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ORIGINAL PAPER

Periphyton nitrogenase activity as an indicator of wetland eutrophication: spatial patterns and response to phosphorus dosing in a northern Everglades ecosystem

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Abstract The use of periphyton nitrogenase activity (biological N_2 fixation) as an indicator of wetland P impact was assessed using patterns of nutrient content (C, N, P, Ca, Mg, K, Fe, and Mn) and acetylene reduction (AR) in floating cyanobacterial periphyton mat (metaphyton) communities of a P-enriched portion of the Florida Everglades, USA (Water Conservation Area-2A, WCA-2A). Spatial patterns of nutrients indicate the enrichment of floating mat periphyton N, P, Fe, and K, and the reduction of Mn and TN:TP in enriched marsh areas. In highly enriched areas, floating mat periphyton AR was approximately threefold greater than that in less enriched, interior marsh zones. Multiple regression models indicated AR dependence on P in eutrophic WCA-2A areas while the AR of more interior marsh periphyton mats was more closely related to tissue levels of Ca and Fe. Nitrogenase activity of floating mat periphyton from P-loaded mesocosms revealed a significant enhancement of N_2 fixation in samples receiving approximately 2–3 mg P m^{-2} of cumulative P dosing or with biomass TP content of 100–300 mg kg^{-1}. At P contents above the optimum, mat periphyton AR was suppressed possibly as a result of changes in species composition or increased levels of NH_4^+. After 3 years of dosing, consistently high AR occurred only at low rates of P enrichment (0.4–0.8 g P m^{-2} yr^{-1}), and the patterns appeared to be seasonal. These findings agree with the hypothesis that P availability is a key determinant of nitrogenase activity in aquatic systems, and thus, may support the use of periphyton nitrogenase to indicate P impacts in P-limited systems. These results also demonstrate the potential existence of a P threshhold for biogeochemical alteration of periphyton mat function in the Everglades, and that cumulative loading of limiting nutrients (i.e., P), rather than instantaneous concentrations, should be considered when evaluating nutrient criteria.

Keywords Metaphyton · Cyanobacterial mat · Nitrogen fixation · Phosphorus · Acetylene reduction

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Introduction

Their importance to ecosystem function combined with their high turnover rate make periphyton communities a sensitive 'indicator' of the nutrient status

and functioning of aquatic ecosystems (McCormick and Cairns 1994). Recently, periphyton has also been proposed as a biotic indicator of impacts to wetland ecosystems such as in the Florida Everglades (McCormick and Stevenson 1998; McCormick et al. 1996). The Everglades is a unique system where periphyton species composition was shown to accurately predict the impacts of phosphorus (P) along the well-studied enrichment gradient of Water Conservation Area 2A (WCA-2A, Fig. 1). WCA-2A is a limestone-based, naturally P-limited wetland similar to other Everglades marshes and those of Central America and the Bahamas (Pinckney et al. 1995a; Rejmankova 2001; Rejmankova and Komarkova 2000). In WCA-2A, more than 40 years of agricultural drainage and surface water discharges have created a 20-km gradient of P and observed effects (Reddy et al. 1993; McCormick et al. 1996). As a result, the WCA-2A gradient is an ideal setting to develop indicators of P impact to wetland ecosystems (Reddy et al. 1999).

In less enriched areas of WCA-2A, periphyton exists as dense calcareous assemblages dominated by the cyanobacteria *Scytonema hofmanii* Agardh and *Schizothrix calcicola* Agardh as well as diatoms (Browder et al. 1994). In eutrophic areas of WCA-2A, there is a visible breakdown of the calcareous periphyton mat structure and a shift to more eutrophic assemblages dominated by cyanobacteria, (e.g., *Lyngbya* sp. and *Oscillatoria* sp.) and filamentous green algae (e.g., *Spirogyra* sp. and *Mougeotia* sp.) (Swift and Nicholas 1987; McCormick et al. 1996). Several studies have shown P enrichment to be a primary cause of the mat breakdown and the shift toward pollution-tolerant taxa (Flora et al. 1988; Hall and Rice 1990; Craft et al. 1995; McCormick and O'Dell 1996).

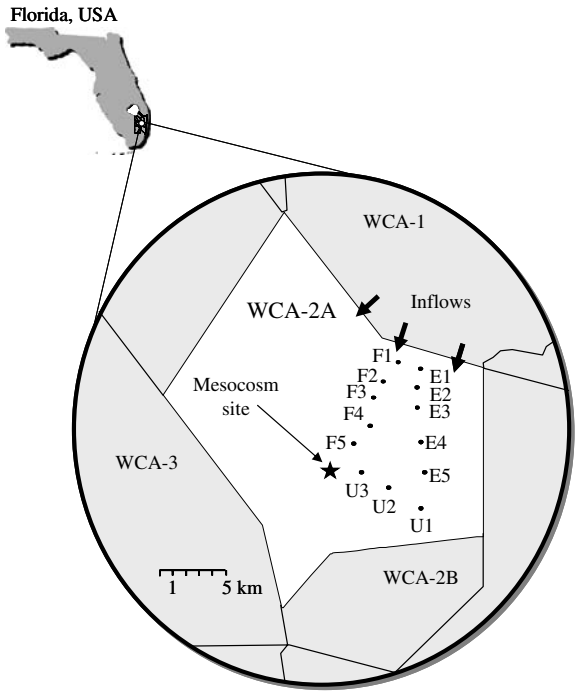

Fig. 1 Location of Water Conservation Area 2A (WCA-2A), transect sampling locations, and the mesocosm site of experimental phosphorus enrichment used in this study

As in other systems dominated by cyanobacteria, the floating mat periphyton in the Everglades has been shown to actively fix atmospheric N_2 via the nitrogenase enzyme complex (N_2 fixation), with rates of acetylene reduction as high as 213 μmol m^{-2} h^{-1} in the non-enriched, floating periphyton of WCA-2A (Inglett et al. 2004). It is widely accepted that under conditions of N limitation, aquatic cyanobacteria capable of N_2 fixation will adjust their nitrogenase activity in accordance with P supply to maintain the appropriate Redfield N:P ratio of their biomass production (Flett et al. 1980; Bergmann and Welch 1990; Hendzel et al. 1994). Thus, in appropriate systems, N_2 fixation rates can be a powerful indicator of N limitation which is often a direct function of P levels. In the case of the WCA-2A eutrophication gradient, the high P levels of the agricultural drainage inputs have significantly increased the demand for nitrogen (N) within the enriched marsh areas. Evidence for this increased N demand is the decrease of water column total N to total P ratios (TN:TP) from >250 in the marsh interior to <50 at the marsh periphery near the drainage inputs as well as algal nutrient limitation assays (McCormick et al. 1996).

The decreases in N:P ratios, as well as the accompanying shift in cyanobacterial species composition would presumably lead to changes in the rates of N_2 fixation by the periphyton communities along the WCA-2A gradient. If so, periphyton nitrogenase activity would be an accurate indicator of nutrient impacts (in particular P) to a wetland ecosystem. To test this hypothesis, the following study was conducted to characterize the N_2 fixation process as it occurs in the floating periphyton communities of the WCA-2A enrichment gradient.

The primary goals of this study were to: (1) document the spatial pattern of floating mat periphyton nitrogenase activity as it relates to nutrient composition along the enrichment gradient, and (2) assess the effects of experimental P loading on the changes in periphyton chemical composition and nitrogenase activity.

