ARTICLE

Biological Nitrogen Fixation in Periphyton of Native and Restored Everglades Marl Prairies

Xiaolin Liao · Patrick W. Inglett

Abstract Periphyton mats are an important component of the Everglades ecosystem. These mats are able to fix atmospheric nitrogen; however little attention has been paid to this function throughout much of the Everglades system. The objective of this study was to characterize and quantify periphyton N_2 fixation in the Hole-in-the-Donut (HID) region of the southern Everglades, where farmed marl prairie wetlands have been restored through complete soil removal to reduce nutrient levels. Significantly higher N_2 fixation rates (measured acetylene reduction) were found in periphyton of the areas cleared in 2000 and 2003 (3–10 nmol g^{-1} DW h^{-1}) compared to the reference wetland site (less than 1 nmol g^{-1} DW h^{-1}). Overall rates were stimulated by light (~2 times the measured dark rates). Areal estimates of fixed N were low compared to other Everglades, ranging from 0.1–0.2 gN m^{-2} yr^{-1} in the restored sites to 0.05 gN m^{-2} yr^{-1} in the reference area. Stable N isotopic ratios (i.e., δ^{15}N) ranged from −1.0‰ to 0.2‰ and were correlated with nitrogenase activity and TN:TP ratios. These findings suggest that periphyton nitrogenase activity and δ^{15}N could serve as indicators of nutrient status and restoration success in these systems.

Keywords Acetylene reduction · Calcareous · Nitrogenase · Restoration · Stable isotopes · Phosphorus · ^{15}N

Introduction

Periphyton mats consist of a complex mixture of algae, heterotrophic microbes, and particles (mineral and detritus), and play an important ecological role in shallow aquatic ecosystems. Periphyton can serve as a major contributor to primary productivity (Dodds et al. 2002), a regulator of water column nutrient levels (Gaiser et al. 2004; Rejmánková and Komárková 2005; Thomas et al. 2006) and benthic fluxes, and based on species composition (McCormick and Stevenson 1998) and enzyme expression (Sharma et al. 2005), can also be a sensitive indicator of water quality (Vis et al. 1998; McCormick 2011). Periphyton is often abundant in shallow wetland systems, including marine tidal flats (Pinckney et al. 2011) and freshwater portions of the limestone based Caribbean (Rejmánková and Komárková 2000; Rejmánková et al. 2004). Similarly, the Florida Everglades (USA) wetland system also maintains an abundance of periphytic assemblages, with biomass estimates ranging between 3 and 6235 g AFDW m^{-2} (Hagerthey et al. 2011).

Periphyton is well described in the Everglades, varying both temporally and spatially in terms of biomass, productivity, and species richness and diversity in relation to macrophyte abundance and nutrients (Browder et al. 1994; McCormick and O'Dell 1996; McCormick et al. 1996, 1998; Gaiser et al. 2006, 2011; Hagerthey et al. 2011). In particular, phosphorus (P) levels are a key regulator of a variety of processes in the Everglades periphyton including species composition (McCormick and O'Dell 1996; McCormick et al. 1998), production and respiration (Iwaniec et al. 2006). Everglades periphyton also show the ability to fix atmospheric N_2 (biological N_2 fixation) (Inglett et al. 2004, 2009). This process was estimated to contribute 10 gN m^{-2} yr^{-1} to a northern oligotrophic Everglades system. Periphyton is abundant throughout much of the Everglades, however, there are few studies to document the significance of biological fixation in other areas of the Everglades, especially in the southern systems (Inglett et al. 2011a).

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One Everglades system where periphyton plays a crucial ecological role is the Hole-in-the-Donut (HID) region of Everglades National Park (ENP) (Fig. 1). This area had a history of farming which disturbed and added excess P to the native pine rockland and marl prairie ecosystems (Smith et al. 2011). Disturbance and excess nutrients led to the invasion of Brazilian pepper (*Schinus terebinthifolius*) after farming ceased (Smith et al. 2011). To restore the HID to marl prairie ecosystem, the technique of complete soil removal, in which all the vegetation and underlying rock-plowed substrate was removed down to bedrock, was adopted (Dalrymple et al. 2003). In this process soils are mechanically cleared to bedrock and allowed to naturally reestablish biotic communities such as periphyton and macrophytes.

During the HID restoration processes, periphyton plays an important role in soil formation, as a source of organic matter and calcium carbonate ($CaCO_3$), both main components of marl soils (Gaiser et al. 2011). Nitrogen fixation also provides an important N source particularly in the recently cleared sites where N is limiting (Smith et al. 2011; Inglett et al. 2011a, b). Though studied intensely in other parts of Everglades, no study has been focused on periphyton in this unique restored calcareous ecosystem where this component is a key target and

evaluation metric for restoration (Gaiser 2009). Furthermore, few studies have assessed N_2 fixation in P-limited ecosystems (Inglett et al. 2004; Rejmánková and Komárková 2000; Rejmánková 2001; Inglett et al. 2009), so it is important to understand the role of periphyton in Everglades marl prairies (Davis et al. 2005). For these reasons, the following study was conducted to (1) characterize periphyton N_2 fixation between the restored and reference wetlands; (2) relate the N_2 fixation with nutrient limitation (i.e., N and P limitation); and (3) assess the potential of N stable isotopes as an indicator of N_2 fixation and nutrient limitation.

