

NITROGEN DYNAMICS DURING THE RESTORATION OF CALCAREOUS
WETLANDS IN THE FLORIDA EVERGLADES

By

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To my parents

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LIST OF ABBREVIATIONS

AR	Acetylene reduction rates
DEA	Denitrification enzyme activity
HID	Hole-in-the-Donut
LAP	Leucine aminopeptidase
MBC	Microbial biomass carbon
MBN	Microbial biomass nitrogen
MBP	Microbial biomass phosphorus
NAG	N-acetyl- β -D- glucosaminidase
PMN	Potentially mineralizable nitrogen
Res00	Wetlands restored in 2000
Res03	Wetlands restored in 2003

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In wetlands, the nitrogen (N) cycle is dynamically controlled by hydrology, fire and phosphorus (P), but despite its role as both a limiting nutrient and pollutant, N cycling has received little attention in most of the Everglades system. This dissertation investigated the spatial and temporal patterns of N cycling in the context of ecosystem restoration using the short-hydroperiod, marl prairies of the Hole-in-the-Donut (HID) region of Everglades National Park. Specifically, two restored sites with high P and a native reference site with low P were followed seasonally and in response to a prescribed fire to assess the role of N in this landscape and in the recovery of these systems following restoration.

N₂ fixation by periphyton was significantly higher in the more N-limited restored sites, and most of this occurred during only 3 months in the early wet season. For the soil, NH₄⁺ and NO_x⁻ dominated the wet and dry seasons, respectively, and accumulated NO_x⁻ levels were quickly consumed by denitrification at the onset of the wet season. N mineralization and enzyme activity also revealed dramatic shifts in the abundance of bacteria and fungal communities during these hydrologic transitions. Fire resulted in immediate increases in soil N, and at the restored sites, increased rates of periphyton

N₂ fixation suggesting either a direct input of N through charcoal or an indirect stimulation of mineralization/nitrification by P in ash. Overall, these findings illustrate the importance of hydrology and fire in maintaining N balance and fate in the Southern Everglades, and an increased need for consideration of N cycle processes during the management of restoration of P-impacted sites.

CHAPTER 1 INTRODUCTION

Background

Nitrogen (N) cycle includes a variety of potential inputs, numerous forms ranging from particulate and dissolved organic to dissolved inorganic (e.g., NO_3^- and NH_4^+) and gaseous species (e.g., N_2 , NH_3 , and N_2O), and a diversity of transformations (e.g., ammonification, N mineralization, nitrification and denitrification). These complexities first involve understanding, describing, and quantifying the natural N cycle at different landscapes. N has ecological significances, for example, it is the limiting nutrient for most plant growth typically in temperate forests, grasslands and the coastal ocean (Vitousek and Howarth 1991). N_2 fixation plays as an important N source especially in a N-limited ecosystem; and the N_2O gas is 298 times more powerful than carbon dioxide as a greenhouse gas (IPCC 2007). There is a large variability on the magnitude of the natural fluxes of N and the storage of N in the N reservoirs. For instance, the estimates of marine N fixation range from 40-200 million metric tons per year, atmospheric deposition of organic N 10-100 million metric tons per year, terrestrial ammonia/ammonium ($\text{NH}_3/\text{NH}_4^+$) emissions 91-186 million metric tons N per year (<http://www.isws.illinois.edu/nitro/hinfl.asp>). It is difficult to precisely quantify the magnitude of the major components of the N cycle since the natural world is large and heterogeneous and moreover, has been widely influenced by a variety of human disturbance (Vitousek and Farrington 1997).

Intensive agricultural activities are believed to be a major factor contributing to the degradation of ecosystems (Hausman 2007; Bending et al. 2000; Kalinina et al. 2009), which input excess nutrients into the native ecosystems, lead to nutrient problems and

invasion of exotic species (Hamilton and Landman 2011). N and phosphorus (P) are responsible for most of the nutrient problems (Conley et al. 2009). Accordingly ecological restoration has attracted increasing attention from the general public, scientists and the government. Many theories, specific cases, mechanisms and techniques of ecosystem restoration have been proposed and studied to restore the degraded ecosystems (Fraser and Kindscher 2001; Mitsch and Day Jr. 2004; Hausman et al. 2007; Kiehl and Pfadenhauer 2007; Smith et al. 2011).

The restoration processes modify the N and P cycle, and the nutrient status would change during the restoration (Davidson et al. 2007). Therefore, a diversity of indicators has been used to demonstrate different nutrient status. For example, field fertilizations are the standard approach to testing nutrient limitation, but these require a long time (Craine and Jackson 2010). TN:TP ratio of plant tissues was then found to be a more simple indicator (van den Driessche 1974; Powers 1980; Koerselman and Meuleman 1996; Vitousek et al. 2010). Microbial parameters recently were paid more attention on its utility as a dynamic indicator of the restoration and nutrient limitation (Olander and Vitousek 2000; Harris 2003, 2009). N_2 fixation (i.e., nitrogenase activity), for example, was reported to negatively related with N availability (Tyler et al. 2003). Extracellular enzyme activities can rapidly respond to different nutrient status (Sinsabaugh et al. 1993, 2009; Garcia-Ruiz et al. 2008; Geisseler and Horwath 2009; Hernández and Hobbie 2010; Tabatabai et al. 2010) and were even suggested as a functional measurement, which can vary independently of microbial community structure (Waldrop et al. 2004; Cusack et al. 2011). Most recently, N stable isotope ratio ($\delta^{15}N$) is being

used as an indicator of plant N demand and N₂ fixation (Davidson et al. 2007; Inglett and Reddy 2006).

Numerous factors regulate different N transformation and N species, such as soil physical properties, nutrient supply, and climate condition (e.g., temperature, permafrost and freeze, and precipitation) (Harrison et al. 2005; Heffernan et al. 2010; Hefting et al. 2004). Spatial and temporal variations of these factors and their interactions often lead to the high variability of soil N cycle (Clément et al. 2002). Hydrology is the major driver of the N cycle in wetland ecosystems (Christense et al. 1990; Heffernan et al. 2010; Hefting et al. 2004). It was reported that water table could enhance nitrification and N₂O fluxes on peatlands (Regina et al. 1996; Elmi et al. 2005). The frequency of dry-rewetting period and the intensity of the wetting pulse have significant effects on microbial biomass N, N mineralization, nitrification, denitrification, and N₂ fixation (Olf et al. 2004; Borcken and Matzner 2009; Muhr et al. 2008; Jeffries et al. 1992). Especially when a globally increasing likelihood of severe drought has been predicted during this century (Meehl et al. 2007), it is emergent to investigate the seasonal changes or hydroperiod effects on the N flux which would further correlate with the primary production of plants, greenhouse gas emission (i.e., N₂O).

Fire is considered as another important disturbance both natural and anthropogenic that can modify the N cycle (Wan et al. 2001). During the combustion, heat releases, transports to the soil and elevates the soil temperature, leading to N loss through volatilization, the transformation of organic N to inorganic forms, and the further biochemical reactions like nitrification and denitrification (Raison 1979). Similarly, the combustion converts the organic P to available forms and deposits as a form of ash.

The P was reported to stimulate the N cycling especially in P-limited ecosystems (Howarth et al. 1988a; White and Reddy 1999, 2000; Inglett et al. 2004). Charcoal as another fire residue, would have the potential of increasing nitrification and N mineralization that has been found in forest ecosystem (Zackrisson et al. 1996; DeLuca et al. 2002; DeLuca and Sala 2006). Compared to the terrestrial ecosystems, little is known about fire effects on nutrient cycle or specifically N cycle in a wetland ecosystem where the interaction between fire and water makes the N cycle more complex. However, fire is widely used as a restoration and management tool in wetlands to reestablish the native vegetation. More is needed to know the underlying mechanisms that how fire could work better.

Everglades Restoration and Nitrogen Cycle

The Everglades is the second largest wetland on the planet and to save it from historical degradation, the largest environmental restoration project in America's history was early performed as the Comprehensive Everglades Restoration Plan (CERP) in which the water including both water quality and quantity is the key issue. The diversity of hydrology across the Everglades featured a variety of spatial and temporal patterns of N cycle. Most existing researches on N cycle focused on the long hydroperiod wetlands (e.g., northern marshes, Shark River and Taylor River Sloughs) that remain flooded for more than 8 months of the year (Rudnick et al. 1999; Sutula et al. 2001, 2003; White and Reddy 1999, 2000, 2003; Inglett et al. 2004, 2011). For example, Sutula et al. (2001) made rough N budgets in the Taylor Slough/C-111 basin wetlands during the wet and dry season and pointed out that high N_2 fixation rates or an underestimation of groundwater N flux may explain the difference between estimates of hydrologic N import and sediment N burial rates. In contrast, N cycle in the short

hydroperiod wetlands that remain inundated for less than 8 months of the year (e.g., southern marl and calcareous wetlands) has rarely received any attention. However, those ecosystems undergo more pronounced dry-rewetting periodic cycles driven by seasonal patterns of rainfall (Ewe et al. 2006), which would lead to distinctive seasonal patterns of N cycle.

Besides reestablishing a more natural hydrology, the establishment of natural fire regime is also a guarantee for the successful Everglades restoration (Lockwood et al. 2003). The Everglades is a fire-dependent landscape during the large-scale and long-term restoration (Beckage et al. 2005). Numerous fire projects have been conducted or been going on in a variety of landscapes across Everglades, such as the River of Grass Prescribed Fire Plan for the wet prairie and sawgrass marsh ecosystems, the Pineland burn plan, the mangrove-marsh ecotone fire, and the fire project in Water Conservation Area 2A (WCA 2A) of the northern Everglades (Spier and Snyder 1998; Qian et al. 2009; Beckage et al. 2005). However, most of the projects focused on restoring native plant species, little is known about the fire effects on nutrient biogeochemistry, let alone on the N cycle that has been ignored across the whole Everglades ecosystem (Miao and Sklar 1998; Miao and Carstenn 2006; Qian et al. 2009).

In addition, with the human disturbance, many areas of Everglades accumulated excess P, forming two contrasting wetlands that is P-enriched versus P-limited ecosystems (Noe et al. 2001). In highly P-impacted areas, N can even be the limiting nutrient (McCormick and O'Dell 1996). As previously discussed that N cycle can be affected by P, the study on N cycle between the high and low P would become more interesting.

Case Study and Objectives

Base on the literature review and those to-be-explored questions in the Everglades, a case study on the N cycle was chosen in a unique ecosystem, the Hole-in-the-Donut (HID), which combined the major features of wetland restoration, contrasting P impacts, short-hydroperiod, and fire disturbance together.

The HID is located in the Miami-Dade County of Miami, Florida. As early as the Everglades National Park (ENP) was established in 1948, farming was allowed in the park. Historic farming enriched the P; and after the farming ceased in 1975, Brazilian pepper (*Schinus terebinthifolius*) was invaded and dominated (Li and Norland 2001). To restore HID to the natural sawgrass (*Cladium jamaicense* Crantz) ecosystem, various methods were tried, and the complete soil removal (CSR) technique that removed all the plants and soils down to the bedrock were proved to be the most efficient (Dalrymple et al. 2003). Then the ENP initiated a large-scale restoration program in spring 1997: during each dry season (December to May), a new area was restored through CSR method. By 2010, about 4,100 acres have been restored (<http://www.nps.gov/ever/naturescience/hidprogram.htm>).

During the restoration to a native marl/calcareous prairie wetland ecosystem, the N and P status would shift from more N-limited immediately after the CSR to more P-limited status (Inglett et al. 2011; Smith et al. 2011). At the very beginning of the restoration, periphyton mats colonize the bedrock, cause the precipitation of the calcite to form soils and simultaneously fix N_2 . With the accumulation of N and the consumption of P, the TN:TP ratio would accordingly vary with time, and become closer to the native calcareous wetlands with low N and P. The comparison of N cycle between the restored and native reference sites could thus help to trace the trajectory of the restoration.

In addition, the hydrology modified by the shallow soil at the restored sites could differ from that at the native reference site, which would affect the seasonal patterns of N cycle when different flooding occurred. Therefore, the specific objectives of the proposed study were to (1) demonstrate the different nutrient status of the restored and native wetlands in the HID using various biogeochemical indicators; (2) investigate the spatial and temporal patterns of N₂ fixation of periphyton in the restored and native wetlands in the HID; (3) determine the seasonal pattern of the soil N dynamics in the restored and native wetlands in the HID; and lastly, (4) explore the effects of fire on the N cycle in the restored and native wetlands in the HID.

Dissertation Format

Chapter 1 provides an introduction for the overall dissertation, beginning with a review of the N cycle, its large variability and the relative controlling factors, then narrowing down to the Florida Everglades, discussing the lack of attention on the N research across the Everglades ecosystems, interpreting the need of the dissertation work, and finally stating the reason why the case study in the HID was selected. Chapter 2–6 are formatted as complete manuscript works intended for subsequent publication with each containing appropriate literature citations for the specified processes discussed. Chapter 2 integrates all the components of the ecosystem (plants, soil, and the periphyton), discussing the potential indicators for nutrient status including not only the commonly reported foliar TN:TP ratio and nutrient use and adsorption efficiency, but also the newly-reported soil enzyme activities, N stable isotopes, and the N₂ fixation of periphyton. Chapters 3 and 4 focus on the component of periphyton, presenting the spatial and temporal patterns of the N₂ fixation of periphyton. Chapter 5 focuses on the component of soil, investigating the temporal pattern of the main N

processes (e.g., denitrification and N mineralization). Chapter 6 explores the effects of fire on the N cycle. Finally, a summary of these studies with concluding remarks as to the limitation and further efforts of the current research is presented in Chapter 7.

CHAPTER 2 INDICATORS OF NUTRIENT STATUS DURING THE RESTORATION OF CALCAREOUS WETLANDS IN THE FLORIDA EVERGLADES

Background

Due to the increase of human disturbance, degradation of various ecosystems has become a serious global issue. Ecological restoration has thus attracted increasing attention from the general public, scientists and governments. Many theories, specific cases, mechanisms and techniques of ecosystem restoration have been proposed and studied (Zedler 2000; Fraser and Kindscher 2001; Hausman et al. 2007; Kiehl and Pfadenhauer 2007). Around the world, there are several ecosystems that are characterized as low-nutrient and species-rich ecosystems. The plants in these nutrient-poor ecosystems are very sensitive even to small changes in nutrient availability (Fischer and Stöcklin 1997). Heathland with acidic soils and calcareous ecosystems with alkaline soils are two typical examples (Gibson et al. 1991; Mitchell et al. 2000; Niinemets and Kull 2005; Diaz et al. 2008; Piqueray et al. 2011). When historical agricultural activities were abandoned in these ecosystems, residual nutrients were left, leading to nutrient problems and invasion of exotic species (Bending et al. 2000; Hausman 2007; Kalinina et al. 2009; Hamilton and Landman 2011). One of the key objectives for ecological restoration of these ecosystems is to reduce the nutrient levels. Nitrogen (N) and phosphorus (P) are responsible for most of the nutrient problems. During the restoration processes, the ecosystem would exhibit different N- and P-cycling patterns, and a specific case is that N could be more limited on young soils, while P could become the limiting element for the ecosystem with old soils (Walker and Syers 1976; Vitousek and Farrington 1997; Davidson et al. 2007).

Field fertilization is the standard approach to testing nutrient limitation, but this approach is time and labor intensive (Craine and Jackson 2010). As a result, many indirect indicators have been discussed and recommended (van den Driessche 1974; Powers 1980; Koerselman and Meuleman 1996; Vitousek et al. 2010; Piqueray et al. 2011). The TN:TP ratio of plant tissues is widely used to determine the nutrient limitation to primary production in terrestrial ecosystems (van den Driessche 1974; Koerselman and Meuleman 1996; Niinemets and Kull 2005). McGroddy et al. (2004) estimated global nutrient ratios for limitation of N and P in terrestrial ecosystems based on molar basis, and reported that global C:N:P ratios for limitations of N and P were 1212:28:1 for foliage. By reviewing data on fertilization studies in a variety of European freshwater wetland ecosystems (bogs, fens, wet heathlands, dune slacks, wet grasslands), Koerselman and Meuleman (1996) generalized that at N:P mass ratios >16 community biomass production is P-limited; at N:P-values <14, N limits plant growth; at N:P ratios between 14 and 16, either N or P may limit plant growth or both elements are equally limiting (co-limitation), but they also pointed out that the method can only be used under conditions where either N or P controls plant growth. It is also argued that the critical ratios have large spatial and temporal variations (Reich and Oleksyn 2004).

In addition to foliar critical values, the Diagnosis and Recommendation Integrated System (DRIS) index is another way to determine nutrient limitations of different ecosystems (Comerford and Fisher 1984). The DRIS method ranks automatically excesses or deficiencies of nutrients in order of importance (Walworth and Sumner 1987) based on a DRIS norm that needs to be established for each ecosystem. Since a norm is usually determined easier for agronomic crops, it has been successfully used to

interpret results of nutrient sufficiency or deficiency of many different crops (Bangroo et al. 2010). Only one DRIS norm has been established for a plantation forest of Loblolly pine in Georgia and the Carolinas (Nguyen 2011).

Other nutrient indicators of plant nutrient status include nutrient use efficiency (NUE) and nutrient resorption use efficiency (NRE). The underlying theory behind these two indicators is that plants themselves develop a physiological strategy to deal with the nutrient limitation or deficiency. They can either use the limiting nutrients more efficiently or withdraw the nutrients from senescing parts to the young tissues. Compared to the plants, no agreement has been made in using soil TN:TP ratio to assess the nutrient status in terrestrial ecosystem, but it somehow indicates a general pattern of nutrient limitation in the sites. For example, Fenn et al. (1998) used both foliar C:N and soil C:N ratios to evaluate N saturation of the mixed forest (Pine, Oak, Fern) in the San Gabriel Mountains, Northeast Los Angeles, CA, and concluded that indications for N saturation in the mixed forest were from 24 to 48 for foliar C:N and from 18.8 to 26.6 for soil C:N. Carlyle and Nambiar (2001) showed that soil C:N ratios for N saturation in the Yellow podsolic, Krasnozern, Siliceous sand soil were 31.3, 34.1, 37.7; Soil N:P ratios for P limitation ranged from 13 to 18.8 and did not differ much among three types of soils. Cleveland and Liptzin (2007) found that similar to the marine phytoplankton, there was a consistent atomic C:N:P ratios in the soil (186:13:1) at the global scale.

Microbial indicators recently were paid more attention on its utility as a dynamic indicator of the restoration and nutrient limitation (Burke et al. 2011; Olander and Vitousek 2000; Harris 2003, 2009). N_2 fixation (i.e. nitrogenase activity), for example, was reported to negatively related with N availability (Tyler et al. 2003). Extracellular

enzyme activities can rapidly respond to different nutrient status (Sinsabaugh et al. 1993; Sinsabaugh et al. 2009; Geisseler and Horwath 2009; Hernández and Hobbie 2010; Tabatabai et al. 2010), and were even suggested as a functional measurement, which can vary independently of microbial community structure (Waldrop et al. 2004; Cusack et al. 2011). Most recently, N stable isotopic ratios ($\delta^{15}\text{N}$) is being used as an indicator of plant N demand (Davidson et al. 2007; Inglett and Reddy 2006).

Sundareshwar et al. (2003) further observed that in the same ecosystem, individual trophic groups could show different nutrient limitation. For example, primary production of plants in coastal wetlands was limited by N; but the bacterial community in the soil was limited by P.

To test those indicators of nutrient status, two restored wetlands with young soils and high P, and a native reference wetland with old soils and low P were selected in the Florida Everglades National Park, USA. The total nutrients of the major components, extractable nutrients, microbial activities, and plant nutrient use and resorption efficiency were measured. The objectives of this study are to 1) describe the nutrient status during the restoration, and 2) to see if different nutrient indicators could give the consistent results.

Methods and Materials

Study Site

Two wetlands restored in 2000 and 2003 as well as an unfarmed reference site adjacent to the restored areas were selected in the HID region of Everglades National Park (Fig. 2-1). In each site, five sampling stations were identified. Soil depth varied among the three sites, 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).

Sampling Methods

Unlike most areas of the United States, the Everglades experiences only two seasons: dry and wet, corresponding to winter and summer. The dry winter season begins from November through April, with cooler and scant rainfall. The wet summer season accounts for approximately 80% of the region's average annual rainfall of 137 cm (54 inches). Rainfall within the Everglades system can vary dramatically from year to year. Historically, some wet years peaked at over 254 cm (100 inches) of rainfall, whereas some dry years received less than 76 cm (30 inches) (<http://www.waterencyclopedia.com/En-Ge/Everglades.html>).

Samples were collected in October 2009 (wet season) and February 2010 (dry season). To estimate soil bulk density, soil cores were collected with three replicates and each depth recorded by driving 3.6 cm diameter aluminum pipe into the soil. The soil depth was measured every 0.5 m along a 10 m line northwards at each transect in each site and then averaged them as the soil depth.

At each of the 15 transect locations three composite samples of surface soil and periphyton were collected. Live healthy leaves of the dominant vegetation were collected by hand at the 15 locations in both wet and dry season. Not all species were sampled, but an effort was made to include species that were present at the most sites. The targeted species included representatives of the genera *Muhlenbergia*, *Cladium*, *Typha*, *Andropogon*, and *Schinus* (Dalrymple et al. 2003). All samples were sealed in plastic bags and kept on ice until their return to the laboratory where the samples were refrigerated at 4°C until subsequent analysis. Soil samples were sieved to remove roots

and rock fragments greater than 2 mm diameter. Sieved soil samples were used in determination of all microbial and enzyme related parameters, while a subsample of sieved soil was oven dried at 105°C for 3 days and ground using a mortar and pestle for moisture content and total nutrient determinations. Plant tissues were oven dried at 65°C for 3 days and ball milled for total nutrient and isotopic ratio determination.

Plant Biomass and Nutrient-Use/Resorption Efficiency

In April, 2010, four replicates of aboveground plant biomass were collected in four 1 m² square plots at two locations in the three sites (Fig. 2-1). After bringing them to the lab, dead and live parts were separated; then oven dried at 65°C for 3 days and ball milled for total nutrient and isotopic ratio determination.

The nutrient use efficiency (NUE) and nutrient resorption efficiency (NRE) of N and phosphorus were determined following methods outlined by Berendse and Aerts (1987), Aerts et al. (1999), and Feller et al. (2002). The live and senescent fractions of each biomass collection were used in the following calculations. The NRE was calculated as the percentage of N (or P) recovered from senescing leaves before stem fall:

$$NRE = (N_{live} - N_{senescent}) / (N_{live}) * 100 (\%)$$

The NUE was calculated as:

$$NUE = A / L_n, (g \text{ biomass } mg^{-1} N)$$

where A is the N productivity, dry matter production per unit of N in the plant and is calculated as:

$$A = \text{biomass production } (g \text{ dry wt } m^{-2} \text{ yr}^{-1}) / \text{biomass N } (mg \text{ N } m^{-2} \text{ yr}^{-1}), (g \text{ dry wt } mg^{-1} N)$$

and L_n is the N requirement per unit of N in the plant and is calculated as:

$$L_n = N_{live} (mg \text{ m}^{-2}) / N_{senescent} (mg \text{ m}^{-2}), (\text{unitless})$$

The NRE is the fraction of N that is remaining in the biomass during a given time period. The NUE at the ecosystem level was taken as biomass production per unit of N in senescent leaves (g dry wt biomass mg⁻¹ N).

Biogeochemical Analysis

Total C and N contents were measured using a Thermo Flash EA 1112 elemental analyzer (CE Elantech, Inc.). Total P was measured colorimetrically using a Shimadzu UV-160 spectrometer (method 365.1, U.S. EPA 1993) following ashing and dissolution in 6M HCl (Anderson 1976). Loss-on-ignition (LOI) was estimated by weight loss after 4-5h combustion in the muffle furnace at 550°C.

Extractable NH₄-N was determined by 0.5M K₂SO₄ extraction and analyzed colorimetrically for NH₄-N using a TechniconTM Autoanalyzer (method 350.1 EPA 1993). Microbial biomass C and N (MBC and MBN) were determined using the chloroform fumigation-extraction technique (Brookes et al. 1985). The extraction efficiency factors for MBC and MBN in the study were 0.37 and 0.42, respectively. These parameters were performed by the Wetland biogeochemistry Laboratory in the Soil and Water Science Department, University of Florida.

Four extracellular enzymes related to the N and P cycles, i.e., alkaline phosphatase (AP, EC 3.1.3.1), phosphodiesterase (BisP, EC 3.1.4.1), N-acetyl-β-D-glucosaminidase (NAG, EC 3.2.1.30), and Leucine aminopeptidase (LAP, EC 3.4.11.1), were measured using fluorogenic enzyme substrates (Hoppe 1983). Methods were modified from Sinsabaugh et al. (1997) to optimize the substrate concentrations in soil samples with fluorogenic substrates methylumbelliferyl for phosphatase and NAG, and L-leucine 7-amido-4-methyl coumarin (AMC) for LAP. Fluorescence was measured at

excitation of 350nm and emission of 450nm using a Bio-Tek Model FL600 fluorometric plate reader (Bio-Tek Instruments, Inc. Winooski, VT).

The measurement of nitrogenase activity (potential N₂ fixation) was using the acetylene (C₂H₂) reduction (AR) assay and the experiment details were according to those of Inglett et al. (2004).