Materials and methods

Site description

Water Conservation Area 2A (WCA-2A) is a large (547 km^2), hydrologically-controlled portion of the once pristine Florida Everglades ecosystem (Fig. 1). Drainage water inputs enter WCA-2A via spillways along the northeastern perimeter. The nutrient enrichment gradient is thus created with high nutrient levels nearer the inflows and non-enriched, background levels in the interior of WCA-2A (Table 1) (Koch and Reddy 1992; Craft and Richardson 1997; Reddy et al. 1998). Among the impacts of the high-nutrient discharges is the development of extensive stands of cattail (*Typha domingensis* Pers.) which have replaced the native Everglades marshes dominated by sawgrass (*Cladium jamaicense* Crantz) and openwater sloughs (dominated by periphyton mats, *Utricularia purpurea* Walt., and *Nymphaea odorata* Ait.) (Davis and Ogden 1994).

Sample collection

Spatial patterns of WCA-2A periphyton mat chemistry and N_2 fixation rates were assessed using floating periphyton mat samples collected from thirteen WCA-2A marsh locations on November 10, 1997, September 19, 1998, and February 8, 1999 (McCormick et al. 1996) (Fig. 1). Ten of these sites spanned the WCA-2A nutrient gradient (E and F transects) from a distance of 1.8 to 10.1 km from the canal inflows, while three additional sites located in the interior of WCA-2A (U transect) were designated as control stations. Due to the large distances between transect stations, helicopter sampling was employed to minimize travel time and subsequent sample storage. Following helicopter touchdown, three replicate grab samples of floating periphyton (metaphyton) was collected at the edge of the nearest macrophyte stand and stored in collected site water prior to analysis (<3 h).

The effect of P enrichment on nitrogenase activity was also tested using floating periphyton mat samples obtained from an experimental mesocosm site in the

Table 1 Mean (±SE) values of selected water quality parameters observed along the WCA-2A transect during the period of this study (November 1997–January 1999)

Parameter	Units	Transect segment		
		<6 km (n = 46) Mean (SE)	6–8 km (n = 18)	>8 km (n = 44)
pH		7.4 (0.0)	7.3 (0.1)	7.5 (0.0)
Conductivity	μS cm^{-1}	1055 (30)	1029 (49)	892 (32)
Alkalinity	mg l^{-1}	288 (7)	276 (12)	238 (8)
Dissolved organic carbon	mg l^{-1}	40 (2)	37 (3)	33 (2)
Total dissolved calcium (Ca)	mg l^{-1}	86 (2)	86 (3)	71 (2)
Total dissolved magnesium (Mg)	mg l^{-1}	9 (2)	7 (4)	12 (2)
Total dissolved potassium (K)	mg l^{-1}	28 (1)	28 (1)	24 (1)
Total dissolved iron (Fe)	μg l^{-1}	7 (0)	8 (0)	6 (0)
Ammonium (NH_4–N)	μg l^{-1}	37 (3)	23 (4)	27 (2)
Nitrate + Nitrite (NO_x–N)	μg l^{-1}	8 (2)	7 (3)	10 (2)
Soluble Reactive Phosphorus (SRP)	μg l^{-1}	25 (3)	8 (4)	7 (3)

Values are based on data collected by the South Florida Water Management District. Dissolved fractions are based on filtration through a 0.45 μm membrane

interior of WCA-2A (Fig. 1). This area was the site of a long-term P dosing experiment by the South Florida Water Management District and consists of 24 individual plots of open-water slough habitat (McCormick and O'Dell 1996). Of these plots, 21 were enclosed with transparent fiberglass cylinders (1.2 m high × 1.5 m diameter) and dosed weekly with ortho-phosphate (NaH_2PO_4) at seven rates (0, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 g P m^{-2} yr^{-1}) beginning in June, 1995. Each rate was replicated three times ($n = 3$). Three additional plots served as open controls.

Initial samplings of floating mat periphyton from these plots were conducted in November of 1996 and 1997. Beginning in 1998, additional samplings were conducted to encompass seasonal variability on the following dates: March 18, May 4, July 6, September 8, November 9, 1998 and January 11, 1999. As for the transect collections, floating periphyton was grab sampled from each mesocosm and briefly stored (<1 h) in site water prior to assessment of nitrogenase activity.

Analytical methods

Nitrogenase activity

Periphyton mat nitrogenase activity was determined using a version of the classic acetylene (C_2H_2) reduction (AR) assay adapted from Stal (1988). Briefly, periphyton mat samples (~3–5 g wet weight) were dissected and inserted into 50-ml, screw-capped culture tubes. The tubes were equipped with an open-top cap containing a teflon-lined, silicone septa (0.120″) to allow insertion of syringe needles. Acetylene gas (6 ml) generated from CaC_2 was injected into each sample tube to initiate the AR incubation. Tubes were then inverted and placed into tube racks, and held in place using a wire screen material (1-cm square mesh openings). The resulting tube/rack assembly floated under its own buoyancy maintaining the samples approximately 1–2 cm below the water surface. The primary focus of this study was the use of nitrogenase as an indicator of P effects. Previous work identified that the majority of N_2 fixation in these mats occurs under light conditions with light AR being approximately twice that observed under dark conditions (Inglett et al. 2004). For this reason, all incubations were conducted under ambient field conditions at approximately midday. Temperature was controlled during the incubation by floating the tube/rack assemblies in marsh water contained in coolers open to ambient light levels. Three incubation blanks (tubes with acetylene and no sample) were included in the incubations.

Following incubation (~2 h), headspace samples (5 ml) of each tube were taken after vigorous shaking (~5 s) of tube contents to equilibrate gas phases. Gas samples were stored in evacuated, 3.5-ml serum vials sealed with gray, butyl-rubber stoppers and aluminum crimp seals. Gas samples were analyzed for ethylene within 36 h of collection using a Shimadzu GC-8A gas chromatograph with a flame ionization detector (110°C) and 6-foot, Poropak-N column (Supelco, Bellefonte, PA) at 80°C using helium as the carrier gas. Standards (prepared using pure (99.5%) ethylene) and a pre-mixed standard concentration gas (Scott Specialty Gases, Inc., Plumsteadville, PA) were used to calibrate the measurement. Rates of ethylene production (acetylene reduction) were calculated using both headspace and aqueous phase (determined using a temperature-corrected solubility constant) ethylene concentrations and corrected for ethylene in gas blanks. Two methods of expressing the nitrogenase rates were explored including that based on dry mass of bulk periphyton (nmol C_2H_4 hr^{-1} g DW^{-1}) or on the mass of organic carbon (nmol C_2H_4 hr^{-1} g OC^{-1}) (determined as difference between periphyton TC and TIC, see below).

Chemical analysis

Samples used for the AR assay were oven dried (70°C), weighed, and ground for analysis of chemical variables. Total N (TN) and total carbon (TC) were measured simultaneously using a Carlo-Erba NA-1500 CNS elemental analyzer (Haak-Buchler Instruments, Saddlebrook, NJ). Total P and metals were determined by inductively coupled argon plasma emission spectroscopy (Thermo Jarrell Ash ICAP 61E; Franklin, MA) following nitric-perchloric acid digestion (Kuo 1996). Total inorganic carbon (TIC) was measured for periphyton mat samples using an acid dissolution/pressure calcimeter method for total carbonate (Loeppert and Suarez 1996). Total organic carbon (TOC) was determined as the difference between TC and TIC.