Methods

Study Site

We selected two wetlands restored in 2000 and 2003, as well as an unfarmed reference site adjacent to the restored areas in the Hole-in-the-Donut region of Everglades National Park (Fig. 1). In each site, we identified five sampling stations (A, B, C, D, E) along a elevation gradient from 0.5 to 1.0 m AMSL (Table 1). Soil depth varied among the three sites,

Fig. 1 Map showing the general area of Everglades National Park and the locations of restored (2000 and 2003) and reference marl prairie sites used in this study
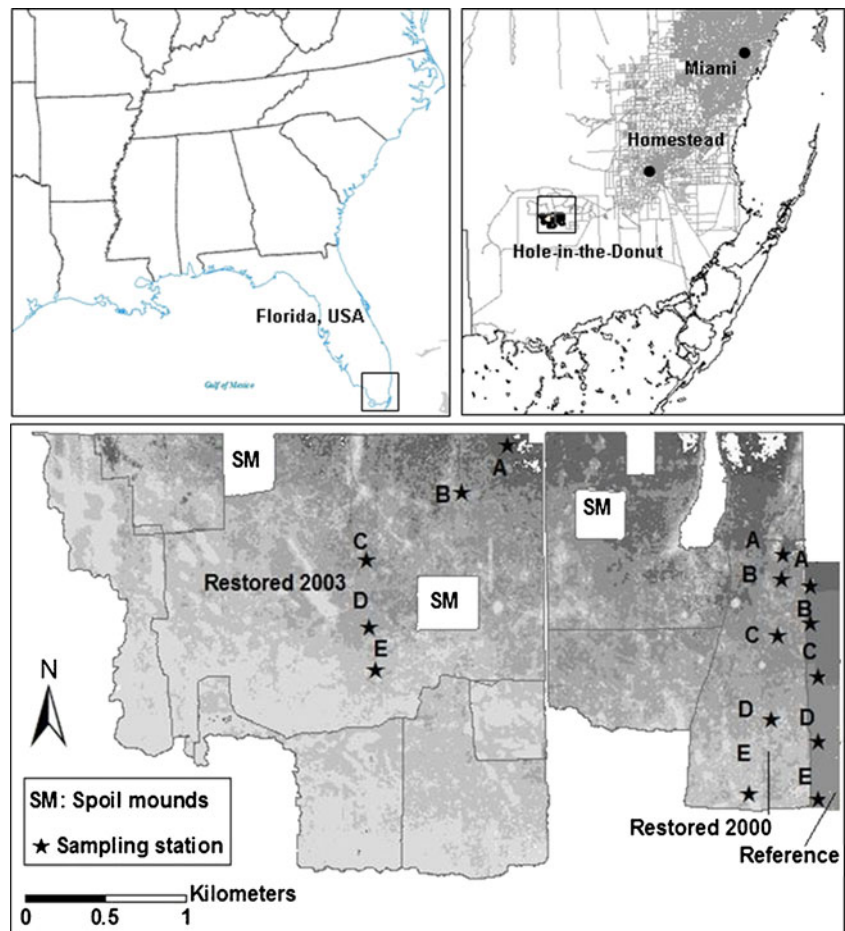
Table 1 Geographical coordinates and the approximate elevation of the sampling sites used in this study

Sampling site	Station	Latitude	Longitude	Elevation (m)
2000-restored	A	N25.38283	W80.67442	0.7–0.8
	B	N25.38150	W80.67452	0.7–0.8
	C	N25.37856	W80.67477	0.6–0.7
	D	N25.37408	W80.67515	0.6–0.7
	E	N25.37019	W80.67652	0.5–0.6
2003-restored	A	N25.38865	W80.69132	0.8–0.9
	B	N25.38618	W80.69414	0.6–0.7
	C	N25.38260	W80.70003	0.6–0.7
	D	N25.37903	W80.69982	0.6–0.7
	E	N25.37678	W80.69946	0.5–0.6
Reference	A	N25.38117	W80.67275	0.8–0.9
	B	N25.37916	W80.67246	0.7–0.8
	C	N25.37635	W80.67224	0.7–0.8
	D	N25.37293	W80.67229	0.6–0.7
	E	N25.36985	W80.67223	0.6–0.7

The ground elevation data extracted from the EDEN DEM model (http://sofia.usgs.gov/eden/models/groundelevmod.php)

with deeper marl soils (Biscayne and Perrine series) in the reference areas (10 cm) and shallower soils (2–3 cm) which have developed after site clearing in the restored areas (Smith et al. 2011). The primary vegetation in the reference site is a mixture of grasses (*Muhlenbergia* sp., *Andropogon* spp.) and sedges (*Cladium jamaicense* Crantz, *Shoenus* sp.) while the restored sites are dominated by grasses such as *Andropogon* spp. and other shrub-like pioneer species including *Ludwigia* spp., and *Baccharis* spp. (Dalrymple et al. 2003). The periphyton in the three sites are all calcitic epilithon or calcareous epiphytic periphyton mats (Gaiser et al. 2006, 2011). The mats in the restored sites were very thin and greenish, while the mats in the reference sites were ~1 cm thick with a dark-colored surface.

Sampling Methods

The Everglades experiences two primary seasons including a mostly dry winter (November through April) and a wet summer (May to October). The wet summer season accounts for approximately 80% of the region's average annual rainfall of 137 cm. Rainfall within the Everglades system can vary dramatically from year to year. Historically, some wet years peaked at over 254 cm of rainfall, whereas some dry years received less than 76 cm (http://www.waterencyclopedia.com/En-Ge/Everglades.html).

We collected samples in October 2009 (wet season) and February 2010 (dry season). At each of the 15 transect locations, three composite samples of surface soil and periphyton were collected. Surface soils were collected using sharpened metal tubes (3.75 cm ID) inserted to bedrock (restored sites) or to a depth of 5 cm (reference area). Periphyton was collected by randomly placing a plastic ring (8 cm ID) and since the study sites were not well flooded, it was easy to remove the periphyton biomass contained within the ring area by hand. This was repeated (up to 5 times) until sufficient biomass had been collected for each composite sample which could then be used to determine periphyton biomass per unit area (g m^{-2}). Samples were stored on ice until their return to the laboratory where the samples were refrigerated at 4°C until subsequent analysis.

Periphyton samples were kept intact (periphyton mat) and inspected to remove large organic debris (plant litter) and soil. Soil samples were sieved to remove roots and rock fragments greater than 2 mm diameter. Sieved soil samples were oven dried at 105°C for three days and ground using a mortar and pestle for moisture content and total nutrient determinations.