Statistical Analysis

Data were analyzed with JMP v.8[®] statistical software (SAS Institute Inc., Cary, NC). Comparisons of means between different sites and seasons were determined using a one-way univariate analysis of variance (ANOVA) with Tukey-Kramer test. Two-way ANOVA was applied to test for difference of the means of various soil properties among sites, seasons and their interaction. Simple regression analysis was applied to evaluate the relationship between different properties. Where applicable variables were log transformed to improve normality. Principal component analysis (PCA) was used to ordinate sites on the basis of the multiple nutrients and microbial parameters. Significances and correlation coefficients are significant at the $P < 0.05$ level, unless otherwise noted.

Results

Basic Properties of the Soil, Periphyton, and the Plants

The two-way ANOVA showed the TC, TN, TP, and TN:TP ratios did not show significant seasonal differences (Table 2-1). For the soil, the pH, TC, and TN at the restored sites and reference site were similar (Table 2-2). However, significantly higher TP values were observed at the restored sites compared to those at the reference site ($P < 0.001$, $n=15$) (Table 2-2). Accordingly, the TN:TP molar ratio at the reference site was markedly higher than those in the two restored site. Moreover, significantly higher

extractable inorganic P and N (i.e., $\text{NH}_4\text{-N}$) were observed in the restored sites compared to the reference site and the magnitudes varied with season (Table 2-1 and 2-3). For the periphyton, TP concentration at the restored sites were approximately three folds higher than that at the reference site; and accordingly the TN:TP molar ratio of the periphyton was significantly higher at the reference site ($P < 0.001$, $n = 15$, Table 2-2).

The foliar TN and TP values were plotted with the two critical TN:TP thresholds reported by Koerselman and Meuleman (1996) across the three sites and species (Fig. 2-2). Foliar TN:TP ratios varied with different plant species, seasons, and sites.

Significantly higher $\delta^{15}\text{N}$ values were measured for the soil at the reference site in both wet and dry seasons (Table 2-4). Similar trends were also found for the periphyton $\delta^{15}\text{N}$ values but were only significantly different in the dry season (Table 2-4). In contrast, the $\delta^{15}\text{N}$ values of plants at the species level were much lower at the reference site relative to the younger restored site (i.e., Res03) except for the *Cladium*. At the community level, the $\delta^{15}\text{N}$ values for the dead fraction of plant aboveground biomass were significantly lower at the reference site compared to the Res03 site, but no significant differences were observed for the live tissues between the three sites (Table 2-4).

Plant Nutrient Use and Resorption Efficiency

The community level nutrient-resorption efficiency of N (NRE-N) was significantly lower for the reference site (approximately 23%) relative to the two restored sites with values above 40%. A significantly lower nutrient-use efficiency of N (NUE-N) was also observed at the reference site with the average of $0.25 \text{ g biomass mg}^{-1} \text{ N}$ compared to the two restored sites with the NUE-N values above $0.4 \text{ g biomass mg}^{-1} \text{ N}$ (Table 2-5).

However, the NRE-P and NUE-P did not show consistent pattern between the three sites (Table 2-5).

Extracellular Enzyme Activities

In general, the activities of the enzymes involved in the N-cycle, i.e., LAP and NAG, were significantly higher at the restored sites compared to the reference site (Fig. 2-3ab). In contrast, activities of the enzymes related to the P cycle, i.e., AP and BisP, were significantly higher at the reference site ($P < 0.05$, $n = 15$) (Fig. 2-3cd).

Significantly higher N_2 fixation rates (measured by the acetylene reduction method) were found in periphyton mats of the sites cleared in 2000 and 2003 ($3 - 10 \text{ nmol g}^{-1} \text{ dw h}^{-1}$) compared to the reference marl prairie wetland site ($< 1 \text{ nmol g}^{-1} \text{ dw h}^{-1}$) (Fig. 2-4, Liao and Inglett 2012).

Multivariate Analyses

The multivariate statistical technique of principal components analysis (PCA) was used to characterize the sites on the basis of the biogeochemical parameters of soil and periphyton (Fig. 2-5). The first two components explained approximately 60% information, and were used to categorize the three sites. In the wet and dry season, restored sites were separated from the reference site predominantly on the basis of PC 1 with major factor loadings of soil and periphyton TP, TC:TP, TN:TP, soil extractable inorganic and organic P. Principal component 2 also contributed to this separation on the basis of soil TN, TC, TC:TN ratio, and MBC.

Discussion

TN:TP Ratio and Nutrient Limitation

Based on the threshold of N:P ratio given by Koerselman and Meuleman (1996), the TN:TP mass ratio for the plant foliar in this study could not tell any solid information

about nutrient limitation. In the wet season, it seemed that most of the TN:TP ratios at the Res03 site fell in the N-limitation zone, and reference site fell in the P-limitation zone. However, all the TN:TP values fell in the P-limitation zone in the dry season (Fig. 2-2a). Moreover, the plant species also have large effects on the TN:TP ratios as shown in the Fig. 2-2b. All of these results suggested that the critical values of TN:TP ratios for diagnosing nutrient limitation would have large temporal and seasonal variation and should be used with caution (Güsewell 2004; von Oheimb et al. 2010).

Comparing with foliar N:P ratio, no agreement has been made in using soil TN:TP ratio to assess the nutrient status in terrestrial ecosystem, but the TN:TP ratios for both periphyton and soil in this study showed consistent trends between the restored sites and the reference site that is significantly higher TN:TP ratios were observed at the reference sites compared to the restored sites (Table 2-2), which attributed to the significantly higher P in the restored sites. As Cleveland and Liptzin (2007) found that similar to the marine phytoplankton, there was a consistent atomic C:N:P ratios in the soil (186:13:1) at the global scale, it was likely to expect that there could potentially be a critical values for different nutrient limitation.

Nutrient Use and Resorption Efficiency

Nutrient use and resorption efficiency (NUE and NRE) have been commonly used to evaluate the response of vegetation to different levels of nutrient availability (Vitousek 1982; Eckstein et al. 1999). It has been suggested that in nutrient limited systems the NRE of senescing leaves will be greater than in nutrient rich environments (Güsewell 2005). In this study, the NRE and NUE of N at the restored sites were significantly higher relative to that at the reference site, which would indicate that the restored sites were more likely to be limited by N. However, the NUE and NRE of P did not show the

patterns as expected and interestingly showed higher numbers in both high P restored sites and low-P reference site (Table 2-5). The inefficiency of NUE and NRE in supporting the nutrient limitation was discussed by earlier reviews of the literature (Aerts 1996; Eckstein et al. 1999), and a study on wetland graminoids by Güsewell (2005) found that NRE of both N and P were on average higher in P-limited systems. More NRE and NUE data were needed for individual species since the index varied widely among and within species (Aerts 1996; Vitousek 1998).

Extracellular Enzyme Activities

In this study, the enzyme activities were reported based on MBC. Many studies have found significant correlations between soil enzyme activities, soil organic matter and microbial biomass (Ajwa et al. 1999). Therefore, sometimes enzyme activities based on dry weight cannot give us the real picture of the microbial activities; the enzyme activities normalized by MBC on the other hand would be a better indicator. There are two categories of enzymes: constitutive enzymes which are always present and active; inducible enzymes which are synthesized or activated when needed. Thus the inducible enzymes related to N- and P-cycling will be produced differently under different N and P availability. Chróst (1991) found out that the activities of aminopeptidases were induced at limited N availability. NAG involved in the degradation of chitin which is a relevant source of N in soils (Hanzlikova and Jandera 1993; Gooday 1994). Sinsabaugh et al. (1993) assumed NAG activity to be induced by low N conditions. The phosphatase was widely discussed as an indicator of P limitation (Olander and Vitousek 2000).

In this study, higher NAG and LAP activities were observed in the restored sites, indicating that the restored sites were lack of available N. Similarly, significantly higher

AP and BisP enzyme activities were observed in the reference site, suggesting that the reference sites were more likely to be limited by P.

N₂ Fixation of Periphyton

Periphyton at the young restored site (Res03) was found to have higher N₂ fixation rates (Res03 area) compared to the reference sites. This agreed with the assumption that the young soils tend to be N limitation. There was a significantly linear positive relationship between N₂ fixation rates and TP, accordingly, significantly negative relationship between N₂ fixation rates and periphyton TN:TP were found in both dry and wet season (Liao and Inglett 2012, also see the details in the Chapter 3), indicating that P may limit the N₂ fixation. Rejmánková and Komárková (2000, 2001) have already demonstrated that addition of P increases N₂-fixation of periphyton in the P-limited Belizean marshes. Inglett et al. (2004, 2009) also found similar results that periphyton N₂ fixation rates were much higher in the areas with high levels of P and low TN:TP ratios in the Everglades.

Stable Isotope Ratios ($\delta^{15}\text{N}$) of Soil, Periphyton, and Plants

The $\delta^{15}\text{N}$ value can be an indicator of N₂ fixation. N₂ fixing cyanobacteria should have $\delta^{15}\text{N}$ close to that of atmosphere (i.e., 0‰) (Goericke et al. 1994). N₂ fixing organisms generally show slight isotope fractionation against ¹⁵N which results in negative $\delta^{15}\text{N}$ (Peterson and Fry 1987; Gu and Alexander 1993). In this study, the stable N isotope signature of the periphyton at the three sites all fell within the range of -2‰ to 2‰ (Table 2-1) as observed by Nadelhoffer and Fry (1994).

The soil $\delta^{15}\text{N}$ values were more positive than periphyton, suggesting that other processes except atmospheric N₂ fixation affected their $\delta^{15}\text{N}$ values. For example coupled nitrification/denitrification and ammonia volatilization or atmospheric deposition

of ^{15}N enriched nitrate or ammonium may have caused shifts in $\delta^{15}\text{N}$ within the soil. Thus, natural ^{15}N abundance of soils is presumed to be an indicator of N cycling (Dawson et al. 2002).

In this study, significant positive correlation between $\delta^{15}\text{N}$ and the TN: TP ratio for both the soil and the periphyton was found (Fig. 2-5ab) though the points were in two clusters along the regression linear line, suggesting the potential of $\delta^{15}\text{N}$ as the indicator of nutrient limitation. Along a chronosequence spanning 3 to 3000 Ky in a California annual grassland, Brenner et al. (2001) found that the mean $\delta^{15}\text{N}$ values of the soil increased by several ‰ from the youngest to oldest sites (3.5 to 6.2 ‰). In Hawaii, the two youngest sites have relatively negative $\delta^{15}\text{N}$ values whereas the 20 Ky or older sites (which should approach steady state) have a peak at 20 Ky (4.8‰) (Vitousek and Farrington 1997). As Vitousek et al. (1989) generalized, lower $\delta^{15}\text{N}$ values would be expected early in soil development since biota are strongly limited by N, microbial immobilization of ^{15}N -enriched N is rapid, and nitrification is very slow. Later in primary succession, N is not limiting, elevated N losses would enrich the remaining N in ^{15}N . In that case, soil $\delta^{15}\text{N}$ will serve as a proxy of the nutrient status or restoration.

Most non-nitrogen-fixing plants have positive $\delta^{15}\text{N}$ values, reflecting the fact that most soils are enriched in ^{15}N compared to atmospheric N_2 (Vitousek et al. 1989). In this study, the soil is indeed enriched ^{15}N compared to the periphyton that fixes atmospheric N, while the native species *Cladium* also showed positive $\delta^{15}\text{N}$ values (Table 2-4). However, strongly negative $\delta^{15}\text{N}$ values have been observed in other non-nitrogen-fixing plants (Table 2-4). It could be caused by the inputs of ^{15}N -depleted N from precipitation coupled with very low N outputs as supposed for the Hawaii forests

(Vitousek et al. 1989). However, in contrast with the results of Hawaiian (Vitousek et al. 1989) and Amazonian (Davidson et al. 2007) forests, most of the species with negative $\delta^{15}\text{N}$ values in this study were found at the old site (i.e., reference site) rather than the early successional sites (i.e., two restored sites). Isotope discrimination can occur during the N uptake, assimilation, and allocation, and different plant parts can have different $\delta^{15}\text{N}$ values (Dawson et al. 2002). Yoneyama et al. (2001) proposed that there may be large whole plant depletion in ^{15}N at high N concentrations. Therefore, it is still needed to be tested if foliar $\delta^{15}\text{N}$ could be a indicator of nutrient status.

Overall, the PCA separated the three sites into two groups based on all the nutrient parameters (Fig. 2-5), i.e., the restored sites and reference site, indicating that the nutrient status between the two groups is different. The results from PCA also showed that the soil physical properties and $\delta^{15}\text{N}$ had weak correlation with the two principal components, suggesting the soil fertility changed more dramatically and rapidly during the restoration.

Conclusions

The restoration in the HID of Florida Everglades through complete soil removal enables a primary succession in the ecosystem. Periphyton primarily colonizes the habitat and facilitates the soil development, followed by pioneer plants expansion. At the same time, the restoration aims in gradually shifting the P-enriched ecosystem to low-nutrient status. In this study, the biogeochemical properties (e.g., total nutrients, available nutrients, microbial activities, and biomass) of the three major components (soil, plants, and periphyton) were measured at two restored and a native reference calcareous wetlands in the wet (October, 2009) and dry season (February, 2010) in the Florida Everglades National Park. The results showed that (1) foliar TN:TP ratio varying

with species and seasons should be carefully used as a indicator of nutrient limitation; (2) the N use efficiency and N resorption efficiency were significantly higher at the restored sites than the reference site suggesting that restored sites were more likely to be limited by N; (3) N enzyme (i.e., NAG and LAP) activities were significantly higher at the restored sites and P-related enzymes (Alkaline phosphatase and phosphodiesterase) were significantly higher at the reference site, suggesting different nutrient demands between the restored and reference wetlands; (4) periphyton at the restored sites fixed more N_2 compared to that at the reference site, especially in the wet season; (5) soil and periphyton $\delta^{15}N$ values could be indicators of the restoration and availability of N but plant $\delta^{15}N$ patterns during restoration still requires more explicit testing. These results indicated that the microbial activities could be more sensitive to the nutrient status, and should be paid more attention during the restoration process.

Table 2-1. Summary of two-way ANOVA tests for effects of season (seas.), site, and their interaction on basic properties of soil and periphyton. ***-P<0.001, **-P<0.01, *-P<0.05, NS-not significant.

Parameter	Soil			Periphyton		
	site	seas.	sitexseas.	site	seas.	sitexseas.
Loss on ignition (LOI)	***	NS	NS	**	**	NS
Total phosphorus (TP)	***	NS	NS	***	NS	NS
Total nitrogen (TN)	**	NS	NS	***	**	**
Total carbon (TC)	**	NS	NS	NS	**	NS
TN:TP ratio	***	NS	NS	***	NS	NS
Extractable inorganic P(P _i)	***	***	*			
Extractable organic P	***	***	*			
Extractable NH ₄ ⁺ -N	***	***	NS			
Extractable organic N	NS	***	***			

Table 2-2. Basic properties of soil and periphyton at Res00, Res03, and Reference sites (mean \pm SE, $n=90$). Different lowercase letters denotes significant difference, $P<0.05$.

Parameter	Unit	Site		
		Res00	Res03	Reference
Soil				
pH		7.61 \pm 0.02	7.55 \pm 0.03	7.98 \pm 0.03
bulk density	g cm ⁻³	0.50 \pm 0.03	0.57 \pm 0.05	0.52 \pm 0.03
soil depth	cm	2.2 \pm 0.2	1.8 \pm 0.2	11.7 \pm 1.2
Loss on Ignition (LOI)	%	18.5 \pm 0.6 a	18.1 \pm 0.5 a	12.9 \pm 0.5 b
Total Phosphorus (TP)	mg kg ⁻¹	630 \pm 32 b	983 \pm 47 a	140 \pm 6 c
Total Nitrogen (TN)	g kg ⁻¹	7.5 \pm 0.3 a	6.3 \pm 0.2 b	6.7 \pm 0.2 b
Total Carbon (TC)	g kg ⁻¹	155 \pm 2 a	143 \pm 3 b	149 \pm 1 ab
TN:TP molar ratio	mol:mol	28 \pm 1 b	15 \pm 1 c	109 \pm 3 a
Periphyton				
Loss on Ignition (LOI)	%	30.0 \pm 1.6 b	38.7 \pm 1.9 b	35.3 \pm 2.2 ab
Total Phosphorus (TP)	mg kg ⁻¹	313 \pm 30 a	314 \pm 34 a	99 \pm 7 b
Total Nitrogen (TN)	g kg ⁻¹	12.9 \pm 0.4 b	13.2 \pm 22.6 b	16.2 \pm 0.6 a
Total Carbon (TC)	g kg ⁻¹	244 \pm 4 a	246 \pm 3 a	250 \pm 6 a
TN:TP molar ratio	mol:mol	121 \pm 12 b	102 \pm 8 b	393 \pm 23 a

Table 2-3. Soil extractable nutrients at Res00, Res03, and Reference sites in the dry and wet season (mean \pm SE, $n=15$). Different lowercase letters denotes significant difference, $P<0.05$. Ext. ON-extractable organic nitrogen; Ext. Pi-extractable inorganic phosphorus; Ext. Po-extractable organic phosphorus.

Parameter	Unit	season	Res00	Res03	Reference
Ext.NH ₃ -N,	mg kg ⁻¹	wet	18.7 \pm 1.1 	29.4 \pm 2.4 a	14.3 \pm 1.7 b
		dry	54.2 \pm 5.3 ab	59.0 \pm 3.8 a	43.1 \pm 2.7 b
Ext. ON	mg kg ⁻¹	wet	54.1 \pm 2.5 b	67.2 \pm 3.8 a	56.6 \pm 4.1 ab
		dry	39.1 \pm 1.8 b	32.7 \pm 1.3 b	51.2 \pm 3.5 a
Ext. Pi	mg kg ⁻¹	wet	8.6 \pm 0.6 b	14.4 \pm 1.3 a	1.5 \pm 0.1 c
		dry	10.9 \pm 0.9 b	19.3 \pm 0.7 a	2.6 \pm 0.2 c
Ext. Po	mg kg ⁻¹	wet	32.0 \pm 3.0 a	39.2 \pm 3.3 a	19.8 \pm 1.1 b
		dry	11.3 \pm 0.9 b	13.9 \pm 0.6 a	4.5 \pm 0.2 c

Table 2-4. Nitrogen stable isotope values ($\delta^{15}\text{N}$) of soil, periphyton, and plants at the three sites (mean \pm SE). Comparisons for all pairs using Tukey's HSD. Different lowercase letters denotes significant difference, $P < 0.05$. For soil, periphyton, and plants at species level, samples were collected in the wet season (October, 2009) and dry season (February, 2010), $n=15$ at each site. For plants at community level, samples were collected in April, 2010, $n=8$ for each site.

Component	season	Res00	Res03	Reference
----- $\delta^{15}\text{N}$ (‰)-----				
Soil	wet	1.71 \pm 0.07 b	1.85 \pm 0.16 b	2.35 \pm 0.06 a
	dry	1.23 \pm 0.10 b	1.42 \pm 0.13 b	2.47 \pm 0.11 a
Periphyton	wet	-0.49 \pm 0.08	-0.61 \pm 0.19	-0.02 \pm 0.14
	dry	-0.75 \pm 0.08 b	-0.97 \pm 0.09 b	0.21 \pm 0.10 a
Plants-dead		-3.05 \pm 0.27 ab	-2.26 \pm 0.23 a	-3.10 \pm 0.17 b
Plants-live		-1.92 \pm 0.32	-1.94 \pm 0.28	-1.45 \pm 0.33
Plant-Species				
<i>Andropogon</i>	wet	-3.13 \pm 0.13 b	-1.85 \pm 0.22 a	-4.11 \pm 0.14 c
	dry	-3.88 \pm 0.42	-3.38 \pm 0.12	-3.86 \pm 0.26
<i>Muhlenbergia</i>	wet	-2.51 \pm 0.20 a	NA	-3.28 \pm 0.16 b
	dry	-3.17 \pm 0.20 b	-2.13 \pm 0.69 a	-4.66 \pm 0.13 c
<i>Cladium</i>	wet	2.43 \pm 0.18 a	0.78 \pm 0.07 b	2.40 \pm 0.17 a
	dry	1.92 \pm 0.09 a	0.70 \pm 0.34 b	1.12 \pm 0.13 b
<i>Typha</i>	wet	1.33 \pm 0.18	1.77 \pm 0.14	NA
	dry	0.46 \pm 0.07 a	-0.21 \pm 0.21 b	-0.22 \pm 0.08 ab
<i>Schinus</i>	wet	-1.82 \pm 0.29 b	2.11 \pm 0.33 a	-1.67 \pm 0.44 b
	dry	-2.48 \pm 0.27 b	-0.85 \pm 0.15 a	-1.77 \pm 0.30 ab

Table 2-5. Nutrient-resorption efficiency and nutrient-use efficiency of nitrogen and phosphorus (NRE-N, NRE-P, NUE-N, and NUE-P) for the community level vegetation (composite biomass). The different letters denote significance among the three sites, mean \pm SE.

Site	NRE_N %	NUE_N g biomass mg ⁻¹ N	NRE_P %	NUE_P g biomass mg ⁻¹ P
Res00	41.3 \pm 1.3 a	0.41 \pm 0.04 ab	63.1 \pm 6.6	7.2 \pm 1.4 b
Res03	42.5 \pm 1.5 a	0.58 \pm 0.08 a	71.0 \pm 3.9	14.0 \pm 2.2 a
Reference	22.9 \pm 2.6 b	0.25 \pm 0.02 b	58.6 \pm 8.0	14.0 \pm 4.3 a

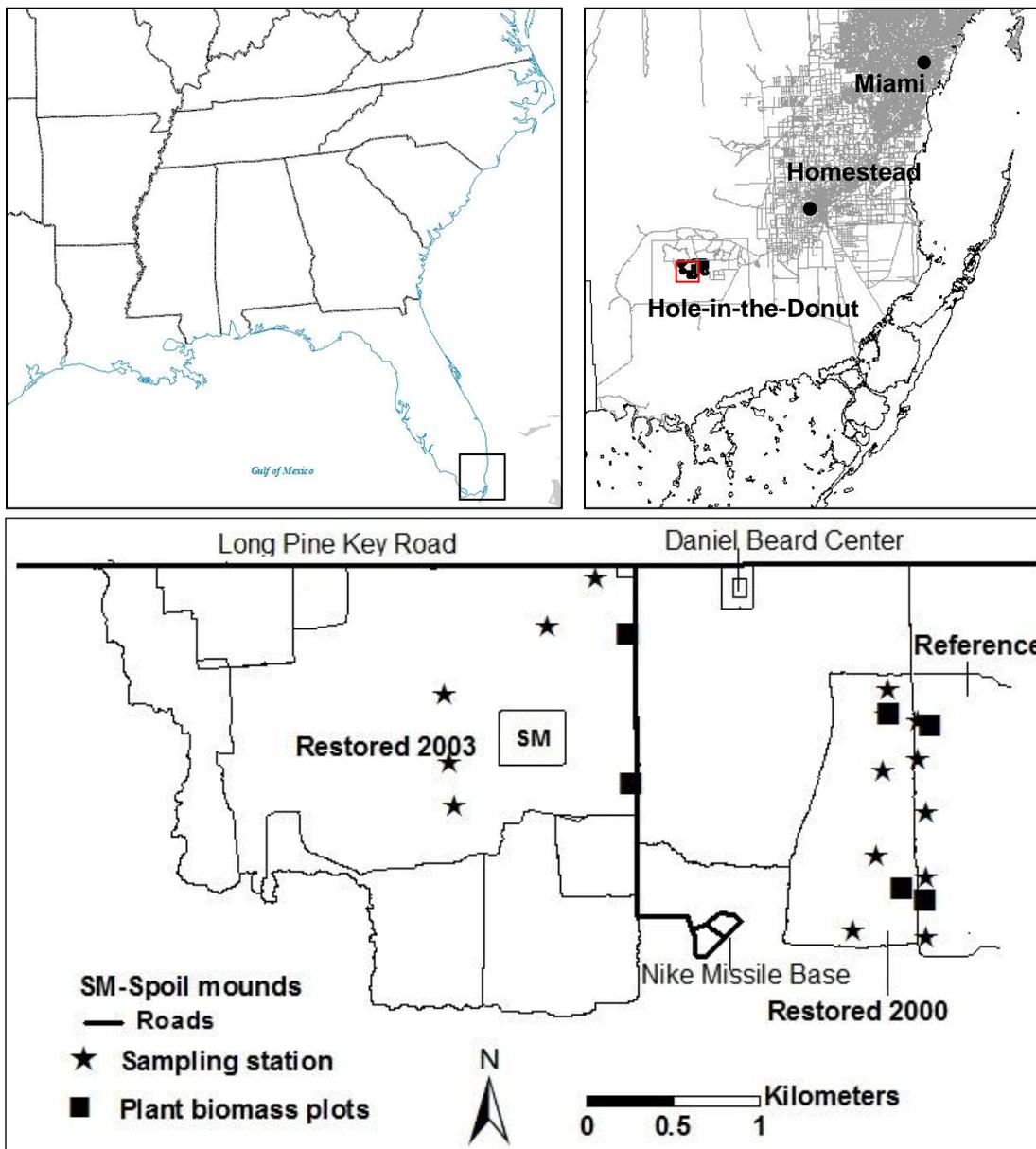


Figure 2-1. Location of the study sites-Hole-in-the-Donut in the Florida Everglades National Park.