Taxonomic identification

Grab samples of floating mat were collected from the WCA-2A mesocosms in July 1998 and January 1999 and stored on ice for transport to the lab. Samples were homogenized and preserved with buffered formalin. Taxonomic analysis was performed by the Florida Department of Environmental Protection Biological Laboratory. For each sample, the relative abundance of functional algal groups (filamentous non-hetrocystous cyanobacteria, filamentous heterocystous cyanobacteria, coccoid cyanobacteria, green algae, and diatoms) was determined on a quantitative subsample by counting representative cells. The number of viable diatoms in the wet mount were counted, but not identified. Non-diatom taxonomic analysis was completed by counting 300 cells within an Utermöhl counting chamber at 420 times magnification using an inverted microscope. Identifications were made at 1,000 times magnification using a compound microscope. A 10 μm length was considered as 1 unit for filamentous species.

Statistical analysis

The importance of the periphyton mat nutrient composition on AR was assessed using separate stepwise multiple regression analyses for the eutrophic (<8 km) and non-enriched (>6 km) transect segments for each sampling date. Samples from the 6–8 km segment were included as a common end member in both regressions. We used this segment approach based on previous studies which identified three groups of WCA-2A periphyton (based on species composition; McCormick et al. 1996) including (1) sites <4 km from the inflows (eutrophic cyanobacteria dominated), (2) sites 4–8 km from the inflow (dominantly green algae), and (3) sites >8 km (consisting of mainly oligotrophic species). An additional predictive regression model for WCA-2A N_2 fixation was developed using the combined data from the November 1997 and February 1999 sampling events. Based on residuals analysis, SQRT-transformed AR data served as the dependent variable for both the individual and combined models. In each case, the final model was selected using the nutrient variables (Ca, Fe, TN, TP, K, and Mn) applying a backward selection technique with variable addition (F-value <5) and removal (F-value <3) criteria. All regression analyses were performed using Statgraphics Plus®, Version 3.1 (Manugistics, Inc., Rockville, MD).

Differences in nutrient variables and AR of samples collected from the experimental WCA-2A mesocosms were tested using a mixed model analysis (proc mixed) with P Loading Rate and Date as fixed main effects, and Date treated as a repeated measure. Mesocosm identity was also included to avoid possible random effects of plot variability. Multiple comparisons of all significant results (P-value <0.1) were conducted using the LSMEANS procedure. These analyses were conducted using SAS version 9.1 (SAS Institute, Cary, NC).

Results and discussion

Spatial trends

Data compiled from the 1997, 1998, and 1999 transect samplings show several trends in WCA-2A periphyton mat chemistry (Fig. 2). Periphyton mats near the inflows was enriched in P, N, and K in relation to the interior, less enriched WCA-2A sites. Periphyton mat Fe concentrations decreased with distance to a minimum at ~8 km. At distances greater than 8 km, periphyton Fe levels increased. In contrast, an increase of periphyton mat Mn was observed with distance from the inflow areas. No simple trend with distance was revealed for Ca and Mg, however, the results were very consistent between samplings and generally revealed a slight elevation of Ca and Mg in the periphyton mats of the more interior marsh zones (>7 km).

Based on previous studies, the distance trends of periphyton mat TN, TP, and Fe (Fig. 2) are likely the result of enrichment from canal discharges (McCormick et al. 1996). In the case of K, however, there was no significant difference between concentrations in canal waters and those of the WCA-2A marsh interior; therefore, the observed increase in periphyton K in the enriched areas appears to be the result of some growth-related factor. A similar pattern of Mn concentration was observed for WCA-2A soil (Craft and Richardson 1997). In that study, dilution was presumed to explain the low Mn levels of the enriched zones, and this could possibly explain the similarly low Mn levels of the periphyton mats reported here. The calcareous nature of Everglades

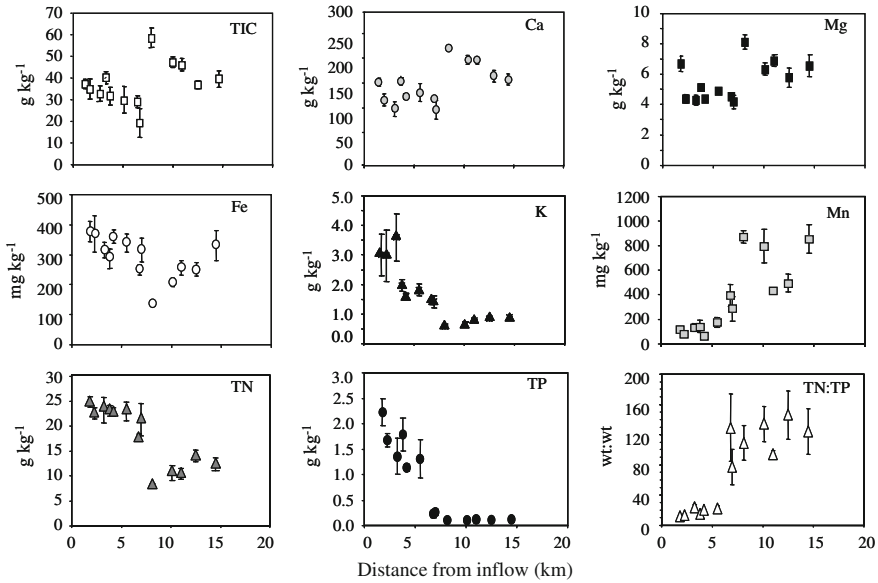

Fig. 2 Spatial patterns of WCA-2A floating periphyton nutrient composition. Points represent the means (±1 SE) of samples from the November 1997, September 1998, and February 1999 transect samplings ($n > 6$). Total Inorganic Carbon (TIC) and Total Organic Carbon (TOC), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), potassium (K), total nitrogen (TN), total phosphorus (TP), and weight TN:TP ratio (TN:TP)

periphyton predominantly explains the high observed concentrations of Mg and Ca, and the high correlation of both Mg and Ca with TIC content strongly indicates the patterns of $CaCO_3$ encrustation in these mats.

Like most microbially-mediated processes, there is a pronounced seasonal effect in nitrogenase activity in WCA-2A periphyton mats (Inglett et al. 2004). Therefore, to visualize the trend along the transect gradient, we expressed nitrogenase activity (AR) as a percent of the maximal activity recorded on each sampling date (Fig. 3a). This normalization allows the combination of data from all sampling times to better establish a predictive relationship in the presence of significant ecosystem variability. Also, because of the variability in the inorganic C fraction, it is more appropriate to express AR on an ash-corrected or organic C basis (g OC^{-1}). The results show a significant trend with distance along the transect (univariate regression, $df = 102$, $R^2 = 0.26$, $P < 0.0001$) with an approximate three-fold enhancement of periphyton mat nitrogenase activity near the inflows compared to the interior zones. The trend of increased nitrogenase activity also coincided with an increased abundance of filamentous heterocystous cyanobacteria (e.g., *Anabaena* spp. and *Calothrix* sp.) in periphyton mats near the inflows (Fig. 3b), indicating that increased N demand favoring dominance by cyanobacteria is potentially a dominant control on N_2 fixation rates.