Nitrogenase Analysis

Nitrogenase activity (N$_2$ fixation) was measured using the acetylene (C$_2$H$_2$) reduction (AR) assay described by Inglett et al. (2004). Wet periphyton (5 g) was placed into 42-mL, screw-capped culture tubes (Kimax^{TM}) with an open-top cap containing a teflon-lined, silicone septa (0.120″ thick). Acetylene gas (generated by adding water to CaC$_2$ in an evacuated serum bottle) was added to each tube (4 ml, approximately 10% headspace) and the tubes were shaken to make sure the gas evenly distributed in the whole space. Tubes containing samples and blanks containing only injected acetylene were incubated at constant temperature (27°C) for up to 3 h under either light (~900 μmol m^{-2} s^{-1} PAR) or dark conditions. After incubation, tubes were shaken to equilibrate gas phases, and gas samples (4 ml) were taken from each tube and stored in evacuated 3.5-ml exetainers.

Gas samples were analyzed for ethylene using a Shimadzu GC-8A gas chromatograph equipped with a flame ionization detector (110°C) and a Poropak-N column (80°C). Two standard gases (1 and 10 ppm; Scott Specialty Gases, Inc., Plumsteadville, PA) were used to calibrate the measurement being expressed as nmol C$_2$H$_4$ g dw^{-1} h^{-1}. Blank corrected AR values were used to estimate actual rates of N$_2$ fixation using a theoretical conversion ratio of three moles of C$_2$H$_2$ reduced to one mole of N$_2$ fixed (Howarth et al. 1988a). When estimating the annual fixed nitrogen, we made the following assumption: (1) there were two seasons with 6 months of wet season represented by the October and 6 months of dry season by February; (2) the whole day was divided by 12 h light condition and 12 h dark condition; and (3) the biomass was constant with the season.

Following the incubation, periphyton contained in each tube was dried at 70°C for 3 days to determine dry weight of periphyton biomass. The dried sample was then ground using a ball mill for chemical and isotopic analysis.

Chemical and Isotopic Analysis

Total C and N content were measured using a Thermo Flash EA 1112 elemental analyzer (CE Elantech, Inc.). Total P of periphyton was measured colorimetrically using a Shimadzu UV-160 spectrometer (method 365.1 U.S. EPA 1993) following ashing and dissolution in 6 M HCl (Anderson 1976). Total organic C was estimated by loss-on-ignition (LOI) at 550°C for 4 h after conversion to organic C with a coefficient factor of 0.51 (Wright et al. 2008). Stable N isotopic ratios were determined using a Finnigan MAT Delta Plus isotopic ratio mass spectrometer (Finnigan Corp. San Jose, CA) (Inglett and Reddy 2006) and expressed as permil (‰) differences from the standard isotopic ratio of atmospheric N_2 (0.3663%) using delta notation (δ) as follows: $\delta^{15}N_{sample} = \frac{\left(^{15}N/^{14}N\right)_{sample} - \left(^{15}N/^{14}N\right)_{standard}}{\left(^{15}N/^{14}N\right)_{standard}} \times 1000.$

Water Chemistry

Water chemistry samples were collected in acid-washed polyethylene bottles in October 2009 when the sites were flooded. The water samples were filtered through 0.45 μm membrane filters, acidified and preserved on ice until their return to the laboratory. We analyzed the total dissolved phosphorus (TDP), soluble reactive phosphorus (SRP, PO_4^{3-}), ammonia-nitrogen (NH_4-N), nitrite-nitrogen plus nitrate-nitrogen (NO_x-N), and total dissolved Kjeldahl Nitrogen (TDKN) for the filtered water samples with standard methods (EPA, US 1983).

Statistical Analysis

All statistical analyses were performed using JMP v.8© statistical software (SAS Institute Inc., Cary, NC). Two-way ANOVA was applied to test for differences of various soil properties between sites, seasons and the interaction of site and season. Comparisons of means for significant effects were determined using Tukey HSD tests. Regression

analysis with Pearson correlation coefficient was used to evaluate the relationship between various nutrient properties, isotopic composition and nitrogenase activity. Differences among means and correlation coefficients were deemed significant at the $P<0.05$ level, unless otherwise noted.

Results and Discussion

Water Chemistry

Results of water chemistry sampling are provided in Table 2. NH_4-N, dissolved inorganic nitrogen (DIN=NO_x-N+NH_4-N), TKN, TDP, and SRP were significantly higher in the 2000-restored site comparing to the reference site and 2003-restored site ($P<0.05$). The water TDP was close to the natural Everglades system below a threshold limit of 10 μg L^{-1} (Thomas et al. 2002). However, the ratio of TDKN: TDP were significantly lower in the restored sites compared to the reference site ($P<0.05$).

Nutrient Composition

A main difference between the reference and restored sites is the nutrient status, in particular the levels of N and P (Smith et al. 2011). Soil TP in the reference site was significantly lower than that in both restored sites (Table 3), contributing to a much higher soil TN:TP molar ratio in the reference site (109) versus that in the 2003-restored (15) or the 2000-restored (28) sites. For the periphyton, the TN content in the reference site was significantly higher ($P<0.05$) with the average of 16 mg g^{-1} comparing to the restored sites with the average of 13 mg g^{-1}. Periphyton TP contents in the 2000- and 2003-restored sites (average of 313 mg kg^{-1}) were approximately three times of that in the reference site (average of 99 mg kg^{-1}; Table 3). Accordingly, the periphyton molar TN:TP ratios in the reference site (average values of 393±23) were approximately three times higher than those in the restored sites (Table 3). Compared with the periphyton in other parts of the Everglades, the TP in the restored HID sites were closer to that of the Water Conservation Area 1 (average of 423 mg kg^{-1}) while the TP in the HID reference site was closer to that in Taylor slough (average of 124 mg kg^{-1} TP; Gaiser et al. 2006). Periphyton

Table 2 Mean water chemistry values at all sites in the October 2009 when the sites was flooded (mean±SE, $n=6$ for 2000-restored site; $n=12$ for 2003-restored site and $n=12$ for the reference site)

