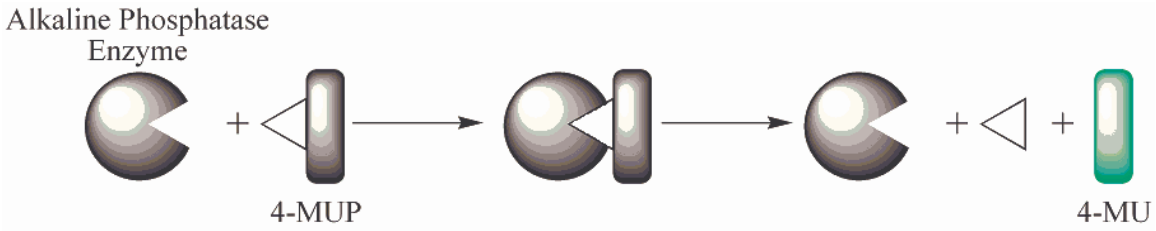

Figure 2.1. Cleavage of phosphate from 4-methylumbelliferyl phosphate (MUP) yielding the fluorescent dephosphorylated product, 4-methylumbelliferone (MU).

The following procedure outlines this method as utilized in this study:

(1) Phosphatase Calibration. An MU stock solution is made by dissolving 10 mg MU in a 1.2% Tris buffer solution (=12 g L^{-1} Tris in deionized water; hereon Tris) in a 1 L flask (= 10 mg L^{-1} MU stock). A 1 mg L^{-1} MU standard is made by diluting the stock with more Tris. From this standard, the following calibration standards (in mg L^{-1}) are made with Tris: 0.00, 0.10, 0.25, 0.50, 0.75, and 1.00. Once fluorescence of each is measured (we used a Turner 10-AU Fluorometer), a graph is made by plotting fluorescence on the y-axis and MU (mg L^{-1}) on the x-axis. A linear regression is applied to the plot. With effective calibration (ie. evidenced by a high r^2 value), the linear equation provided by the regression is used to estimate the concentration of MU using the fluorescence values measured from the samples.

(2) *MUP solution*. A 0.2 mM MUP (or 2 x 10^{-4} M MUP) solution is desired. Therefore, to a 250 ml volumetric flask, 13.26 mg MUP is diluted to mark with 1.2% Tris buffer solution and dissolved.

(3) *Sample Processing*. A 10 ml volume of site water and 30 ml 1.2% Tris buffer solution is added to an empty Micromate® Popper® 50 ml syringe with a closed 3-way stopcock with Luer lock attached. Then, the sample FGD (with periphyton community attached) is carefully placed inside the syringe with the effort of minimizing disturbance. At this point, gas should be released out of the syringe by opening the stopcock and carefully pushing it out the syringe. To begin the analysis, the syringe is injected with 4 ml of the 0.2 mM MUP solution. The injection time is recorded. Approximately 5 ml of the syringe solution is released into a test tube shortly after injection. Fluorescence and time are recorded. The fluorescence and time are measured again in the same way periodically (at least two more times) over a 30-45 minutes period. Numerous samples are allowed to run in a staggered fashion in the process.

(4) *Calculation of APA*. The fluorescence reading obtained at each elapsed time is converted to an estimate of the concentration of MU (mg L^{-1}) using the linear equation determined in the phosphatase calibration. Time readings are converted to the number of minutes elapsed (i.e. minutes following injection of MUP). The estimated concentrations of MU are then plotted against the number of minutes elapsed. A line through the plot should generally be straight (Healey and Hendzel 1979), and the slope of this line (in units of mg MU $min.^{-1}$) is an estimate of APA for a sample. However, we have found that a straight line will not effectively be approximated when saturation occurs. In this case, the point(s) past an arbitrary point of saturation can be dropped to achieve a better estimate. Alternatively, the points can be kept, and maximum APA rate estimation can be utilized. This would require the use of a nonlinear regression and determination of the maximum slope on the curve. If the latter APA slope estimation method is chosen for

handling saturation, it should be used for the entire dataset and reported. In this study, we chose to drop the point(s) past an arbitrary point of saturation using a rule-of-thumb: when $r^2 < 0.95$ in the linear equation, the point was dropped to improve this value.

N_2 Fixation Potential: Acetylene-Reduction Technique

A classical method for determination of nitrogen fixation potential in algal communities is the acetylene-reduction technique (Flett et. al 1976). Although the nitrogenase enzyme normally functions to fix dinitrogen (N_2) gas (Figure 2.2), the acetylene-reduction technique takes advantage of the fact that the nitrogenase enzyme can also reduce acetylene to ethylene (Figure 2.3).

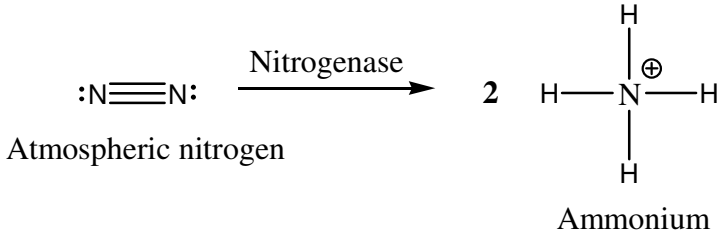

Figure 2.2. Chemical transformation of atmospheric nitrogen to ammonium by nitrogenase.

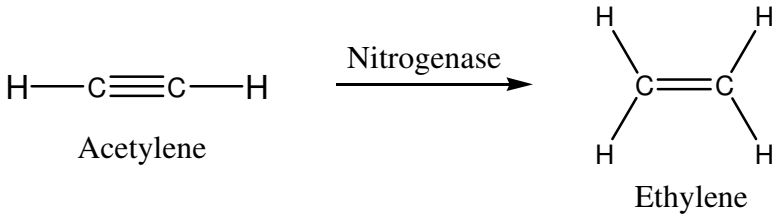

Figure 2.3. Chemical transformation of acetylene to ethylene by nitrogenase.

FGD were transferred into Popper® Micromate 50-ml syringes with a closed 3-way stopcock with Luer lock attached along with 30-ml of site water. The syringes were inoculated with 5 ml acetylene and gently mixed to allow rapid dissolution of acetylene

gas as in Scott et al. (2005). Syringes were placed in randomly assigned positions into a photosynthetically active radiation incubation apparatus for approximately 5 hours. Dark incubated replicates (n = 2) were utilized only to confirm light stimulated N_2 fixation rates. All rates were corrected to plankton N_2 fixation rates also measured (n = 2). The remainder of the acetylene reduction method follows that of Flett et al. (1976), thus will not be described in further detail. This technique requires the use of a gas chromatographer (GC) to measure ethylene production. We used a Carle® AGC Series 100 for gas chromatography.

Data Analysis

PROC TTEST with PAIRED statement was used to perform a paired t-test in SAS 9.1.3 (SAS Institute Inc. 2004).to compare water-column NO_3-N and PO_4-P concentrations upstream from the NDS rack with those downstream from the rack.

Agar depletion analyses of NO_3-N and PO_4-P from the respective N and P treatment agar were performed using nonlinear regression models of nutrient concentration plotted against days of retrieval in SigmaPlot 9.0 (Systat Software Inc. 2004).

Accuracy and precision of the more time-efficient method of measuring APA and N_2 fixation on same-sample FGD was estimated by regressing mean APA following N_2 fixation (hereafter, APA(N_2 fixation)) against APA in SigmaPlot 9.0. Means were paired by nutrient treatment (control, N, and P) and date (6, 18, and 29) and weighted by 1/y to account for increasing variance as mean APA values increased (Draper and Smith 1998).

A two-factor analysis of variance (ANOVA in PROC GLM) using day of retrieval and nutrient treatment as factors was performed on each of the following response

variables: AFDM, Chl.*a*, % Chl.*a*, N_2 fixation potential, and APA. Post hoc comparisons were performed using LSMEANS with Tukey adjustments. Each normalization option was utilized in separate tests, and evaluated for its ecological applicability and effectiveness. Since two Chl.*a* samples were lost during the centrifugation step of the ethanol extraction method, post hoc comparisons were performed by the Tukey-Kramer adjustment of LS MEANS, which like the Tukey adjustment, also has an experiment-wise error rate. Due to this occurrence, any data normalized to or utilizing Chl.*a* in its dimensions also had to alternatively utilize the Tukey-Kramer adjustment in post hoc comparisons of LS MEANS. Type III sums of squares calculations in PROC GLM can handle datasets with unequal sample sizes (SAS Institute Inc. 2004), thus no alternative ANOVA procedure needed to be utilized.

Because group variances tended to increase with mean response magnitude, log transformations were applied to most datasets before respective statistical analyses. Prior to log-transformation, each of these datasets were anchored with the minimum data value at 1.0 by adding or subtracting a constant to the dataset utilized in any data analysis. This technique standardizes the effect of various transformations (Osborne 2002) and keeps the resultant log transformations positive.

Results

Water Chemistry and Temperature

Mean concentrations of NO_3-N during the study period were significantly higher downstream from the NDS rack during the study period ($t_{0.05,4} = 2.46$, $p = 0.03$; Table 2.1). , TN, TP, and PO_4-P concentrations were not significantly higher downstream at α

= 0.05 ($t_{0.05,4}$ = 1.70, p = 0.08), but means of these three parameters were elevated relative to the upstream location (Table 2.1). Water temperature averaged 25.5°C with a range of 23-28°C.

Table 2.1. Water column nitrogen and phosphorus content upstream (= ambient) and downstream from the NDS rack, and results of a paired t-test of the respective means (all n = 5).

	Mean ± 1 S.E. in (µg L^{-1})			
Analyte	Upstream	Downstream	paired t	one-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_3-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

Field Diffusion Rates of Nitrogen and Phosphorus

Mean (± S.E.) agar concentrations of NO_3-N and PO_4-P plotted with different forms of exponential loss equations for the N and P treatments respectively both yielded equations with greater than 99% reduction in squared prediction error, indicating that the equations produced are highly accurate equations for agar nutrient concentration over the 29 day study period (Figure 2.4). NO_3-N concentrations of the control and P treatment were close to zero (Figure 2.4a). PO_4-P concentrations of the control and N treatment were also low, though the PO_4-P concentrations of the N treatment appear to have trended slightly higher than the control (Figure 2.4b).

The first derivatives of the prediction equations (Figure 2.4a,b) predict the release rates in units of g L^{-1} d^{-1} for NO_3-N and PO_4-P of the N and P treatments respectively. These equations were converted to prediction equations of the nutrient release rates in units of mg cm^{-2} d^{-1} of an NDS. The resultant equations are displayed in the release rate plots in Fig.2.5. The predicted release rate of NO_3-N from the N treatment falls by >50%

by day 2, but the falling release rate becomes a more gradual decline after day 3, still releasing 2,875 µg NO_3-N cm^{-2} d^{-1} on day 6, 1,230 µg NO_3-N cm^{-2} d^{-1} on day 18, and 569 µg NO_3-N cm^{-2} d^{-1} on day 29 (Figure 2.5a). The release rate of PO_4-P from the P treatment does not have the same rapid initial decline, and, on the same respective days, had predicted release rates of 410, 255, and 178 µg PO_4-P cm^{-2} d^{-1} (Figure 2.5b).

Alkaline Phosphatase Activity following acetylene-reduction vs. analyzed alone

APA measured following acetylene reduction (N fixation potential) was positively correlated to APA measured without the intervening step of estimating N fixation (Figure 2.6). The r^2 values ranged from 0.86-0.91, depending upon areal or biomass standardizations. However, APA following acetylene reduction exhibited greater variability among FGDs of the same treatment than did APA alone. Moreover, APA following acetylene reduction was either higher (when standardized to Chl.*a* or AFDM) or lower (when standardized to surface area) than APA alone, suggesting that the acetylene reduction procedure was influencing APA in an inconsistent manner, possibly through physical disturbance of the outer layer of attached mat. Because of the greater variability, moderate error (~10-15%) and inconsistent slope following acetylene reduction, APA values that were not subjected to acetylene reduction were used in subsequent analysis of nutrient treatment effects.

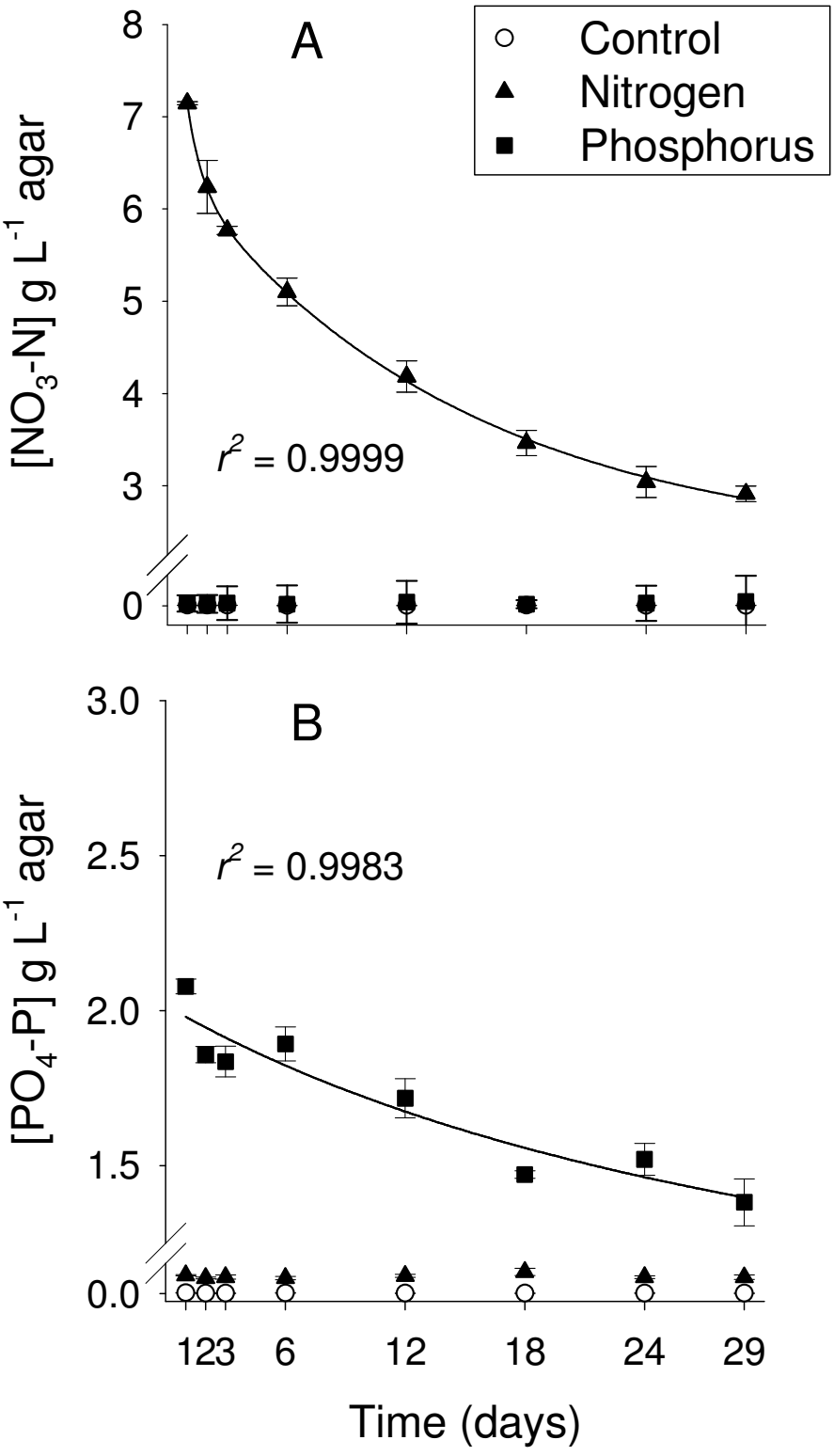

Figure 2.4. Mean (± S.E.) agar concentrations of a) NO_3-N and b) PO_4-P plotted against time of the study period. The double, 5 parameter exponential loss equation $f(x) = 2.3063 + 3.417e^{(-1.3458x)} + 4.2387e^{(-0.0701x)}$ was applied to a), and the modified single, 3 parameter exponential loss equation $f(x) = 0.6216e^{(75.299/(x+63.9791))}$ was applied to b).