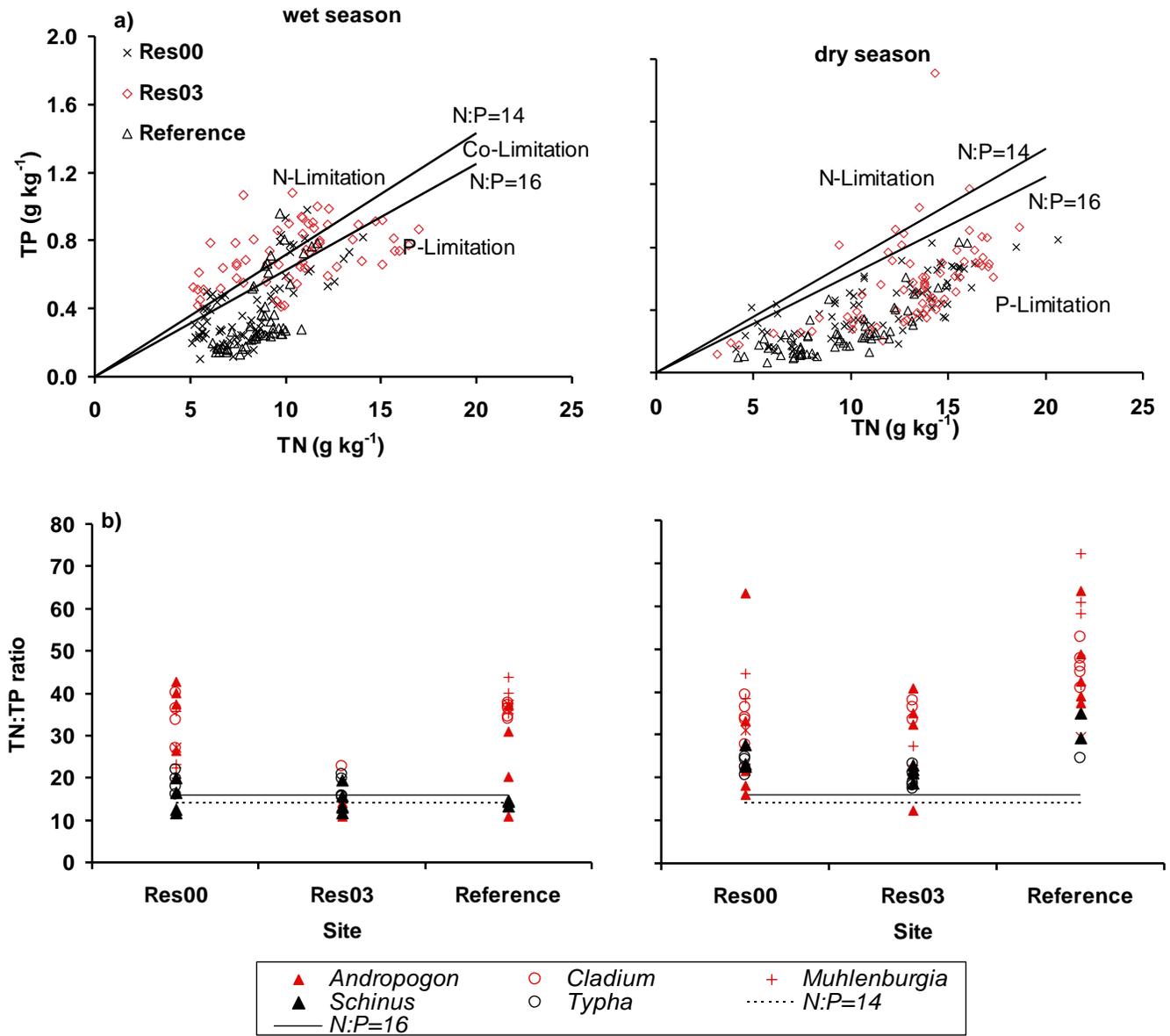


Figure 2-2. Relationship between plant TN and TP concentration for the Res00, Res03, and Reference sites(a); plant TN:TP mass ratios sorted by different species at the three sites (b) The critical TN:TP ratios were from Koerselman and Meuleman (1996).

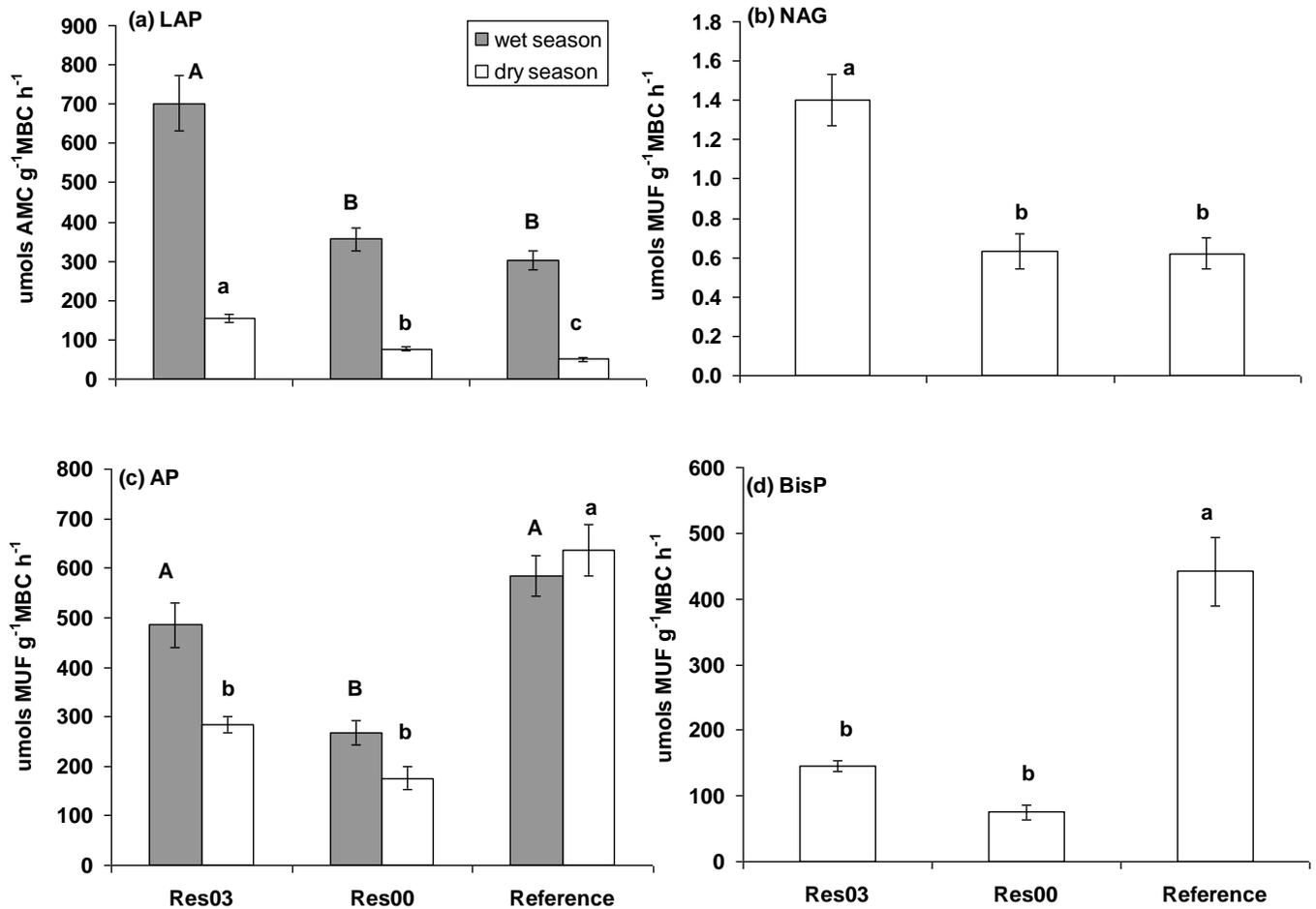


Figure 2-3. Activities of N-related enzymes (a) Leucine-aminopeptidase, LAP, (b) N-acetyl- β -D-glucosaminidase, NAG (only measured in the dry season), and P-related enzymes (c) Alkaline phosphatase, AP, (d) phosphodiesterase, BisP (only measured in the dry season) at the Res00, Res03, and the reference sites in different seasons. The different lowercase and uppercase letters denote significance in the wet (grey bars) and dry season (white bars), respectively.

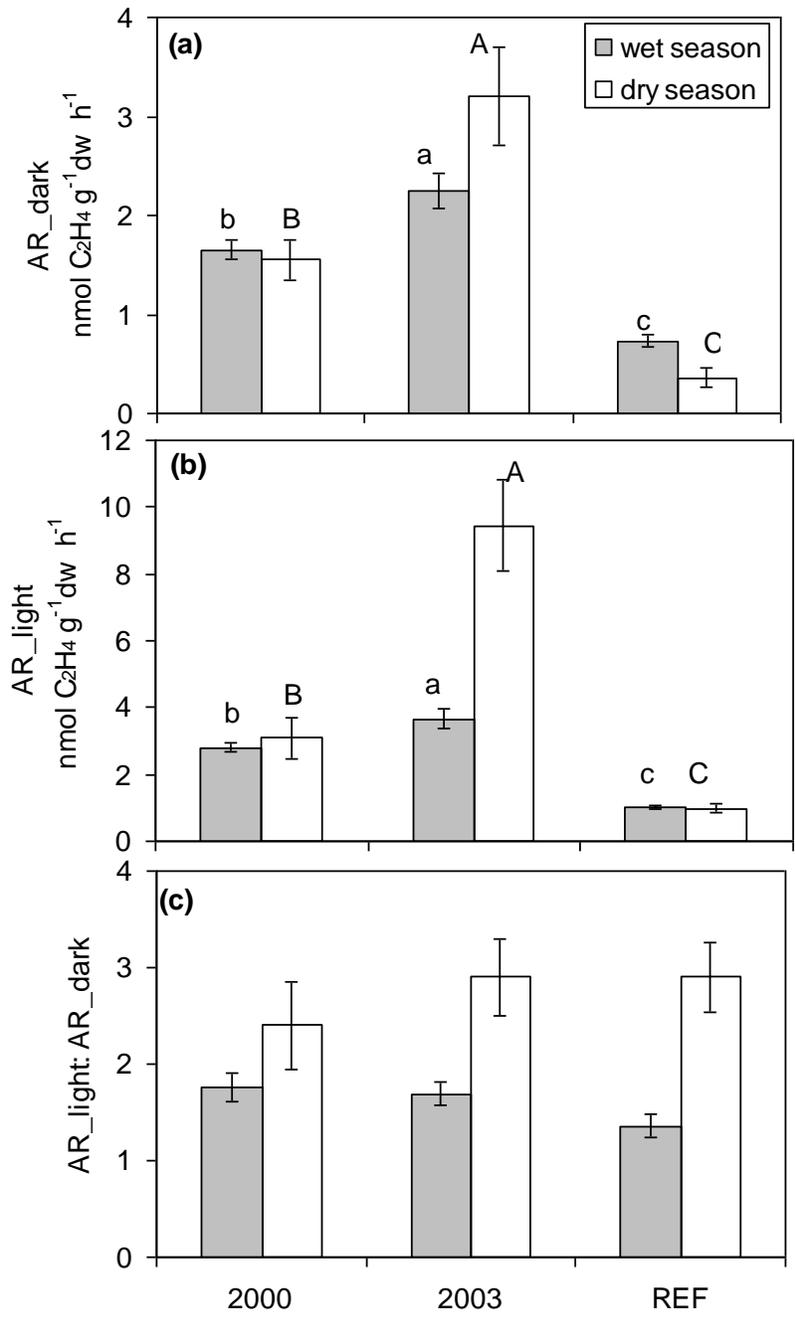
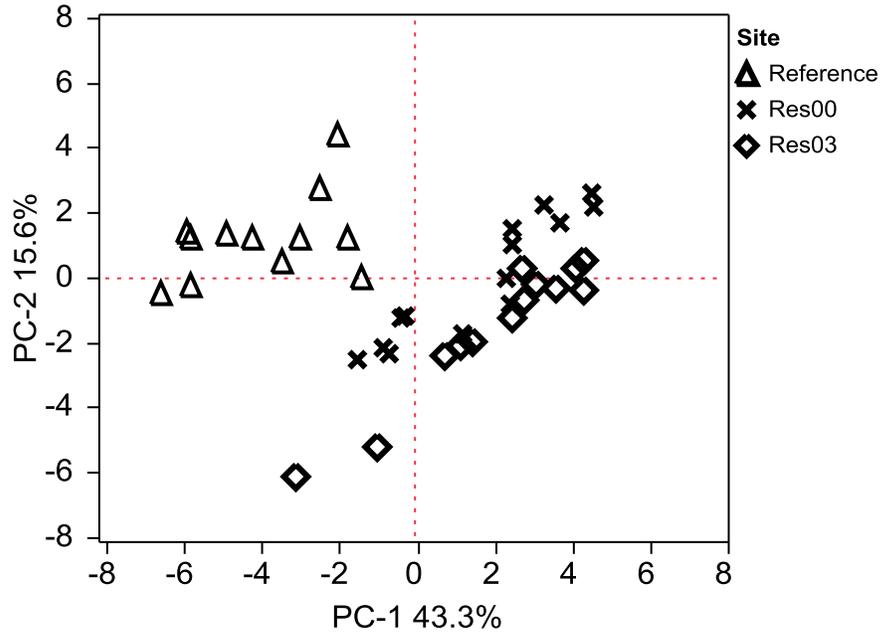


Figure 2-4. Periphyton acetylene reduction (AR) rates under dark (a), light (b) incubation, and the ratio of AR rates under light and dark conditions (c) determined in each of the three Everglades sites in the dry (white bars) and wet seasons (shaded bars). The different lowercase and uppercase letters denote significant differences between the sites (P<0.05).

(a) wet season



(b) dry season

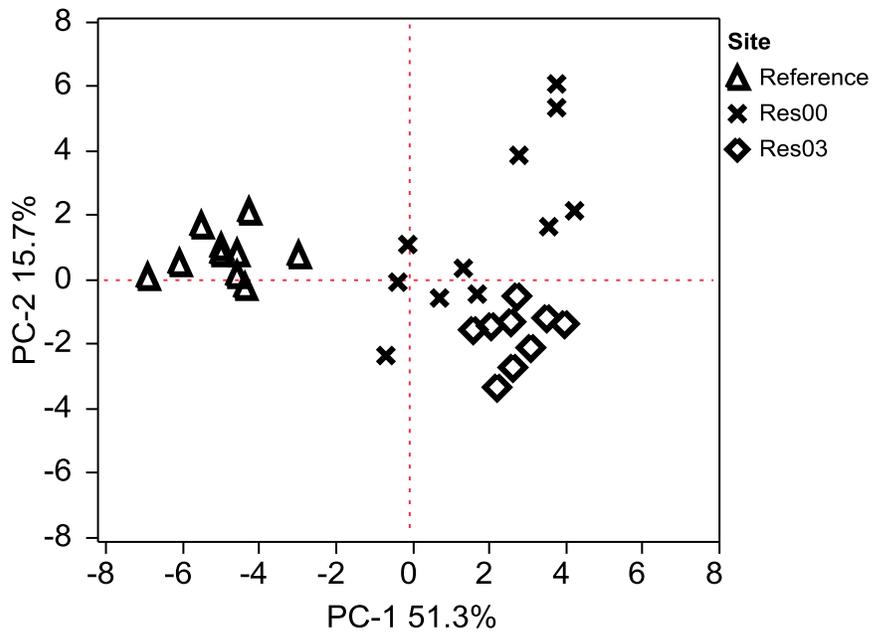


Figure 2-5. Score plots of principal components analysis on the biogeochemical properties of soil and periphyton in the wet and dry season.

CHAPTER 3
SPATIAL PATTERNS OF PERIPHYTON NITROGENASE ACTIVITY IN THE
CALCAREOUS WETLANDS

Background

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; Thomas et al. 2006; Rejmánková and Komárková 2005) 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 et al. 2000, 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

Reprinted with permission from Liao X, Inglett PW (2012) Biological nitrogen fixation in periphyton of native and restored everglades marl prairies. *Wetlands*, 32(1):137-148

periphyton also show the ability to fix atmospheric N₂ (biological N₂ fixation) (Inglett et al. 2004, 2009). This process was estimated to contribute 10 g N 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. 2011).

One Everglades system where periphyton plays a crucial ecological role is the Hole-in-the-Donut (HID) region of Everglades National Park (ENP) (Fig. 3-1). 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 (CaCO₃), both main components of marl soils (Gaiser et al. 2011). N₂ fixation also provides an important N source particularly in the recently cleared sites where N is limiting (Smith et al. 2011; Inglett et al. 2011). 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 been done on the N₂ fixation in P-limited ecosystem (Inglett et al. 2004, 2009; Rejmánková and Komárková 2000; Rejmánková 2001). It is important and deserving of attention to understand the role of periphyton in the marl prairie (Davis et al. 2005). For these reasons, the following study was conducted to (1) characterize periphyton N₂ fixation between the restored and reference wetlands; (2) relate the N₂ fixation with nutrient limitation (i.e., N and P limitation); and (3) assess the potential of N stable isotopes as an indicator of N₂ fixation and nutrient limitation.

Materials and Methods

Study Site

The Everglades experiences two primary seasons including a mostly dry winter (November through May) and a wet summer (June to October). The wet summer season accounts for approximately 80 percent 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>).

Two wetlands restored in 2000 and 2003 (referred to as Res00 and Res03) as well as an unfarmed reference site adjacent to the restored areas were selected in the HID region of Everglades National Park (Fig. 3-1). In each site, five sampling stations (A, B, C, D, E) were identified along a elevation gradient from 0.5 m to 1.0m AMSL (Table 3-1). Soil depth varied among the three sites, 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* sp.) and sedges (*Cladium jamaicense* Crantz, *Shoenus* sp.) while the restored sites are

dominated by pioneer species such as *Ludwigia* spp., *Baccharis* spp., and *Andropogon* spp. (Dalrymple et al. 2003). The periphyton in the three sites are all calcitic epilithon or calcareous epiphytic periphyton mat (Gaiser et al. 2006, 2011). The mats in the restored site were very thin and greenish and the periphyton in the Res03 site is more disaggregated compared to that in Res00 site; while the mats in the reference sites were 1 cm thick with dark color.

Sampling Methods

Samplings were conducted 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 3 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_2H_2) reduction (AR) assay described by Inglett et al. (2004). Wet periphyton (5 g) were placed into 42-mL, screw-capped culture tubes (KimaxTM) 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 hours under either light ($\sim 900 \mu\text{mol m}^{-2}\text{s}^{-1}$ PAR) or dark conditions. After incubation, 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 10ppm; Scott Specialty Gases, Inc., Plumsteadville, PA) were used to calibrate the measurement being expressed as $\text{nmol C}_2\text{H}_4 \text{ g dw}^{-1} \text{ h}^{-1}$. The gas dissolved in the liquid phase was ignored since there was no accumulated water in the tube. Blank corrected AR values were used to estimate actual rates of N_2 fixation using a theoretical conversion ratio of 3 moles of C_2H_2 reduced to 1 moles of N_2 fixed (Howarth et al. 1988a). When estimating the annual fixed N, the following assumptions were made: (1) there were two seasons with six months of wet season represented by the October and 6 months of dry season by February; (2) the whole day was divided by 12 hours light condition and 12 hours dark condition; (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 nutrient and isotopic analysis.

Chemical and Isotopic Analysis

Total C and N content were measured using 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 6M 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 (Goldin 1987; 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 N₂ (0.3663‰) using delta notation (δ) as follows:

$$\delta^{15}N_{sample} = \frac{(^{15}N/^{14}N)_{sample} - (^{15}N/^{14}N)_{standard}}{(^{15}N/^{14}N)_{standard}} \times 1000$$

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 0.45µm membrane filter and acidified to preserve on ice until their return to the laboratory. The total dissolved phosphorus (TDP), soluble reactive phosphorus (SRP, PO₄³⁻), ammonia-nitrogen (NH₄-N), nitrite-nitrogen plus nitrate-nitrogen (NO_x-N), total dissolved Kjeldahl Nitrogen (TKN) were analyzed 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 3-2. $\text{NH}_4\text{-N}$, dissolved inorganic nitrogen ($\text{DIN} = \text{NO}_x\text{-N} + \text{NH}_4\text{-N}$), TKN, TDP and SRP were significantly higher in the Res00 site comparing to the reference site and Res03 site ($P < 0.05$). The water TDP was close to the natural Everglades system below a threshold limit of $10 \mu\text{g L}^{-1}$ (Thomas et al. 2002). However, the ratio of DIN: SRP and TDKN: TDP were significantly lower in the restored site compared to the reference site.

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). 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 restored sites with the average of 313 mg kg^{-1} were approximately three times of that in the reference site with the average of 99 mg kg^{-1} (Table 2-2). Accordingly, the

periphyton molar TN:TP ratios in the reference site were approximately three times higher than those in the restored sites, with average values of 393 ± 23 (Table 2-2). Compared to the periphyton in other parts of Everglades, the TP in the restored sites were closer to that of the Water conservation Area 1 (WCA-1) with the average of 423 mg kg^{-1} and the TP in the reference site was comparable to the values in the Taylor slough (TS) that has similar calcareous mats with a average of 124 mg kg^{-1} TP (Gaiser et al. 2006). Periphyton TN was significant higher in the dry season for the Res00 site (with the average of 15 mg g^{-1}) and Res03 site (with the average of 14 mg g^{-1}) compared to the wet season with the average of 11 mg g^{-1} in the Res00 site and 12 mg g^{-1} in the Res03 site. No significant seasonal difference of periphyton TN was observed in the reference site; neither was 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. 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 two restored wetlands at different age (Res03 vs Res00) as expected; the possible explanation is that the gaps between the two restored sites were small (Table 2-3). However, the soil TP and TN:TP ratio somehow distinguished the two sites

Nitrogenase Activity

Acetylene reduction (AR) rates under both light and dark conditions were significantly higher ($P < 0.05$) in the restored sites (Fig. 2-4ab). For the Res00 and reference sites, there were not significant differences in the AR rates between the two

seasons; while for the Res03 site, the AR rates were significantly higher in the dry season. Rejmánková et al. (2004) reported that the N₂ 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 N₂ fixation. Vargas and Novelo (2007) also found that the highest AR rates of periphyton appeared during the rainy season in the Yucatan peninsula.

In this study, the contrasting seasonal patterns of periphyton N₂ fixation between Res00, reference sites and Res03 site could be the result of the 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 N₂ 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, the periphyton biomass was found to be 155 ± 16 g AFDW m⁻² in the Res00 area, 208 ± 28 g AFDW m⁻² in the Res03 area and 295 ± 33 g AFDW m⁻² in the reference site, which fell 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). Periphyton production in marl prairies, especially in the wet season, was much higher than the long-hydroperiod mats of periphyton, such as the periphyton mats in the water conservation area (McCormick et al. 1998; Hagerthey et al. 2011).

Using the theoretical ratio of 3 moles of C_2H_4 produced per mole of N_2 fixed, it is possible to estimate the amount of N_2 fixed for a given AR rates. Although the empirical ratio vary among different ecosystems, the theoretical ratio of 1:3 has proven reasonable for cyanobacterial mats (Howarth et al. 1988a; Doyle and Fisher 1994). In this study, significantly higher N_2 fixation rates of periphyton were observed in the Res03 site with average of $0.2 \pm 0.03 \text{ g N m}^{-2} \text{ yr}^{-1}$ compared to the reference sites with average of $0.05 \pm 0.01 \text{ g N m}^{-2} \text{ yr}^{-1}$ ($P < 0.05$) (Fig. 3-2b). These values are much smaller in comparison with the unimpacted Water Conservation Area-2A (WCA-2A) in Florida Everglades, where the N_2 fixation rates ranged from 1.8-18 $\text{g N m}^{-2} \text{ yr}^{-1}$ (Inglett et al. 2004).

In this regard, a rough estimate of the time for the Res03 site to reach the same level of N storage as the reference site was made. The N storage for the Res03 and reference site is 35 g m^{-2} and 125 g m^{-2} , respectively (Inglett et al. 2011, in review). Assumed that all the N fixed by the periphyton in the Res03 site was absorbed by the soil, the time when the TN storage in the Res03 site was close to the reference site could be roughly calculated, which is $(125-35) \text{ g N m}^{-2} / 0.2 \text{ g m}^{-2} \text{ yr}^{-1} = 450 \text{ yr}$. It was a long time even without considering any pathways of N loss.

There was no significant difference in the light:dark AR ratio among the three sites (Fig. 2-4c) 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 this 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 Res03 and reference sites ($P < 0.05$), likely reflecting seasonal changes in the species composition of the periphyton communities. McCormick et al. (1998) observed that oligotrophic periphyton assemblages exhibited strong seasonal shifts in species composition and were dominated by cyanobacteria during the wet season and diatoms during the dry season.

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

N_2 fixation by cyanobacteria is accompanied by relatively little isotopic fractionation and as a result, N_2 -fixing cyanobacteria should have $\delta^{15}\text{N}$ close to 0‰ (Goericke et al. 1994; Kline and Lewin 1999). In this study, the $\delta^{15}\text{N}$ of periphyton over all three sites all fell within the range of -2‰ to 2‰ (Table 2-3), which agrees with observations from other studies (Nadelhoffer and Fry 1994; Gu and Alexander 1993; 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 the freshwater metaphyton (1-12‰, Scott et al 2007). Differences in periphyton $\delta^{15}\text{N}$ also correspond to differences in N_2 fixation measured between the three sites, with the highest $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ of six N_2 -fixing blue-green algae was $1.0 \pm 1.3\%$, whereas the $\delta^{15}\text{N}$

of six green algae showed an average of 6.6 ± 4.5 ‰. The higher $\delta^{15}\text{N}$ of non- N_2 -fixing algae reflected the utilization of dissolved inorganic N.