Site	SRP μg L^{-1}	NH_4-N μg L^{-1}	NO_x-N μg L^{-1}	TDP μg L^{-1}	TDKN mg L^{-1}	DIN:SRP mol:mol	TDKN:TDP mol:mol
2000-restored	6.6 (2.1)	250 (62)	5.4 (0.4)	9.9 (1.4)	1.6 (0.2)	103 (29)	364 (81)
2003-restored	3.0 (0.1)	47 (6)	3.3 (0.4)	4.4 (0.7)	0.8 (0.1)	37 (5)	456 (40)
Reference	2.7 (0.1)	88 (12)	4.2 (0.9)	2.6 (0.6)	0.6 (0.0)	78 (10)	914 (228)

Table 3 Basic soil and periphyton properties in the three sites (average±S.E., $n=30$). Different lowercase letters denotes significant difference ($P<0.05$)

Component/Parameter	Unit	Site		
		2000-restored	2003-restored	Reference
Periphyton				
Loss on ignition	%	30±2b	39±2a	35±2ab
Total phosphorus (TP)	mg kg^{-1}	313±30a	314±34a	99±7b
Total nitrogen (TN)	g kg^{-1}	13±0b	13±0b	16±1a
Total carbon	g kg^{-1}	244±4a	246±3a	250±6a
TN:TP	molar	121±13b	102±8b	393±23a
Soil				
Loss on ignition	%	19±1a	18±1a	13±1b
Total phosphorus (TP)	mg kg^{-1}	630±32b	983±47a	140±6c
Total nitrogen (TN)	g kg^{-1}	7.5±0.3a	6.3±0.2b	6.7±0.2b
Total carbon	g kg^{-1}	155±3a	143±3 b	149±1ab
Total organic carbon	g kg^{-1}	94±3a	92±2a	66±2b
TN:TP	molar	28±1b	15±1c	109±3a

TN was significantly higher in the dry season for the 2000-restored site (average of 15 mg g^{-1}) and 2003-restored site (average of 14 mg g^{-1}) compared to the wet season where the average was 11 mg g^{-1} and 12 mg g^{-1} in the 2000- and 2003-restored sites, respectively. No significant seasonal difference was observed in periphyton TN in the reference site or in periphyton TP and TN:TP ratio in all of the three sites.

The ratio of N:P is often used as an indicator of nutrient limitation, with the value of 16:1 (molar) being the theoretical threshold between N and P limitation for oceanic phytoplankton (Redfield 1973). Other studies have reported optimal N:P of 20 (Hecky and Kilham 1988) or 30 (Smith 1983) for freshwater phytoplankton. Cleveland and Liptzin (2007) found that similar to the marine phytoplankton, there was a consistent atomic C:N:P ratios in both the soil (186:13:1) and the soil microbial biomass (60:7:1) at the global scale. These nutrient values suggest that the HID system is primarily N-limited in the 2003 restored site, co-limited by N and P in the 2000-restored site, and strongly limited by P in the reference marl prairie (Smith et al. 2011). However, other studies didn't think this indicator is conclusive (Scott et al. 2005). There were not significant differences in the nutrient parameters between the restored wetlands of different age (2003-restored vs 2000-restored) with the possible explanation that the age difference between these two sites was small. However, the soil TP and TN:TP ratio somehow distinguished the two sites (Table 3).

Nitrogenase Activity

Acetylene Reduction (AR) rates under both light and dark conditions were significantly higher ($P<0.05$) in the restored sites (Fig. 2a, b). For the 2000-restored and reference sites, there were no significant differences in the AR rates between the two seasons; while for the 2003-restored site, the AR rates were significantly higher in the dry season. Rejmánková et al. (2004) reported that the N$_2$ fixation of periphyton in the tropic marsh of Belize was higher in the wet season (i.e., July and September), and they speculated that the warmer temperature and higher solar radiation in the wet season would facilitate nitrogen fixation. Vargas and Novelo (2007) also found that the highest AR rates of periphyton appeared during the rainy season in the Yucatan peninsula.

In our study, the contrasting seasonal patterns of periphyton N$_2$-fixation between 2000-restored, reference sites and 2003-restored site could be the result of our sampling time at the end of the wet season (i.e., October) and the dry season (i.e., February), when the environmental factors may not have been dramatically different. Actually, precipitation in October 2009 was even lower than that in February 2010 (NOAA Daily Surface Meteorologic Data). A number of other factors could contribute to observed patterns of N$_2$-fixation including temperature, solar intensity, different vegetation in different seasons (resulting in shading), but without experimentally testing these effects we can only speculate about the possible factors for the difference.

In this study, periphyton biomass was found to be 155± 16 g AFDW m^{-2} in the 2000-restored area, 208±28 g AFDW m^{-2} in the 2003-restored area, and 295±33 g AFDW m^{-2} in the reference site. All of these values fall in the range of those reported for the marl prairies of Florida Everglades (Gottlieb et al. 2005; Iwaniec et al. 2006; Gaiser et al. 2011), which are greater than those of the long-hydroperiod Everglades periphyton (McCormick et al. 1998; Hagerthey et al. 2011).

Using the theoretical ratio of 3 moles of C$_2$H$_4$ produced per mole of N$_2$ fixed, it is possible to estimate the amount of N$_2$ fixed for a given AR rate. Although the empirical ratio varies among different ecosystems (Doyle and Fisher 1994), the theoretical ratio of 1:3 has proven reasonable for cyanobacterial mats (Howarth et al. 1988a). In our study, significantly higher N$_2$ fixation rates of periphyton were observed in the 2003-restored site with average of 0.2±0.03 gN m^{-2} yr^{-1} compared to the reference sites with average of 0.05±0.01 gN m^{-2} yr^{-1} ($P<0.05$) (Fig. 3b). These rates are much lower in comparison with the unimpacted Water Conservation Area-2A (WCA-2A) of northern Everglades, where the N$_2$ fixation rates ranged from 1.8–18 gN m^{-2} yr^{-1} (Inglett et al. 2004).

With regard to restoration, we could make a very rough estimate of the time required for the 2003-restored site to reach the same level of nitrogen storage as the reference site. The TN storage for the 2003-restored and reference site is 35 gm^{-2} and 125 gm^{-2}, respectively (Inglett et al. 2011b).