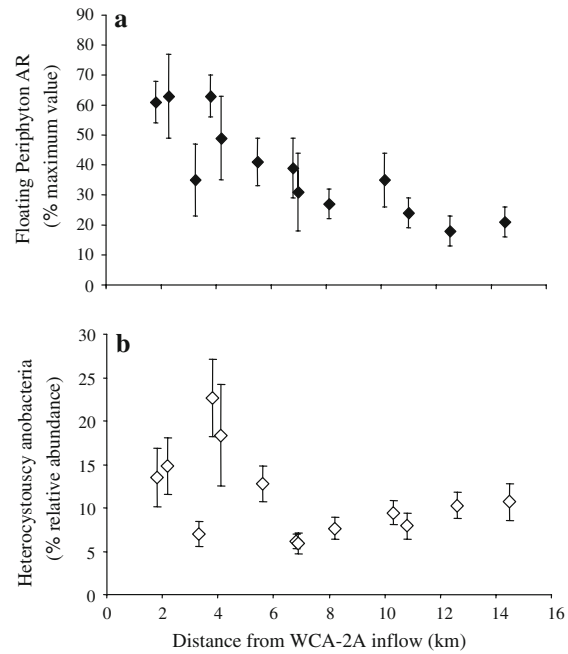

Fig. 3 Spatial patterns of nitrogenase activity measured as acetylene reduction (AR) (**a**) and percent abundance of heterocystous cyanobacteria (**b**) of WCA-2A floating periphyton communities. AR values are expressed as the percent of the maximum AR value recorded for each sampling date (1997 = 565 nmol g OC^{-1} h^{-1}, 1998 = 1157 nmol g OC^{-1} h^{-1}, 1999 = 2415 nmol g OC^{-1} h^{-1}). Points represent the mean ± SE of at least 6 measurements of AR or 12 counts for heterocystous cyanobacterial abundance

Based on the range reported for non-enriched WCA-2A slough periphyton mats by Inglett et al. (2004), the observed three-fold enhancement would equate to yearly AR reduction rates of 57–315 nmol g dw^{-1} h^{-1} in periphyton near the WCA-2A inflows. This range in AR is high compared to that of natural systems, but is very low in relation to other systems enriched by nutrient discharges (e.g., Scott et al. 2005). Given the low estimates for periphyton biomass in inflow areas (McCormick et al. 1998), it is also unlikely that periphyton mats contribute a significant portion of fixed N to the enriched WCA-2A areas (Inglett et al. 2004).

To explore potential nutrient control of nitrogenase activity in these periphyton mats, stepwise multiple regression models of AR were developed for the eutrophic (<8 km) and non-enriched (>6 km) transect portions (Table 2). These two transect ranges were chosen to maximize the number of observations and still adequately characterize the non-linearity of the periphyton mat nutrient patterns (Fig. 2). During the August 1998 sampling, Ca was the primary element showing a significant relationship with non-enriched periphyton mat AR, while the AR of the eutrophic stations was essentially constant. The negative coefficient of Ca and the lack of importance of any other chemical parameters make the models of this sampling date difficult to interpret. Based on the seasonal trends observed for non-enriched WCA-2A periphyton mats (Inglett et al. 2004), it is possible that during this late wet season event, periphyton senescence was occurring, and thus, N_2 fixation was characteristically low and poorly correlated with tissue chemistry variables.

In the interior zone regressions, the importance of P in determining AR rates was evident only during the November 1997 sampling where it was the dominant predicting variable. More consistently, Fe and Ca were significant explanatory variables of AR. This contribution of Ca and Fe in explaining AR is likely a function of the degree of mat calcification where the positive coefficient of Ca and the large negative coefficient of Fe are indicative of a positive effect of mat encrustation. In this case, the high levels of mat encrustation may indicate high periphyton growth activity with increased demand for N derived from N_2 fixation. In the calcification process, Fe appears to better predict the AR of non-enriched WCA-2A mat periphyton, however, the negative coefficient for Fe in the model is contrary to previous work showing Fe stimulation of nitrogenase (Paerl et al. 1994). It is well known that nitrogenase has a high Fe requirement (Paerl 1990); therefore, it could be concluded that the observed correlation between

Table 2 Model parameters for stepwise multiple regression analysis (see text) of square root transformed acetylene reduction rates of eutrophic (<8.0 km) and non-enriched (>6.0 km) WCA-2A transect segments

Sampling date	Distance <8.0 km			Distance >6.0 km		
	Model R^2 (n)	Variables	Estimate (SE)	Model R^2 (n)	Variables	Estimate (SE)
November 1997	0.818 (24)	Constant	28.3 (1.3)	0.678 (21)	Constant	22.7 (1.8)
		Ca	−0.2 (0.0)		Fe	−194 (57)
		TN	−4.9 (0.9)		TN	−4.6 (1.8)
		TP	50.4 (7.2)		TP	227 (54)
August 1998	NA (17)	Constant	20.9 (1.4)	0.467 (12)	Constant	20.5 (3.6)
					Ca	−0.43 (0.15)
February 1999	0.733 (16)	Constant	43.1 (9.9)	0.828 (20)	Constant	14.1 (4.9)
		TN	−14.5 (4.0)		Ca	0.6 (0.1)
		TP	99.8 (17.0)		Fe	−365 (142)
November 1997/ February 1999	0.693 (40)	Constant	32.4 (3.2)	0.748 (41)	Constant	25.1 (1.9)
		TN	−10.6 (1.8)		Fe	−375 (61)
		TP	96.7 (10.6)		Mn	47 (16)
					TN	−3.5 (1.1)
					TP	118 (47)

Models are presented for individual and combined (November 1997/February 1999) transect samplings

Fe and WCA-2A N_2 fixation is likely an artifact of the periphyton calcification process.

The multiple regression results for the enriched zones consistently identified TP as the dominant variable explaining observed AR rates (Table 2). This agrees with studies noting the importance of P availability to rates of planktonic nitrogenase activity (e.g., Flett et al. 1980) and microbial mat N_2 fixation (Pinckney et al. 1995b). The inclusion of TN in the eutrophic models also suggests a significant negative influence of N and agrees with previous studies showing N-suppression of nitrogenase activity (Horne et al. 1979; Diaz et al. 1990; Pinckney et al. 1995b). The magnitude of this N effect was much lower than that observed for P clearly indicating that N_2 fixation of the eutrophic WCA-2A slough periphyton mats is largely governed by P supply.

Phosphorus dosing experiment

The effects of experimental P enrichment on interior WCA-2A periphyton nutrient chemistry are shown in Fig. 4. Phosphorus loading significantly increased the P content of the periphyton mats from 75 mg kg^{-1} in the control mesocosms to 3,552 mg kg^{-1} in the 12.8 g m^{-2} yr^{-1} loading rate. Associated with this P increase were significant increases in biomass N (10–23 g kg^{-1}), and lowering of the periphyton mass TN:TP ratio from 158 to <7. Consistent with the breakdown in the calcareous mat structure, P loading also resulted in decreases in mat TIC, Ca, and Mg. Overall there was a decrease in periphyton mat Mn content with increased P loading, while in contrast, increased Fe concentration was only observed for the single periphyton sample taken at the highest P loading rate in 1996. Many of these trends in periphyton mat chemistry were similar to those observed along the WCA-2A transect. The trends of periphyton mat K in the mesocosm samples, however, were different showing a much more rapid increase from the control to the low to mid-level P loading rates (0.7–5.5 g kg^{-1}), and then slightly declining at the highest P loading rates (3.9 and 3.5 g kg^{-1} in the 6.4 and 12.8 g P m^{-2} yr^{-2}, respectively).