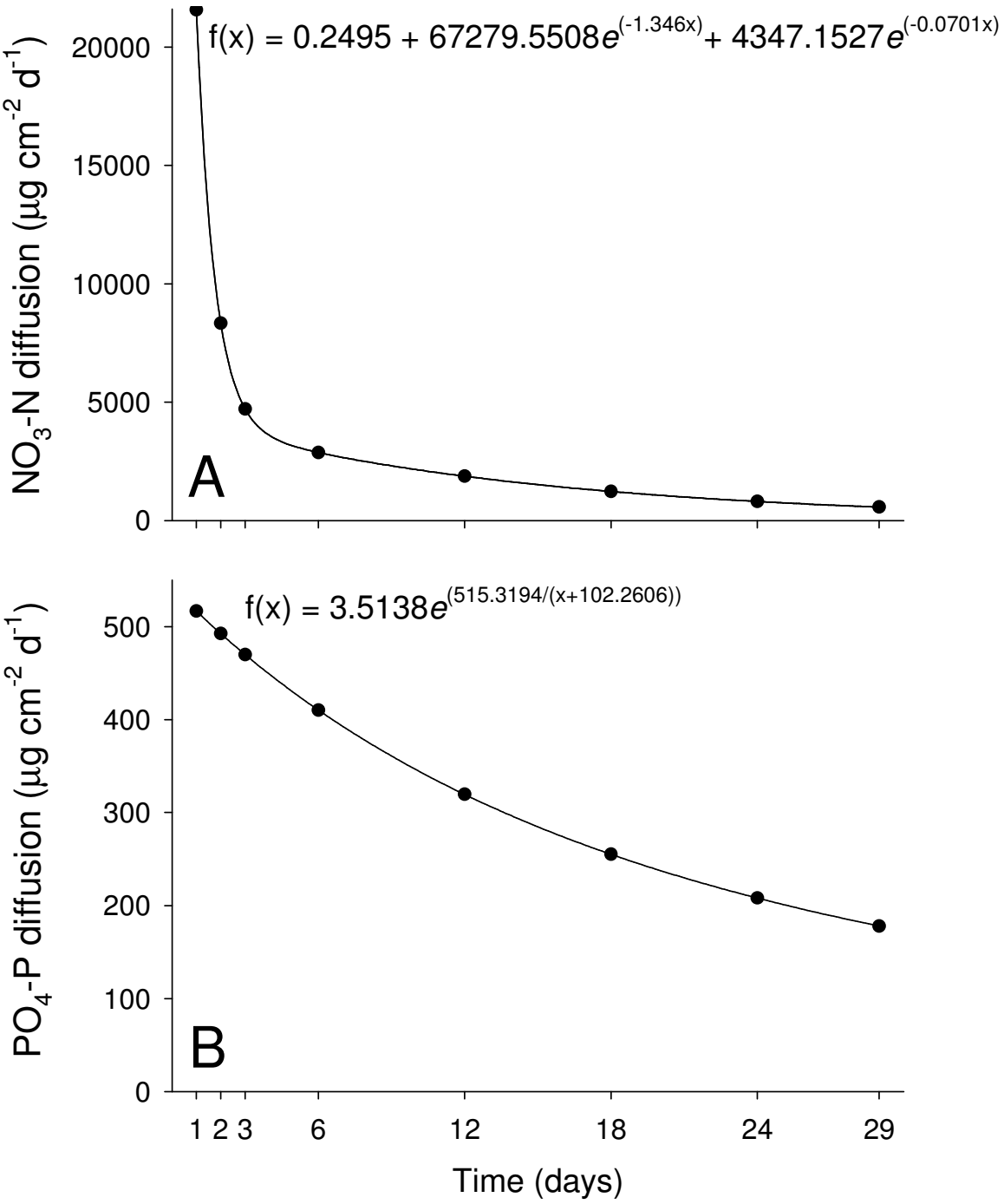

Figure 2.5. NDS nutrient release rates of a) NO_3-N and b) PO_4-P of the N and P treatments respectively. First derivatives of equations in Fig.8a,b were converted to prediction equations of the nutrient release rate (µg cm^{-2} d^{-1}) of an NDS. The resultant plots and equations are displayed below.

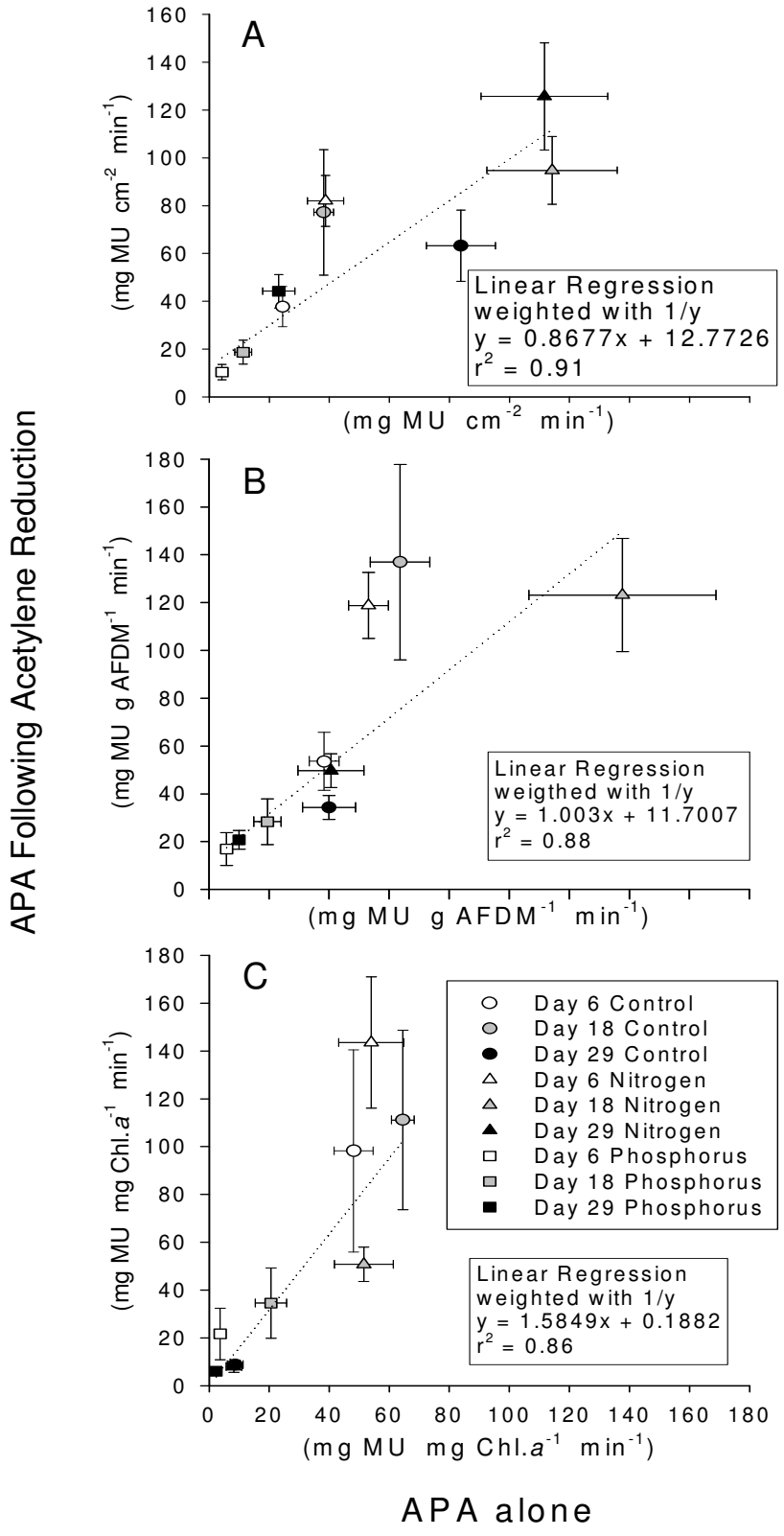

Figure 2.6. Plots of paired means (± S.E.) of APA following the acetylene-reduction technique against APA measured alone with linear, 1/y weighted regression equations and r^2 values. Plots are of APA normalized to a) surface area, b) AFDM, and c) Chl.*a*.

Effects of N and P Enrichment on Periphyton Accrual

Periphyton accrual measured as log-transformed, anchored (LTA) AFDM increased during the study period ($p < 0.0001$), and this trend is also observed in the untransformed means (± SE) displayed in Figure 2.7. However, there was no significant effect by treatment ($p = 0.2751$), and no significant interaction ($p = 0.9056$). Accrual from days 1 to 6 remained somewhat linear and equivalent for all treatments. Using combined treatment untransformed means on days 1 and 6, the accumulation rate of AFDM was 0.1197 mg AFDM cm^{-2} d^{-1} from days 1-6. Calculating this rate again in the same manner from days 24-29, the accumulation rate of AFDM was 0.2197 mg AFDM cm^{-2} d^{-1}.

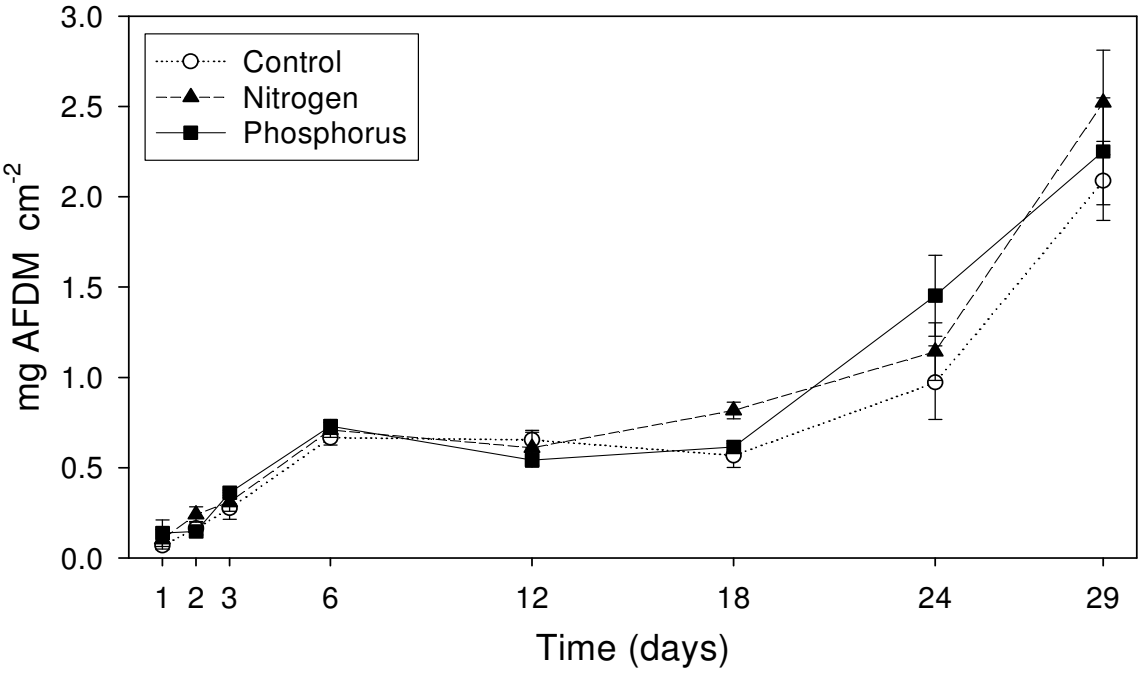

Figure 2.7. Periphyton accumulation measured as mean (± S.E.) surface area specific AFDM of each treatment over the number of days following deployment. A two factor ANOVA from log transformed anchored values indicated a significant main effects of days ($F_{7,129} = 92.88$, $p < .0001$), but no significant main effect of treatment ($F_{2,129} = 1.30$, $p = 0.2751$) and no significant interaction ($F_{14,129} = 0.54$, $p = 0.9056$).

Periphyton accrual results in the form of LTA % Chl.*a* and surface-area specific Chl.*a* are displayed in Figure 2.8a,b respectively. On days 18 and 29, the N treatment had significantly greater % Chl.*a* than the control and P treatment. Percentage Chl.a also more than doubled from retrieval day 18 to 29, indicative of a shift to greater overall autotroph abundance by day 29. Overall, surface-area specific Chl.*a* also more than doubled from retrieval day 18 to day 29, which coincides with the observations mentioned above for periphyton accumulation measured as AFDM. Unlike AFDM, there was a significant interaction between retrieval day and treatment in Chl.*a* ($F_{4,97} = 5.12$, $p = 0.0009$). The treatments did not significantly differ on day 6, but by day 18, the addition of N stimulated Chl.*a*. Chl.*a* of the N treatment remained significantly higher than the P treatment on day 29, though it was no longer statistically higher than the control.

Effects of N and P Enrichment on APA and N_2 fixation

Interactions between days and treatments significantly affected APA when normalized to AFDM or Chl. *a* (both respective $p < 0.05$), though the significance level of this interaction for APA normalized to surface area was also low ($p = 0.0734$; Figure 2.9). P enrichments always decreased APA response on each day of retrieval, regardless of the normalization option, with the exception of day 18 normalized by Chl a (Fig.2.9c; N to P comparison: $p = 0.0980$). On day 18, the N treatment stimulated areal APA (Fig.2.9a). APA standardized by area increased over time across all treatments, and this trend was most apparent in the P treatment. When normalized to AFDM or Chl.*a* (Figure 2.9b,c), APA time series were generally the same, except there was no significant