The $\delta^{15}\text{N}$ of periphyton and soil were significantly correlated with each other (Fig.3-4), suggesting the N source in soil is derived from that fixed by the periphyton. At a given site, the soil had a higher $\delta^{15}\text{N}$ 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 $\delta^{15}\text{N}$ within the soil (Aranibar et al. 2003; Evans and Ehleringer 1993).

Relationships between Nutrients, Nitrogenase Activity, and $\delta^{15}\text{N}$

Various factors affect N_2 fixation, including physical factors such as light intensity and temperature, and nutrient status (Howarth et al. 1988b). N and P concentrations and loadings are two mostly-discussed factors. Scott et al. (2007) showed that periphyton N_2 fixation decreased as reactive N accumulated in the periphyton matrix. In this 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, this study showed that increase TN content in February corresponded to increase AR rates for the Res03 site.

Ammonium concentrations can be quite 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 HID sites averaged from 47 to $250 \mu\text{g L}^{-1}$ (Table 3-2), which was much higher

than the values reported by Inglett et al. (2004) ($38 \mu\text{g L}^{-1}$ for the WCA-2A of northern Florida Everglades). This would explain why the AR rates of periphyton in this study were extremely lower than their values, even though the TN:TP ratio in the restored sites was similar with the northern Everglades by Inglett et al. (2004).

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. 3-3), indicating that P likely controls N_2 fixation. P-controlled N_2 fixation has been reported by many researchers (Howarth 1988b; Smith 1990; Rejmánková and Komárková 2000, 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 N information, another controlling factor of N_2 fixation and would further indicate nutrient limitation (Howarth 1990). In this 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 suppressed N_2 fixation.

There was a significantly negative correlation between N_2 fixation rates and the $\delta^{15}\text{N}$ in both the wet and dry season (Fig. 3-5a), which was similar to the finding of Rejmánková et al. (2004) and Inglett et al. (2004). Scott et al. (2007) also found that in the freshwater marsh, the metaphyton $\delta^{15}\text{N}$ decreased with increasing N_2 fixation during May through September sampling. Like the correlation of nitrogenase activity with TN:TP ratio and ^{15}N , significant relationships between $\delta^{15}\text{N}$ and the periphyton TP, TN:TP ratio were also found (Fig. 3-5b,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 other reports of calcareous periphyton mats, nitrogenase activity in this study was stimulated by light 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 less attention. For the young restored ecosystems, N_2 fixation would be an important N source and thus a key target or evaluation metric for restoration. The seasonal pattern of N_2 fixation and $\delta^{15}N$ were not consistent in the three sites, while $\delta^{15}N$ has a potential of characterizing the N_2 fixation and tracing the N-cycle, more information such as the ^{15}N signature for other inorganic nitrogen form, N_2 -fixing species composition were needed to explain the differences. The periphyton community is sensitive to nutrient status, and in this study, N_2 fixation, TN:TP ratio and the $\delta^{15}N$ were correlated in both wet and dry season. These findings suggest that N_2 fixation and $\delta^{15}N$ could also be employed as indicators of nutrient limitation and patterns of nitrogenase activity.

Table 3-1. Geographical coordinates and the approximate elevation of the sampling sites used in this study.

Sampling site	Station	Latitude	Longitude	Elevation (m)
Res00	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
Res03	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

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

Table 3-2. Mean water chemistry values at all sites in the October 2009 when the sites was flooded (mean \pm SE, n=6 for Res00 site; n=12 for Res03 site and n=12 for the reference site)

Site	SRP $\mu\text{g L}^{-1}$	$\text{NH}_4\text{-N}$ $\mu\text{g L}^{-1}$	$\text{NO}_x\text{-N}$ $\mu\text{g L}^{-1}$	TDP $\mu\text{g L}^{-1}$	TDKN mg L^{-1}	DIN:SRP mol:mol	TDKN:TDP mol:mol
Res00	6.6 (2.1)	250 (62)	5.4 (0.4)	9.9 (1.4)	1.6 (0.2)	103 (29)	364 (81)
Res03	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)

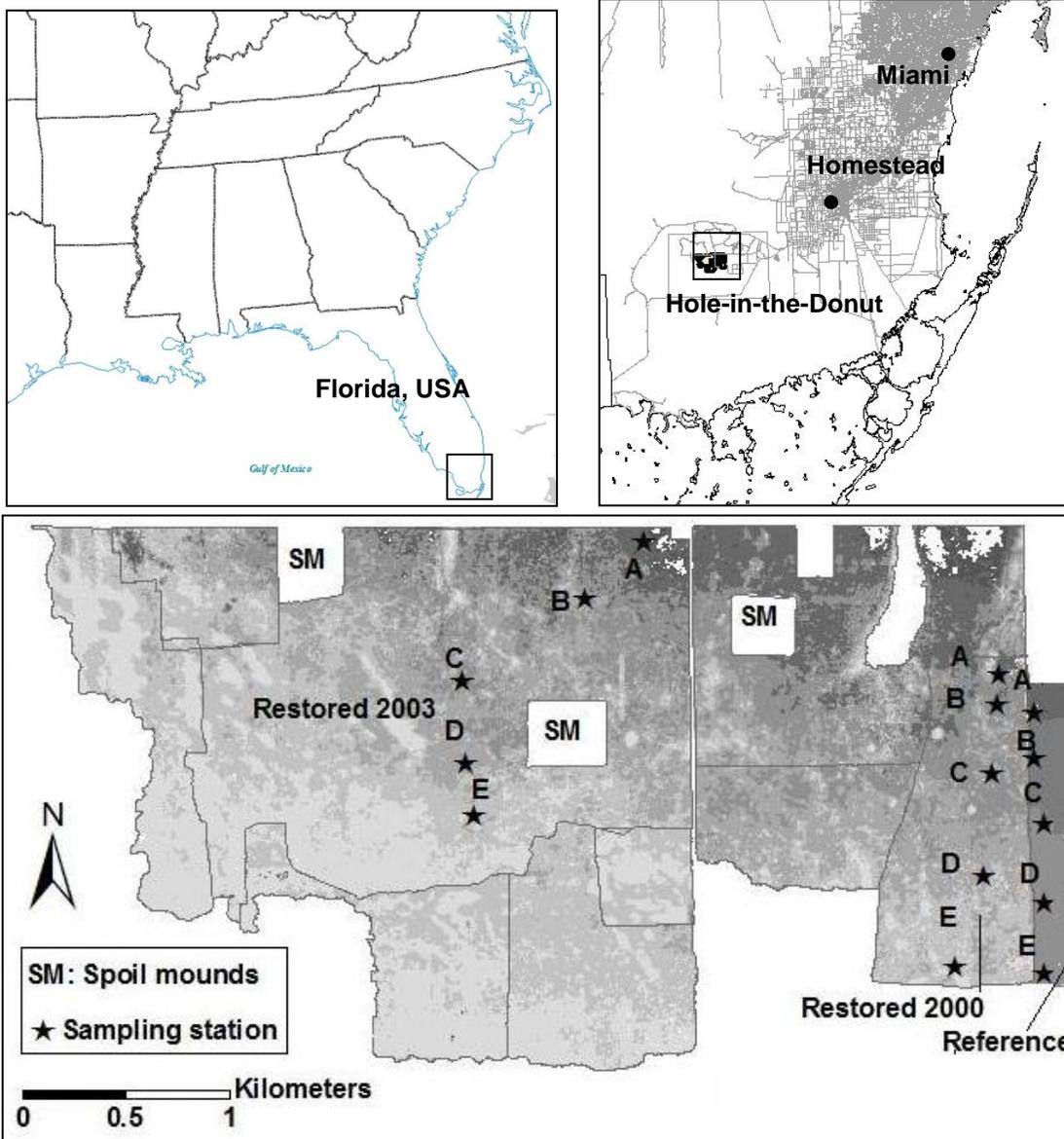


Figure 3-1. Map showing the general area of Everglades National Park and the locations of restored and reference marl prairie sites used in this study.

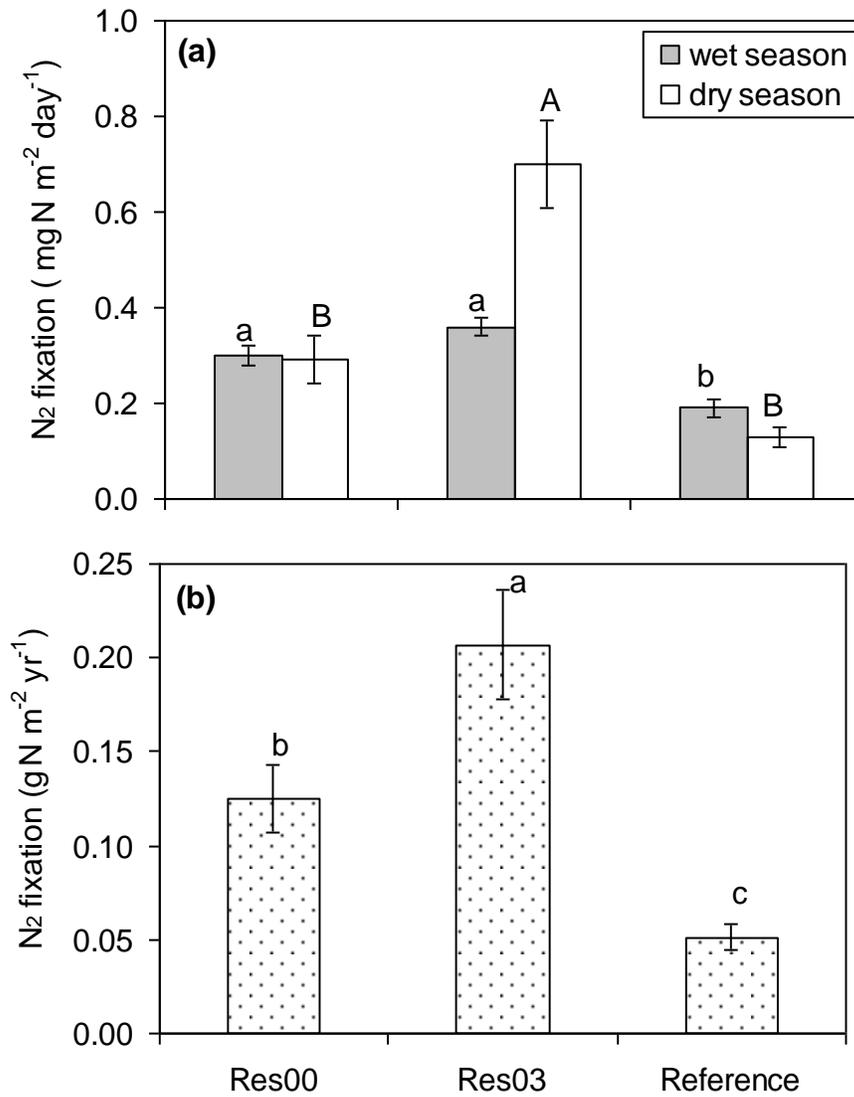


Figure 3-2. Areal estimates of N₂ fixation in the restored and reference sites (see text for calculation details). The white bars represent the data in the dry season, the shaded bars represent the data in the wet season; and the dash bars represent the whole year estimate. The different lowercase letters denote significant differences ($P < 0.05$) between the three sites.

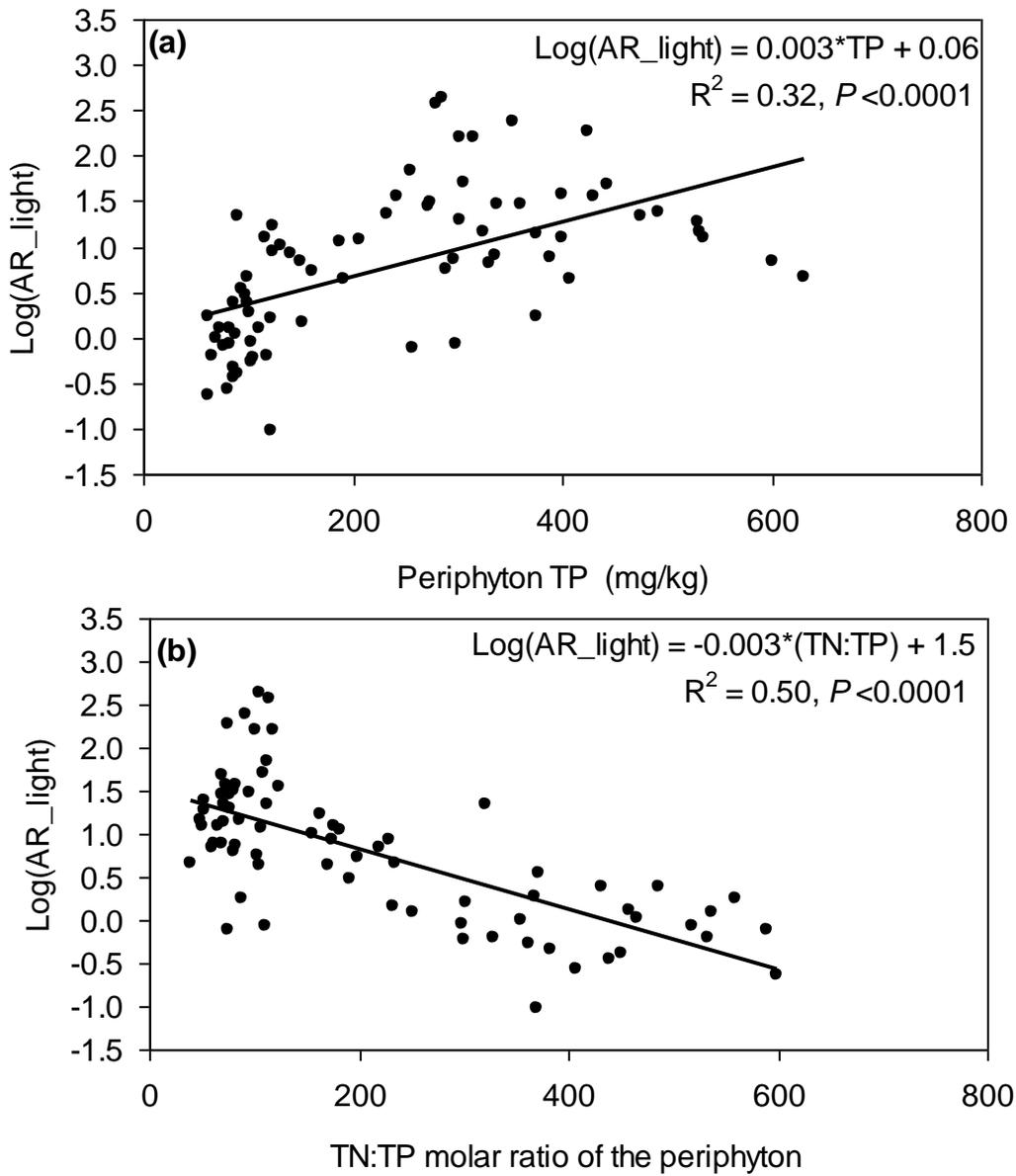


Figure 3-3. Relationships between periphyton acetylene reduction (AR) under light conditions and periphyton TP (a) and TN:TP ratio (b) in the dry and wet seasons.

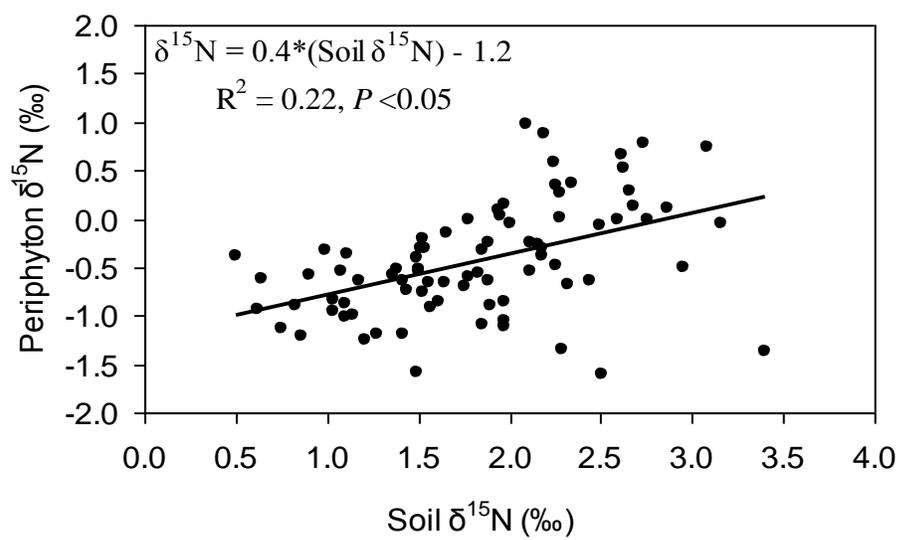


Figure 3-4. Relationship of periphyton $\delta^{15}\text{N}$ (‰) with soil $\delta^{15}\text{N}$ (‰).

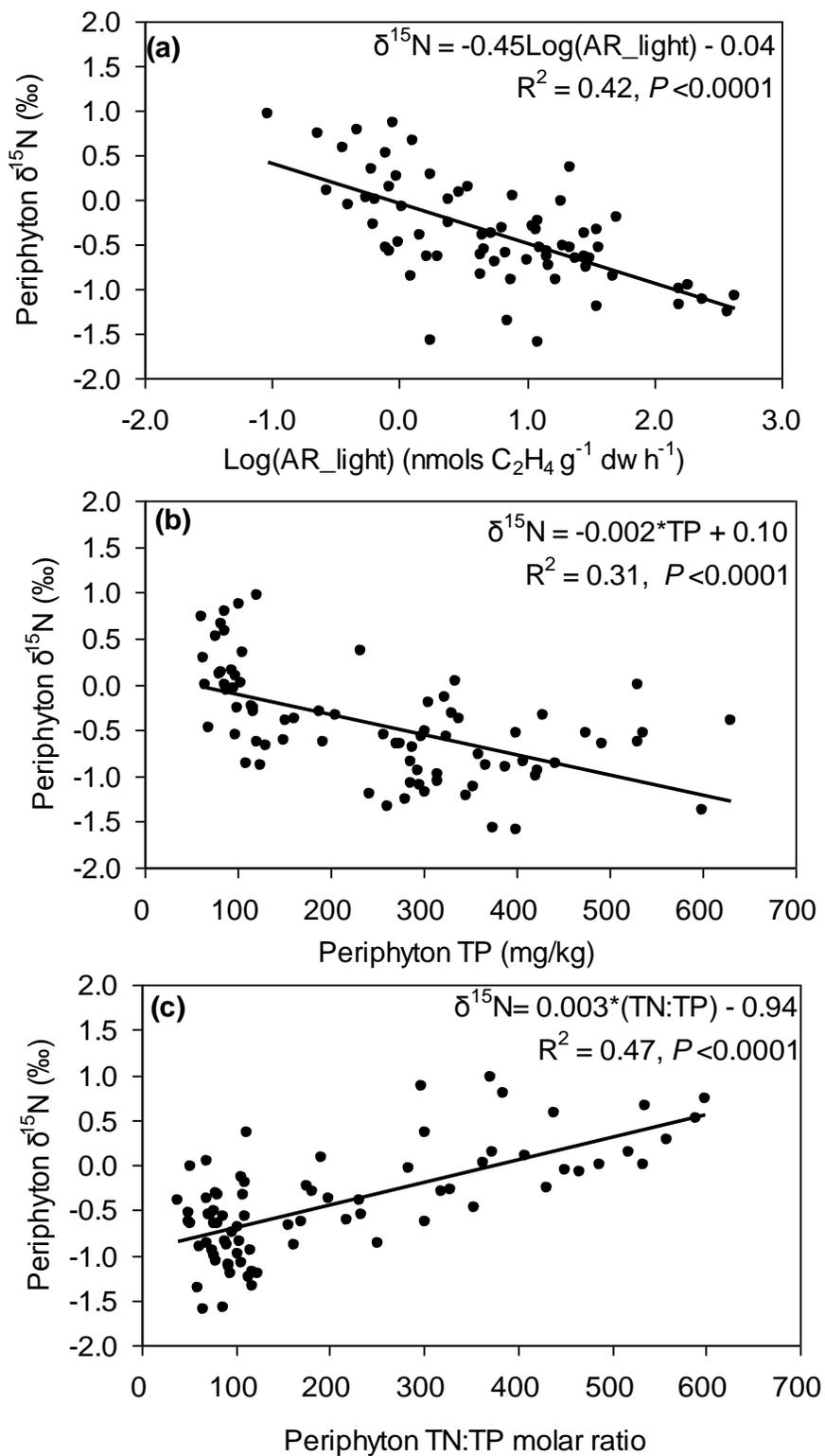


Figure 3-5. Relationship between periphyton $\delta^{15}\text{N}$ (‰), acetylene reduction rates (AR), TP and TN:TP ratio

CHAPTER 4 SEASONAL PATTERN OF PERIPHYTON NITROGENASE IN THE SHORT- HYDROPERIOD, CALCAREOUS WETLANDS

Background

Periphyton are complex assemblages of cyanobacteria, algae, heterotrophic microbes, microfauna and detritus growing attached to a substrate, and can also be called cyanobacteria mats. Periphyton are widely distributed in aquatic systems, limestone-based tropical wetlands in the Caribbean, Florida Everglades, Yucatan Peninsula of Mexico and Belize alkaline marshes (Rejmánková and Komárková 2000; Gaiser et al. 2011; Vargas and Novelo, 2007; Rejmánková et al. 2004). As an important component of these ecosystems, periphyton perform a range of vital functions including: facilitating and stabilizing soil formation through calcium carbonate precipitation (Hagerthey et al. 2011); serving as a main contributor to primary productivity (McCormick and O'Dell 1996; Gaiser et al. 2011); regulating water column nutrient levels and benthic fluxes; and based on species composition and microbial activities, serving as a sensitive indicator of water quality and nutrient status (McCormick et al. 1996; McCormick and Stevenson 1998; Sharma et al. 2005; Jasrotia and Ogram 2008; Scott et al. 2007, 2008, 2009). In addition, periphyton can be an important nitrogen (N) source as diazotrophs in the periphyton convert atmospheric N₂ to bioavailable N forms. However, not many studies have focused on the contribution of N₂ fixation by these organisms, especially in tropical wetlands (Vargas and Novelo 2007).

Periphytic mats are a ubiquitous feature of the Florida Everglades, USA, and can be categorized into four distinct habitats including the marl prairies and rocky glades, the ridge and slough of the central Everglades, the soft-water marshes (such as Water Conservation Area-1, WCA-1), and the coastal mangroves along Florida and Biscayne

Bays (Gaiser et al. 2011). Periphyton can be further classified into two habitats, based on hydroperiod including long (flooded for more than 8 months of the year) and short (inundated for less than 8 months of the year) hydroperiod marshes (Acosta and Perry 2000). Accordingly, spatial patterns of periphyton species composition, abundance, and appearance with water quality and hydrologic conditions have been well described (Browder et al. 1994; Gaiser et al. 2011; Gottlieb et al. 2005; Iwaniec et al. 2006). In comparison, the temporal dynamics of periphyton has received less attention.

McCormick et al. (1998) described the changes of the periphyton biomass and species composition during the wet and dry season in the long-hydroperiod northern Everglades, finding that epiphyton and metaphyton biomass was seasonal and peaked during the wet season. In contrast, seasonal variation of periphyton has been rarely studied in short hydroperiod marshes (Gottlieb 2003). These short-hydroperiod periphyton have a rapid response to the dry-rewetting cycle similar to cyanobacterial crust communities found in desert environments (Gottlieb 2003; Belnap 2004; Aranibar et al. 2003; Pócs 2009). Gottlieb (2003) found that the dry short hydroperiod mats became net primary producers within a short time after hydration. Similarly, respiration and photosynthesis of soil biological crusts in the southern Utah begin almost immediately upon rewetting; but there is generally a lag phase between wetting and the initiation of N_2 fixation, with a further lag time before maximal fixation rates are reached (Belnap 2001). The longer they are desiccated, the more time it takes for N_2 fixation to begin and to reach maximal rates after rewetting (Belnap 2001). Therefore, sampling strategy with finer time span is needed to know more details about the temporal pattern of periphyton especially in the short hydroperiod landscape.

It is well known that periphyton can be used as an indicator of restoration in the Florida Everglades since it responds very rapidly to the changes in the two dominant drivers of wetland structure and function, i.e., hydrology and water quality (McCormick et al. 1996; Gaiser et al. 2004; Gaiser 2009). Specifically, phosphorus (P) the limiting nutrient in most areas of the Everglades controls the abundance and community of the periphyton (McCormick et al. 1996). However, few people have investigated the response of periphyton N₂ fixation to the nutrient status in Everglades (Inglett et al., 2004, 2009, 2011; Jasrotia and Ogram 2008) though in other ecosystems like lakes, numerous evidences have shown that P regulates N₂ fixation. Moreover, some fractions of Everglades have been experiencing a shift from P limitation to N limitation, such as the downstream areas towards Florida Bay where P derive from the Gulf of Mexico is much more abundant (Childers et al. 2006; Inglett et al. 2011). In those areas under the N limitation, periphyton N₂ fixation will function as an important N source and serves as another attribute of periphyton except biomass and species composition in indicating the restoration process.