Fig. 2 Periphyton acetylene reduction (AR) rates under dark (**a**), light (**b**) incubation, and the ratio of AR rates under light and dark conditions (**c**) determined in each of the three Everglades sites in the dry and wet seasons. The different lowercase and uppercase letters denote significant differences between the sites ($P<0.05$)
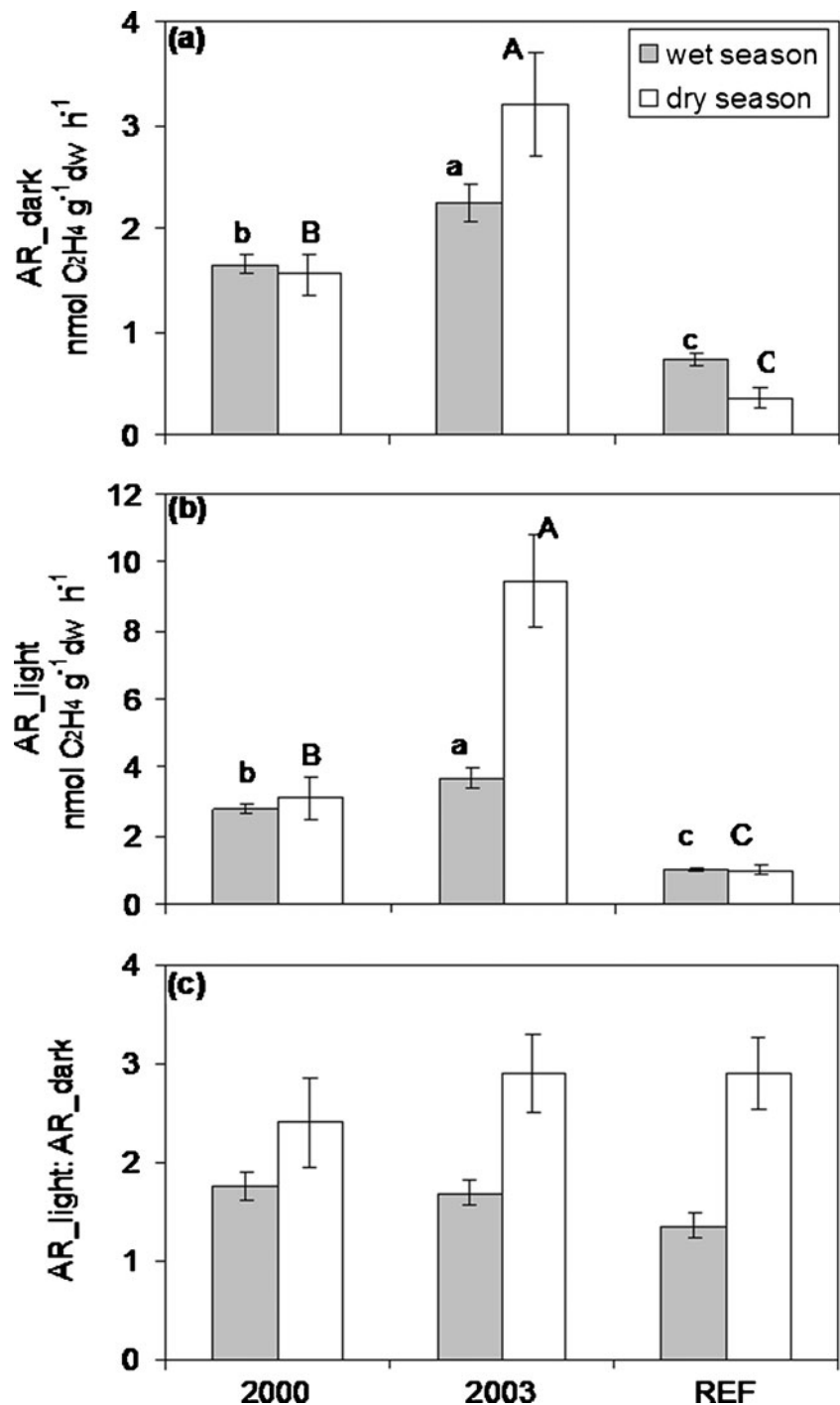
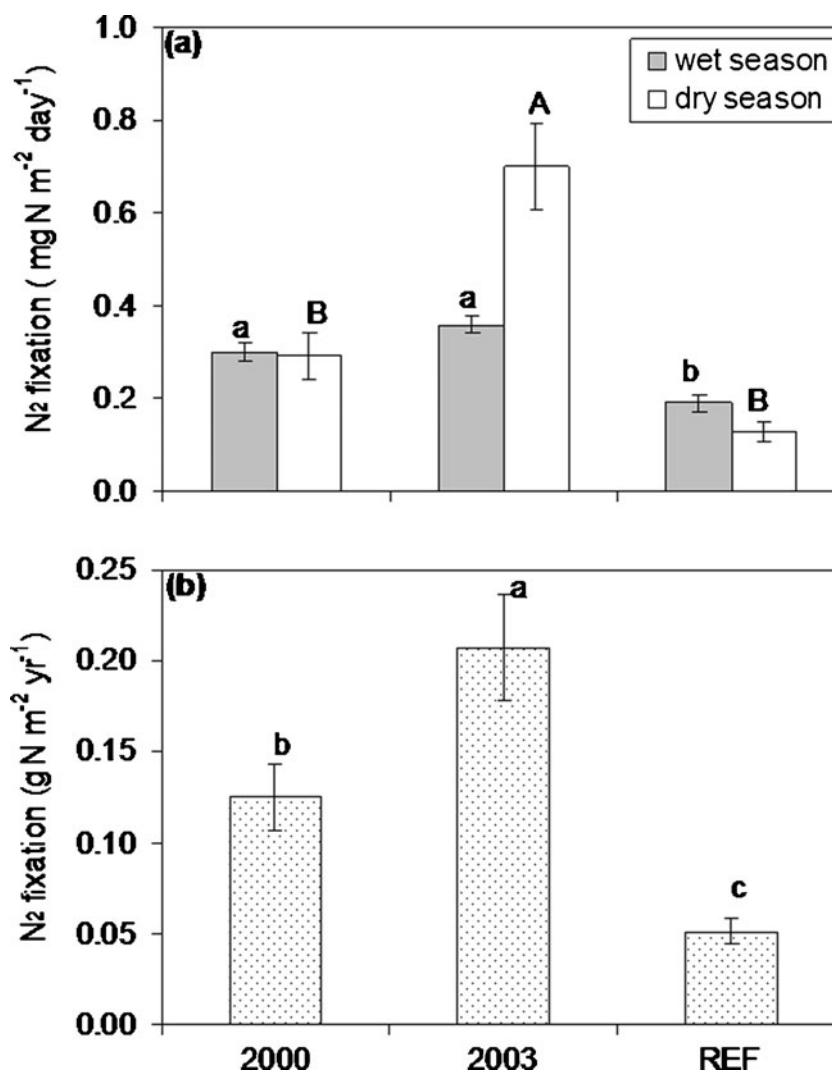Assuming that all N fixed by the periphyton in the 2003-restored site was absorbed by the soil, we estimate that it would require 450 yr to accumulate the amount of N contained in the reference site soils.