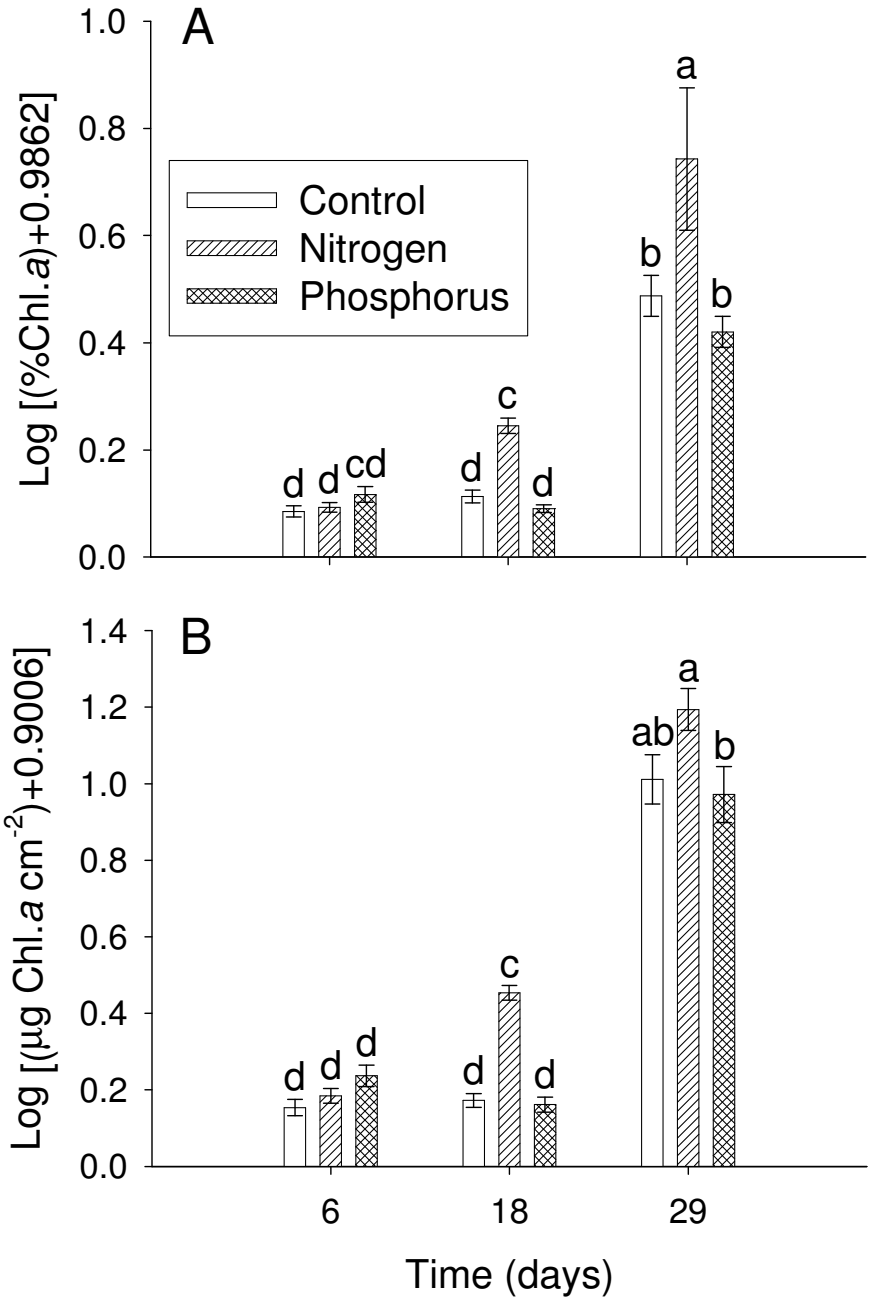

Figure 2.8. a) Means (± S.E.) of log-transformed anchored values of %Chl.*a* of each treatment on retrieval days. The two factor ANOVA indicated significant main effects of days ($F_{2,97}$ = 124.28, p < .0001) and treatment ($F_{2,97}$ = 10.68, p < .0001) and a significant interaction ($F_{4,97}$ = 3.60, p = 0.0088). Same letter indicates no significant difference in Tukey-Kramer adjusted post hoc comparisons of LSMEANS. b) Means (± S.E.) of log transformed anchored values of surface area specific Chl.*a* of each treatment on retrieval days. The two factor ANOVA indicated a significant main effects of days ($F_{2,97}$ = 406.32, p < .0001) and treatment ($F_{2,97}$ = 14.91, p < .0001) and a significant interaction ($F_{4,97}$ = 5.12, p = 0.0009). Same letter indicates no significant difference in Tukey-Kramer adjusted post hoc comparisons of LSMEANS.

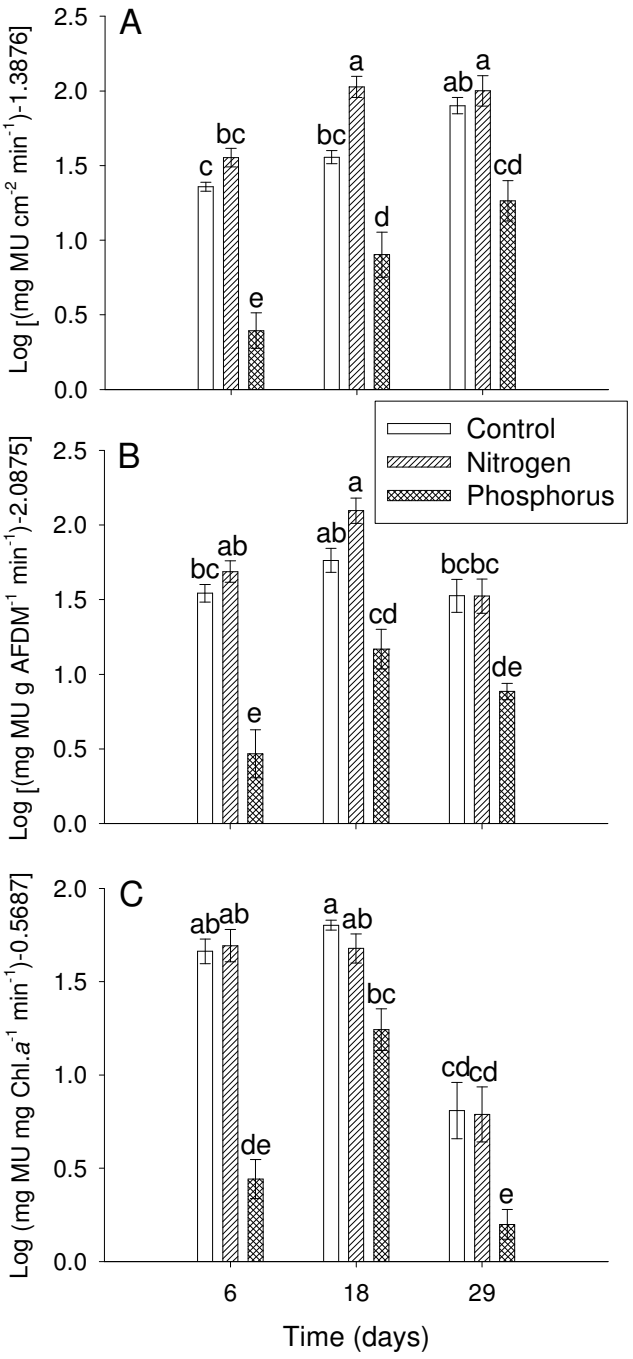

Figure 2.9. Means (± S.E.) of log-transformed anchored values of APA measured alone normalized to a) surface area, b) AFDM, and c) Chl.*a* of each treatment on retrieval days. The two factor ANOVA of a) indicated significant main effects of days ($F_{2,36}$ = 32.99, p < .0001) and treatment ($F_{2,36}$ = 91.67, p < .0001) and an interaction not significant at α = 0.05 ($F_{4,36}$ = 2.34, p = 0.0734), b) indicated significant main effects of days ($F_{2,36}$ = 15.97, p < .0001) and treatment ($F_{2,36}$ = 70.68, p < .0001) and a significant interaction ($F_{4,36}$ = 2.92, p = 0.0343), and c) indicated significant main effects of days ($F_{2,35}$ = 71.39, p < .0001) and treatment ($F_{2,35}$ = 57.17, p < .0001) and a significant interaction ($F_{4,35}$ = 5.19, p = 0.0022). Same letter indicates no significant difference in Tukey adjusted post hoc comparisons of LSMEANS (in c), Tukey-Kramer adjustments were used).

decrease in APA of the control and P treatment time series from days 24 to 29 when normalized to AFDM.

N_2 fixation was significantly affected by an interaction between retrieval day and treatment, regardless of normalization ($p < 0.001$; Figure 2.10). On day 6, no treatment differed. However, N_2 fixation of the N treatment was significantly lower days 18 and 29 relative to the control and P treatments. However, there was no stimulation of N_2 fixation by the P treatment relative to the control and the P treatments actually trended towards lower N_2 fixation rates than controls on days 18 and 29 (Figure 2.10). Chl.*a* normalized rates trended down with time between days 18 to 29. Although AFDM and Chl.*a* normalized rates were generally the same, they did differ in one aspect. In AFDM normalized rates, there were no significant time series decreases in control and P treatment N_2 fixation rates from days 18 to 29.

Discussion

Field diffusion rates of NO_3-N and PO_4-P from respective N and P treatments of this novel NDS design were effectively approximated in this experiment. Fitted exponential decay functions predicted that by days 18 and 29, release rates of the N and P treatments had fallen to 1,230 and 569 µg NO_3-N cm^{-2} d^{-1} and 255 and 178 µg PO_4-P cm^{-2} d^{-1} respectively from release rates of 21,565 µg NO_3-N cm^{-2} d^{-1} and 517 µg PO_4-P cm^{-2} d^{-1} at day 1 of deployment. Thus, diffusion was still a substantially active process throughout the study period, and may substantiate the use this NDS design in longer term studies. However, the ratio of N:P released apparently decreases with time, thus treatments of N+P utilized in studies will experience a decreasing ratio of N and P

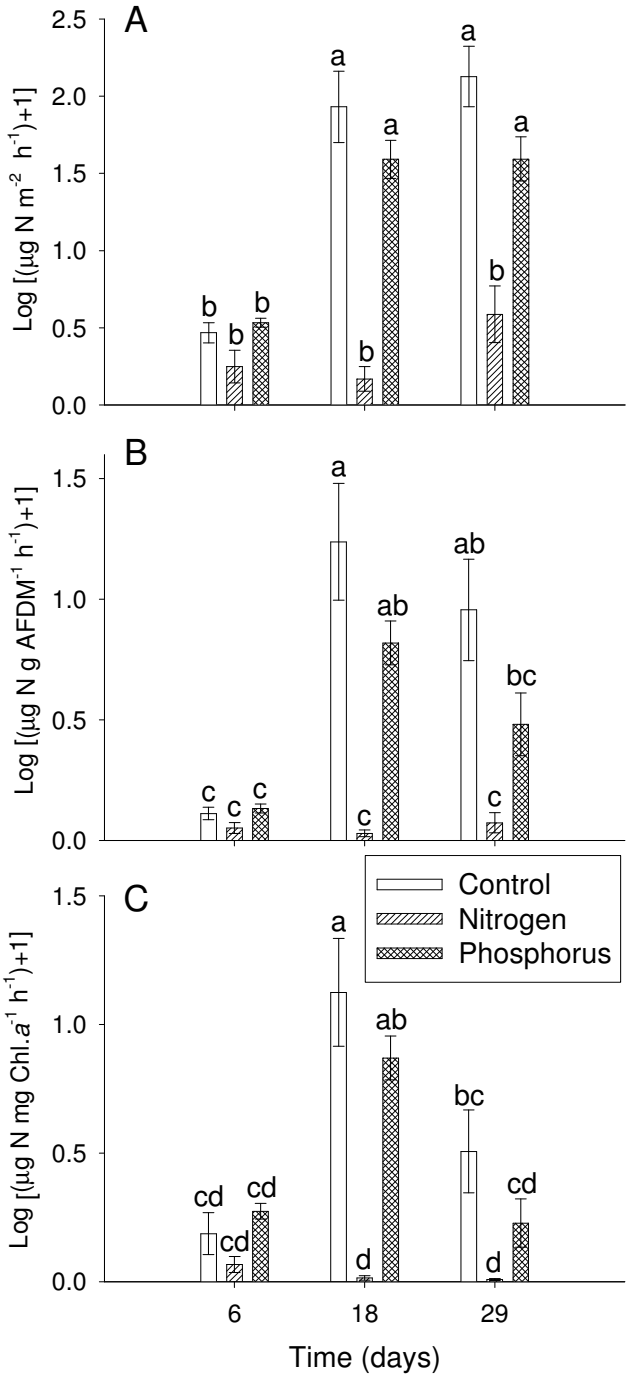

Figure 2.10. Means (± S.E.) of log-transformed anchored values of N_2 fixation normalized to a) surface area, b) AFDM, and c) Chl.*a* of each treatment on retrieval days. The two factor ANOVA of a) indicated significant main effects of days ($F_{2,36}$ = 42.67, p < .0001) and treatment ($F_{2,36}$ = 55.52, p < .0001) and a significant interaction ($F_{4,36}$ = 9.14, p < .0001), b) indicated significant main effects of days ($F_{2,36}$ = 19.06, p < .0001) and treatment ($F_{2,36}$ = 26.75, p < .0001) and a significant interaction ($F_{4,36}$ = 6.27, p = 0.0006), and c) indicated significant main effects of days ($F_{2,35}$ = 21.42, p < .0001) and treatment ($F_{2,35}$ = 24.18, p < .0001) and a significant interaction ($F_{4,35}$ = 6.75, p = 0.0004). Same letter indicates no significant difference in Tukey adjusted post hoc comparisons of LSMEANS (in c), Tukey-Kramer adjustments were used).

reduced APA, thus the effects of an N+P treatment would likely yield these same reductions in response in studies of this duration. However, in long term studies, this issue of N:P release ratio may become increasingly important with deployment duration, particularly in N+P treatments, and the plausible implications on N_2 fixation rates and APA as time progresses in N+P treatments are intriguing. With this NDS design, the effects of nutrient enrichment could be evaluated on many different types of longer term issues of interest, such as nutrient recycling, successional patterns of community composition and structure, and stoichiometry.

In this experiment, we attempted to minimize any differential exposure of periphyton to nonnutrient factors by entering NDS into a completely randomized order in a slot number assigned NDS rack. However, by doing so, the results of water column nutrient content comparisons of upstream and downstream water samples suggest that NDS may have been exposed to elevated concentrations of NO_3-N, and quite possibly PO_4-P, though this result was not significant (Table 2.1). Because the downstream water samples were taken up to 0.5 m downstream from the rack, it is also likely that nutrient exposure of an NDS could have been even higher than these downstream samples depending on the proximity of other NDS, and, of particular importance, what the treatment type was of neighboring NDS. However, since there were measurable differences in the response variables, it is clear that nutrient exposure was substantially influenced by the assignment of the NDS treatment itself. Moreover, the much greater magnitude of concentrations of the NDS treatment relative to the slight elevations in ambient water caused by enrichment of the surface water from neighboring NDS units suggest that downstream exposure probably had a minor influence on results.

Nevertheless, the effect of differing ambient nutrient concentrations and ratios on APA and N_2 fixation is of great importance in the context of numerical nutrient criteria and improving our understanding of nutrient enrichment and ecosystem processes. Evaluating the complementary use of these nutrient limitation indicators is an area in need of future research.

I evaluated the relationship between paired mean values of APA following acetylene reduction (i.e., APA(N_2 fixation)) and APA measured alone for each normalization option (surface-area, AFDM, and Chl.*a*), and found that APA(N_2 fixation) values become too inaccurate and increasingly variable with increasing response magnitude. It is expected that there would be a positive correlative relationship between the two measures, but a prediction equation that could be utilized to correct for any differences in APA would have needed to be highly accurate to be reliable. Therefore, based on these results, APA values measured after the acetylene reduction technique are not sufficient for utility when accuracy and precision are high priority. We speculate that this difference in APA could be due to the shaking step of the acetylene reduction technique, which breaks the periphyton mat apart, thus resulting in a condition not representative of that found in nature.

Periphyton accrual in the form of surface-area specific AFDM was not a sensitive indicator of nutrient limitation. AFDM did increase during the study period ($p < 0.0001$), but there was no effect of treatment. There appear to have been two primary stages of AFDM accrual. An initial stage of colonization and growth occurred from deployment to day 6, with an accumulation rate of 0.12 mg AFDM cm^{-2} d^{-1} from days 1-6. From a successional standpoint, early colonization such as this often includes base level adnate

diatoms, which can remain attached to substrates even following a sloughing event (Johnson 1996, Tuchman 1988).

Following day 6, periphyton accrual appeared to enter a plateau-period lasting until at least day 18. This plateau coincided with a noted grazer presence observed by day 12. Grazers have been shown to affect periphyton nutrient content (Evans-White and Lamberti 2005), constrain periphyton biomass (Power 1992, Rosemond et al. 2000), and alter community composition (McCormick and Stevenson 1989, McCormick and Stevenson 1991). However, grazers were not quantified in this study, thus additional studies incorporating the effect of grazers and nutrient enrichments on N_2 fixation and APA could prove useful in understanding how these factors could interplay. It would also be interesting to investigate the potential for selective grazing on these NDS based on nutrient content or differing species assemblages caused by nutrient enrichment.