The Hole-in-the-Donut (HID) located in the Everglades National Park is such a case that P was accumulated from the historical agricultural activities (Smith et al. 2011, Fig. 4-1). To restore the HID to its originally low nutrient status, the technique of complete soil removal was adopted (Li and Norland 2001; 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 (CaCO₃) both main components of marl soils (Gaiser et

al. 2011). N_2 fixation of periphyton also provides an important N source particularly in the recently cleared sites where N is limiting (Smith et al. 2011; Inglett et al. 2011). Liao and Inglett (2012) described the spatial pattern of periphyton N_2 fixation in two restored and one reference wetlands in this area. However, temporal dynamics of periphyton N_2 fixation is still lack of understanding. Therefore, the following questions were proposed in this study: (1) Does N_2 fixation vary with time in the subtropical, oligotrophic, calcareous prairie wetlands? (2) Are the seasonal patterns for the restored and native wetlands the same? and (3) What nutrient parameters control seasonal changes of periphyton N_2 fixation in these wetlands.

Materials and Methods

Study Site

Two restored wetlands (cleared in 2000 and 2003, and referred to as Res2000 and Res2003, respectively) as well as an unfarmed reference site adjacent to the restored areas were selected in the HID region of Everglades National Park (Fig. 4-1). In each site, two sampling stations with different elevation were identified (Fig. 4-1). The soil depth, vegetation composition, and the periphyton description were the same as the previous chapter.

Precipitation, water depth, and solar radiation for this location are shown in Fig. 4-2. The mean daily precipitation and temperature were obtained from the Royal Palm Ranger Station of South Florida published by National Oceanic and Atmospheric Administration (NOAA). Water depth was estimated by subtracting the water level from site elevation. The water level was provided by the Everglades National Park from a nearby station and corrected based on a common datum (NAD88). Seasonal levels of photosynthetically reactive radiation (PAR, $\mu\text{mol m}^{-2}\text{s}^{-1}$) were estimated by multiplying the

solar radiation ($W\ m^{-2}$) observed at the weather station in the Florida International University with conversion factor 1.93 in the winter and 1.95 in the summer (Lee and Downum 1991) (Fig. 4-2).

Field Sampling

The study sites were visited in the wet and dry season during 2010 and 2011 (April, May, June, July, and September in 2010 and January, May in 2011). Based on water levels and rainfall patterns, the seven sampling dates were classified into wet and dry season, where the dry season included the April, May, June 2010 and January in 2011, and the wet season included the July and September samplings.

A grid sampling system was used in this study as follows. Three 10×30 m plots were designated in each sampling station. Each of these plots was further divided into seventy-five 2 m×2 m sampling squares. At each sampling, three 2 m×2 m grid squares were randomly sampled within each 10×30 m plot and composited to form one replicate. Thus, for each sampling, nine 2 m×2 m sampling squares were composited to form three replicates. All samples were sealed in plastic bags and kept on ice until their return to the laboratory where the samples were refrigerated at 4°C until subsequent analysis.

Nitrogenase Analysis

Nitrogenase activity (N_2 fixation) was measured using the acetylene (C_2H_2) reduction (AR) assay described by Inglett et al. (2004). The details are the same as the previous chapter, also could referred to Liao and Inglett (2012).

When estimating the annual fixed N, I (1) used the periphyton biomass data from the previous study (February 2010, Liao and Inglett 2012) and assumed the biomass was constant with the season; (2) averaged the N_2 fixation rates from April, May,

January and June sampling as the N₂ fixation rate in the dry season (7 months), the rates from July and September sampling as the N₂ fixation rate in the wet season (5 months); (3) day length was considered as 12 hours of light and 12 hours dark.

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

Chemical and Isotopic Analysis

When surface water was present (e.g., in July and September), triplicate water samples from the sites were randomly collected, which were filtered through the 0.45 µm membrane, acidified, and stored on ice until their return to the laboratory.

Ammonium (NH₄⁺) and soluble reactive phosphorus (SRP) were measured by flow injection with a Bran+Luebbe Auto Analyzer 3 Digital Colorimeter (Bran+Luebbe, Norderstedt, Germany) for NH₄⁺ (EPA Method 350.1) and nitrate (NO₃⁻) was measured on a Alpkem Rapid Flow Analyzer 300 Series (Alpkem Corp., Clackamas, Oregon) using EPA Method 353.3. Total Kjeldahl nitrogen (TKN) was determined via Kjeldahl block digestion and analyzed by flow injection with a Bran+Luebbe Auto Analyzer 3 Digital Colorimeter for NH₄⁺ (EPA Method 350.1). Total dissolved phosphorus (TDP) was measured as SRP on a Bran+Luebbe Auto Analyzer 3 Digital Colorimeter after digestion with H₂SO₄ and potassium persulfate (EPA Method 365.1).

Periphyton total carbon (C) and N content were measured using Thermo Flash EA 1112 elemental analyzer (CE Elantech Inc., USA). Total P of periphyton was measured colorimetrically on a Shimadzu UV-160 spectrometer (Shimadzu Cor., Kyoto, Japan) using EPA method 365.1 following ashing and dissolution in 6M HCl (Anderson 1976). Loss-on-ignition (LOI) was obtained by combustion in the muffle furnace at 550°C for 4

h. 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 N₂ (0.3663‰) using delta notation (δ) as follows:

$$\delta^{15}N_{sample} = \frac{(^{15}N/^{14}N)_{sample} - (^{15}N/^{14}N)_{standard}}{(^{15}N/^{14}N)_{standard}} \times 1000.$$

Statistical Analysis

Data were analyzed with JMP v.8[®] statistical software (SAS Institute Inc., Cary, NC). Shapiro–Wilk test was used to test for normality, and data were log transformed when necessary. A three-way ANOVA was applied to analyze the main effects of site, elevation and time and their interactions on periphyton properties, followed by Tukey’s tests ($P < 0.05$) for means comparisons. Regressions between the nitrogenase activity and the nutrients of periphyton were performed using the linear least-squares method for the wet and dry season.

Results

Periphyton Properties

Results of the three-way ANOVA demonstrated that the factors of time and site had significant effects on almost all the periphyton parameters (Table 4-1), while elevation significantly affected TP, TN:TP molar ratio, δ¹⁵N and the N₂ fixation rates (Table 4-1). For some parameters, such as TP, TN:TP, and the N₂ fixation rates, there was a significant interaction between time, site and elevation (Table 4-1).

In all the samplings, TN of periphyton was significantly higher ($P < 0.05$) in the reference site, followed by the Res2000 site and the lowest TN values in the Res2003 site (Fig. 4-3). Converting concentration to an areal basis, TN storage in periphyton was

even higher at the reference site (mean of 15.6 g N m^{-2}) compared to either the Res2000 or Res2003 wetlands with an average of 8.0 g N m^{-2} and 6.4 g N m^{-2} , respectively (Fig. 4-6).

Both site and elevation were significant factors affecting TP and TN:TP ratio (Table 4-1). TP was 2–3 times higher in the restored sites than the reference sites, but the specific magnitudes and orders among the three sites were variable between the high and low elevation (Fig. 4-3). In the Res2000 site, significantly higher TP values were observed in the high ($181\text{--}532 \text{ mg kg}^{-1}$) compared to the low elevation ($75\text{--}171 \text{ mg kg}^{-1}$). However, no significant differences of TP were found between the high and low elevation in either of the Res2003 or reference sites. Accordingly, significantly lower TN:TP ratios of periphyton were found in the restored areas ($88\text{--}336$ in the 2003-restored site) compared to the reference site ($199\text{--}840$) ($P < 0.05$) (Fig. 4-3). The effect of elevation was significant only for the TN:TP molar ratio of the Res2000 site where significantly higher ratios were observed in the low elevation ($163\text{--}382$) than in the high elevation ($24\text{--}154$) ($P < 0.05$).

The stable N isotope ratios ($\delta^{15}\text{N}$) fell in the range of -1.9 to 0.9‰ among the three sites (Fig. 4-4). There were not consistent orders in magnitude among the three sites. During most of the sampling dates for all the three sites, the $\delta^{15}\text{N}$ was higher in the low elevation than the high elevation (Fig. 4-4).

Nitrogen Fixation

Nitrogen fixation rates measured by acetylene reduction (AR) for the periphyton in the reference site were significantly lower than the restored sites ($P < 0.05$); especially during the wet season (e.g., July), where periphyton AR rates under light condition were approximately 10 times that in the reference site (Fig. 4-5). Using the biomass

estimated from a previous study (Liao and Inglett, 2012) and the theoretical ratio of three moles of C_2H_4 produced per mole of N_2 fixed, it was estimated that approximately $0.4 \text{ g N m}^{-2} \text{ yr}^{-1}$ was fixed by the periphyton in the Res2003 site compared to $0.2 \text{ g N m}^{-2} \text{ yr}^{-1}$ in the reference site (Fig. 4-6).

Seasonal Patterns

In all sites, concentrations of major nutrients showed contrasting seasonal patterns during the study (Fig. 4-3). TN showed a general decrease throughout the summer from May to July followed by an increase from September to the next May in the Res2000 site and reference sites (Fig. 4-3). The seasonal patterns of TP and TN:TP molar ratio in the Res2000 and reference sites were similar and more apparent than the pattern observed in the Res2003 site (Fig. 4-3). The TP peak and minimum for the reference site appeared in September ($147 \pm 6 \text{ mg kg}^{-1}$) and April ($63 \pm 5 \text{ mg kg}^{-1}$). Accordingly, the TN:TP ratio showed in a inverse way with the peak in April (595 ± 56) and the minimum in September (221 ± 6). Similarly, for the Res2000 site, the TP varied from 165 ± 4 (September) to $91 \pm 5 \text{ mg kg}^{-1}$ (April) in the low elevation station while from 523 ± 9 (September) to $263 \pm 82 \text{ mg kg}^{-1}$ (July) in the high elevation station. Inversely, the peak and minimum of TN:TP ratio appeared in April (352 ± 11 and 112 ± 21 for the low and high elevation, respectively) and September (171 ± 6 for the low elevation and 43 ± 10 for the high elevation), respectively. In contrast, the TP and TN:TP ratio in the Res2003 site did not vary as much with season as in the other two sites, with the peak and minimum of TP in July ($192 \pm 31 \text{ mg kg}^{-1}$) and May ($118 \pm 11 \text{ mg kg}^{-1}$); and TN:TP ratios in April (228 ± 35) and July (144 ± 25).

Despite the appearance of similar trends, there was not a clear seasonal pattern of periphyton $\delta^{15}N$ in any of the three sites (Fig. 4-4). Overall, there was an increase from

the end of the dry period (i.e., April and May) into the beginning of the wet season (i.e., June). Values remained stable during the flooded time period of July and September, but did not return back to the initial level as a cycle in the next May 2011.

The seasonal patterns of N_2 fixation rates for the periphyton under both light and dark conditions (the dark condition is not shown) in all the three sites appeared to follow a unimodal curve (Fig. 4-5). The peak of periphyton AR rates in the Res2000 and Res2003 areas appeared in July within the range of 20–79 $\text{nmol g}^{-1}\text{dw h}^{-1}$ and 31–53 $\text{nmol g}^{-1}\text{dw h}^{-1}$, respectively. In contrast, the peak of periphyton AR rate at the reference site was observed in September with the range of 2–5 $\text{nmol g}^{-1}\text{dw h}^{-1}$.

Relationship between N_2 Fixation, TN, TP, TN:TP and $\delta^{15}\text{N}$

Throughout the seasonal sampling, there were strong correlations between nitrogenase activity and measured physical, chemical, and isotopic variables (Table 4-2, Fig.4-6). Periphyton moisture contents displayed a significantly positive relationship with the AR rates in the dry season ($R^2=0.5$, $P<0.0001$) but not in the wet season ($R^2=0.002$, $P=0.78$). Overall, nitrogenase activity was negatively related with TN:TP ratio, while correlations with other parameters were seasonal. For example, in the dry season there was no significant correlation between the TN content, but rates of AR were positively correlated with TP ($R^2= 0.25$, $P<0.05$) and negatively correlated with TN:TP molar ratio ($R^2= 0.27$, $P<0.05$) (Table 4-2). In contrast, for the wet season, TN had a significantly negative correlation with the N_2 fixation rates ($R^2= 0.42$, $P<0.05$), while TP did not ($R^2= 0.14$, $P=0.23$) and TN:TP molar ratio again showed a significant negative correlation with AR rate ($R^2= 0.62$, $P<0.05$) (Table 4-2). Overall, $\delta^{15}\text{N}$ had an inverse pattern of the AR rates through the three sites with higher AR rates and correspondingly lower $\delta^{15}\text{N}$

values in the restored sites (Fig. 4-6). Within a given site, however, periphyton $\delta^{15}\text{N}$ changed little (less than 0.5‰) despite an approximately 10 fold change in AR rates.

Discussion

Spatial and Seasonal Patterns of Periphyton N_2 Fixation

The site differences in nitrogenase activity agreed with previous studies conducted in this area (Liao and Inglett 2012) where higher N_2 fixation rates were found in the restored sites than in the reference site. I also found in this study that AR rates were often greater in the high elevation sites, but in July when the sites were flooded, the AR rates were higher in the low elevations sites (Fig. 4-5). Higher P levels in the high elevations (Fig. 4-3) could explain the greater AR rates in that location; however, in these shallow wetland systems, elevation is also a surrogate of hydrology (Smith et al. 2011) which could regulate periphyton productivity and nitrogenase activity through patterns of flooding and drought. The low elevation is more frequently inundated and the water depth tends to be higher than the high elevation station. In this regard, the light availability for the periphyton in the low elevation would decline more quickly with the deeper water depth than the high elevation (Hagerthey et al. 2011; Kahn and Wetzel 1999), leading to more flood-tolerant N_2 -fixers dominated in the low elevation stations which could fix N_2 more efficiently in the wet season.

I observed very pronounced seasonal patterns of N_2 fixation with overall higher N_2 fixation in the wet season (e.g., July and September) (Fig. 4-5). These patterns in the restored sites (Fig. 4-5) were similar with those observed in Water Conservation Area-2A (WCA-2A) of the northern Everglades (Inglett et al. 2004), in which the AR rates of the floating mats showed a peak in July and then dropped dramatically in September and continued the decline to a minimum in November. In contrast, for the reference site,

the nitrogenase pattern was much more gradual increasing to a later peak in September.

Considering the seasonality of N₂ fixation, the estimated areal annual N fixed by periphyton in this study was much higher with the average of 0.4 and 0.2 g N m⁻² yr⁻¹ compared to the previous estimate with the average of 0.1 and 0.05 g N m⁻² yr⁻¹ in the restored and reference sites, respectively (Liao and Inglett 2012). This study resulted in a larger estimate primarily because the previous study missed the dramatic high peak of nitrogenase activities observed in the early wet season.

A variety of factors have been used to explain patterns of nitrogenase activity in algal systems. Rejmánková et al. (2004) reported that N₂ fixation of periphyton in a tropical marsh of Belize was higher in the wet season (i.e., July and September), and they speculated that warmer temperatures and higher solar radiation facilitate N₂ fixation. Vargas and Novelo (2007) also found that the highest AR rates of periphyton appeared during the rainy season in the Yucatan peninsula. In the northern Everglades, Inglett et al. (2004, 2009) suggested that nutrient levels in combination with elevated water temperatures and patterns of exposure to UV light at the water surface could explain relative rates of N₂ fixation in floating periphyton mats.

In the extreme dry season (e.g., May and January), the periphyton in my study sites was more like the soil biological crusts in arid or semiarid ecosystems where water availability is highly influential on periphyton activity (Garcia-Pichel and Pringault 2001; Stradling et al. 2002; Belnap et al. 2004). In this study, moisture content explained approximately 50% of the variation in N₂ fixation rates ($R^2=0.51$, $P<0.0001$). Nitrogenase activity is absent during dry periods but recovers rapidly following

rehydration in the arid ecosystems (Evans and Johansen 1999; Jeffries et al. 1992; Rychert and Skujinš 1974; Skujinš and Klubek 1978). It was similar in this study with a dramatic pulse of AR rates in July after the dry season for the restored sites (Fig. 4-5).

In addition, light intensity could exert an influence in N₂ fixation by photosynthetically-driven N₂ fixers (Howarth et al. 1988b; Rejmánková and Komárková 2000). In contrast with other parts of the Everglades, the periphyton of this marl prairies was benthic. In September, light available for the inundated periphyton was decreased both as incident solar radiation and by the increase of water depth (more flooded than July) (Fig. 4-2). Less light penetrating to the benthos could explain the decline of AR rates after July.

Relationship of N₂ Fixation with Nutrient Status

The spatial pattern of periphyton TN and TP between the restored and reference sites agreed with a previous study conducted in this system (Liao and Inglett 2012). The significant effects of elevation on the TP and TN:TP ratios in the Res2000d site were unclear, however the enrichment of P in the Res2000 high station may be the result of this site being farmed more frequently throughout its history prior to restoration (Smith et al. 2011). In this study, periphyton TN:TP ratios were in a wide range from 24 to 840 and the values for the reference site were consistently higher than those in the restored sites, which may suggest that reference site would be strongly limited by P, while restored areas would be either N-limited or co-limited by N and P. In this regard, the result that periphyton in the restored sites with lower N content tend to fix more N would be an internal mechanism of overcoming N limitation (Scott et al. 2007). If ignored other N sources and the N loss from periphyton, to accumulate the similar amount N in the

reference site (15.6 g N m^{-2}), it would take 38.8 years for the Res2000 wetland and 37.7 years for the Res2003 wetland.

The seasonal pattern of TN was in contrast with the results reported by Scott et al. (2007). In their study, they found that TN was increasing throughout the summer (from May to July) before falling down in September. It was found that there was a gradual increase of TP after the dry period (i.e., April and May) with peaks appearing at different times for different sites (Fig. 4-3). This could be explained by the dry-rewetting theory summarized by Gottlieb et al. (2005) and Thomas et al. (2006) where it was found that after two days flooding, rapid recovery allowed the re-absorption of 90% of released TP from periphyton mats. In all of the three sites, an increase of TP in the wet season but time-delayed in the reference site and Res2000 site were observed, which would be the different recovery time and P-uptake and release rates in the different sites.

Furthermore, since the increase of TP could elevate the N_2 fixation as discussed before, the dry-rewetting regime could explain the increase of AR rates in the flooded July and September.

Nitrogenase activity will be stimulated by higher P and inhibited by excess N (Howarth et al. 1988a, 1990; Smith 1990; Rejmánková and Komárková 2000, 2001; Inglett et al. 2004, 2009). For this study, in the dry season with lower P and higher N content, periphyton nitrogenase activity was only significantly positively correlated with TP. In the wet season when P increased and N content declined, nitrogenase activity significantly correlated with N content instead (Table 4-2). Through both seasons, TN:TP ratio was significantly negatively correlated with nitrogenase activity (Table 4-2). It seems to indicate that during the development of periphyton, P would become the

primary control factor of N₂ fixation at the very beginning with lower nitrogenase activity; then with the time when fixed N is high (i.e., higher nitrogenase activity), the N₂ fixation tends to be inhibited by the availability of an alternative N source (Doyle and Fisher 1994; Crews et al. 2001ab), such as extractable inorganic N from attached soil mineralization.

Water column NO₃-N in this study was very low (1.0 to 28 µg L⁻¹) while dissolved NH₄-N was higher (11 µg L⁻¹ to 63 µg L⁻¹). However, Horne et al. (1972, 1979) found that planktonic N₂ fixation was regulated in part by NO₃⁻ and NH₄⁺ concentrations; heterocyst formation by cyanobacteria was suppressed at nitrate concentrations of 2.0–2.2 µg L⁻¹ and NH₄-N concentrations of 20–168 µg L⁻¹ (Horne et al. 1979). This may explain why N₂ fixation rates for the reference site did not reach the peak in July where the dissolved NO₃⁻ was significantly higher with the average of 15 ± 4 and 20 ± 4 µg L⁻¹ in the high and low elevation, respectively, compared to the two restored sites (Table 4-3).

Relationship of N₂ Fixation with δ¹⁵N

In general, N₂ fixation by cyanobacteria is accompanied by relatively little isotopic fractionation and as a result, N-fixing cyanobacteria should have δ¹⁵N close to 0 ‰ (Goericke et al. 1994; Kline and Lewin 1999). In this study, the δ¹⁵N of periphyton over all three sites fell within the range of -2‰ to 2‰ (Fig. 4-4), which agrees with observations from other studies (Nadelhoffer and Fry 1994; Gu and Alexander 1993; Rejmánková et al. 2004).

One source of evidence to substantiate a seasonal N₂ fixation pattern could be the seasonal trend of periphyton δ¹⁵N where it is widely regarded that relative increases in N₂ fixation coincide with lower values of δ¹⁵N (Rejmánková et al. 2004, Inglett et al.

2004, Scott et al. 2007). However, in this study, the $\delta^{15}\text{N}$ did not agree with the results from the northern Everglades (Inglett et al. 2004) where $\delta^{15}\text{N}$ had a significant seasonal pattern with a recorded maximum in January ($\delta^{15}\text{N}=2.7\text{‰}$) and minimum in July ($\delta^{15}\text{N}=1.1\text{‰}$). The ^{15}N depletion also did not correspond with the seasonal peak in nitrogenase activity, where in July, near maximum $\delta^{15}\text{N}$ coincided with the highest nitrogenase activity (Fig. 4-4).

As discussed before, $\delta^{15}\text{N}$ had an obvious inverse pattern with the N_2 fixation across the sites (Fig. 4-6), suggesting it is a good indicator of N_2 fixation for the different sites; however, it may not be as a reliable indicator through different season. These results were also supported by Scott et al. (2007) that metaphyton $\delta^{15}\text{N}$ decreased with increasing N_2 fixation during the May, July and September sampling at the Lake Waco Wetland complex, near Waco, Texas, US, but the correlation between these variables did not appear robust among months.

The possible explanation for this contradicted observation is that the N isotopic signatures would depend on other environmental factors. For example, MacLeod and Barton (1998) found that the isotopic signatures for N in samples of periphyton in a small headwater stream varied with light intensity and season. Kendall et al. (2001) observed a strong negative correlation between periphyton N isotopic composition and water depth. Inglett et al. (2004), however, found a positive relationship between $\delta^{15}\text{N}$ and water depth. The $\delta^{15}\text{N}$ values of inorganic N in the rainwater and the surroundings could also affect the final N stable isotopic signature of the periphyton (Scott et al. 2007), which would weaken the correlation with nitrogenase activity (Rolf et al. 2008).

Denitrification could also raise the $\delta^{15}\text{N}$ values of periphyton (Kendall and McDonnell 1998; Triska and Oremland 1981). Independent measurements of soil at these sites showed high denitrification potential in the July and September (unpublished data), which could enrich the N stable isotope signature of available N and weaken the correlation of the $\delta^{15}\text{N}$ with nitrogenase activity. Hagerthey et al. (2011) also emphasized that although nitrification and denitrification has not been directly measured, they cannot be easily discounted because anoxia does occur in periphyton. In addition, the final $\delta^{15}\text{N}$ of periphyton is an integration of N isotopic signatures of all N sources, thus the seasonal changes of other components attached with periphyton, such as soils and plants, could also affect the $\delta^{15}\text{N}$ natural abundance (Handley and Raven 1992; Nadelhoffer and Fry 1994; Inglett and Reddy 2006).

Conclusions

Periphyton has important ecological functions in the Everglades, especially through N_2 fixation which is considered as a major part in the N budget (Wozniak et al. 2008; Inglett et al. 2011). Of the two main periphyton habitats in the Florida Everglades, few records on the spatial and temporal pattern of periphyton N_2 fixation exist in the short-hydroperiod wetland ecosystem, which responds more rapidly to the dry-rewetting regime and is therefore highly seasonal (Gaiser et al. 2011). This study focused on the seasonal pattern of nitrogenase activity of periphyton in native and restored marl prairie wetlands of the Hole-in-the Donut region of the Southern Everglades.

Similar seasonal patterns of N_2 fixation were observed among the natural and restored sites, with higher rates in the wet season, especially in the flooded period from July through September. The peak of N_2 fixation differed between native and sites restored by complete soil removal. In native reference areas, the seasonal pattern was

a gradual increase and decrease throughout the year with a peak observed in September, while most N₂ fixation occurred during a brief 2–3 month period at the onset of flooding and a peak in July for the two restored sites. Calculated annual rates from the seasonal patterns were higher compared to the previous results (Liao and Inglett 2012) when real peaks were included in this study.

In this study, N₂ fixation was controlled by both physical (moisture) and chemical (N and P) parameters. Dry season rates were a function of both moisture content (hydrating periphyton) and P content, while during flooded months excess N levels appeared to suppress nitrogenase activity. Unlike other studies, seasonal patterns of periphyton $\delta^{15}\text{N}$ did not strictly correlate with N₂ fixation. In general, $\delta^{15}\text{N}$ in the three sites showed inverse pattern of the N₂ fixation rates, but the negative relationship was much weaker in the wet season (e.g., July and September). It is suggested that $\delta^{15}\text{N}$ could be a good indicator of the relative N₂ fixation between sites, but would not be as robust in determining seasonal N₂ fixation since the final $\delta^{15}\text{N}$ values of periphyton integrate a variety of N sources and processes (e.g., denitrification).

Results of this study have important implications for the N budget of the HID wetlands and other wetlands with extreme seasonal dry/wet cycles. The dynamic interaction between flooding, nutrient availability, and periphyton N₂ fixation warrant further study in light of N cycling in both native and restoring systems. More work is also needed to relate seasonal patterns of species composition to those observed for nutrients and nitrogenase activity in this study. Understanding the seasonal dynamics of these parameters can help improve the models for N dynamics and our understanding

of the role of periphyton in Southern Everglades marshes and other similar wet prairie wetlands.