Concurrent with the chemical changes, P addition also resulted in increasing periphyton mat nitrogenase activity (as AR), though the observed effect was quite variable (Figs. 5, 6). Because the inorganic C fraction is known to decrease with P loading (Fig. 4), we again express AR on an ash-corrected or organic C basis (g OC^{-1}). With this correction, AR was generally lowest in samples from the control and low loading rates (<0.4 g P m^{-2} yr^{-1}), achieved a maximal value in the intermediate loading rates (0.8–3.2 g P m^{-2} yr^{-1}, depending on sampling date), and declined at the highest loading rates (>3.2 g P m^{-2} yr^{-1}) (Fig. 5).

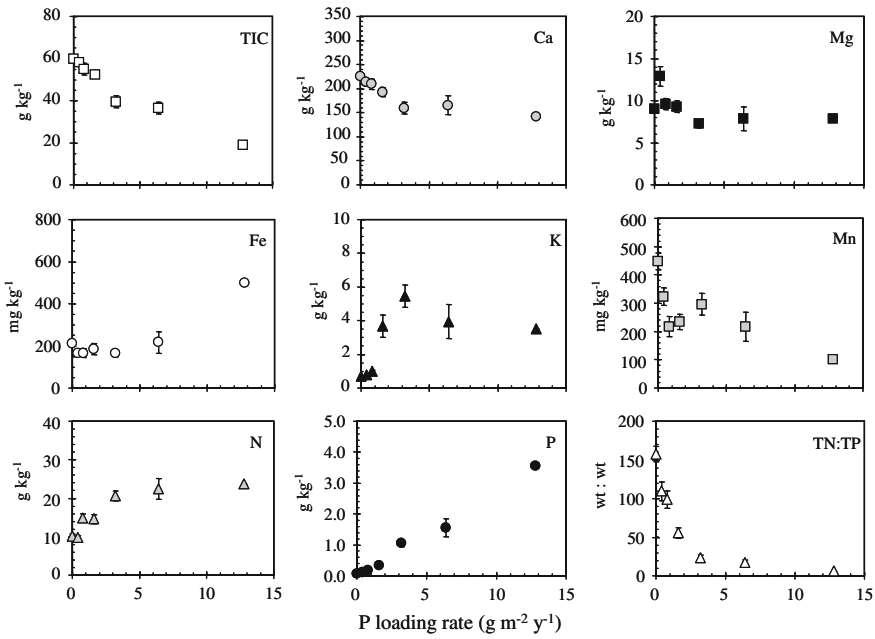

Fig. 4 Phosphorus dosing effects on floating periphyton mat nutrient composition (inorganic carbon (TIC), organic carbon (TOC), calcium (Ca), and magnesium (Mg). iron (Fe), manganese (Mn), potassium (K), total nitrogen (TN), total phosphorus (TP), and weight-based TN:TP) of samples collected from 1996 to 1999 from the WCA-2A mesocosm enclosures. Points represent the mean (±1 SE) of at least 8 measurements except in the 12.8 g P m^{-2} yr^{-1} level where $n = 1$

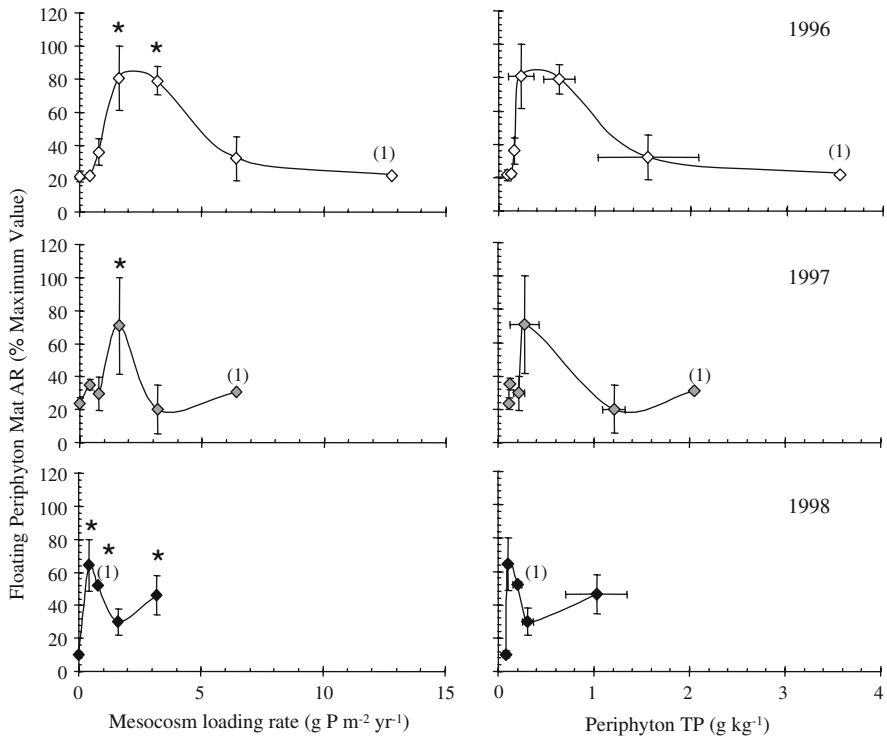

Fig. 5 Effect of phosphorus loading on nitrogenase activity (AR) for samples of floating periphyton mats collected from the mesocosm dosing experiment in November 1996, November 1997, and November 1998. Points represent the mean (±1 SE) of three measurements unless noted (values in parentheses). Means which are significantly different from control (0.0 g P m^{-2} yr^{-1}) were determined using 90% confidence interval estimates and are denoted by (*). Missing points for high P dosing levels are the result of a loss of floating periphyton in these mesocosms. Lines connecting points are for illustrative purposes only and do not imply a statistical relationship or model fit

Maximum recorded rates were approximately three to fourfold greater than those observed in control samples. This level of enhancement is similar to the threefold enhancement observed near the inflows on the transect (Fig. 3), and seemingly confirms the original hypothesis that increased P availability will result in higher N_2 fixation in WCA-2A periphyton.

Interestingly, the P enhancement of AR varied with loading rate and time during the course of this study (Fig. 5). In November 1996, approximately 17 months after the first P dosing, maximum rates of AR were observed in the samples from the 3.2 and 1.6 g P m^{-2} yr^{-1} loading rates. During the same season in 1997, however, peak AR occurred in floating periphyton mats from the 1.6 g P m^{-2} yr^{-1} loading rate while in 1998, the peak was observed in the 0.8 and 0.4 g P m^{-2} yr^{-1} mesocosms (Fig. 5). In this manner, the stimulation of nitrogenase progressed to lower levels of P enrichment with increased duration of P loading. This suggests the potential for an optimum level of P for enhancement of nitrogenase activity in WCA-2A periphyton mats. Based on the 3 years of data in this study, the optimum for periphyton mat nitrogenase activity occurred with the loading of approximately 2–3 g P m^{-2} (calculated based on cumulative loading rates) or biomass TP content of 0.1–0.3 g kg^{-1} (Fig. 5).