Accumulation again became highly active from days 24-29, with a substantial accumulation rate of 0.2197 mg AFDM cm^{-2} d^{-1}. *Cladophora* presence was noted by day 24, and this rate likely corresponds with the increased *Cladophora* growth from days 24-29. As succession occurs in benthic algal communities, low-profile species become topped with upright or stalked species (Steinman 1996). Filamentous algal species, such as *Cladophora*, can emerge from the mat, thus occupying the upper level (Steinman 1996). This final accumulation stage observed in this experiment appears to fit this latter successional pattern of filamentous algal growth.

Periphyton accrual in the form of surface-area specific Chl.*a* did respond to N enrichment. Combined with the results of % Chl.*a*, which was also stimulated by N enrichment, this suggests that N enrichment primarily stimulated autotrophic growth.

Filamentous green algae, such as *Cladophora*, can become a nuisance in nutrient-rich streams (Welch et al. 1989, Biggs 1985, Wharfe et al. 1984). Time series increases from days 18-29 in %Chl.*a* and surface-area specific Chl.*a* are likely a result of observed *Cladophora* presence in this nutrient-rich, stream-like wetland outflow.

Since APA results were different when periphyton where analyzed following the acetylene-reduction assay, only those results of APA measured alone were utilized for comparison of periphyton APA. P enrichment almost always decreased APA response on each day of retrieval, regardless of the normalization option. On day 18, N enrichment stimulated areal APA, suggesting that the N-limited component of the periphyton community may have been stimulated by N enrichment to a point where P became limiting. In the areal rate, APA of the P treatment increased over time, likely reflective of increasing P limitation with decreasing P diffusion. APA of the N treatment was never significantly higher when normalized to AFDM, though the trend remains. Although similar in overall trends with AFDM normalized APA, when normalized to Chl.*a*, the time series from days 18-29 showed a significantly reduced response, likely due to increasingly abundant *Cladophora* presence.

The normalization of APA to Chl.*a* is probably the least ecologically valid normalization option of the three. Periphyton communities are not entirely autotrophic, and recent research has shown that bacteria can function to provide a substantial amount of the APA in periphyton (Sharma et al. 2005). One argument for the use of surface area as a normalization option is that it is unlikely that substrates can penetrate far into a periphyton mat during incubation (Newman et al. 2003). However, FGD in this study were not always homogeneously covered by periphyton, especially in earlier retrievals.

For heterogeneously covered, thin periphyton mats, such as in this study, AFDM is at least equally valid, if not a better normalization option since AFDM also accounts for biomass of the entire community. It is well understood that periphytic algae often have a spatially heterogeneous distribution within and on substrata (Round and Happey 1965, Cattaneo 1978, Pringle 1985, Cazaubon and Loudiki 1986, Miller et al. 1987, Sinsabaugh et al. 1991, Goldsborough 1994). However, if surface-area specific APA were chosen to be utilized using this NDS design, perhaps due to thick, fairly homogeneous periphyton coverage, it may be safe to assume that FGD surfaces are relatively similar, thus colonization differences would not be based on differences in substrata.

N_2 fixation rates also served as an effective indicator of nutrient limitation. N_2 fixation potential was low in all normalization options and treatments on day 6 (Figure 2.10), perhaps indicative that sufficient growth had not occurred for allotment of resources to such a metabolically expensive process. Under every normalization option, the N treatment always decreased N_2 fixation by days 18 and 29, indicative that N enrichment had capably suppressed N limitation within the periphyton community. There was never a P stimulation of N_2 fixation beyond the control, though both of their rates were typically higher than the N treatment. Interestingly, the P treatments actually trended towards lower N_2 fixation rates than the control on days 18 and 29. It is possible that the absence of P stimulation of N_2 fixation potential in this study was attributed to luxury uptake and storage of P, as speculated before (Horne and Commins 1987).

All normalization options demonstrated similar differences in N_2 fixation on retrieval days 6 and 18, but N_2 fixation normalized to Chl.*a* had comparably lower respective rates by day 29 than the other normalization options (Figure 2.10c). Most

aquatic N_2 fixation is performed by Chl.*a* containing organisms such as cyanobacteria (Vitousek et al. 2002) and diatoms with endosymbionts of cyanobacterial origin (Prechtl et al. 2004, Steiner et al. 2002). Thus, Chl.*a* is also evidenced here as the more sensitive normalization option for measures of N_2 fixation potential.

APA and N_2 fixation proved to be sensitive indicators of nutrient limitation, and showed greatest separation in response between treatments on the day 18 retrieval. N_2 fixation rates were effective in demonstrating N limitation of periphyton grown on controls and P treatments, and APA rates were effective in demonstrating P limitation of periphyton grown on controls and N treatments. Indirectly, this suggests that the periphyton communities were both N and P limited, consistent with the belief that a single nutrient limiting paradigm is not entirely valid for application to multispecies algal communities (Francoeur 2001, Borchardt 1996, Tilman et al. 1982).

CHAPTER THREE

Effects of Experimental Nutrient Enrichment on Periphyton Alkaline Phosphatase Activity and Nitrogen Fixation along a Phosphorus Gradient in Four Streams

Introduction

Nutrient pollution can degrade water quality. Excessive inputs of nutrients, such as nitrogen (N) and phosphorus (P), lead to eutrophication, and ecological consequences almost always follow. Eutrophication can result in harmful algal blooms and hypoxia, which can suffocate many forms of aquatic life in freshwater ecosystems. In addition, many algae and other microbes produce harmful and/or bad-tasting compounds, thus further degrading water quality.

Of the nutrients required by algal communities, it is most often N and P that are in shortest supply (Borchardt 1996). In response to N-limitation, capable species may fix atmospheric dinitrogen (N_2) gas. This process is energetically expensive, and will not occur if other sources of available N are in the environment (Howarth et al. 1988). N_2 fixation can be performed by both autotrophs and heterotrophic bacteria. Heterotrophic N_2 fixation typically dominates in sediments of marine and estuarine ecosystems (Howarth et al. 1988, Sundareshwar et al. 2003, Tyler et al. 2003). However, most aquatic N_2 fixation is performed by Chl.*a* containing organisms such as cyanobacteria (Vitousek et al. 2002) and diatoms with endosymbionts of cyanobacterial origin (Prechtl et al. 2004, Steiner et al. 2002).

In response to P-limitation, capable microbes may increase alkaline phosphatase activity (APA). APA has been utilized as an indicator of nutrient status in both classical

studies (e.g. Kuenzler and Perras 1965, Fitzgerald and Nelson 1966, Healey and Hendzel 1979, Bothwell 1985, Jansson et al. 1988) and more recent studies (Bowman et al. 2005, Newman et al. 2003, Kahlert et al. 2002). In the aquatic environment, alkaline phosphatases catalyze the hydrolysis of dissolved organic phosphate esters (Kuenzler and Perras 1965). Although most microbes are capable of directly utilizing APA as a means of overcoming phosphorus deficiency, in periphyton mats, symbioses between bacteria and algae may exist in which cyanobacteria provide carbon to bacteria via photosynthesis and bacteria provide phosphorus via APA (Sharma et al. 2005, Scott and Doyle 2006). In periphyton, bacterial production and photosynthetic production can be positively correlated (Scott and Doyle 2006). However, algal production can increase more rapidly than bacterial production with increasing nutrients (Carr et al. 2005).

Gradients of N and/or P availability can develop in nature as nutrients are depleted along the flow path of water. In the Everglades, a shift from P-limitation to those of other nutrients occurred as distance from nutrient-rich inflows from agricultural canals increased (McCormick et al. 1996). Changes in periphyton community composition were also strongly related to distance from the inflows (McCormick et al. 1996). Similar changes have also been observed in periphytic assemblages along experimental P-depletion gradients in mesocosms (Yangdong et al. 2000). In a mesocosm study in the Everglades, surface-area specific APA decreased in higher P loadings, while productivity rapidly increased (Newman et al. 2003). Trends in lakes have shown that shifts to N limitation usually favor nitrogen fixing organisms (Levine and Schindler 1999). This trend has also been observed in a constructed freshwater marsh (Scott et al. 2005). In

this constructed wetland, N_2 fixation rates were greatest at the outflow, where lower total nitrogen (TN) concentration and lowest N:P ratio was observed (Scott et al. 2005).

In streams, relatively few studies have utilized APA and N_2 fixation rates to study effects of nutrient ratios and concentrations on periphyton. Recently, Marcarelli and Wurtsbaugh (2006) effectively demonstrated that P availability and temperature interplay to control N_2 fixation and periphyton community composition in oligotrophic streams. In another recent study, APA was found to be inversely related to TP and soluble reactive phosphorus concentrations and levels of cellular P (Bowman et al. 2003). APA has also been used as an indicator of P limitation across streams of different watershed land use and geology (Klotz 1985), and can be directly related to the N:P ratios in ambient stream water (Klotz 1992).

Using nutrient-diffusing substrata (NDS), we investigated the effect of ambient N and P concentrations on periphyton accrual, APA, and N_2 fixation using a P gradient of selected stream sites. The objectives of this study were to assess these periphyton response variables under differing ambient nutrient concentrations and nutrient treatments (N, P, N+P, and controls). A set of central Texas stream sites were selected based upon historical ambient P concentrations, which consequentially made the selection of sites into an incidental N gradient as well. Periphyton accrual variables, in the form of AFDM and Chl.a, were quantified. In addition, APA and N_2 fixation rates were estimated. We hypothesized that (i) periphyton accrual of the controls would increase up the P gradient of sites, (ii) greatest periphyton accrual would occur on N+P treatments, though both N and P treatments would have greater biomass accumulation than the control, (iii) APA of the controls would decrease up the P gradient of sites,

(iv) APA of N treatment would be highest and also decrease up the P gradient of sites, (v) APA would be similarly low in both P and N+P treatments, (vi) N_2 fixation potential of the controls would decrease up the P gradient of sites, (vii) N_2 fixation potential of P treatment would be highest and also decrease up the P gradient of sites, (viii) and N_2 fixation potential would be similarly low in both N and N+P treatments.

Methods

Study Region

Stream sites selected for this experiment are located in the Cross Timbers Ecoregion within the Brazos River watershed of Central Texas (Figure 3.1). Four stream sites of similar size, substrate, and canopy cover were selected from this ecoregion to establish a P gradient of sites based on historical data and current monitoring sites (R. S. King unpublished data). In increasing order of historical P concentrations, the stream sites selected were the Paluxy River near Glen Rose, Texas (Site code: PALU-01), the North Bosque River in Meridian, Texas (Site code: NBOS-04), the Leon River near Hamilton, Texas (Site code: LEON-01), and the Leon River in Gatesville, Texas (Site code: LEON-02). Although two Leon River sites were used, the distance between these sites is >65 km, and there are historically large differences in ambient N and P concentrations.

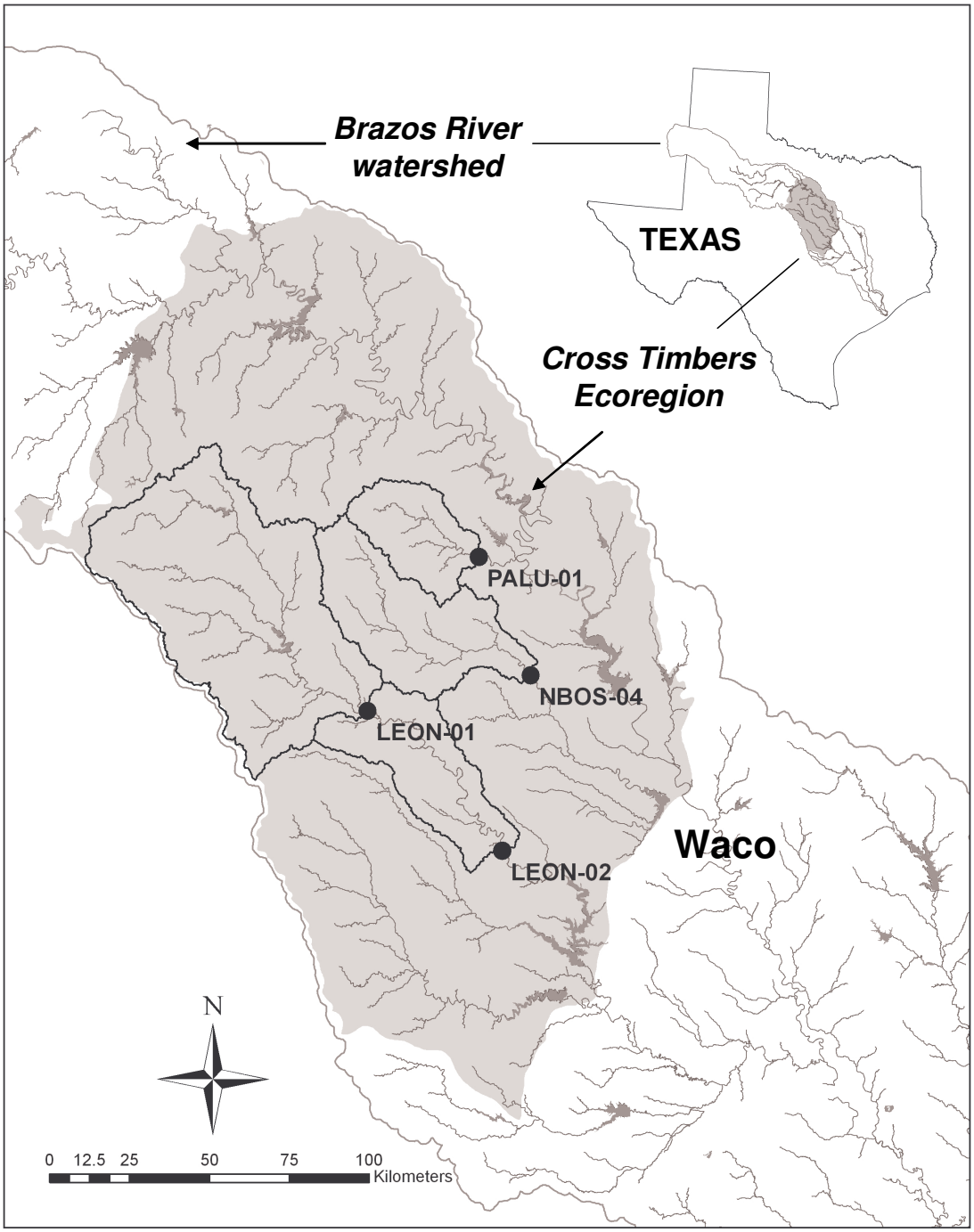

Figure 3.1. Map of study region and the four selected stream locations and their watersheds.

Nutrient-Diffusing Substrata and Rack Design

NDS were constructed based on the design of Gibeau and Miller (1989), but similar to that of Marcarelli and Wurtsbaugh (2006) by use of a FGD. Four large batches of 20 g L^{-1} agar were made. Controls were poured from a batch with no added nutrients. N treatments were made from a batch with 0.5 M $NaNO_3$. P treatments were made from a batch of 0.05 M $Na_2HPO_4 \cdot H_2O$. N+P treatments were made from a batch with additions of both 0.5 M $NaNO_3$ and 0.05 M $Na_2HPO_4 \cdot H_2O$. After being mixed, agar treatments were poured into individual 50 ml centrifuge tubes (Carolina Biological Supply, http://www.carolina.com). The cap of each tube was drilled in the center to produce a 7/8 inch (~ 22 mm) diameter hole. A Danco #42 Cap Thread Gasket and a 25 mm fritted glass disc (FGD) of fine porosity (4 - 5.5 microns) (Wilmad LabGlass, http://www.wilmad.com) were rested atop the rim of the centrifuge tube while submerged in a tub of deionized water at deployment, and capped. Although this thread gasket may not have been a necessary component of the NDS, by applying the gasket, the tighter fit of FGD within may guide diffusion through FGD pores. FGD also provide substrate for the attachment of periphyton.