Table 4-1. Summary of three-way ANOVA tests for main effects and interactions of time, site, and elevation on periphyton parameters: loss of ignition (LOI, %), total nitrogen (TN, g kg⁻¹), total phosphorus (TP, mg kg⁻¹), TN:TP molar ratio, N stable isotopic signature ($\delta^{15}\text{N}$, ‰), N₂ fixation measured by acetylene reduction rates under light condition (AR_light, nmol C₂H₄ g⁻¹ dw⁻¹ hr⁻¹) and the ratios of AR under light and dark conditions (AR_L:D). *-*P*<0.05; **-*P*<0.01, ***-*P*<0.001, NS-no significant difference.

Source	LOI	TN	TP	TN:TP	$\delta^{15}\text{N}$	AR_light	AR_L:D
time	**	*	*	**	**	**	NS
site	**	**	**	**	**	**	**
elevation	NS	NS	**	*	**	*	NS
timexsite	**	NS	NS	*	NS	**	NS
timexelevation	NS	NS	NS	NS	NS	**	*
elevationxsite	NS	NS	**	**	NS	NS	NS
timexsitexelevation	NS	NS	NS	NS	*	NS	NS

Table 4-2. Mean water chemistry values observed at the two restored wetlands (Res2000 and Res2003) and the native reference wetland (Reference) in different elevation. during the wet flooded sampling time (July and September, 2010). Data were represented as the mean (standard error). DOC-dissolved organic carbon, TDP-total dissolved phosphorus, TDKN-total dissolved Kjeldahl nitrogen. N=3.

Site	Elevation	DOC mg L ⁻¹	NH ₄ -N µg L ⁻¹	NO ₃ -N µg L ⁻¹	TDP µg L ⁻¹	TDKN mg L ⁻¹	TDKN:TDP mol:mol
July, 2010							
Res2000	High	9.5 (1.9)	41 (7)	4.2 (0.1)	4.4 (0.4)	0.7 (0.1)	437 (43)
	Low	14 (0.4)	42 (2)	5.1 (0.3)	4.4 (0.3)	1.4 (0.0)	696 (40)
Res2003	High	5.3 (0.5)	19 (0)	5.4 (0.9)	3.9 (2.0)	0.5 (0.1)	541 (26)
	Low	7.8 (0.2)	25 (1)	5.9 (0.2)	4.4 (1.8)	0.6 (0.0)	542 (104)
Reference	High	5.8 (2.0)	46 (9)	15 (4.3)	4.0 (1.0)	0.9 (0.3)	464 (134)
	Low	7.4 (0.9)	34 (4)	20 (4.1)	1.8 (0.2)	0.9 (0.1)	1124 (206)
Sep., 2010							
Res2000	High	4.2 (0.2)	12 (1)	1.0 (-)	3.4 (0.4)	0.8 (0.0)	516 (27)
	Low	3.6 (0.2)	13 (1)	2.1 (-)	5.9 (2.5)	0.8 (0.1)	506 (8)
Res2003	High	2.9 (0.1)	19 (5)	4.8 (0.3)	2.9 (0.1)	0.6 (0.1)	475 (36)
	Low	4.4 (0.6)	20 (9)	4.8 (0.4)	4.4 (0.9)	0.8 (0.0)	436 (73)
Reference	High	5.3 (0.2)	16 (0)	1.2 (0.2)	7.1 (0.4)	0.9 (0.0)	267 (17)
	Low	4.4 (0.5)	21 (0)	1.8 (0.4)	5.9 (0.9)	1.0 (0.2)	386 (44)

Table 4-3. Correlation between the N₂ fixation measured by acetylene reduction (AR) rates and total nitrogen (TN), total phosphorus (TP) and TN:TP molar ratio in the wet (right side, N=22) and dry season (left side, N=12). The AR rates satisfied the normalization after the log transformation (Y=log (AR)). The wet season included the July and September sampling results and the dry season included the April, May, June and January sampling results.

Parameters (X)	Dry season	Wet season
TN	$Y=0.03X+0.8, R^2=0.01, P=0.59$	$Y=-0.7X+10, R^2=0.42, P=0.02$
TP	$Y=0.002X+0.1, R^2=0.25, P=0.02$	$Y=0.005X+1.3, R^2=0.14, P=0.23$
TN:TP molar ratio	$Y=0.001X+0.9, R^2=0.27, P=0.01$	$Y=-0.02X+4.2, R^2=0.62, P=0.003$

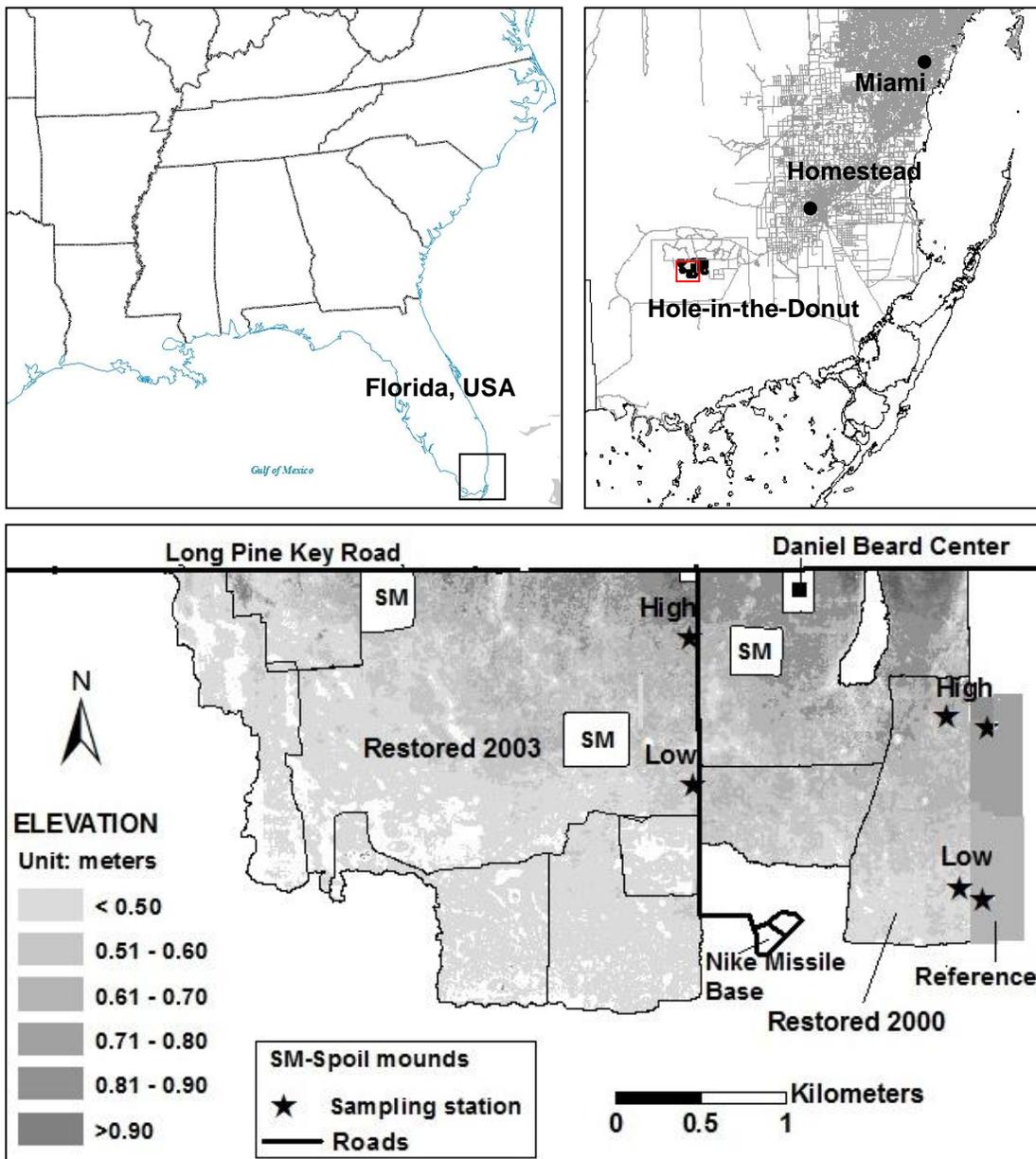


Figure 4-1. Location of sampling sites in the two restored wetlands (restored in 2000 and 2003) and a native reference wetland of Hole-in-the-Donut in the Florida Everglades, US.

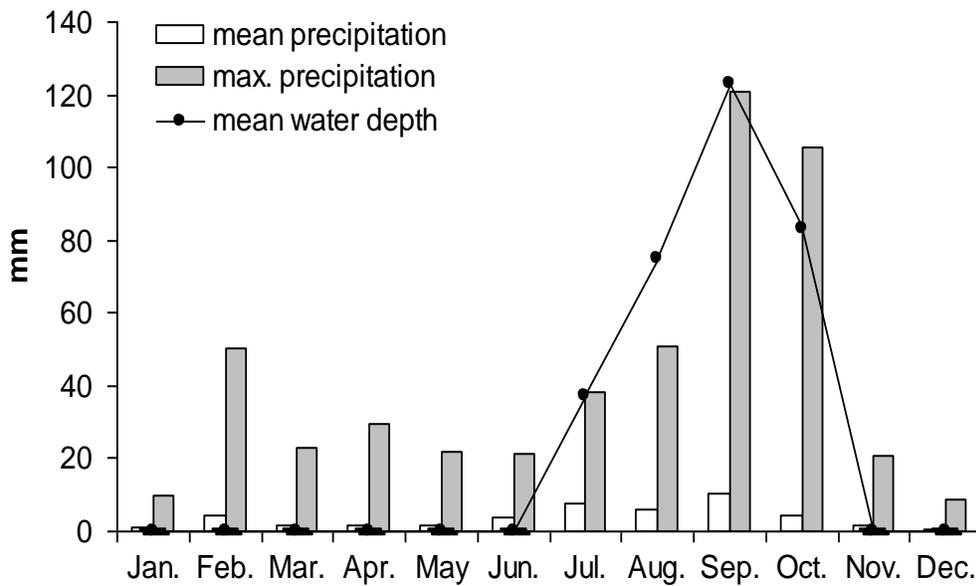
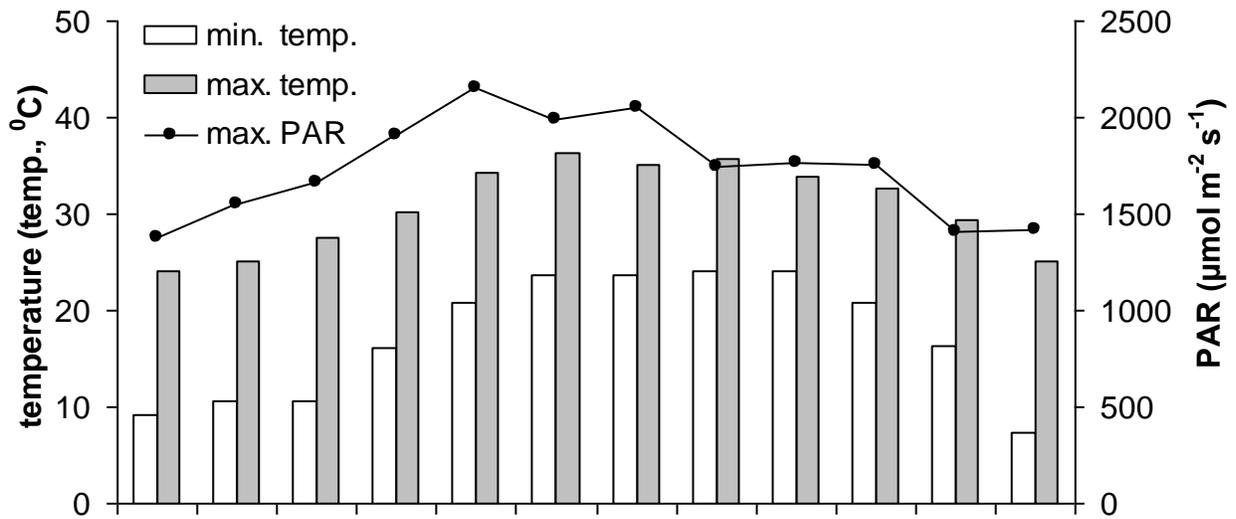


Figure 4-2. Mean maximum (max.) and minimum (min.) daily temperature (temp, °C) and mean maximum daily photosynthetically active radiation (PAR, $\mu\text{mol m}^{-2} \text{s}^{-1}$), mean water depth (WD, mm) (from Jan. to Jun., Nov. to Dec., the water depth was negative values), and the daily mean and maximum precipitation for the study site in 2010. Date source in details seen in the text.

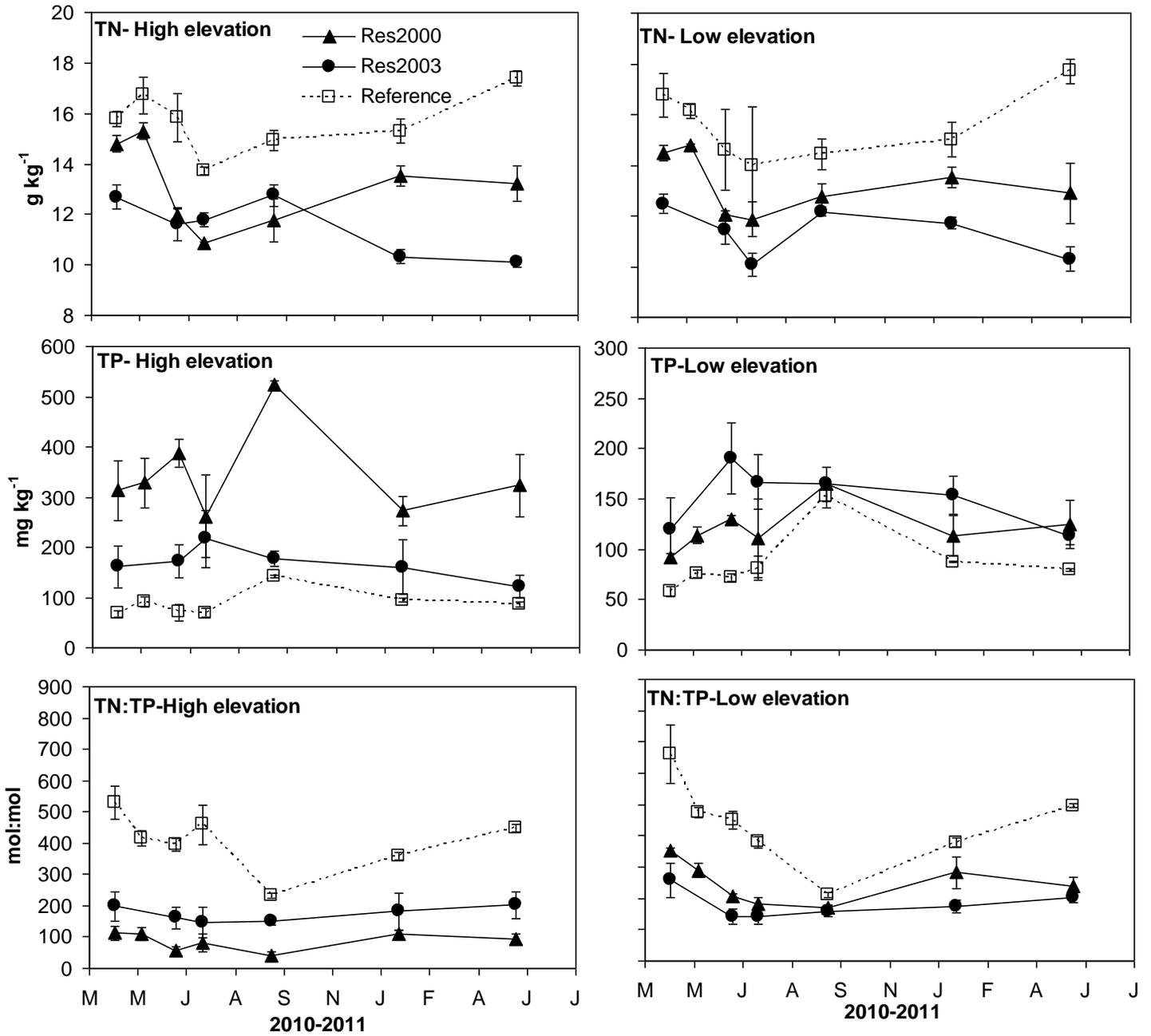


Figure 4-3. Seasonal patterns of periphyton nutrient in the two restored wetlands (Res2000 and Res2003) and the native reference wetland (Reference) during 2010 and 2011. Elevation factor has a significant effects on the total phosphorus (TP) and the TN:TP molar ratio, so the seasonal patterns of TP and TN:TP were separate from the high and low elevation.

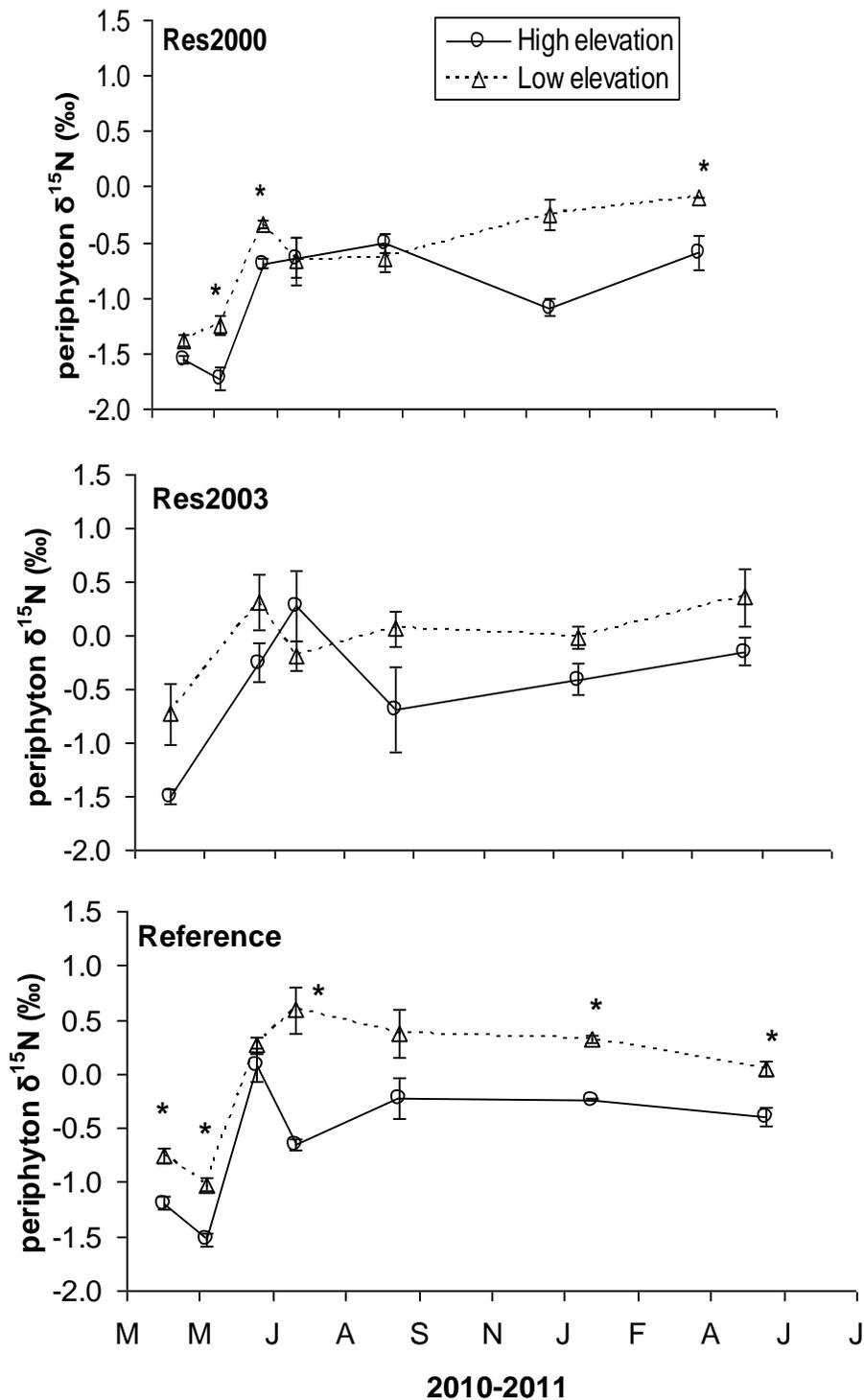


Figure 4-4. Seasonal changes of periphyton $\delta^{15}\text{N}$ (‰) in the two restored wetlands (Res2000 and Res2003) and the native reference wetland (Reference) in different elevation. (* denotes significant elevation difference, $P < 0.05$).

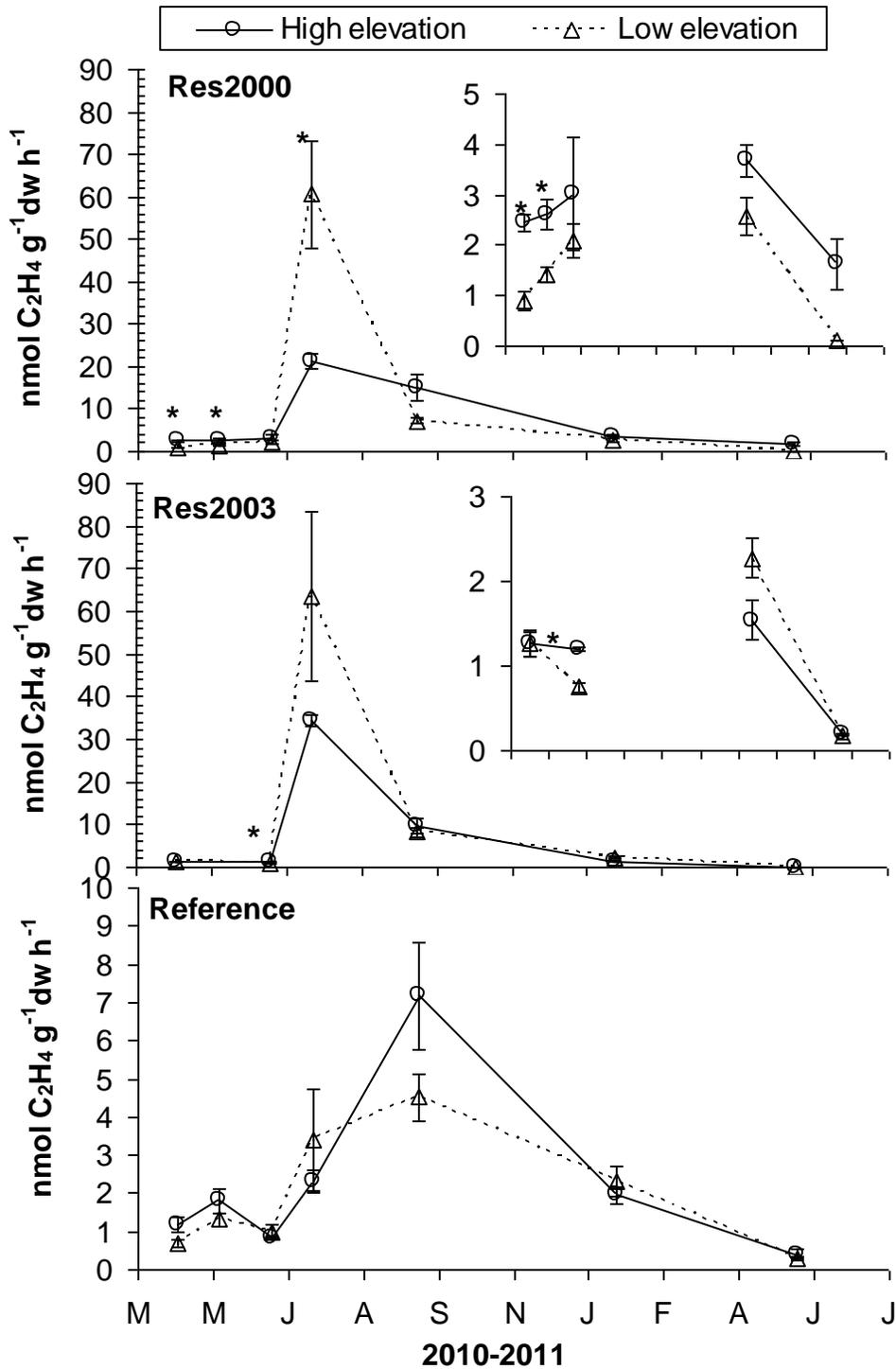


Figure 4-5. Seasonal changes of periphyton acetylene reduction (AR) rates under light condition in the two restored wetlands (Res2000 and Res2003) and the native reference wetland (Reference) in different elevation. (* denotes significant elevation difference, $P < 0.05$)

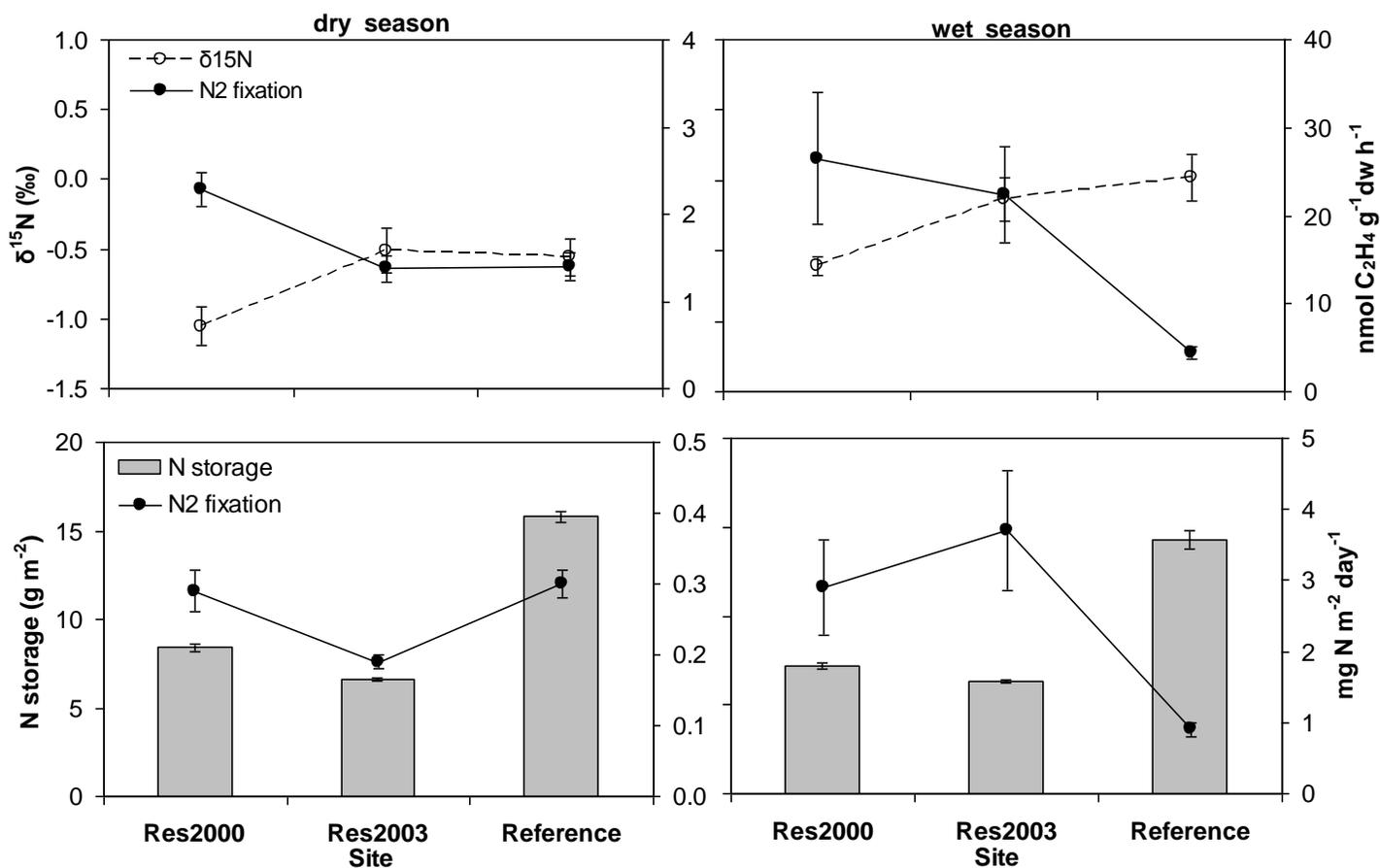


Figure 4-6. Nitrogen fixation measured by acetylene reduction rates (AR) ($\text{nmol C}_2\text{H}_4 \text{ g}^{-1} \text{ dw h}^{-1}$), estimated fixed nitrogen per day using the 3:1 ratio and assuming 12 hours light condition and 12h dark condition ($\text{mg N m}^{-2} \text{ day}^{-1}$), nitrogen storage (g N m^{-2}), and nitrogen stable isotopic signature of periphyton in the dry (including the April, May, June and January, on the left side) and wet (including the July and September, on the right side) season for the Res2000, Res2003 and reference site. Error bars represent standard error (S.E.).