The presence of an optimum P level for nitrogenase activity in the mesocosm experiment stands in sharp contrast to the nitrogenase activity observed along the WCA-2A transect which consistently increased with biomass P concentrations up to >2 g kg^{-1} and showed no such optimum P concentration (Figs. 2, 3). Several factors may explain the observed optimum P level for nitrogenase enhancement in WCA-2A periphyton. At levels below the optimum P concentration, P likely limits mat productivity and lowers N demand resulting in low rates of N_2 fixation. Therefore, as P is increased and

TN:TP ratios lowered, we would expect an increase in periphyton AR such as that of the low loading rate mesocosms (Fig. 5). This was also the general trend observed along the WCA-2A transect, where AR continually increased with increasing TP content in excess of 2.0 g kg^{-1} (Figs. 2, 3). In contrast, periphyton mat AR in the highest loaded mesocosms (>6.4 g P m^{-2}) seemed suppressed relative to the peak observed rate even despite having increased TP content and lowered biomass TN:TP ratios similar to the periphyton mats of the transect (Fig. 4). Ultimately, continued P dosing at the highest rates resulted in the loss of floating mat periphyton from the mesocosm enclosures (Figs. 5, 6), and thus, prevent any further comparisons with the results from the transect samples.

The low TN:TP ratios of periphyton mats in the highest loading rates (6.4 and 12.8 g P m^{-2}) indicate nitrogenase suppression is not likely the result of an absence of potential N limitation. Rather, decreased AR above these mesocosms could be the result of limitation by another nutrient, for example Fe, which showed lower biomass concentrations in samples from the mesocosms (Fig. 4) compared to those of the WCA-2A transect stations (Fig. 2). The presence of high NH_4^+ concentrations could also affect the nitrogenase patterns observed in the WCA-2A mesocosms (Rejmankova and Komarkova 2000). One source of this NH_4^+ could be through increased mineralization of the peat soils following P addition (White and Reddy 2000). Newman et al. (2004) did observe NH_4–N levels as high as 9 mg l^{-1} in the porewaters of the WCA-2A mesocosms during the P dosing experiment, and profile measurements indicated a potentially significant flux of porewater NH_4–N into the watercolumn.

If these NH_4^+ concentrations were sufficiently increased, watercolumn N_2 fixation could have been suppressed. For example, in mats similar to those of WCA-2A, small additions of NH_4^+ (<1 mg N l^{-1}) were sufficient to reduce nitrogenase activity to almost half that of samples amended with P alone (Rejmankova and Komarkova 2000). In this study, however, we measured the highest nitrogenase activity in November, 1996 (~week 70 of Newman et al. 2004) in the 3.2 g P m^{-2} treatment with the highest levels of NH_4^+ production. Also, Newman et al. (2004) noted after 71 weeks of dosing at the 12.8 g P m^{-2} yr^{-1} level, that porewater NH_4^+ levels were actually reduced below those of the controls, indicating that high P dosing resulted in increased N demand. For this reason, elevated NH_4^+ does not fully

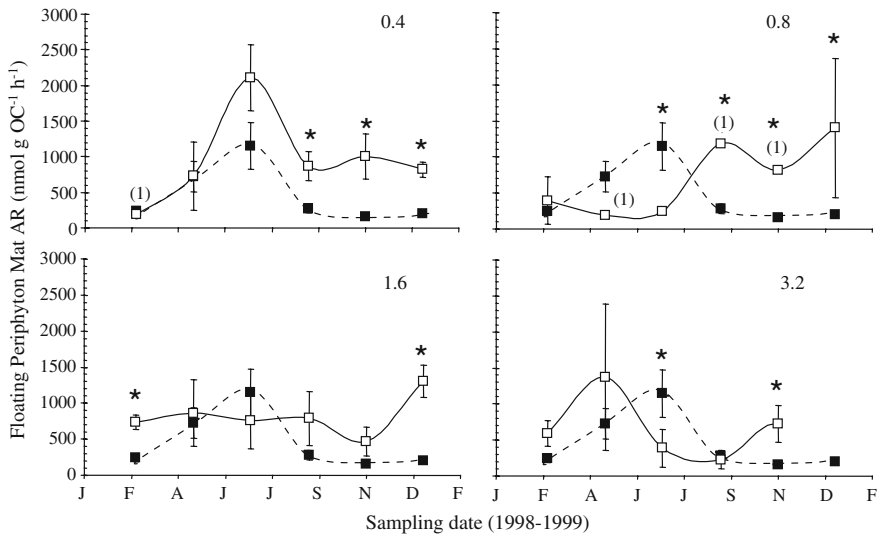

Fig. 6 Seasonal patterns of floating periphyton mat nitrogenase activity (AR) for samples collected from the control (closed squares) and each of four levels (0.4, 0.8, 1.6, and 3.2 g P m^{-2} yr^{-1}) of P-enriched (open squares) WCA-2A mesocosm enclosures. Points represent the mean (±1 SE) of three measurements unless noted (values in parentheses). Means which are significantly different from control on a given date were determined using 90% confidence interval estimates and are denoted by (*)

explain reduced AR in the 12.8 g P m^{-2} yr^{-1} mesocosm in the1996 sampling.

Alternatively, species shifts favoring the dominance of non-N_2 fixing taxa could also affect periphyton nitrogenase patterns. In their study utilizing these same WCA-2A mesocosm enclosures, McCormick and O'Dell (1996) and McCormick et al. (2001) observed a rapid (within 4 weeks) change in the 6.4 and 12.8 g P m^{-2} yr^{-1} mesocosms leading to a transient increase in filamentous chlorophytes (e.g. *Spirogyra*) followed by the dominance of eutrophic cyanobacteria (e.g. *Plectonema wollei* and *Oscillatoria princeps*). This transition toward green algae could explain lower AR rates of some treatments in this study, however, it is insufficient to explain low AR in the 6.4 and 12.8 g P m^{-2} yr^{-1} treatments in 1996 which by that time, were already shown to be dominated by eutrophic cyanobacteria (McCormick and O'Dell 1996). Also, an abundance of green algae does not explain the very high nitrogenase activity observed in the 3.2 g P m^{-2} yr^{-1}, a treatment dominated by filamentous green algae in December, 1995 (McCormick and O'Dell 1996).

Species composition of samples in 1998 (Table 3) showed the highest composition of non-cyanobacterial algae (as "other" in Table 3) occurred in the 3.2 g P m^{-2} yr^{-1} mesocosms. The exact species composition during the 1996 and 1997 mesocosm samplings is not known, but based on the TP content of samples used in the McCormick et al. (2001) work, the shift to dominance of green algae occurred at a periphyton biomass P concentration of between $\sim$0.5 and 1 g P kg^{-1} DW. In this study, this range of P concentration was only observed in samples at dosing rates above 1.6 g P m^{-2} yr^{-1} (Figs. 4, 5) indicating that, in agreement with the species composition in Table 3, the 3.2 g P m^{-2} yr^{-1} treatment level would still have been dominated by filamentous chlorophytes.