Racks were constructed to hold NDS using wire poultry netting (Keystone Steel & Wire Red Brand) framed with plant stakes for stability. Although slight, NDS of this design can increase water column nutrient concentrations in proximities <0.5 meters downstream (D. A. Lang unpublished data). Therefore, within the racks, controls were placed upstream, N and P treatments were placed on opposing sides downstream from the controls, and N+P treatments were positioned at the downstream end to minimize contamination (Tate 1990). For each rack, four anchors were constructed to hold the rack

in place under field conditions. Each anchor consisted of a garden pavestone with an attached garden sprinkler pipe section. Multiple pipe section lengths were on-hand in the field so that an appropriate length could be used in consideration of depth and evenness of site substrate. Each rack corner was attached by steel wire at a distance of ~30 cm to the upper pipe section of an anchor.

Environmental Factors

Rack deployment locations were chosen that minimized differences in light intensity, canopy cover, substrate type, and water velocity. Since light can affect algal growth and consequential nutrient demand (Tett et al. 1985, Goldman 1986, Sterner et al. 1997), racks were deployed where no canopy cover was detectable by densiometer, and submerged at 15 cm depths. These 15 cm depths were achieved by adjusting respective rack to anchor attachments. Maximum light intensities were measured at deployment depth at each site during the respective deployment period to confirm similarly experienced light intensities. Substrate type at site deployment locations were largely sand with <50% gravel. Although there was no to low flow at each site during the study period, positions within pools were chosen that had no detectable velocity at the racks' upstream facing edge, which not only kept experienced velocity relatively similar, but might provide some level of buffering to changes in flow that can occur from events such as rainstorms while deployed.

Study period total nitrogen (TP), total phosphorus (TP), NO_3-N, and PO_4-P concentrations were estimated at day 0, 6, 12, and 18 of the 18 day study period at each site. A Lachat® auto analyzer was used to determine these water chemistry levels. To determine study period water temperature at each site, a HOBO® Water Temp Pro

temperature data logger was attached to each NDS rack at deployment. These temperature data loggers recorded temperature readings every 5 minutes, and were retrieved at the end of a deployment along with NDS. Maximum light intensity (μmols m^{-2} s^{-1}) readings were taken at each site at mid-day during the middle of the study period.. Readings were recorded while there was no cloud cover in front of the sun using a LiCor light meter.

Periphyton Sampling

Ten replicates of each treatment (control, N, P, and N+P) were deployed at each site. Deployment periods of 18 days were implemented 3-days staggered in random order of sites to allow for practical deployment and retrieval processing times. Upon retrieval, NDS were immediately placed in centrifuge tube racks, which were transported back to the laboratory for analyses inside an ice chest lined with a damp towel (no ice). Since transit times varied between 45-90 minutes, drops of filtered site water were carefully given to periphyton every 20-30 minutes to keep the community wet during transport.

Micromate® Popper® 50 ml syringes with a closed 3-way stopcock with Luer lock attached were used for assays of both APA and N_2 fixation potential. Following estimation of APA and N_2 fixation, periphyton were scraped off FGD into its respective assay solution and diluted to 50 ml using deionized water. This 50 ml solution was homogenized using a vortex until well-mixed. Before settling could occur in each NDS, 15 ml and 25 ml aliquots were removed for Chl.*a* and AFDM estimations respectively.

Periphyton Accrual

The following steps were taken to determine ash-free dry mass: (1) 25 ml aliquots were filtered onto pre-ashed, pre-weighed 0.45 micron glass fiber filters (GFF). (2) GFF and sample thereon were desiccated in a drying oven set at 60^{o}C for 24-48 hours. (3) Dried GFF and sample thereon were re-weighed, and placed into a muffle furnace for 1 hour set at 500^{o}C. (4) Final weights were then taken. By subtracting final weights from dried weights in (3), the ash-free dry mass (AFDM) of the 25 ml aliquot was determined. An estimate of total AFDM of a sample was then calculated by doubling this value.

The ethanol extraction method, as described in detail by Biggs and Kilroy (2000), was followed for extraction and determination of Chl.*a* content. Alcohol solvents, such as ethanol in particular, have proven to be more efficient solvents than acetone at extracting pigments from algae (Holm-Hansen and Reimann 1978, Riemann and Ernst 1982, Sartory 1982). Although grinding can assist with extractions in acetone (Marker 1972), it may not compensate for the poor extraction efficiency of acetone (Biggs and Kilroy 2000). Additionally, grinding is an unnecessary additional step not required for extraction in ethanol (Sartory and Grobbelaar 1984).

An autotrophic index (AI) is sometimes applied to datasets that contain measures of AFDM and Chl.*a* of samples, and is simply the value of AFDM (mg cm^{-2}) divided by Chl.*a* (mg cm^{-2}) (APHA et al 1992). A low AI would be indicative of a higher autotrophic composition in a sample with high viability (APHA et al 1992). Since Chl.*a* amount per unit of biomass is of interest in this study, we opted to utilize %Chl.*a* as a more intuitive approach. %Chl.a estimations in this study were calculated as (mg Chl.*a* cm^{-2} mg AFDM cm^{-2}), or the inverse of AI, multiplied by 100%.

Alkaline Phosphatase Activity: Fluorometric Technique

When APA readings are taken after the acetylene-reduction technique for measuring N_2 fixation potential, estimations of actual APA is inaccurate (D. A. Lang, unpublished data). Therefore, replicates at each site had to be split for estimation of APA and N_2 fixation potential. For 5 replicates of each treatment, syringes were filled with a 10 ml volume of site water and 30 ml 1.2% Tris for preparation of the APA assay. Syringes of the other 5 replicates of each treatment were filled only with 30 ml of site water. Then, respective FGD were carefully transferred into the syringes.

APA rates were determined using a method based on Healey and Hendzel (1979). This method involves measure of fluorescence, caused by phosphate cleavage of a compound which yields a highly fluorescent molecule (Healey and Hendzel 1979). The substrate used was 4-methylumbelliferyl phosphate (MUP). Its dephosphorylated product, 4-methylumbelliferone (MU), is highly stable and fluorescent (Fernley and Walker 1965). A calibration equation was utilized to relate fluorescence to the concentration of MU. To begin the analysis, each syringe was injected with 4 ml of the 0.2 mM MUP solution. Fluoresence readings were taken periodically over a 30-45 minute period. A plot of relative MU concentration over time was used to calculate APA in mg MU min^{-1}.

Periphyton communities are not entirely autotrophic, and recent research has shown that bacteria can function to provide a substantial amount of the APA in periphyton (Sharma et al. 2005). Dry mass (DM) has proven to be a suitable for normalization of APA provided there are no problems separating mineral materials (Hernández 1996). AFDM has also proven to be suitable for normalization of APA

(Chapter 2), and is recommended over the use of DM to measure periphyton biomass since silt can be a substantial component of DM (Barbour et al. 1999). When periphyton coverage is thick, surface area may be a better normalization option since it is unlikely that substrates can penetrate far into a periphyton mat during incubation (Newman et al. 2003). However, FGD in this study were not always homogeneously covered by periphyton, and did not grow thicker than ~1mm. Therefore, APA was normalized to AFDM in this study.

N_2 Fixation Potential: Acetylene-Reduction Technique

To begin analysis of N_2 fixation rates, syringes were inoculated with 5 ml acetylene and gently mixed to allow rapid dissolution of acetylene gas as in Scott et al. (2005). Syringes were placed in randomly assigned positions into a photosynthetically active radiation incubation apparatus for approximately 5 hours. All rates were plankton-corrected. The remainder of the acetylene reduction method follows that of Flett et al. (1976), thus will not be described in further detail here. This technique requires the use of a gas chromatographer (GC) to measure ethylene production. We used a Carle® AGC Series 100 for gas chromatography. N_2 fixation rates were normalized to Chl.*a*.

Data Analysis

Separate statistical analyses were performed for response variables surface-area specific AFDM, %Chl.*a*, surface-area specific Chl.*a*, APA normalized to AFDM, and N_2 fixation normalized to Chl.*a* content. Using stream site and nutrient treatment as factors, a two factor ANOVA in PROC MIXED using type III sums of squares was performed on each of the response variables in SAS 9.1.3 (SAS Institute Inc. 2004). Post hoc

comparisons using Tukey adjustments of LSMEANS were utilized to determine where differences occurred in the analyses. P-values less than $\alpha = 0.05$ were considered significant. Additional graphs were made for APA and N_2 fixation response variables plotting response over nutrient concentration to evaluate the pattern of response.

Results

Environmental Factors

Study period environmental factors of ambient nutrient concentrations, water temperature, and maximum light intensity are summarized in Table 3.1. The established P gradient of sites represented the order predicted during selection of stream sites. The sites also spanned an N gradient. Based on TP concentrations, the site order, from low to high P, was PALU-01, NBOS-04, LEON-01, and LEON-02. LEON-02 had extremely high ambient nutrient concentrations. Temperature ranges experienced at each stream site overlapped substantially. Same day maximum light intensity readings at the stream sites ranged from 1297-1481 µmol m^{-2} s^{-1}, and these modest differences almost certainly reflected differences in time at which light intensity was measured at each site.

Table 3.1. Environmental factors at each site. Ambient total nitrogen, total phosphorus, NO_3-N, and PO_4-P concentrations (mean ± 1 S.E.) at each stream site (all n = 4) during the study period. Mean water temperature (range) at each site during the study period. Maximum light intensity at rack deployment depths based on same day measurements.

Factor	PALU-01	NBOS-04	LEON-01	LEON-02
TN (µg N L^{-1}) ± 1 S.E.	278.8 ± 106.2	372 ± 152.3	722.3 ± 81.4	6520 ± 1705.7
TP (µg P L^{-1}) ± 1 S.E.	13.7 ± 1.1	24.2 ± 4.8	40.1 ± 5.2	2402.5 ± 423.3
NO_3-N (µg N L^{-1}) ± 1 S.E.	5.7 ± 1.6	5.1 ± 1.5	299.5 ± 98.1	4460.5 ± 1682.3
PO_4-P (µg P L^{-1}) ± 1 S.E.	0.5 ± 0.3	0.5 ± 0.5	2.2 ± 0.4	1720.5 ± 382.2
Water Temp. (^oC) (range)	16.5 (14-18.5)	17.5 (14-25)	16.1 (14.6-19.6)	16 (15-19.7)
Light Intensity (µmol m^{-2} s^{-1})	1447	1430	1481.0	1297

Periphyton Accrual

There was a significant interaction between site and treatment in surface-area specific AFDM (Fig.3.2). At PALU-01, there was no significant difference between treatments. At NBOS-04, only the N treatment stimulated surface-area specific AFDM above the control. Surface-area specific AFDM of treatments at LEON-01 were not significantly different. However, at the high nutrient site, LEON-02, surface-area specific AFDM of the N+P treatment was significantly lower than all other treatments. At this site, surface-area specific AFDM of the N treatment was not stimulated above the control, but was significantly greater than both the P and N+P treatments. Surface-area specific AFDM of controls also significantly increased up the P gradient of stream sites.

Periphyton accrual results in the form of %Chl.*a* and surface-area specific Chl.*a* are displayed in Figure 3.3a,b respectively. A significant interaction was present between site and treatment in both %Chl.a and surface-area specific Chl.*a* results. N+P enrichment stimulated %Chl.*a* above every other treatment at all sites except LEON-02, where it was actually significantly lower than the control and N treatment. Likewise, the P treatment at LEON-02 was also significantly less than the control and N treatment. N enrichment alone did not stimulate %Chl.a at PALU-01, but did so at NBOS-04 and LEON-01. Surface-area specific Chl.*a* exhibited similar responses as % Chl.*a*, except that N+P enrichment did not yield a stimulation response at PALU-01, and, at LEON-01, the N treatment did not either.

Alkaline Phosphatase Activity

The two factor ANOVA (factors: site, treatment) indicated that there was a significant interaction between site and treatment in APA normalized to AFDM (Figure

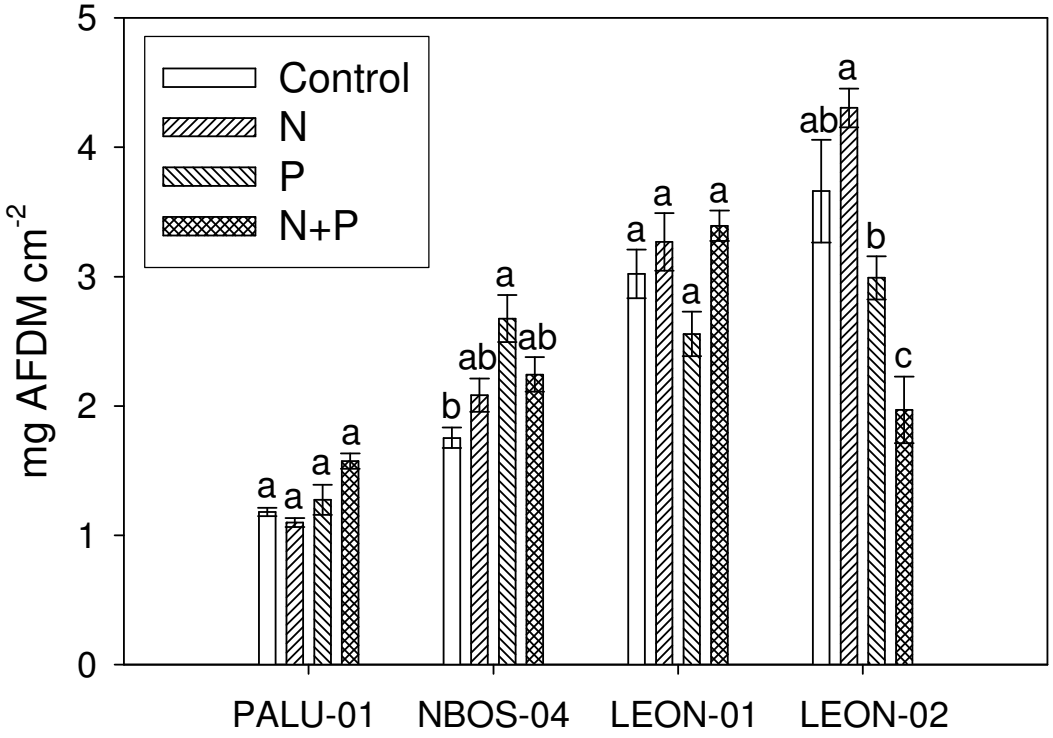

Figure 3.2. Surface-area specific AFDM (mean ± 1 S.E.) of each treatment at stream sites. The two factor ANOVA indicated significant main effects of site ($F_{3,144}$ = 107.7, p <.0001) and treatment ($F_{3,144}$ = 3.95, p = 0.0096) and a significant interaction ($F_{9,144}$ = 13.23, p <.0001). Same letter indicates no significant difference in within-site comparisons in Tukey adjusted post hoc comparisons of LSMEANS. Comparisons among sites were excluded for clarity.