There was no significant difference in the light:dark AR ratio among the three sites (Fig. 2c) with the average of 2 and 3. Overall, the ratio was similar to that of periphyton at unimpacted interior of WCA-2A with the ratio of 3.3 ± 0.5 (Inglett et al. 2004). The response of nitrogenase activity to

light intensity can give us information of the N_2 fixing species in a microbial community (Fay 1992). The light:dark AR ratios in our study were greater than 1, suggesting that photosynthetically-driven microbes such as heterocystous cyanobacteria were largely responsible for the observed rates. The light:dark AR ratio was significantly higher in the dry season for the 2003-restored and reference sites ($P<0.05$), likely reflecting seasonal changes in the species composition of the periphyton communities (McCormick et al., 1998).

Periphyton and Soil $\delta^{15}N$

Nitrogen fixation by cyanobacteria is accompanied by relatively little isotopic fractionation and as a result, nitrogen-fixing cyanobacteria should have $\delta^{15}N$ close to 0‰ (Goericke et al. 1994; Kline and Lewin 1999). In our study, the $\delta^{15}N$ of periphyton over all three sites all fell within the range of −2–2‰ (Table 4) reported in other studies (Gu and Alexander 1993; Nadelhoffer and Fry 1994; Rejmánková et al. 2004), but is on average lower than the range reported for northern Everglades marshes (1.1–2.7‰, Inglett et al. 2004) and other freshwater metaphyton (1–12‰, Scott et al. 2007). Differences in periphyton $\delta^{15}N$ also correspond to differences in N_2 fixation measured between the three sites, with the highest $\delta^{15}N$ corresponding to the lowest nitrogenase activity observed in the reference site.

Stable isotope ^{15}N signatures are often used to characterize N source and the mechanism of algal and plant N metabolism (Handley and Raven 1992; Nadelhoffer and Fry 1994; Inglett and Reddy 2006). Differences in ^{15}N natural abundance among freshwater N_2-fixing cyanobacteria and non-fixing green algae were reported by Gu and Alexander (1993) as a reliable indicator of N_2-fixation. They found that the natural abundance of ^{15}N of six N_2-fixing blue-green algae was 1.0±1.3‰, whereas the $\delta^{15}N$ of six green algae showed an average of 6.6±4.5‰. The higher $\delta^{15}N$ of non-N_2-fixing algae reflected the utilization of dissolved inorganic N.

The $\delta^{15}N$ of periphyton and soil were significantly correlated with each other (Table 4, Fig. 4), suggesting the N source in soil is derived from that fixed by the periphyton. At a given site, the soil had a higher $\delta^{15}N$ than the periphyton, suggesting that other processes in addition to atmospheric N fixation affected their ^{15}N composition. For example, coupled nitrification/denitrification, ammonia volatilization, or atmospheric deposition of ^{15}N enriched N may have caused increases in $\delta^{15}N$ within the soil (Evans and Ehleringer 1993; Aranibar et al. 2002).

Fig. 4 Relationship of periphyton $\delta^{15}N$ (‰) with soil $\delta^{15}N$ (‰)

$$\delta^{15}N = 0.4*(\text{Soil }\delta^{15}N) - 1.2$$
$$R^2 = 0.22, P < 0.05$$
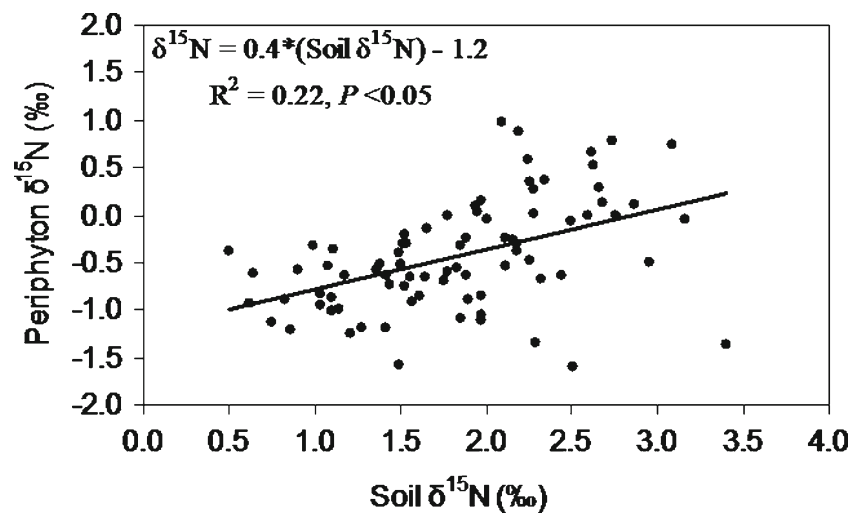
Relationships between Nutrients, Nitrogenase Activity, and $\delta^{15}N$

Various factors affect N_2 fixation, including physical factors such as light intensity and temperature, and nutrient status (Howarth et al. 1988b). Nitrogen and phosphorus concentrations and loadings are two commonly discussed factors. Scott et al. (2007) showed that periphyton N_2 fixation decreased as reactive N accumulated in the periphyton matrix. In our study, the N_2 fixation of periphyton was negatively and weakly correlated with the TN content of periphyton ($R^2 = 0.2$, $P < 0.05$); however, significantly higher TN content in the reference site corresponded with significantly lower AR rates. In contrast with the results of Scott et al. (2005) that decreasing N_2 fixation was related to the increase in metaphyton N content, our study showed that increased TN content in February corresponded to increased AR rates for the 2003-restored site.