CHAPTER 5
SEASONAL DYNAMICS OF SOIL NITROGEN PROCESSING DURING THE
RESTORATION IN SHORT-HYDROPERIOD, CALCAREOUS WETLANDS

Background

Nitrogen (N) and phosphorus (P) are critical for primary production (Hecky and Kilham 1988; Elser et al. 1990; Vitousek and Howarth 1991). Compared to P cycle, the N cycle is more complex especially in wetlands including a diversity of transformations (e.g., ammonification, N mineralization, nitrification and denitrification) and numerous forms ranging from particulate and dissolved organic to dissolved inorganic and gaseous species. The extractable ammonium (NH_4^+) and nitrate (NO_3^-) as the products of N mineralization can be adsorbed by the plants and thus enhance their primary production. Microbial groups actively involve in these processes and can decompose complex and refractory organic matters into available N by releasing enzymes. Denitrification is the major pathway leading to the N loss and potentially contributes to the greenhouse gas emission (i.e., the intermediate product N_2O).

Accordingly, a variety of factors regulate N cycling such as temperature, moisture content, plant growth. Spatial and temporal variations of these factors and their interactions often lead to the high variability of soil N cycle. For example, available N to plants (NH_4^+ and NO_3^-) tend to pulse after a rain event (Cain et al. 1999), and are taken up and depleted by plants (Evans et al. 2001; Giese et al. 2011). Temporal pattern of soil N cycle can simultaneously reflect the effects of temperature, moisture contents and plant growth (Parker and Schimel 2011), thus it is important and necessary to understand the temporal or seasonal patterns of N cycle.

In addition, the temporal patterns of N cycle vary with different climate regime, soil types and landscapes. The seasonal pattern in Mediterranean-type climate of California

grasslands with wet-and-cool winter and hot-and-dry summer would be expected to greatly differ from that found in other grassland systems (Parker and Schimel 2011). The seasonal pattern in the tropic or subtropical wetlands would be driven more by precipitation rather than temperature in the northern peat wetlands. The acidic and calcareous soil would also show a distinct seasonal trend of availability of N (Taylor et al. 1982; Van Hoeyk et al. 2000). Many of the N processes are governed by oxidation-reduction reactions, which in wetlands are mainly caused by hydrologic fluctuations.

Specifically for the Florida Everglades wetland ecosystem, little has been done on the N cycle compared to P (White and Reddy 1999, 2003; Sutula et al. 2001; Inglett et al. 2004, 2006, 2009). However, an understanding of N dynamics could be crucial to understanding the spread of P impacts in the Everglades systems (Inglett et al. 2011). For example, White and Reddy (2000) found that the increase of potentially mineralized nitrogen (PMN) was linked to the P enrichment in the northern Everglades. Newman et al. (2001) further confirmed their results and concluded P enrichment could enhance soil porewater NH_4^+ regeneration. N_2 fixation, an important function of periphyton, is a significant N source in Everglades (Craft et al. 1995) and was reported to be stimulated by P (Inglett et al. 2004, 2009).

Moreover, the diversity of hydrology across the Everglades featured a variety of spatial and temporal patterns of N cycle. Most existing researches on N cycle has focused on the long hydroperiod wetlands (e.g., northern marshes) that remain flooded for more than 8 months of the year (White and Reddy 2000, 2003; Inglett et al. 2004, 2006). Comparatively less attention has been paid on the short hydroperiod wetlands that remain inundated for less than 8 months of the year (e.g., southern marl and

calcareous wetlands) (Gottlieb et al. 2005; Gaiser et al. 2011; Sutula et al. 2001). Those short-hydroperiod, marl/calcareous wetlands undergo more pronounced dry-rewetting periodic cycles that is directly pulsed by seasonal changes of rainfall (Ewe et al. 2006), which would lead to distinctive seasonal patterns of N cycle. However, little is known about importance of these processes.

For those reasons, three calcareous wetlands with short-hydroperiod, in southern Everglades of Florida, i.e., two restored wetlands with high P and one native reference wetlands with low P, were selected to investigate the seasonal patterns of soil N cycling. The following questions were proposed: (1) how do soil N availability, the internal processes and microbial activities vary with the time? (2) Do the temporal patterns of N cycle differ between the restored and reference wetlands? I measured extractable inorganic N pools, potentially mineralizable nitrogen (PMN), potential rates of denitrification, microbial biomass carbon and nitrogen (MBC and MBN), and N-related enzymes seven times during 2010 to 2011. I expected that during the wet season when soils are moist and microbes are active, N processing rates and microbial activities would be higher. Under the influence of different P concentration, the restored and reference sites would show some differences in the seasonal patterns.

Methods and Material

Study Site

The climate of the south Florida mainland is sub-tropical with a rainy season that extends from the end of May to October followed by a pronounced dry season from November to most of May (Ross et al. 2006). Plant growth also exhibit different seasonal patterns. The study site was located in the Hole-in-the-Donut (HID) in the Everglades National Park (ENP) which had a long history of farming (Smith et al. 2011).

Disturbance and excess nutrients by the agricultural activities led to the invasion of Brazilian pepper (*Schinus terebinthifolius*) after farming ceased (Li and Norland 2001). To restore the HID to marl prairie ecosystem, complete soil removal that is removing all the vegetation and soil down to the bedrocks was adopted (Dalrymple et al. 2003). During the soil development, the young restored sites would be more N limited with high P while the reference site tend to limited by P (Smith et al. 2011).

I selected two wetlands restored in 2000 and 2003 (referred to as Res00 and Res03) as well as an unfarmed reference site adjacent to the restored areas in the Hole-in-the-Donut region of Everglades National Park (Fig. 4-1). The description of the study sites including the vegetation, and soil depth were the same as the Chapter 2.

Field Sampling

This section is the same as the Chapter 4

Soil Physical and Chemical Analysis

Soil samples were sieved to remove roots and rock fragments greater than 2 mm diameter. Sieved soil samples were used in determination of all microbial and enzyme related parameters, while a subsample of sieved soil was oven dried at 105°C for 3 days and ground using a mortar and pestle for moisture content and total nutrient determinations.

The measurement of Loss-on-ignition (LOI), total carbon (TC), total nitrogen (TN), total phosphorus (TP), extractable ammonium (NH₄-N) and nitrite/nitrate (NO_x-N), microbial biomass C and N (MBC and MBN) follow the same methods as described in the Chapter 2.

To measure soil potential mineralizable nitrogen (PMN), a procedure modified from White and Reddy (2000) was used. The soil was incubated under anaerobic

condition at 40°C for 10 days, followed by KCl extraction, and then the NH₄-N was determined colorimetrically using a Technicon™ Autoanalyzer (EPA 350.1, 1993). On the same day as the preparation of the incubated samples, time zero control samples were extracted and the NH₄-N was measured in the same way. The PMN was calculated as the difference between the final and time zero NH₄-N content.

Denitrification Enzyme Activities

Denitrification enzyme assay (DEA) was modified from Smith and Tiedje (1979), using the acetylene block technique (yielding N₂O production) and adding sufficient NO₃⁻ and carbon source. Triplicate, 5 g wet soil samples were sealed with rubber septa stoppers in a 30ml tube; 3ml distilled deionized (DDI) water were added to the tube and purged with N₂ to maintain anaerobic conditions. 2 ml acetylene gas (C₂H₂, approximately 10% headspace) was injected to the tubes to block the conversion from N₂O to N₂, and the tubes were then shaken for 1h to disperse the gas. After shaking, DEA solution (0.202 g KNO₃ L⁻¹, 0.25 g chloramphenicol L⁻¹, and 0.360 g C₆H₁₂O₆ L⁻¹) purged with oxygen free-N₂ gas was added to the tubes. Samples were shaken and incubated at room temperature. Headspace gas was collected at 1h interval for about 5 hours. The potential denitrification rate was calculated from the steepest portion of curve produced when cumulative N₂O evolution was plotted against time. The sampled gas was injected in a Shimadzu GC-14-A ECD gas chromatograph equipped with an electron capture detector (ECD) and Porapak Q packed column. The operation temperatures for the column, injection port, and detector were set at 70, 120, and 230 °C respectively. A 10 ppm standard concentration gas (Scott Specialty Gases, Inc., Plumsteadville, PA) was used to calibrate the measurement, and results were reported as N₂O-N produced per gram of dry weight soil per hour.

Extracellular Enzyme Activities

Two N-related enzymes, i.e., N-acetyl- β -D-glucosaminidase (NAG, EC 3.2.1.30) and Leucine aminopeptidase (LAP, EC 3.4.11.1), were measured using fluorogenic enzyme substrate (Hoppe 1983). Methods were modified from Sinsabaugh et al. (1997) to optimize the substrate concentrations in soil samples with fluorogenic substrates 4-Methylumbelliferyl N-acetyl- β -D-glucosaminide (MUF-N) for NAG, and L-leucine 7-amido-4-methyl coumarin (AMC) for LAP. Enzyme substrates MUB-N and AMC produce fluorochrome methylumbelliferone (MUF) and aminomethylcoumarin (AMC) once they are hydrolyzed by the enzyme NAGase and LAPase respectively. The fluorescence of samples and standards was measured at excitation of 350nm and emission of 450nm using a Bio-Tek Model FL600 fluorometric plate reader (Bio-Tek Instruments, Inc. Winooski, VT). The potential enzyme activity was expressed as $\mu\text{mols MUF or AMC released g}^{-1}\text{dw h}^{-1}$.

Statistical Analysis

Data were analyzed with JMP v.8[®] statistical software (SAS Institute Inc., Cary, NC). A Three-way ANOVA was applied to analyze the main effects of site, elevation and time and their interactions on soil properties, followed by Tukey's tests ($P < 0.05$). When there were significant site and date interactions, separate one-way analyses for the different sites and dates were performed. All results are reported as significant when $P < 0.05$. Multivariate analysis with Pearson product-moment correlation coefficients was used to evaluate the relationship between different soil properties. Data were log transformed when necessary to improve normality.

Results

Spatial Patterns of Soil Properties

All the three sites have alkaline soil with pH ranging from 7.4 to 8.5 (Fig. 5-1). In general, pH at the reference site was significantly higher than the restored sites. The soil moisture contents were significantly lower at the reference site compared to the restored sites (Fig. 5-1, $P < 0.05$). Interestingly, the total nitrogen (TN) was higher at the Res00 site compared to the reference and Res03 sites. During all the samplings, the total phosphorus (TP) at the reference site was significantly lower within the range from 93 to 199 mg kg⁻¹ compared to the Res00 site from 256 to 888 mg kg⁻¹ and the Res03 site from 377 to 905 mg kg⁻¹ ($P < 0.05$). Accordingly, the TN:TP molar ratios at the reference site were significantly higher than the values in the restored sites ($P < 0.05$). In addition, the TOC:TN molar ratios at the three sites were all below 25 with significantly higher values at the restored sites compared to the reference site ($P < 0.05$).

In general, the extractable NO_x-N and extractable NH₄-N was significantly higher at the restored sites compared to the reference site ($P < 0.05$, Fig. 5-2). Patterns of the MBC and MBN at the three sites was consistent with Res00 site the highest and the reference site lowest; while the MBC:MBN ratio was significantly higher at the reference site than those at the restored sites ($P < 0.05$, Fig. 5-3). Overall, significantly higher PMN was found at the restored sites than the reference site ($P < 0.05$, Fig. 5-4).

Significantly higher potential denitrification enzyme activity rates were found at the restored sites compared to the reference site ($P < 0.05$) during all the sampling times (Fig. 5-5). The two N-related enzymes (LAP and NAG) were significantly higher at the restored sites than the values at the reference site during most of the sampling times ($P < 0.05$, Fig. 5-6).

Seasonal Patterns of Soil Properties

The total nutrients did not vary significantly with time (Fig. 5-1). Soil moisture contents showed a pronounced seasonal pattern at all the three sites with higher values in the wet season (i.e., June, July and September) than those in the dry season, especially in the May 2011, the soil was extremely dry falling down to 10%-20% moisture content (Fig. 5-1).

The seasonal pattern of extractable $\text{NO}_x\text{-N}$ was inverse with $\text{NH}_4\text{-N}$ that is higher values of $\text{NO}_x\text{-N}$ appeared in the dry season while higher values of the extractable $\text{NH}_4\text{-N}$ occurred in the wet season (Fig. 5-2). The dominant inorganic N form in the HID was $\text{NH}_4\text{-N}$ indicated by high $\text{NH}_4\text{-N}:\text{NO}_x\text{-N}$ ratio in most sampling times. However, in the extreme dry sampling time (May 2011), the extractable $\text{NO}_x\text{-N}$ exceeded $\text{NH}_4\text{-N}$ concentration, and higher PMN was observed in the dry season or the spring rather than the wet season (Fig. 5-4).

The two restored sites demonstrated more similar seasonal patterns and different from the pattern at the reference site. Interestingly, the peaks of the MBC and MBN for the reference site both appeared in the September while for the restored site, the peaks appeared in June or July and January. The ratio of MBC:MBN dropped to the minimum values in July.

The LAP at the three sites showed similar seasonal patterns with significantly higher values in September ($P < 0.05$, Fig. 5-5). There were no consistent seasonal trends of NAG among the three sites. For the restored sites, the NAG activities dropped to the lowest point in July and then increased to the highest point in September; but there were not apparent seasonal changes for the reference site compared to the restored sites.

There was a pronounced seasonal pattern for DEA at all the three sites with higher denitrification rate in the wet season and the peak appearing in July. The DEA in the May, 2011 was unexpectedly higher comparing with other sampling time in the dry season.

Discussion

Seasonal Pattern of Available Nitrogen

In this study, $\text{NH}_4\text{-N}$ concentration was consistently higher than the $\text{NO}_x\text{-N}$ in most of the sampling dates. The $\text{NH}_4\text{-N}$ showed inverse seasonal patterns as $\text{NO}_x\text{-N}$, which may contribute to the different properties of the two ions. NH_4^+ tends to bind to the soil's negatively-charged cation exchange complex; while NO_x^- does not bind to the soil solids because of its negative charges. NO_x^- is usually more subject to loss than is NH_4^+ . Significant loss mechanisms include leaching and denitrification. The NO_x^- is so soluble that it leaches easily when excess water percolates through the soil. In this regard, very low $\text{NO}_x\text{-N}$ was measured in the wet season with abundant rainfall in this study (Fig. 5-2). High $\text{NO}_x\text{-N}$ accumulations during the extreme dry May 2011 was explained by reduced ion mobility in thin water films of dry soils (Giese et al. 2011) or as a result of high net nitrification (Hefting et al. 2004).

Different seasonal patterns of available inorganic N and N mineralization rates were observed in different ecosystems. Jackson et al. (1998) observed that since plant accumulation of N was mainly confined to two short periods of the year: fall and early spring in California annual grassland, the soil nitrate was low or depleted. However, peak values of available N and mineralization rates were reported in spring and autumn in different British grasslands (Morecroft et al. 1992). Taylor et al. (1982) showed mineralization rates and instant inorganic N concentrations were maximum in late winter

or early spring in an acidic and calcareous soil; and further concluded from other researches that overall, the spring peak in N mineralization has been observed by many workers. They attribute the high N availability in spring to the 'partial sterilization' effect of the winter climate where protein substrate accumulates from the death of soil organisms and can be utilized when warmer conditions allow the development of the appropriate microbial populations. The decline in the N mineralization rates during late spring and early summer may be due to the lowest soil water contents.

In this study, N mineralization did not exhibit a pronounced seasonal pattern but lower rates were observed in the summer period (wet season) and increased in the end of the winter (January) and the spring (April). However, in the summer, the moisture contents were not low; the 'partial sterilization' theory otherwise would not be possible to explain the pattern in this study since the Florida winter was still warm with the mean daily temperature dropping to 15°C (grey line at the bottom of Fig. 5-3). The results here contradict the results reported by Sing and Kashyap (2007) that during an annual cycle the maximum N-mineralization rates were recorded in the rainy season in a seasonally dry tropical forest and savanna ecosystems in Vindhyan region, India. However, as they suggested, the variations in rates are related to differences in soil moisture content, nutrient status and vegetation cover in combination with other environmental factors.

In addition, since TP and TN:TP ratio did not change significantly with the time, it is unlikely that the TP or TN:TP ratio would indirectly affect the seasonal patterns of potentially mineralization even though White and Reddy (2000) pointed out that high P could enhance the N mineralization in a northern Everglades ecosystem.

Seasonal Patterns of Microbial Biomass Carbon and Nitrogen

Microbial biomass is the labile portion of the organic fraction in soils and serves as both an important source of and sink for plant available nutrients (Jenkinson and Ladd 1981; Garcia and Rice 1994).

Moisture condition is a major factor controlling survival and activity of microorganisms in the soil. The results showed different seasonal patterns between the restored sites and reference site. Generally, at the reference site the seasonal patterns of MBC and MBN corresponded very closely with the patterns of soil moisture content increasing across the wet season from April to September with the peak appearing in the September (Fig. 5-1 and Fig. 5-3). However, for the restored sites, when the soil moisture content increased over 60% in July and September, the MBC and MBN showed a sudden drop especially for the Res03 site (Fig. 5-3). This contrasting results between the reference and restored sites would suggest that adequate soil moisture increases microbial biomass and activity whereas beyond some point (e.g., field capacity), microbial activity decreases with increasing moisture, due to limited oxygen availability (Killham 1994). My results also supported the review by Wardle (1998) who selected 58 published studies on the temporal patterns of soil MBC and MBN to generalize the controls of the temporal variability. In the review, it was concluded that the microbial biomass peaks appeared in different seasons and showed both positive and negative responses to temporal patterns of soil moisture.

The MBC:MBN ratio is considered to be an indicator of the microbial composition that is bacteria have low MBC:MBN ratio than fungi (Anderson and Domsch 1980). Enhanced root growth and increased root exudates are thought to stimulate bacterial growth (Clarholm 1985), which have a low C:N ratio. In this study, a decreasing

microbial C:N ratio from spring to summer was observed (Fig. 5-2), which agree with the results reported by Corre et al. (2002). As vegetation expanded from spring towards summer, root exudates might have also increased and favored the growth of bacteria.

Seasonal Pattern of Extracellular Enzyme Activities

Soil enzymes play an essential role in catalyzing reactions necessary for organic matter decomposition and nutrient cycling and are often used as indices of microbial activity and soil fertility (Dick 1994; Tabatabai 1994; Ajwa et al. 1999). The two N-acquisition enzymes measured in this study, i.e., LAP and NAG, are inducible enzymes synthesized or activated when needed. Chróst (1991) found the activities of LAP were induced at limited N availability. NAG involved in the degradation of chitin which is a relevant source of N in soils. The results in this study showed that significantly higher N-related enzyme activities were found at the restored sites, indicating more N-limitation at those sites (Smith et al. 2011).

Various factors affect the enzyme activities such as the temperature, moisture, and nutrient demand and availability (Tabatabai 1994). Seasonality of enzyme activity can be understood as a driver that integrates simultaneous variance in those factors (Weedon et al. 2011). Studies that measured seasonal variation in potential enzyme activities have typically found large intra-annual differences (Saiya-Cork et al. 2002; Boerner et al. 2005; Wallenstein et al. 2009; Kluber et al. 2011). For example, in the Arctic and sub-Arctic environments, the highest enzyme activities were observed around the spring thaw which would be contributed to their temperature sensitivity (Wallenstein et al. 2009). However, in this study sites, the temperature seems not play that significant role in controlling the enzyme activities.

The key to understanding seasonality in enzyme activity may be in the factors that regulate various systems (Boerner et al. 2005; Weedon et al. 2011). Chitinase (NAG) and acid phosphatase were reported to be regulated by primarily microclimate and soil chemical factors, whereas lignocellulose degrading enzymes such as glucosidase and phenol oxidase are more regulated by substrate availability (Sinsabaugh et al. 1992, 1993). In this study, LAP was significantly correlated with moisture content ($r=0.64$, $P<0.001$) but did not correlate with LOI (a surrogate of organic matter) ($r=0.19$). In contrast, the seasonal pattern of NAG did not exactly follow the soil moisture content and even showed lowest values in the very wet July (Fig. 5-5). In this regard, NAG activities were significantly correlated with LOI ($r=0.51$, $P<0.001$), suggesting that NAG would be more regulated by substrate availability. This result was different from other studies in which moisture content primarily controlled the NAG activities. For example, Kluber et al. (2011) observed lowest levels of NAG activity in summer with low water content in the coniferous forests of the Pacific Northwest (USA).

Seasonal Patterns of Denitrification

In this study, the potential denitrification activity *ex situ* was measured under idealized conditions, i.e. constant room temperature and with enough carbon and nitrate supply. Though the results may overestimate the real activities, it is still considered to be effective to compare the difference between different sites (Hernandez and Mitsch 2007; Kjellin et al. 2007). Despite having less than 5 cm of soil and moisture contents similar to the reference site, relatively higher DEA activities were observed at the two restored sites, which was also reported by Smith and Ogram (2008).

The trend of increasing denitrification rates in the wet season (from May to July) could be explained by the optimum water moisture content that created anaerobic

condition for the denitrification. Most studies (Blackmer et al. 1980; Ruz-Jerez et al. 1994; Ashby et al. 1998; Shelton et al. 2000; Machefert and Dise 2004) have shown that with increasing moisture contents, the denitrification and the production of N_2O increased. In this study, the average precipitation in May 2010 was only 2 cm with the soil moisture contents less than 45% and in July, the rainfall exceeded 8 cm with the soil moisture contents over 60%. Consequently, denitrification was stimulated and a drastic pulse of N_2O emission occurred through a dry-rewetting cycle (Muhr et al. 2008; Davidson et al. 1993). Accordingly, higher denitrification would further deplete the soil NO_3^- which was the case in this study (Fig. 5-2). The result from Hefting et al. (2007) that higher denitrification reduced the N availability in soils also supported my finding.

However, in the last sampling, the moisture content was extremely low (~ 20% gravimetric water content) but unexpectedly higher potentially denitrification rates were measured (Fig. 5-5). The high temperature could be a reason. Another possible explanation was in the extreme dry condition, the fungi would contribute more to the denitrification since its more tolerance of drought than bacteria (Shoun et al. 1992; Crenshaw et al. 2008; Yuste et al. 2011). For example, recent work suggests that fungal rather than bacterial pathways dominate denitrification in semiarid and desert soils (McLain and Martens 2005, 2006; Crenshaw et al. 2008).