3.4). Both controls and N treatments had significantly higher APA than the P and N+P treatments at every site except LEON-02, where all treatment APA responses were similarly low. APA of the controls and N treatments was highest at PALU-01. APA of these treatments was 187-211% higher than the APA responses exhibited by these respective treatments at NBOS-04 and LEON-01. APA of the controls and N treatments was not significantly different between the NBOS-04 and LEON-01 sites. Plots of treatment APA against TP and TN, in Figure 3.5a,b respectively, display the trends of APA on control and nutrient treatments up respective P and N gradients. APA of the P and N+P treatment remained low regardless of the concentrations of TP and TN. APA of both the control and the N treatment appears to decrease after the lowest TP and TN

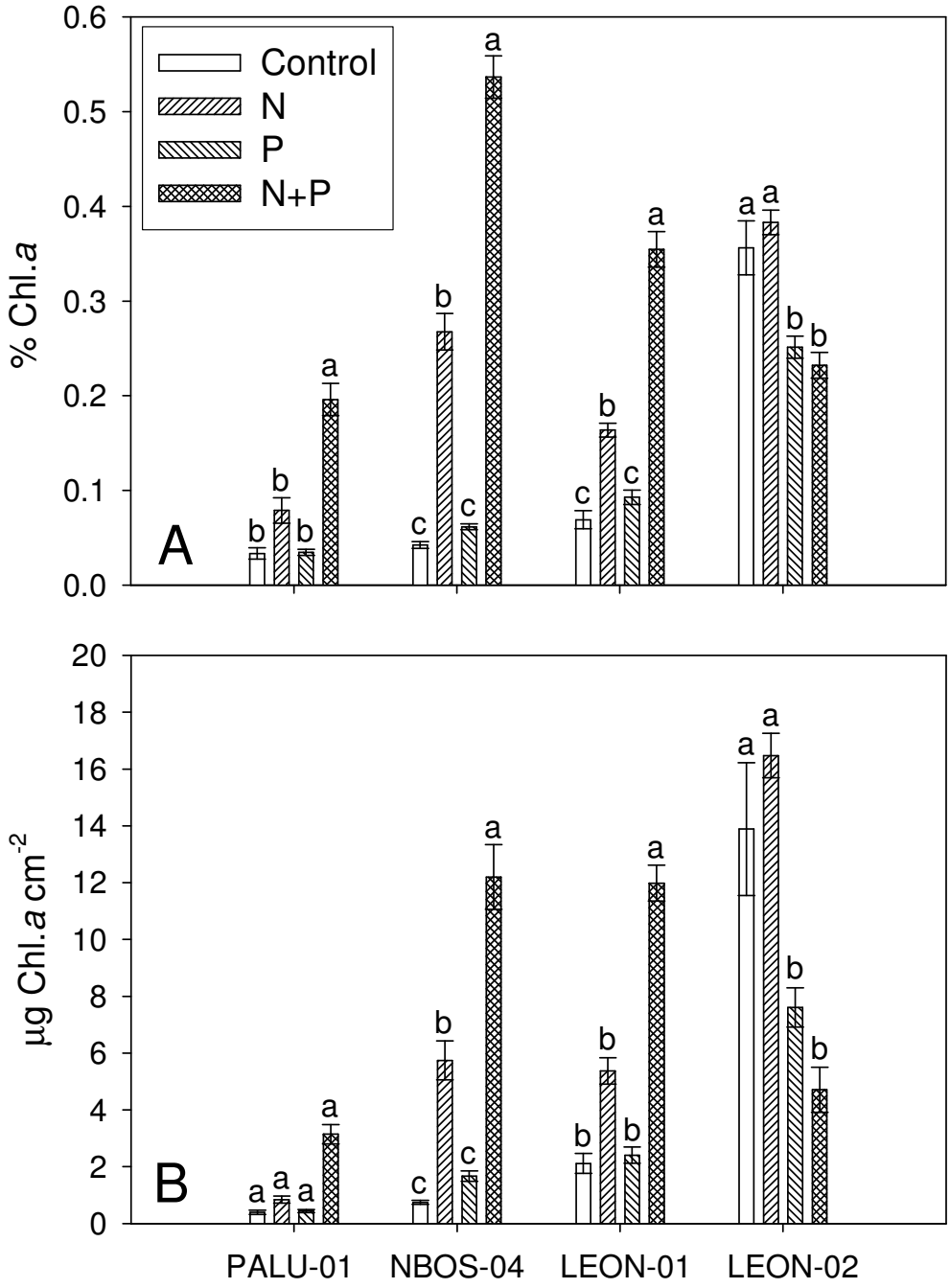

Figure 3.3. a) %Chl.*a* (mean ± 1 S.E.) of each treatment at stream sites. The two factor ANOVA indicated significant main effects of site ($F_{3,144}$ = 168.04, p < .0001) and treatment ($F_{3,144}$ = 201.93, p < .0001) and a significant interaction ($F_{9,144}$ = 64.80, p <.0001). Same letter indicates no significant difference in within-site comparisons in Tukey adjusted post hoc comparisons of LSMEANS. Comparisons among sites were excluded for clarity. b) Surface-area specific Chl.*a* (mean ± 1 S.E.) of each treatment at stream sites. The two factor ANOVA indicated significant main effects of site ($F_{3,144}$ = 97.10, p < .0001) and treatment ($F_{3,144}$ = 35.20, p < .0001) and a significant interaction ($F_{9,144}$ = 30.94, p <.0001). Same letter indicates no significant difference in within-site comparisons in Tukey adjusted post hoc comparisons of LSMEANS. Comparisons among sites were excluded for clarity.

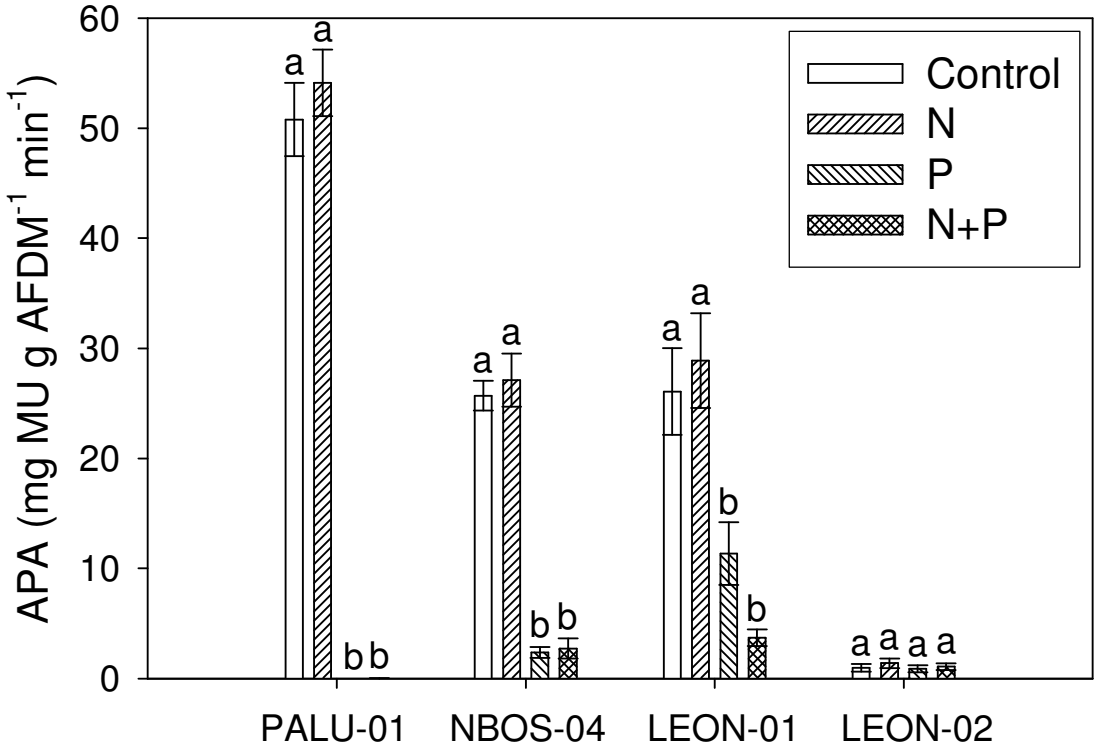

Figure 3.4. APA normalized to AFDM (mean ± 1 S.E.) of each treatment at stream sites. The two factor ANOVA indicated significant main effects of site ($F_{3,64}$ = 96.24, p <.0001) and treatment ($F_{3,64}$ = 172.56, p <.0001) and a significant interaction ($F_{9,64}$ = 34.84, p <.0001). Same letter indicates no significant difference in within-site comparisons in Tukey adjusted post hoc comparisons of LSMEANS. Comparisons among sites were excluded for clarity.

values, and plateau in mid-nutrient levels until again falling to extremely low values when TP and TN are high. Although APA of the N treatment appears to have trended higher than the control at TP and TN concentrations less than 50 and 400 µg L^{-1} respectively (Figure 3.5a,b), there was not a significant difference between them at any site (Figure 3.4).

N_2 Fixation Potential

Results of a two factor ANOVA (factors: site, treatment) on N_2 fixation potential normalized to Chl.a indicated a significant interaction between site and treatment (Figure 3.6). There was no stimulation of N_2 fixation potential by P enrichment at any site. N_2

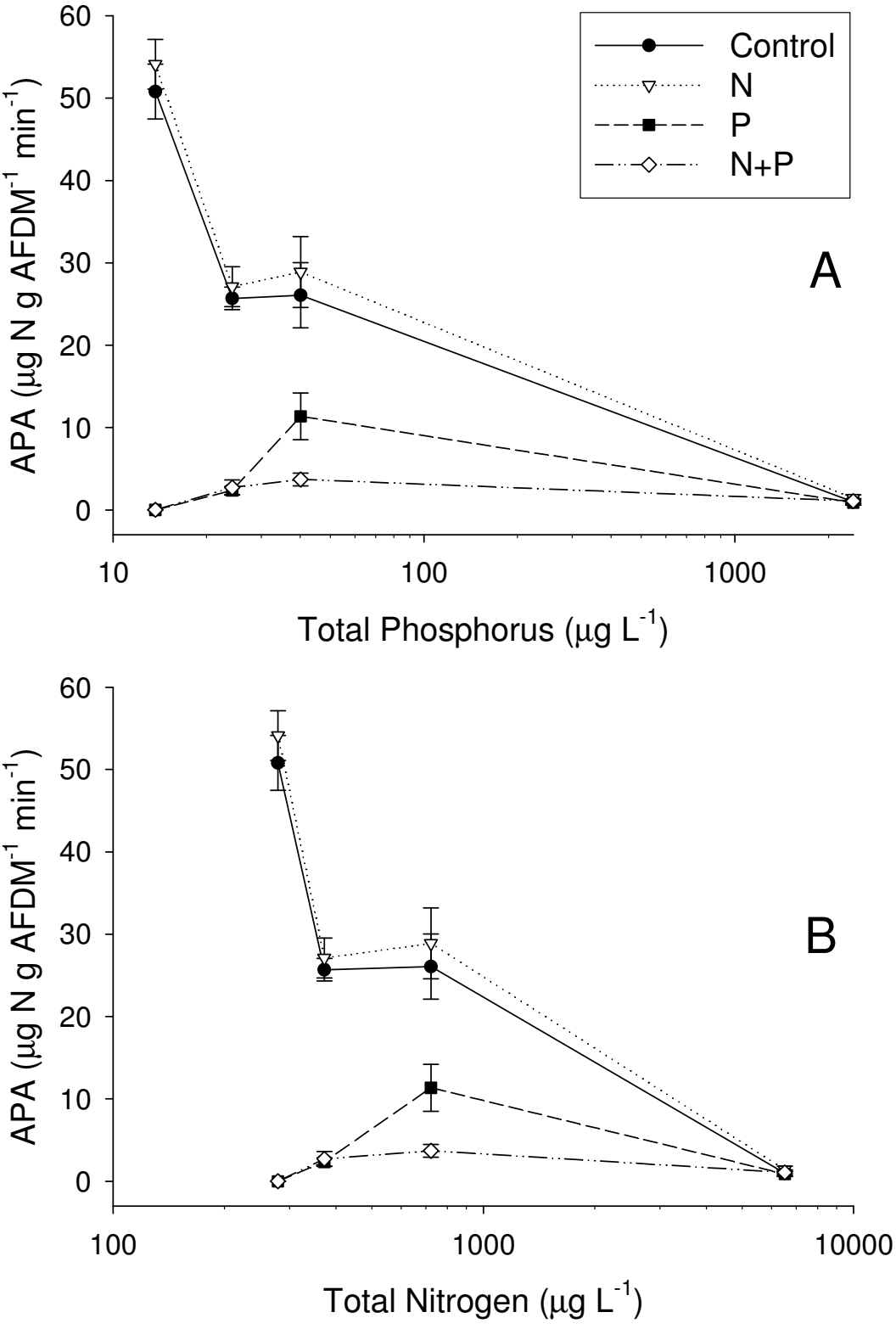

Figure 3.5. a) APA normalized to AFDM (mean ± 1 S.E.) of each treatment at ambient PO_4-P concentrations. b) APA normalized to AFDM (mean ± 1 S.E.) of each treatment at ambient NO_3-N concentrations.

fixation potential of the P treatment was not significantly different from the controls at any site. N_2 fixation potential of the controls was greater than the N and N+P treatments at every site except LEON-02, where all N_2 fixation potential was low. N_2 fixation potential on the P treatment was significantly higher than the N and N+P treatments at PALU-01 and NBOS-04 sites only. N_2 fixation potential of the controls and nutrient treatments plotted against TP and TN are displayed in Fig.3.7a,b respectively. Although controls do appear to have some level of decrement in N_2 fixation potential with increasing TP and TN, N_2 fixation of the P treatments appears to respond more readily to increasing TP and TN.

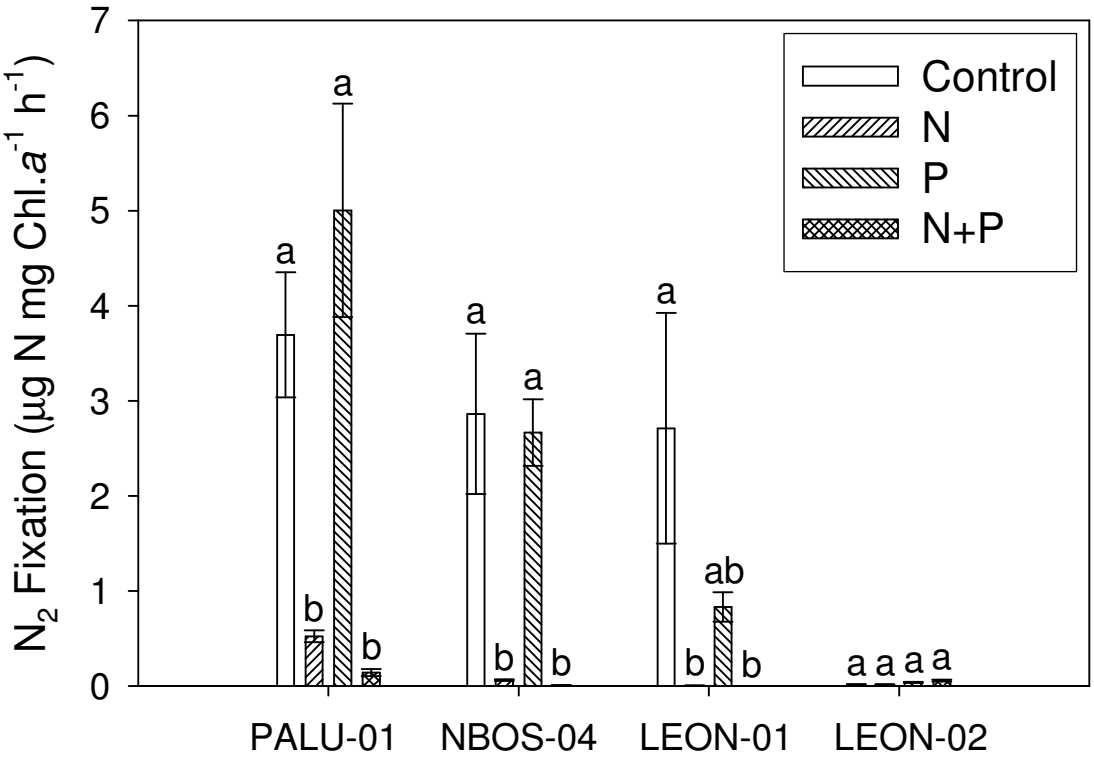

Figure 3.6. N_2 fixation potential normalized to Chl.*a* (mean ± 1 S.E.) of each treatment at stream sites. The two factor ANOVA indicated significant main effects of site ($F_{3,64}$ = 14.85, p <.0001) and treatment ($F_{3,64}$ = 24.08, p <.0001) and a significant interaction ($F_{9,64}$ = 4.97, p <.0001). Same letter indicates no significant difference in within-site comparisons in Tukey adjusted post hoc comparisons of LSMEANS. Comparisons among sites were excluded for clarity.