Ammonium concentrations can also be important in regulating fixation rates in sediments since they can

inhibit the synthesis of new nitrogenase (reviewed by Howarth et al. 1988b). The ammonium concentrations of the water samples collected in October 2009 in our sites averaged from 47 to 250 µg L^{-1} (Table 2), which was much higher than the values reported by Inglett et al. (2004) (38 µg L^{-1} for the WCA-2A of northern Florida Everglades). This may explain why our AR rates of periphyton were lower even though the TN:TP ratio in the HID restored sites was similar with those of the northern Everglades.

There was a significantly positive linear relationship between N_2 fixation rates and TP; accordingly, significantly negative relationship between N_2 fixation rates and periphyton TN:TP were found in both dry and wet season (Fig. 5), indicating that P likely controls N_2 fixation. Phosphorus-controlled N_2 fixation has been reported by many researchers (Howarth et al. 1988b; Smith 1990; Rejmánková and Komárková 2000; Rejmánková 2001; Inglett et al. 2004, 2009). However, TN:TP ratio is regarded as more reasonable factor than TP in regulating the N_2 fixation since it integrates the nitrogen information, another controlling factor of N_2 fixation and would further indicate nutrient limitation (Howarth 1990).

In our study, the restored sites with lower TN:TP ratio were more likely to be N-limited, which then induced higher nitrogenase activities; while the reference site with higher TN:TP ratio was P-limited and had lower activities. In this regard, it should be noted that the use of nutrient ratios as an indicator of nutrient limitation varies between ecosystems. Field fertilization or nutrient enrichment experiments are the standard approach to testing nutrient limitation, but these are often laborious (Craine and Jackson 2010). Other studies have shown that periphyton N fixation rates are correlated with nutrient limitation status that is measured directly through nutrient bioassays (Scott et al. 2005).

Table 4 Soil and periphyton $\delta^{15}N$ values in the three sites and different seasons ($n = 15$, mean±S.E). Different letter denotes the significant differences

Season	Site	$\delta^{15}N$ (‰)	
		Periphyton	Soil
Wet	2000-restored	−0.5±0.1a	1.7±0.1b
	2003-retored	−0.6±0.2a	1.9±0.2b
	Reference	0.0±0.1a	2.4±0.1a
Dry	2000-restored	−0.8±0.1b	1.2±0.1b
	2003-restored	−1.0±0.1b	1.4±0.1b
	Reference	0.2±0.1a	2.5±0.1a