Because our first measurements of nitrogenase were obtained over 1 year following the first dosing event, it is reasonable to expect that a stimulation of AR would also have occurred in the highest loading rate mesocosms (6.4 and 12.8 g P m^{-2} yr^{-1}) during the initial weeks to months of dosing. Based on changes in periphyton TP content, the maximum AR in these high loading treatment levels would have occurred within the first 5 weeks of P dosing (Newman et al. 2004). After 3 years of loading, P enhancement of AR was still observed, but only in the mesocosms of the lowest loading rates (Fig. 5), and there was a definite seasonality in the effect of P (GLM Factorial ANOVA using log transformed data, $df = 19$, $F = 1.98$, $P = 0.026$). Only at the lowest rates of P enrichment (0.4 and 0.8 g P m^{-2} yr^{-1}) was floating mat AR consistently at or above that of control

Table 3 Mean (SE) relative abundance counts of dominant taxonomic groups in floating periphyton mat samples obtained from the WCA-2A experimental mesocosms during July, 1998 and January, 1999

Date (P load)	Cyanobacteria					
	n	Filament. non-heterocyst.	Filament. heterocyst.	Coccoid	Diatoms	Other
July 1998						
(0.0 g m^{-2} y^{-1})	3	74.2 (3.4)	0.7 (0.7)	19.1 (3.0)	5.2 (1.0)	0.8 (0.1)
(0.4 g m^{-2} y^{-1})	3	80.7 (2.7)	2.2 (0.5)	11.5 (1.0)	4.2 (1.0)	1.5 (0.3)
(0.8 g m^{-2} y^{-1})	3	54.3 (4.5)	2.2 (1.8)	39.0 (3.6)	2.6 (0.6)	1.9 (0.8)
(1.6 g m^{-2} y^{-1})	3	69.8 (5.9)	8.4 (2.1)	17.4 (4.7)	0.8 (0.5)	3.6 (0.8)
(3.2 g m^{-2} y^{-1})	3	63.3 (5.5)	0.8 (0.6)	32.8 (5.2)	0.7 (0.1)	2.4 (0.4)
January 1999						
(0.0 g m^{-2} y^{-1})	2	68.7 (7.9)	0.2 (0.2)	28.0 (8.2)	2.3 (0.0)	0.8 (0.0)
(0.4 g m^{-2} y^{-1})	2	75.3 (3.9)	3.5 (2.2)	19.5 (0.8)	1.2 (0.5)	0.6 (0.3)
(0.8 g m^{-2} y^{-1})	2	70.1 (3.1)	13.2 (7.9)	12.9 (8.5)	1.9 (1.6)	1.9 (0.5)
(1.6 g m^{-2} y^{-1})	2	69.4 (1.4)	13.0 (3.1)	13.5 (0.9)	1.0 (0.3)	3.2 (0.4)
(3.2 g m^{-2} y^{-1})	1	66.9	3.5	10.1	2.6	17.0

mesocosms throughout the year (Fig. 6). In the 0.4 g P m^{-2} yr^{-1} treatment, AR enhancement was observed primarily during the July 1998–January 1999 sampling events and had a similar trend to that of the control mesocosms. In contrast, the seasonal AR pattern was more variable in the higher loading rate mesocosms where it was either similar to the controls (3.2 g P m^{-2} yr^{-1}), completely opposite to the controls (0.8 g P m^{-2} yr^{-1}), or almost constant throughout the year (1.6 g P m^{-2} yr^{-1}) (Fig. 6).

Some of the discrepancies in seasonal patterns of periphyton mat AR between P treatments could undoubtedly be explained by effects of P loading on periphyton species composition. Table 3 results demonstrate the fluctuation of some periphyton mat groups between winter/summer or dry/wet seasons, particularly in the percentages of heterocystous cyanobacteria and coccoid cyanobacteria. Unlike the patterns of species composition and AR of the transect (Fig. 3b), however, it is unclear to what degree the abundance of heterocystous cyanobacteria coincide with AR patterns observed in the mesocosms. Also, as previously mentioned, higher NH_4^+ levels in the water column could also explain lowered AR activity, and may interact with P levels to influence species shifts affecting the presence and activity of N_2-fixing taxa. It is also likely that the seasonal AR patterns of some mesocosms could reflect the early effects of P enhancement. This possibility is particularly visible in the 0.4 g P m^{-2} yr^{-1} which began the year with AR rates similar to the controls, but by July, had become enhanced above controls (Fig. 6). This mid season increase may indicate that the effect of P was beginning to peak in the 0.4 loading rate during this time period.

One possibility which may hinder the interpretation of the mesocosm results is the age of the mesocosm experiment at the time of this study. For more than 1 year prior to this study, the WCA-2A mesocosm enclosures were experimentally enriched with P. During this time, changes (e.g., increased macrophyte growth) may have occurred within the enclosures which could have interfered with the response of periphyton mat nitrogenase to P enrichment. For example, greatly increased macrophyte density and heavy periphytic growths on enclosure walls could have the varied effects of restricting light penetration to the benthic surfaces, disruption of benthic and water column nutrient exchanges, and the concentrating of phytotoxic chemicals present in plant biomass (e.g., *Nymphaea* sp.) (Elakovich et al. 1999).

Conclusions

Thus far, the bulk of Everglades research has focused on the importance of P as a limiting nutrient and its importance to periphyton productivity and species composition (reviewed by Noe et al. 2001). The results of this and previous research show an increasing importance of N_2 fixation in the Everglades both as a potential control of nutrient limitation in the P-enriched areas near the inflows, and as a natural process characterizing the non-enriched ecosystem. Eutrophic WCA-2A periphyton is characterized by higher rates of N_2 fixation than the periphyton WCA-2A interior zones, but shading by dense macrophyte cover (which reduces periphyton biomass) likely diminishes the importance of this process as a potential input of N in the eutrophic marsh zones. Nutrient content of the periphyton biomass indicate the increased nitrogenase activity is primarily the result of increased N limitation in the highly eutrophic areas of WCA-2A.

The spatial patterns from the WCA-2A transect and the temporal patterns from the P dosing mesocosm study also demonstrate that periphyton nitrogenase activity increases in response to P loading. In this manner, nitrogenase activity, like other periphyton enzyme activities, can provide a useful biogeochemical indicator in the Everglades and other wetland ecosystems. The controlled mesocosm experiment revealed that nitrogenase activity became enhanced at periphyton P contents between 100 and 300 mg P kg^{-1} DW, and changes with continued loading demonstrated that the effect of P loading on nitrogenase activity is cumulative. This finding agrees with previous work illustrating how even low rates of P loading can be detrimental to long term periphyton mat stability in systems like the Everglades (Gaiser et al. 2005). The differing response of nitrogenase to increased P from periphyton samples collected along the WCA-2A transect likely represents differences between the transect and mesocoms in environmental conditions (light, macrophyte species compositions, etc.) and nutrient sources (agricultural drainage along the transect, and phosphate for the mesocosms).

As yet, the involvement of N_2 fixation in the processes related to periphyton mat disintegration and species shifts remains uncertain, and more study is needed to fully relate the short-term, long-term, and seasonal patterns in nitrogenase activity to patterns in species composition. More research is also needed to more accurately determine the coupling of C, N, and P cycles within the periphyton mat to better determine the biogeochemical processes influencing mat stability and functioning. This research demonstrates that N_2 fixation may have a significant role in this process, but currently, little emphasis is being placed on the potential interaction between nitrogenase and enzymes of other elemental cycles such as phosphatase, glucosidase, etc. Further establishment of such linkages between nutrient cycles, could vastly improve our ability to understand and predict changes in periphyton systems as they relate to ecosystem conditions and periphyton function.