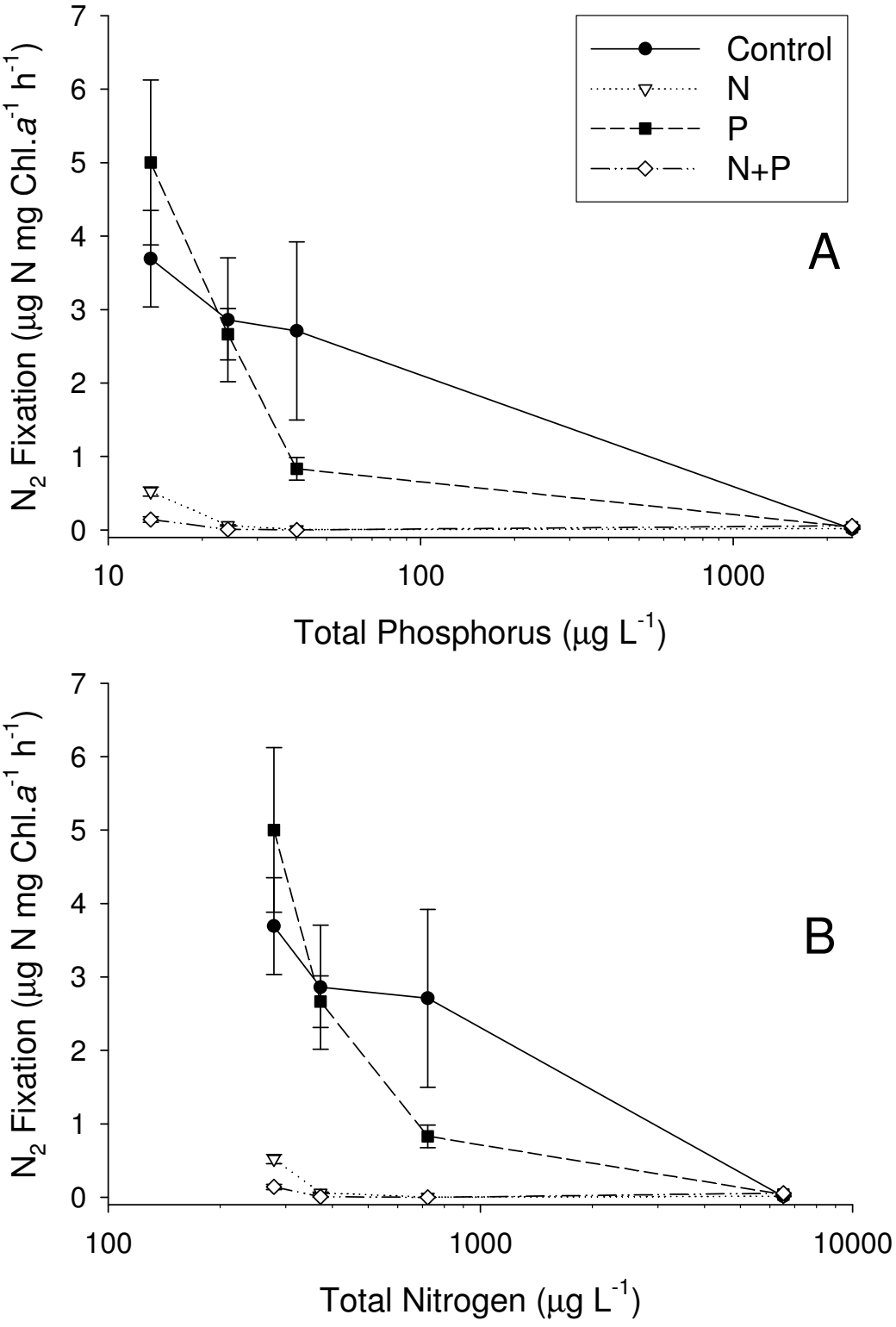

Figure 3.7. a) N_2 fixation potential normalized to Chl.*a* (mean ± 1 S.E.) of each treatment at ambient PO_4-P concentrations. b) N_2 fixation potential normalized to Chl.*a* (mean ± 1 S.E.) of each treatment at ambient NO_3-N concentrations.

Discussion

Surface-area specific AFDM of controls apparently increased up the P gradient of sites, indicative of the stimulatory effect of ambient N and P increases on periphyton community biomass (Figure 3.2). However, contrary to my predictions, nutrient enrichment rarely stimulated biomass as surface-area specific AFDM at any site. However, periphyton accrual in the form of surface-area specific Chl.*a* was stimulated by N+P enrichment at NBOS-04 and LEON-01 (Figure 3.3b). %Chl.*a* was an even more sensitive response variable, and showed that N+P enrichment effectively stimulated %Chl.*a* at all sites except LEON-02 (Figure 3.3a). The N treatment had also stimulated %Chl.a at NBOS-04 and LEON-01 above both the control and P treatment (Figure 3.3a). Combined with the lack of N+P stimulation in AFDM results, this suggests that N+P enrichment stimulated the autotroph community within the periphyton more than fungal and bacterial components at the first three sites in the gradient, and, though not as high as the response in the N+P treatment, this also suggests that N enrichment similarly led to this community shift. In addition, the relative insensitivity of AFDM to nutrient enrichment at each site, but apparent increase in AFDM of controls with ambient nutrients by site, suggests that NDS do not simulate a nutrient gradient like site ambient nutrients. Available species pools at sites likely influence how periphyton communities respond to nutrient enrichments.

At LEON-02, the N+P treatment had significantly less AFDM, %Chl.*a*, and Chl.*a* than the control, and P treatment results from surface-area specific Chl.*a* and %Chl.*a* were also significantly lower than the control (Figures 3.2 & 3.3). Since both treatments inherently enrich the periphyton community with P, this significant reduction may be a

direct or indirect consequence of P enrichment. This significant reduction in the response variables at LEON-02 may have been due to an inhibitory response from excessive P additions to already saturated conditions experienced, contaminants from the upstream effluent discharge that interacted with P enrichment, sloughing as a result of community structure changes from P enrichment, or from an act of selective grazing.

To our knowledge, no studies have shown significant reductions in periphyton AFDM or Chl.*a* to NO_3 or PO_4 enrichments. However, there is abundant literature on the effects grazers on periphyton. Fish such as *Campostoma anomalum* can significantly reduce periphyton biomass in streams (Power et al. 1985), and it is an abundant taxa across all of these sites (R. S. King unpublished data). Even with nutrient enrichment, *Campostoma* grazing can significantly suppress periphyton growth (Stewart 1987). Snails are also a common trophic group that may graze upon periphyton. Grazer snails have been shown to affect periphyton nutrient content (Evans-White and Lamberti 2005), constrain periphyton biomass (Power 1992, Rosemond et al. 2000), and alter community composition (McCormick and Stevenson 1989, McCormick and Stevenson 1991). In addition, it has been found that at least one grazer snail (*Elimia* sp.) may selectively graze upon cyanobacteria (Tuchman and Stevenson 1991). However, grazers were not quantified in this study. Investigations into the possibility of selective grazing upon periphyton of NDS could prove useful in understanding the dynamics of nutrient enrichment on both bottom-up and top-down ecological processes.

APA, as a general pattern, was high at lower ambient nutrients and decreased with increasing ambient nutrients (Figure 3.5), but, contrary to our predictions, N enrichment was incapable of significantly stimulating APA above that of the control (Figure 3.4). It

was found that this stimulation can occur, though a statistical significance was rarely detected (Chapter 2). P or N+P enrichment always reduced APA below controls and N treatments at the three lower ambient nutrient sites (Figure 3.4). At the site with high ambient N and P (LEON-02), APA was not stimulated by any treatment (Figure 3.4). In addition to the utility of APA as an indicator of P-limitation, then, lack thereof can be indicative of P saturation within a periphyton community.

Like APA, N_2 fixation potential of the control decreased with increasing ambient nutrient concentrations (Figure 3.7). At the site with high ambient N and P (LEON-02), N_2 fixation was not stimulated by any treatment (Figure 3.6). Thus, in addition to the utility of N_2 fixation as an indicator of P-limitation, then, lack thereof can be indicative of N saturation within a periphyton community. Interestingly, P enrichment did not stimulate N_2 fixation potential above the control at any site (Figure 3.6). However, it did appear that N_2 fixation potential of the P enrichment at the lowest ambient nutrients trended highest, and decreased more dramatically with increasing ambient nutrients than did the control (Figure 3.7). P enrichment may satisfy much of the P-limitation within the community, and, at the lowest of ambient nutrient concentrations, this may give that proportion of the community incentive to focus more energy into fixing N. It is possible that the absence of P stimulation of N_2 fixation potential in this study was attributed to luxury uptake and storage of P, and has been used as an explanation for lack of P stimulation of N_2 fixation in at least one other study (Horne and Commins 1987). However, at sites with much lower nutrients, P-limitation was greatest, as indicated by the high APA of controls at sites with lower ambient N and P (Figure 3.4). In addition, it is also important to note that although P enrichment can stimulate N_2 fixation in

cyanobacteria (Sanudo-Wilhelmy et al. 2001), in at least coastal wetlands, heterotrophic N_2 fixation can actually be inhibited by P enrichment (Sundareshwar et al. 2003). Still, it is generally accepted that most aquatic N_2 fixation is performed by Chl.*a* containing organisms such as cyanobacteria (Vitousek et al. 2002) and diatoms with endosymbionts of cyanobacterial origin (Prechtl et al. 2004, Steiner et al. 2002). However, shifts in periphyton community composition from high cyanobacterial abundance to other taxa can occur with higher water-column N and P concentrations (Swift 1981, 1984, Swift and Nicholas 1987, Hall and Rice 1990, McCormick and O'Dell 1996). Seasonality of periphyton community structure adds another level of complexity to the issue (Vymazal and Richardson 1995).

Although more research is needed to effectively identify the contributions of these taxa to N_2 fixation rates, this study does lend some surrogate measure of the relative contributions of trophic groups within the periphyton community based on %Chl.a (Figure 3.3a). As evidenced by increases in %Chl.*a* with N addition, it is likely that it was the photoautotrophic subset of the periphyton community that was limited by N. Since P stimulation of %Chl.*a* was lacking, yet APA decreased with P addition, it is also likely that it was the heterotrophic subset of the periphyton community that was limited by P. Therefore, the differential nutrient limitation indicative in this experiment was likely resultant of differences in photoautotrophic and heterotrophic nutrient requirements. Combined, the results of this study provide substantial evidence that differential nutrient limitation occurs within periphyton communities, consistent with the belief that a single nutrient limiting paradigm is not entirely valid for application to multispecies algal communities (Francoeur 2001, Borchardt 1996, Tilman et al. 1982).

CHAPTER FOUR

Conclusions

Field diffusion rates of NO_3-N and PO_4-P from respective N and P treatments of this novel NDS design were effectively approximated in this experiment. APA and N_2 fixation proved to be sensitive indicators of nutrient limitation, and showed greatest separation in response between treatments on the day 18 retrieval. Although not accurate if measured on same NDS used to estimate N_2 fixation potential, the separate but dual usage of APA and N_2 fixation potential as nutrient limitation indicators proved to be a valuable method for determining actual nutrient limitation within periphyton communities. In the second study (Chapter 3), substantial evidence was provided that differential nutrient limitation indeed occurs within periphyton communities. APA and N_2 fixation potential responses were both substantial in controls at low ambient TP and TN, and it was speculated that the subset of the community that was P-limited were mostly heterotrophs, whereas the subset that was N-limited were autotrophs.

Together, these experiments have provided valuable scientific information about methodology concerning this NDS design and the value of its usage in field studies. This study has provided evidence that this NDS design can be used as an effective tool for evaluation of nutrient pollution on key ecosystem processes, and that the dual use of APA and N_2 fixation rates as nutrient limitation indicators can provide valuable information about actual nutrient status in streams.

REFERENCES

American Public Health Association (APHA), 1992. Standard methods for the examination of water and wastewater. American Public Health Association, American Water Works Association and Water Pollution Control Federation. Washington, D.C., 874 pp.

American Public Health Association (APHA). 1995. Standard methods for the examination of water and wastewater. American Public Health Association, American Water Works Association, and Water Pollution Control Federation. 19th edition, Washington, D.C.

Barbour MT, Gerritsen J, Snyder BD, Stribling JB. 1999. Rapid Bioassessment Protocols for Use in Streams and Wadeable Rivers: Periphyton, Benthic Macroinvertebrates and Fish, Second Edition. EPA 841-B-99-002. U.S. Environmental Protection Agency; Office of Water; Washington, D.C.

Barnese LE, Schelske CL. 1994. Effects of nitrogen, phosphorus and carbon enrichment on planktonic and periphytic algae in a softwater, oligotrophic lake in Florida, U.S.A. Hydrobiologia 227:159-170.

Biggs BJF. 1985. Algae: A blooming nuisance in rivers. Soil Water 21:27-31.

Biggs BJF, Kilroy C. 2000. Stream periphyton monitoring manual. Christchurch, New Zealand: NIWA. 226 p.

Borchardt MA. 1996. Nutrients. In: Stevenson RJ, Bothwell ML, Lowe RJ, editors, Algal Ecology: Freshwater Benthic Ecosystems. San Diego (CA): Academic Press, Inc. p. 183-227.

Bothwell ML. 1985. Phosphorus limitation of lotic periphyton growth rates: An intersite comparison using continuous-flow troughs (Thompson River system, British Columbia). Limnology and Oceanography 30(3):527-542.

Bowman MF, Chambers PA, Schindler DW. 2005. Epilithic algal abundance in relation to anthropogenic changes in phosphorus bioavailability and limitation in mountain rivers. Can. J. Fish. Aquat. Sci. 62:174-184.

Carr GM, Morin A, Chambers PA. 2005. Bacteria and algae in stream periphyton along a nutrient gradient. Freshwater Biology 50:1337-1350.

Cattaneo A. 1978. the microdistribution of epiphytes on the leaves of natural and artificial macrophytes. British Phycological Journal 13:183-188.

Cazaubon A, Loudiki M. 1986. Microrépartition des algues épilithiques sur les cailloux d'un torrent Corse, le Rizzanése. Annales de Limnologie 22:3-16.

Corkum LD. 1996. Patterns of nutrient release from nutrient diffusing substrates in flowing water. Hydrobiologia 333:37-43.

Currin CA, Paerl HW. 1998. Environmental and physiological controls on diel patterns of N_2 fixation in epiphytic cyanobacterial communities. Microbial Ecology 35:34-45.

DeYoe HR, Lowe RL, Marks JC. 1992. The effect of nitrogen and phosphorus on the endosymbiont load of *Rhopalodia gibba* and *Epithemia turgida* (Bacillariophyceae). Journal of Phycology 28:773-777.

Draper NR, Smith H. Applied regression analysis. 3rd ed. New York (NY): Wiley. 706 p.

Evans-White MA, Lamberti GA. 2005. Grazer species effects on epilithon nutrient composition. Freshwater Biology 50:1853-1863.