Fig. 5 Relationships between periphyton acetylene reduction (AR) under light conditions and periphyton TP (**a**) and TN:TP ratio (**b**) in the dry and wet seasons
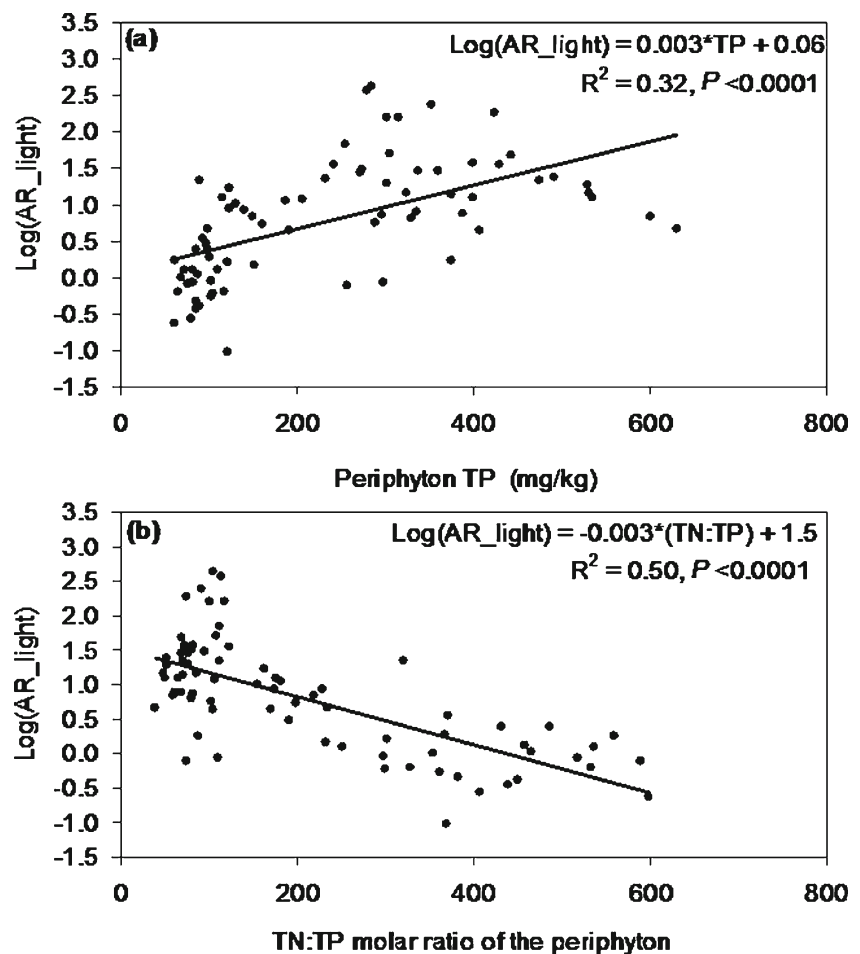
There was a significantly negative correlation between N_2 fixation rates and the $\delta^{15}N$ in both the wet and dry season (Fig. 6a), which was similar to the finding of Rejmánková et al. (2004) and Inglett et al. (2004). Scott et al. (2007) also found that in a freshwater marsh, the metaphyton $\delta^{15}N$ decreased with increasing N_2 fixation during May through September sampling. Like the correlation of nitrogenase activity with TN:TP ratio and ^{15}N, we also found a significant relationship between ^{15}N and both the periphyton TP content and TN:TP ratios (Fig. 6b,c).
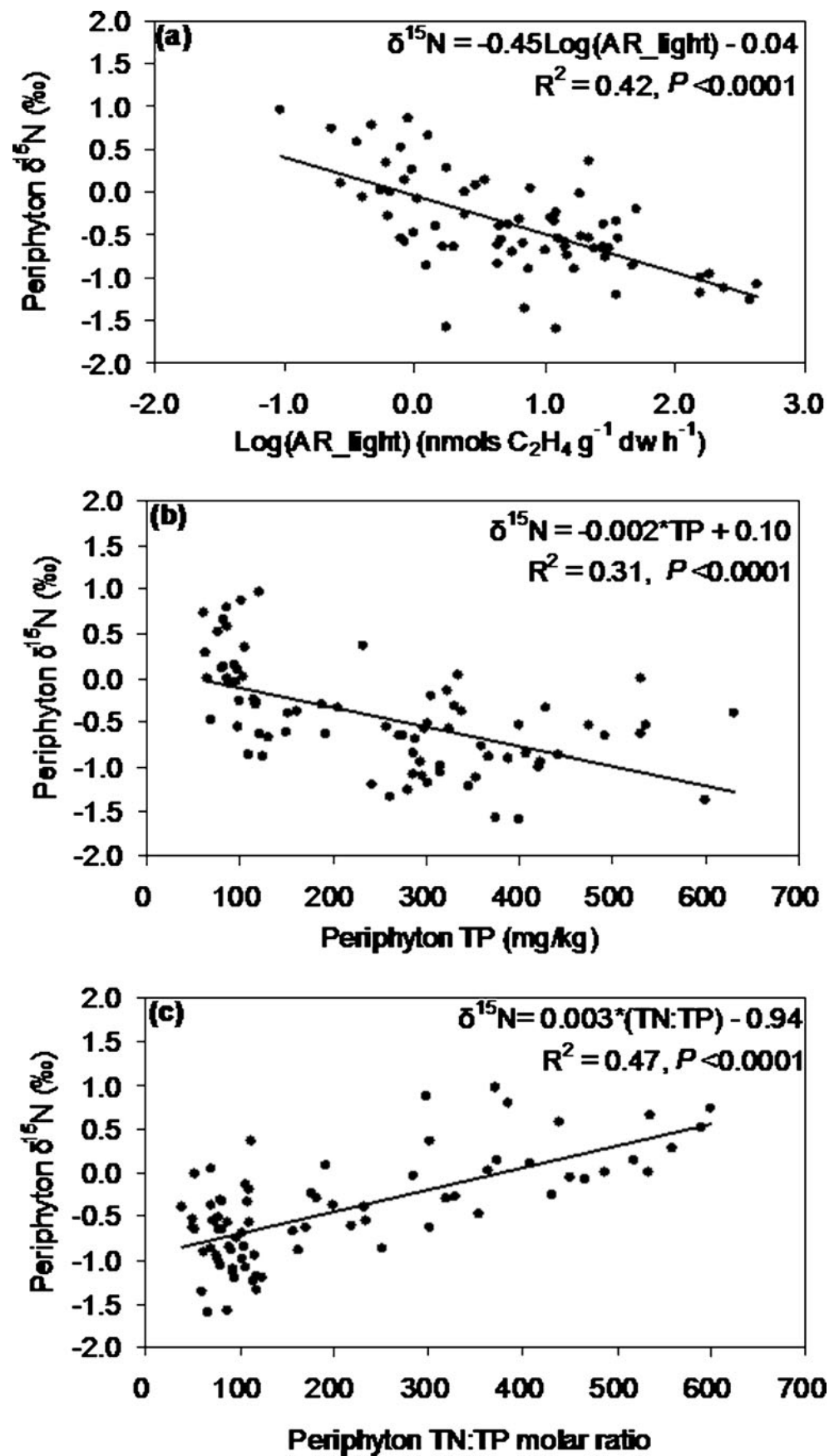
Conclusions

Periphyton plays a significant role in the Everglades, especially in the restoration of the calcareous Hole-in-the-Donut wetland ecosystem. This study documented the presence of nitrogenase activity in calcareous periphyton mats of the Southern Everglades (marl prairie) and estimated its annual contribution to the N budget of these wetlands.

Overall rates of the reference marl prairie system were low compared to published estimates of northern Everglades systems. Periphyton in restored sites exhibited significantly higher AR rates than the reference marl prairie system, serving as evidence of N limitation in the developing systems following soil removal. Like the other reports of calcareous periphyton mats, nitrogenase activity in this study was higher under light condition indicating the primary N_2 fixing community was cyanobacterial in nature.

Periphyton N_2 fixation is considered as a major part in the N budget in the oligotrophic Everglades that has received little attention. For the young restored HID ecosystems, N_2-fixation can be an important N source and thus a key target or evaluation metric for restoration. The periphyton community is also sensitive to nutrient status, and in this study, N_2-fixation, TN:TP ratio and ^{15}N were correlated in both wet and dry seasons. There was also a relationship between $\delta^{15}N$ in the periphyton and soil indicating this parameter has a potential of characterizing the importance N accumulation via N_2 fixation and tracing periphyton N. These findings suggest that N_2 fixation and ^{15}N could also be useful indicators of nutrient limitation and ecosystem recovery in other wetland systems.

Fig. 6 Relationship between periphyton δ¹⁵N (‰), Acetylene Reduction rates (AR), TP and TN:TP ratio

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