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References

Bergmann MA, Welch HE (1990) Nitrogen fixation by epilithic periphyton in small arctic lakes in response to experimental nitrogen and phosphorus fertilization. Can J Fish Aquat Sci 47:1545–1550

Browder JA, Gleason PJ, Swift DR (1994) Periphyton in the Everglades: spatial variation, environmental correlates, and ecological implications. In: Davis SM, Ogden JC (eds) Everglades: the ecosystem and its restoration. St. Lucie Press, Delray Beach, FL

Craft CB, Richardson CJ (1997) Relationships between soil nutrients and plant species composition in everglades peatlands. J Environ Qual 26:224–232

Craft CB, Vymazal J, Richardson CJ (1995) Response of Everglades plant communities to nitrogen and phosphorus additions. Wetlands 15:258–271

Davis SM, Ogden JC (eds) (1994) Everglades: the ecosystem and its restoration. St. Lucie Press, Del Ray Beach, FL

Diaz MR, Corredor JE, Morell JM (1990) Nitrogenase activity of *Microcoleus lyngbyaceus* mat communities in a eutrophic tropical marine environment. Limnol Oceanogr 35:1788–1795

Elakovich SD, Spence S, Yang J (1999) Phytochemical inhibitors from Nymphaeceae: *Nymphaea odorata* and *Nuphar lutea*. In: Cutler HG, Cutler SJ (eds) Biologically active natural products: agrochemicals. CRC Press, pp 49–56

Flett RJ, Schindler DW, Hamilton RD, Campbell NER (1980) Nitrogen fixation in Canadian Precambrian shield lakes. Can J Fish Aquat Sci 37:494–505

Flora MD, Walker DR, Scheidt DJ, Rice RG, Landers DH (1988) The response of Everglades marsh to increased nitrogen and phosphorus loading. Part I: nutrient dosing, water chemistry, and periphyton productivity. Everglades National Park, Homestead, FL, USA

Gaiser EE, Trexler JC, Richards JH, Childers DL, Lee D, Edwards AL, Scinto LJ, Jayachandran K, Noe GB, Jones RD (2005) Cascading ecological effects of low-level phosphorus enrichment in the Florida everglades. J Environ Qual 34:717–723

Hall GB, Rice RG (1990) Response of the Everglades marsh to increased nitrogen and phosphorus loading. Part III: periphyton community dynamics. Everglades National Park, Homestead, FL, USA

Hendzel LL, Hecky RE, Findlay DL (1994) Recent changes of N_2-fixation in Lake 227 in response to reduction of the N:P loading ratio. Can J Fish Aquat Sci 51:2247–2253

Horne AJ, Sandusky JC, Carmiggelt CJW (1979) Nitrogen fixation in Clear Lake, California. 3. Repetitive synoptic sampling of the spring Aphanizomenon blooms. Limnol Oceanogr 24:316–328

Inglett PW, Reddy KR, McCormick PV (2004) Periphyton chemistry and nitrogenase activity in a northern Everglades ecosystem. Biogeochemistry 67:213–233

Koch MS, Reddy KR (1992) Distribution of soil and plant nutrients along a trophic gradient in the Florida Everglades. Soil Sci Soc Am J 56:1492–1499

Kuo S (1996) Phosphorus. In: Sparks DL (ed) Methods of soil analysis, Part 3: chemical analysis. Soil Science Society of America Book Series No. 5. Madison, WI

Loeppert RH, Suarez DL (1996) Carbonate and gypsum. In: Sparks DL (ed) Methods of soil analysis, Part 3: chemical analysis. Soil Science Society of America Book Series No. 5, Madison, WI

McCormick PV, Cairns J Jr (1994) Algae as indicators of environmental change. J Appl Phycol 6:509–526

McCormick PV, O'Dell MB (1996) Quantifying periphyton responses to phosphorus in the Florida Everglades: a synoptic-experimental approach. J North Am Benthol Soc 15:450–468

McCormick PV, Stevenson RJ (1998) Periphyton as a tool for ecological assessment and management in the Florida Everglades. J Phycol 34:726–733

McCormick PV, Rawlik PS, Lurding K, Smith EP, Sklar FH (1996) Periphyton-water quality relationships along a nutrient gradient in the northern Florida Everglades. J North Am Benthol Soc 15:433–449

McCormick PV, Shuford RBE, Backus JB, Kennedy WC (1998) Spatial and seasonal patterns of periphyton biomass and productivity in the northern Everglades, Florida, USA. Hydrobiologia 362:185–208

McCormick PV, O'Dell MB, Shuford RBE, Backus JG, Kennedy WC (2001) Periphyton responses to experimental phosphorus enrichment in a subtropical wetland. Aquat Bot 71:119–139

Newman S, McCormick PV, Miao SL, Laing JA, Kennedy WC, O'Dell MB (2004) The effect of phosphorus

enrichment on the nutrient status of a northern Everglades slough. Wetlands Ecol Manage 12:63–79

Noe GB, Childers DL, Jones RD (2001) Phosphorus biogeochemistry and the impact of phosphorus enrichment: why is the Everglades so unique? Ecosystems 4:603–624

Paerl HW (1990) Physiological ecology and regulation of N_2 fixation in natural waters. Adv Microbiol Ecol 11:305–344

Paerl HW, Prufert LE, Bebout B, Guo CZ (1994) Iron-stimulated N_2 fixation and growth in natural and cultured populations of the planktonic marine cyanobacteria *Trichodesmium* spp. Appl Environ Microbiol 60: 1044–1047

Pinckney J, Paerl HW, Fitzpatrick M (1995a) Impacts of seasonality and nutrients on microbial mat community structure and function. Mar Ecol-Prog Ser 123:207–216

Pinckney J, Paerl HW, Reid RP, Bebout B (1995b) Ecophysiology of stromatolitic microbial mats, Stocking-Island, Exuma-Cays, Bahamas. Microb Ecol 29:19–37

Reddy KR, DeLaune RD, DeBusk WF, Koch MS (1993) Long term nutrient accumulation rates in the Everglades. Soil Sci Soc Am J 57:1147–1155

Reddy KR, Wang Y, Debusk WF, Fisher MM, Newman S (1998) Forms of soil phosphorus in selected hydrologic units of the Florida Everglades. Soil Sci Soc Am J 62:1134–1147

Reddy KR, White JR, Wright A, Chua T (1999) Influence of phosphorus loading on microbial processes in the soil and water column of wetlands. In: Reddy KR, O'Connor GA, Schelske C (eds) Phosphorus biogeochemistry in subtropical ecosystems. Lewis Academic, Boca Raton, FL, pp 249–273

Rejmankova E (2001) Effect of experimental phosphorus enrichment on oligotrophic tropical marshes in Belize, Central America. Plant Soil 236:33–53

Rejmankova E, Komarkova J (2000) A function of cyanobacterial mats in phosphorus-limited tropical wetlands. Hydrobiologia 431:135–153

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

Stal LJ (1988) Nitrogen fixation in cyanobacterial mats. Methods Enzymol 167:474–484

Swift DR, Nicholas RB (1987) Periphyton and water quality relationships in Everglades water conservation areas, 1978–1982. South Florida Water Management District #87-2, West Palm Beach, FL

White JR, Reddy KR (2000) Influence of phosphorus loading on organic nitrogen mineralization of Everglades soils. Soil Sci Soc Am J 64:1525–1534