Fairchild BW, Lowe RL. 1984. Algal substrates which release nutrients: Effects on periphyton and invertebrate succession. Hydrobiologia 114:29-37.

Fairchild BW, Lowe RL, Richardson RB. 1985. Algal periphyton growth on nutrient-diffusing substrates: an *in situ* bioassay. Ecology 66:465-472.

Fairchild BW, Sherman JW. 1993. Algal periphyton response to acidity and nutrients in softwater lakes: lake comparison vs. nutrient enrichment approaches. Journal of the North American Benthological Society 12:157-167.

Fairchild BW, Sherman JW, Acker FW. 1989. Effects of nutrient (N, P, C) enrichment, grazing and depth upon littoral periphyton of a softwater lake. Hydrobiologia 173:69-83.

Fernley HN, Walker PG. 1965. Kinetic behaviour of calf-intestinal alkaline phosphatase with 4-Methylumbelliferyl phosphate. Biochem. J. 97:95-103.

Fitzgerald GP, Nelson TC. 1966. Extractive and enzymatic analyses for limiting or surplus phosphorus in algae. Journal of Phycology 2:32–37.

Flett RJ, Hamilton RD, Campbell NER. 1976. Aquatic acetylene reduction techniques: solutions to several problems. Canadian Journal of Microbiology 22:43-51.

Foy CD, Chaney RD, White MC. 1978. The physiology of metal toxicity in plants. Annual Review of Plant Physiology 29:511-566.

Francoeur SN. 2001. Meta-analysis of lotic nutrient amendment experiments: detecting and quantifying subtle responses. Journal of the North American Benthological Society 20(3):358-368.

Gibeau GG Jr., Miller MC. 1989. A micro-bioassay for epilithon using nutrient-diffusing substrata. J. Freshw. Ecol. 5:171-176.

Goldman JC. 1986. On phytoplankton growth rates and particulate C:N:P ratios at low light. Limnology and Oceanography 31:1358-1363.

Goldsborough LG. 1994. Heterogeneous spatial distribution of periphytic diatoms on vertical artificial substrata. Journal of the North American Benthological Society 13(2):223-236.

Hall GB, Rice RG. 1990. Response of the Everglades marsh to increased nitrogen and phosphorus loading. Part 3. Periphyton community dynamics. 47 pp., Everglades National Park, Homestead, Florida.

Healey FP, Hendzel LL. 1979. Fluorometric measurement of alkaline phosphatase activity in algae. Freshwater Biology 9:429-439.

Hein M. 1997. Inorganic carbon limitation of photosynthesis in lake phytoplankton. Freshwater Biology 37(3):545-552.

Hernández I. 1996. Analysis of the expression of alkaline phosphatase activity as a measure of phosphorus status in the red alga *Porphyra umbilicalis* (L.) Kützing. Bot. Mar. 39:255-262.

Hernández I, Neill FX, Fernández JA. 1996. Alkaline phosphatase activity of the red alga *Corallina elongate* Ellis et Solander. Scientia Marina 60:297-306.

Holm-Hansen O, Riemann B. 1978. Chlorophyll a determination: improvements in methodology. Oikos 30:438-447.

Horne AJ, Commins ML. 1987. Macronutrient controls on nitrogen fixation in planktonic cyanobacterial populations. New Zealand Journal of Marine and Freshwater Research 21:413-423.

Howarth RW, Marino R, Lane J, Cole JJ. 1988. Nitrogen fixation in freshwater, estuarine, and marine ecosystems. 1. Rates and importance. Limnology and Oceanography 33(4, part 2):669-687.

Jansson M, Olsson H, Pettersson K. 1988. Phosphatases: origin, characteristics, and functions in lakes. Hydrobiologia 170:157-175.

Johnson RE. 1996. The effects of density-dependent resource limitations on the vertical distribution of diatoms in periphyton mats. Master's Thesis, Loyola University of Chicago, Chicago.

Kahlert M, Hasselrot AT, Hillebrand H, Pettersson K. 2002. Spatial and temporal variation in the biomass and nutrient status of epilithic algae in Lake Erken, Sweden. Freshwater Biology 47:1191-1215.

Klotz RL. 1985. Factors controlling phosphorus limitation in stream sediments. Limnology and Oceanography 30(3):543-553.

Klotz RL. 1992. Factors influencing alkaline-phosphatase activity of stream epilithon. Journal of Freshwater Ecology 7:233-242.

Kuenzler EJ, Perras JP. 1965. Phosphatases of marine algae. Biological Bulletin 128:271-284.

Levine SN, Schindler DW. 1999. Influence of nitrogen:phosphorus supply ratios and physicochemical conditions on cyanobacteria and phytoplankton species composition in Experimental Lakes Area, Canada. Can. J. Fish. Aquat. Sci. 56:451-466.

Lowe RL, Golladay SW, Webster JR. 1986. Periphyton response to nutrient manipulation in streams draining clearcut and forested watersheds. Journal of the North American Benthological Society 5:221–229.

Marcarelli AM, Wurtsbaugh WA. 2006. Temperature and nutrient supply interact to control nitrogen fixation in oligotrophic streams: An experimental examination. Limnology and Oceanography 51(5):2278-2289.

Miller AR, Lowe RL, Rotenberry JT. 1987. Succession of diatom communities on sand grains. Journal of Ecology 75:693-709.

Marker AFH. 1972. The use of acetone and methanol in the estimation of chlorophyll in the presence of phaeophytin. Freshwater Biology 2:361-385.

Matlock MD, Matlock ME, Storm DE, Smolen MD, Henley WJ. 1998. Limiting nutrient determination in lotic ecosystems using a quantitative nutrient enrichment periphytometer. Journal of the American Water Resources Association 34:1141-1147.

McCormick PV, O'Dell MB. 1996. Quantifying periphyton response to phosphorus enrichment in the Florida Everglades: a synoptic-experimental approach. Journal of the North American Benthological Society 15:450-468.

McCormick PV, O'Dell MB, Shuford RBE III, Backus JG, Kennedy WC. 2001. Periphyton responses to experimental phosphorus enrichment in a subtropical wetland. Aquatic Botany 71:119-139.

McCormick PV, Rawlik PS, Lurding K, Smith EP, Sklar FH. 1996. Periphyton-water quality relationships along a nutrient gradient in the Northern Everglades. Journal of the North American Benthological Society 15:433-449.

McCormick PV, Stevenson RJ. 1989. Effects of snail grazing on benthic algal community structure in different nutrient environments. Journal of the North American Benthological Society 8(2):162-172.

McCormick PV, Stevenson RJ. 1991. Grazer control of nutrient availability in the periphyton. Oecologia 86:287-291.

Newman S, McCormick PV, Backus JG. 2003. Phosphatase activity as an early warning indicator of wetland eutrophication: problems and prospects. Journal of Applied Phycology 15:45-59.

Osborne J. 2002. Notes on the use of data transformations. Practical assessment, Research, & Evaluation 8(6).

Ott RL, Longnecker M. 2001. An introduction to statistical methods and data analysis. 5th ed. Pacific Grove (CA): Duxbury. 1152 p.

Power ME. 1992. Habitat heterogeneity and the functional significance of fish in river food webs. Ecology 73:1675-1688.

Power ME, Matthews WJ, Stewart AJ. 1985. Grazing minnows, piscivorous bass, and stream algae: dynamics of a strong interaction. Ecology 66(5):1448-1456.

Prechtl J, Kneip C, Lockhart P, Wenderoth K, Maier U. 2004. Intracellular spheroid bodies have nitrogen-fixing apparatus of cyanobacterial origin. Molecular Biology and Evolution. 21(8):1477-1481.

Pringle CM. 1985. Effects of chironomid (Insecta: Diptera) tube-building activities on stream diatom communities. Journal of Phycology 21:185-194.

Pringle CM, Bowers JA. 1984. An in situ substratum fertilization technique: diatom colonization on nutrient-enriched, sand substrata. Can. J. Fish. Aquat. Sci. 41:1247-1251.

Reddy KJ, Haskell JB, Sherman DM, Sherman LA. 1993. Unicellular, aerobic nitrogen-fixing cyanobacteria of the genus *Cyanothece*. Journal of Bacteriology 175:1284-1292.

Redfield AC. 1958. The biological control of chemical factors in the environment. American Scientist 46:205-222.

Reuter JG. 1983. Alkaline phosphatase inhibition by copper: Implications to phosphorus nutrition and use as a biochemical marker of toxicity. Limnology and Oceanography 28(4):743-748.

Rhee G-Y, Gotham IJ. 1980. Optimum N:P ratios and coexistence in phytoplankton. Journal of Phycology 16:486-489.

Riemann B, Ernst D. 1982. Extraction of chlorophylls a and b from phytoplankton using standard extraction techniques. Freshwater Biology 12:217-223.

Rosemond AD, Mulholland PJ, Brawley SH. 2000. Seasonally shifting limitation of stream periphyton: response of algal populations and assemblage biomass and productivity to variation in light, nutrients, and herbivores. Can. J. Fish. Aquat. Sci. 57(1):66-75.

Round FE, Happey CM. 1965. Persistent vertical-migration rhythms in benthic microflora. IV. A diurnal rhythm of the epipelic diatom association in non-tidal flowing water. British Phycological Bulletin 2:463-471.

Sanudo-Wilhelmy SA, Kustka A, Gobler CJ, Hutchins DA, Capone DJ, Raven JA, DiGiacomo PM. 2001. Phosphorus limitation of nitrogen fixation by Trichodesmium in the central Atlantic Ocean. Nature 411:66-69.

Sartory DP. 1982. Spectrophotometric analysis of chlorophyll a in freshwater phytoplankton. Hydrological Research Institute Technical Report TR 115, Pretoria, South Africa.

Sartory DP, Grobbelaar JU. 1984. Extraction of chlorophyll *a* from freshwater phytoplankton for spectrophotometric analysis. Hydrobiologia 114:177-187.

SAS Institute Inc. 2004. SAS® Help and Documentation. Cary, NC, USA.

Scott JT, Doyle RD. 2006. Coupled photosynthesis and heterotrophic bacterial biomass production in a nutrient-limited wetland periphyton mat. Aquatic Microbial Ecology 45:69-77.

Scott JT, Doyle RD, Filstrup CT. 2005. Periphyton nutrient limitation and nitrogen fixation potential along a wetland nutrient-depletion gradient. Wetlands 25:439-448.

Schindler DW. 1977. Evolution of phosphorus limitation in lakes. Science 195:260-262.

Scrimgeour GJ, Chambers PA. 1997. Development and application of a nutrient-diffusing bioassay for large rivers. Freshwater Biology 38:221–231.

Sharma K, Inglett PW, Reddy KR, Ogram AV. 2005. Microscopic examination of photoautotrophic and phosphatase-producing organisms in phosphorus-limited Everglades periphyton mats. Limnology and Oceanography 50(6):2057-2062.

Sinsabaugh RL, Weiland T, Linkins AE. 1991. Epilithon patch structure in a boreal river. Journal of the North American Benthological Society 10:419-429.

Steiner J, Pfanzagl B, Ma Y, Löffelhardt W. 2002. Evolution and biology of cyanelles. Biology and Environment: Proceedings of the Royal Irish Academy 102B(1):7-9.

Steinman AD. 1996. Effects of grazers on freshwater benthic algae. In: Stevenson RJ, Bothwell ML, Lowe RJ, editors, Algal Ecology: Freshwater Benthic Ecosystems. Academic Press, Inc., San Diego, CA, USA. p. 341-373.

Stelzer RS, Lamberti GA. 2001. Effects of N:P ratio and total nutrient concentration on stream periphyton community structure, biomass, and elemental composition. Limnology and Oceanography 46(2):356-367.

Sterner RW, Elser JJ, Fee EJ, Guildford SJ, Chrzanowski TH. 1997. The light:nutrient ratio in lakes: The balance of energy and materials affects ecosystem structure and process. The American Naturalist 150(6):663-684.

Stewart AJ. 2004. Responses of stream algae to grazing minnows and nutrients: a field test for interactions. Oecologia 72(1):1-7.

Sundareshwar PV, Morris JT, Koepfler EK, Fornwalt B. 2003. Phosphorus limitation of coastal ecosystem processes. Science 299:563-565.

Swift DR. 1981. Preliminary investigations of periphyton and water quality relationship in the Everglades Water Conservation Areas. 83 pp., Tech. Publ. 81-5, South Florida Water Management District, West Palm Beach, Florida.

Swift DR. 1984. Periphyton and water quality relationship in the Everglades Water Conservation Areas. In: Gleason PJ, editor, Environments of South Florida: Present and Past II. Coral Gables (FL): Miami Geological Society. p. 97-117.

Swift DR, Nocholas RB. 1987. Periphyton and water quality relationship in the Everglades Water Conservation Areas 1978-1982. 44 pp., Tech. Publ. 87-2, South Florida Water Management District, West Palm Beach, Florida.

Systat Software Inc. 2004. SigmaPlot 9.0®.

Tate CM. 1990. Patterns and controls of nitrogen in tallgrass prairie streams. Ecology 71(5):2007-2018.

Tett P, Heaney SI, Droop MR. 1985. The Redfield ratio and phytoplankton growth rate. Journal of the Marine Biological Association of the United Kingdom 65:487-504.

Tilman D, Kilham SS, Kilham P. 1982. Phytoplankton community ecology: The role of limiting nutrients. Annual Review of Ecology and Systematics 13:349-372.

Tuchman NC. 1988. Effects of different intensities and frequencies of disturbance by snail herbivory on periphyton succession. Ph.D. Dissertation, University of Louisville, Louisville, KY.

Tuchman NC, Stevenson RJ. 1991. Effects of selective grazing by snails on benthic algal succession. Journal of the North American Benthological Society 10:430-443.

Tyler AC, Mastronicola TA, McGlathery KJ. 2003. Nitrogen fixation and nitrogen limitation of primary production along a natural marsh chronosequence. Oecologia 136:431-438.

Vallee BL. 1959. Metal and enzyme interactions: Correlation of composition, function and structure. Physiol. Rev. 39:443.

Vitousek, P. M., K. Cassman, C. Cleveland, T. Crews, C. B. Field, N. B. Grimm, R. W. Howarth, R. Marino, L. Martinelli, E. B. Rastetter, and J. I. Sprint. 2002. Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry 57/58:1-45.

Vymazal J, Richardson CJ. 1995. Species composition, biomass, and nutrient content of periphyton in the Florida Everglades. Journal of Phycology 31:343-354.

Welch EB, Horner RR, Patmot CR. 1989. Prediction of nuisance periphytic biomass: A management approach. Water Res. 23:401-405.

Wharfe JR, Taylor KS, Montgomery HAC. 1984. The growth of *Cladophora glomerata* in a river receiving sewage effluent. Water Res. 18:971-979.

Winterbourn MJ. 1990. Interactions among nutrients, algae, and invertebrates in a New Zealand mountain stream. Freshwater Biology 23:463-474.

Winterbourn MJ, Fegley A. 1989. Effects of nutrient enrichment and grazing on periphyton assemblages in some spring-fed, South Island streams. New Zealand Natural Sciences 16:57-65.

Wuhrmann K, Eichenberger E. 1975. Experiments on the effects of inorganic enrichment of rivers on periphyton primary production. Int. Ver. Theor. Angew. Limnol. Verh. 19:2028-2031.

Wurtsbaugh W, Horne A. 1983. Iron in eutrophic Clear Lake, California: its importance for algal nitrogen fixation and growth. Can. J. Fish. Aquat. Sci. 40:1419-1429.

Yangdong P, Stevenson RJ, Vaithiyanathan P, Slate J, Richardson CJ. 2000. Changes in algal assemblages along observed and experimental phosphorus gradients in a subtropical wetland, U.S.A. Freshwater Biology 44(2):339-353.