Moreover, this study showed a marked accumulation of nitrate in the dry season at all the three sites (Fig. 5-2), and followed a increase of denitrification at the beginning of the wet season (Fig. 5-5). The high amount of nitrate and rapid consumption could mean a pulse of N_2O is produced at the transit during the dry-rewetting cycle of a short-hydroperiod ecosystem.

Conclusions

N cycle is involved with a diversity of input, transformation, and export, which is regulated by numerous factors. Temporal patterns of the soil N cycle can simultaneously reflect the impacts of these variables like temperature, moisture contents and plant growth. In this study, the seasonal dynamics of soil N availability and soil mineralization in the short-hydroperiod ecosystem did not follow that of plant growth but were more regulated by microclimate (i.e., soil moisture content). In the wet season (June to September), available nitrate/nitrite (NO_x^-) decreased while available ammonium (NH_4^+) increased in the wet season, suggesting that the NO_x^- may be lost by leaching or consumed by denitrification especially in the rainy seasons. Moreover, the accumulation of nitrate in the dry season closely corresponded with the increasing of denitrification at the beginning of the following wet season, which indicated that the short-hydroperiod systems would be a potential N_2O hot spot in particular at the alternating point from dry to wet period.

When further correlating this output pathway with the input of N_2 fixation (Chapter 4), we could consider more interesting things about the N budget. At the beginning of the wet season, periphyton N_2 fixation also showed a peak. It is possible that the soil could fix N_2 fixation and show the same seasonal pattern. In this regard, the same peak of N_2 fixation and denitrification would cancel one another, and it is uncertain what the net effect of these two processes will have on the annual N budget.

In addition, the decreasing MBC:MBN ratio from spring to summer would tell us the changes of microbial composition with the time that is in the summer, more root exudes would favor the growth of bacteria. More solid evidence from molecular perspective is needed to confirm this inference.

In general, the season patterns of N cycle at the three sites were similar except for some microbial properties like MBC and MBN. The different seasonal patterns of MBC and MBN would contribute to the slight hydrological difference between the reference and restored sites or the buffer capability of deeper soil profile at the reference site. It would be expected that with the restoration and soil accumulation, the seasonal patterns of the restored sites will be closer to that in the reference site.

Table 5-1. Summary of three-way ANOVA tests for main effects and interactions of time, site, and elevation on soil parameters: soil moisture content (MC),pH, loss of ignition (LOI), total nitrogen (TN), total phosphorus (TP), TN:TP ratio, total organic carbon (TOC): TN ratio, microbial biomass carbon, nitrogen and phosphorus (MBC, MBN,MBP), extractable NH₄-N, extractable NO_x-N, potentially mineralized nitrogen (PMN), denitrification (DEA), Leucin-aminopeptidase (LAP) and N-acetyl-glucosaminidase (NAG). Significant correlations are marked: *-P<0.05; **-P<0.01, ***-P<0.001, NS-no significant difference.

Factors	LOI	TN	TP	TOC: TN	TN: TP	NO _x	NH ₄	NH ₄ : NO _x	DEA	PMN	LAP	NAG	MBC	MBN	MBC: MBN	LAP:NAG
time	***	***	NS	***	***	***	***	***	***	***	***	**	***	***	***	***
site	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	NS
elev.	NS	NS	***	NS	***	**	NS	NS	NS	**	NS	NS	*	***	***	NS
time*site	NS	NS	NS	NS	***	***	*	***	***	***	***	NS	***	***	***	NS
time*elev.	NS	NS	NS	NS	NS	***	NS	***	NS	NS	***	NS	*	NS	NS	NS
site*elev.	NS	NS	***	NS	**	NS	NS	NS	NS	*	NS	NS	*	***	*	NS
time*site*elev.	NS	NS	*	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

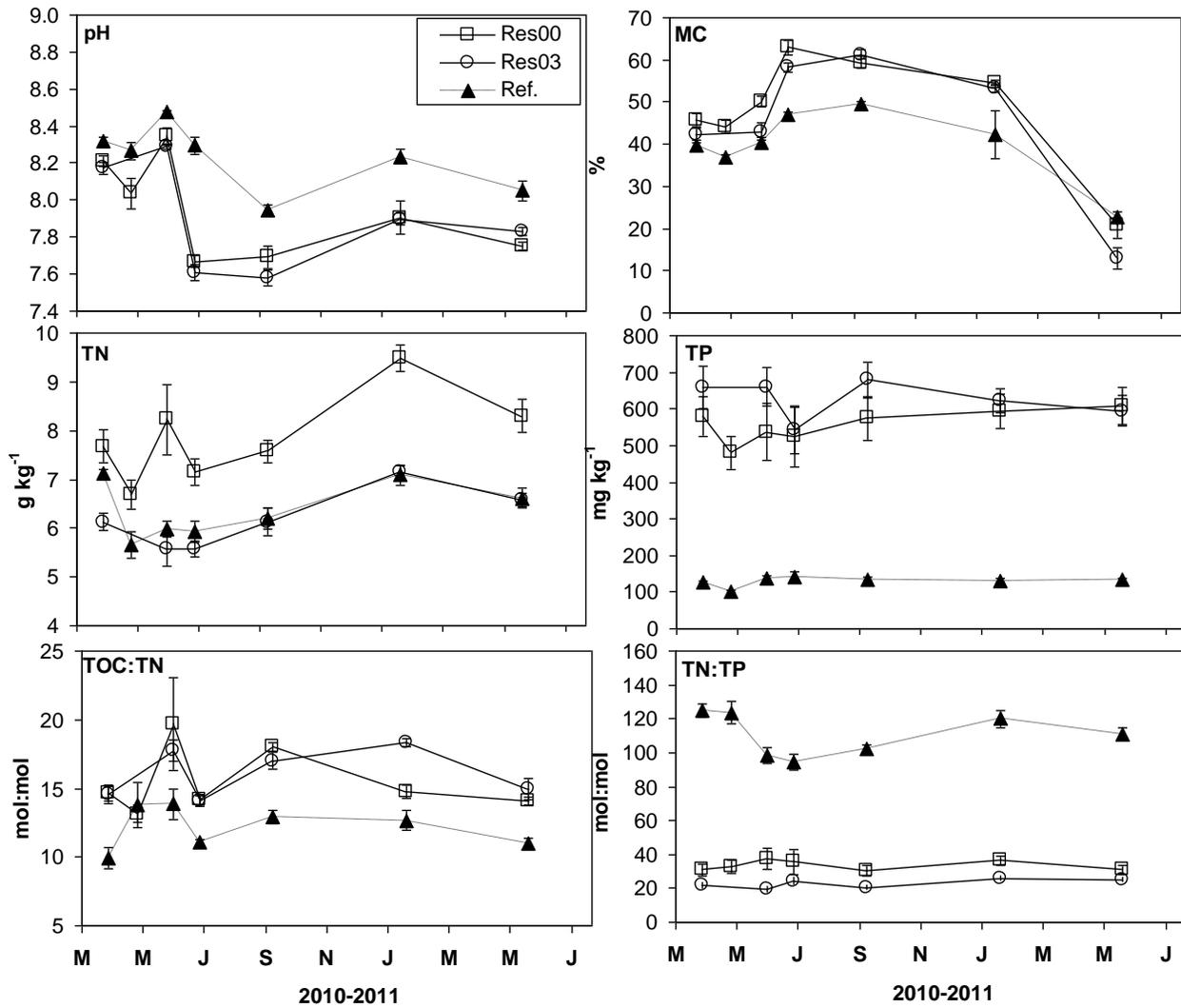


Figure 5-1. pH, soil moisture content, total nitrogen (TN), total phosphorus (TP), total organic carbon: total nitrogen (TOC:TN) and TN:TP over two years (2010-2011) in the restored (Res00 and Res03) and reference (Ref.) sites in the Hole-in-the-Donut. Error bars represent ± 1 standard error of the mean for each sampling date (N=6).

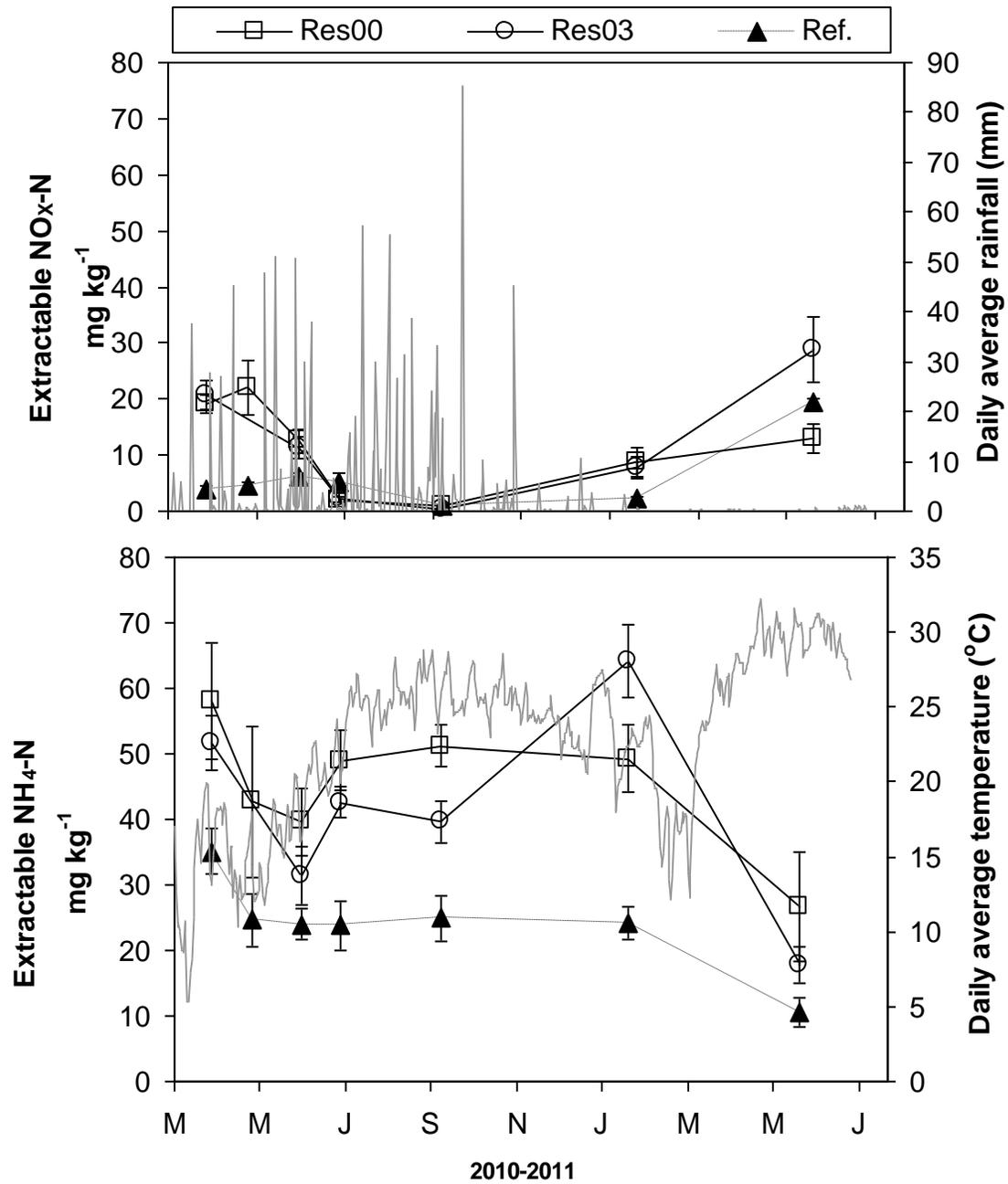


Figure 5-2. Extractable NO_x-N and NH₄-N with temperature and rainfall events (light grey line) over two years (2010-2011) in the restored (Res00 and Res03) and reference (Ref.) sites in the Hole-in-the-Donut. Error bars represent ± 1 standard error of the mean for each sampling date (N=6).

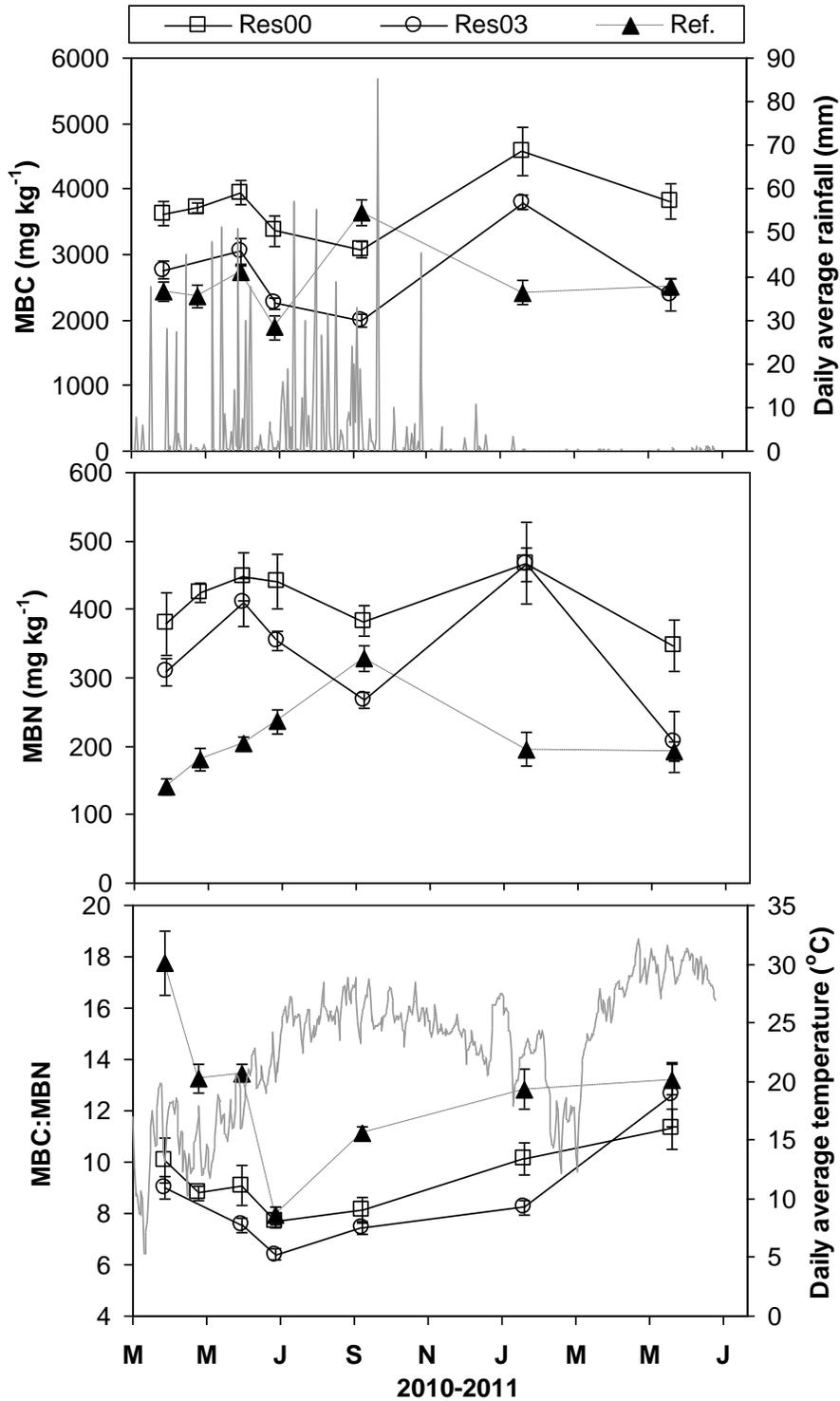


Figure 5-3. Microbial biomass carbon, nitrogen and their ratio (MBC, MBN and MBC:MBN) ratios with temperature and rainfall events (light grey line) over two years (2010-2011) in the restored (Res00 and Res03) and reference (Ref.) sites in the Hole-in-the-Donut. Error bars represent ± 1 standard error of the mean for each sampling date (N=6).

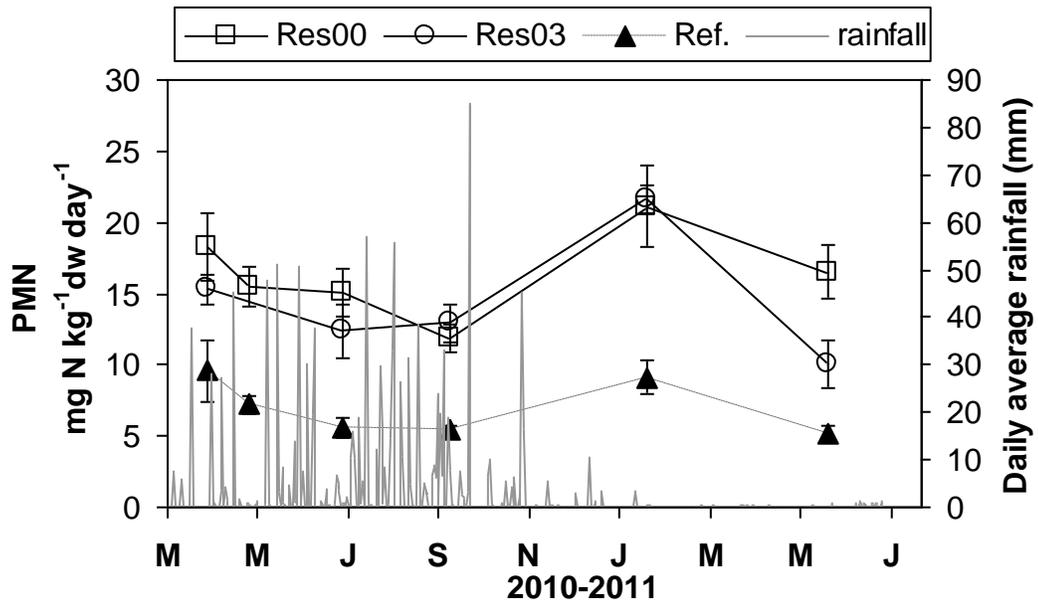


Figure 5-4. Potentially mineralized nitrogen (PMN) with rainfall events (light grey line) over two years (2010-2011) in the restored (Res00 and Res03) and reference (Ref.) sites in the Hole-in-the-Donut. Error bars represent ± 1 standard error of the mean for each sampling date (N=6)

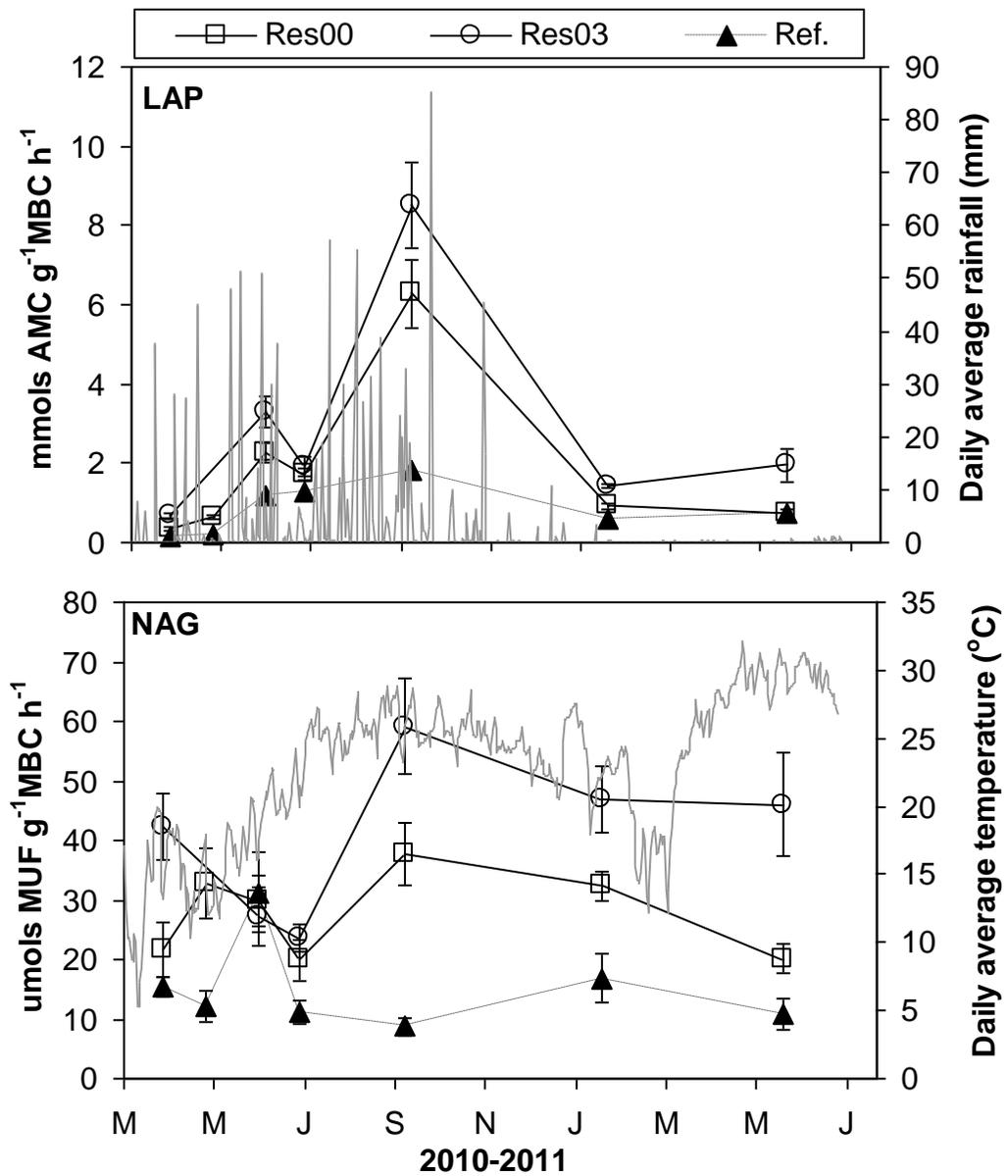


Figure 5-5. Leucine aminopeptidase (LAP) and N-acetyl- β -D-glucosaminidase (NAG) activities with rainfall events and temperature (light grey line) over two years (2010-2011) in the restored (Res00 and Res03) and reference (Ref.) sites in the Hole-in-the-Donut. Error bars represent ± 1 standard error of the mean for each sampling date (N=6).

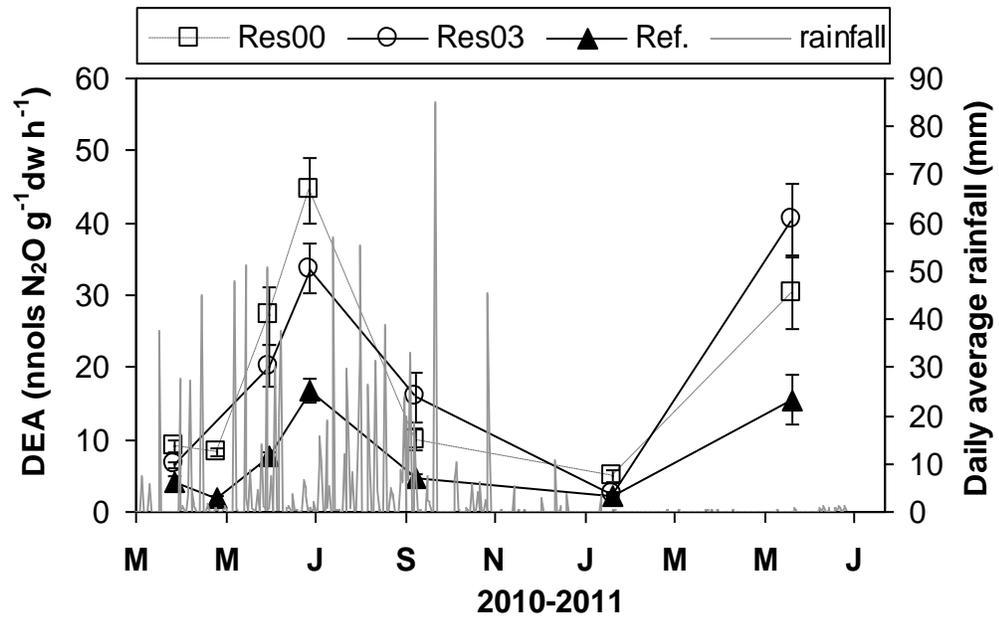


Figure 5-6. Denitrified enzyme activities (DEA) with rainfall events (light grey line) over two years (2010-2011) in the restored (Res00 and Res03) and reference (Ref.) sites in the Hole-in-the-Donut. Error bars represent ± 1 standard error of the mean for each sampling date (N=6).

CHAPTER 6 FIRE EFFECTS ON THE NITROGEN CYCLE IN CALCAREOUS WETLANDS

Background

Fire is widely used as a primary restoration technique in various ecosystems and has ecological significance. It was reported that fire can reduce fuel loads which then reduce the risk caused by the wildfire; prepare sites for seeding and planting and improve wildlife habitat (Heisler et al. 2004). Moreover, fire also has great influences on nutrient cycles in ecosystem by changing the form, distribution and amount of nutrient as well as by changing species composition (Bissett and Parkinson 1980; Brown et al. 2004; Certini 2005; Dumontet et al. 2006; Grogan et al. 2000; Raison 1979).

The effect of fire on the nitrogen (N) cycle is highly important because N is often the limiting nutrient for primary productivity. Compared to P, the N cycle is more complex and involving various processes, for example, N can be easily lost through denitrification, ammonification and volatilisation. Since N is important for plants, many studies have focused on the available N status before and after fire, trying to know the effects of the fire on subsequent primary production.

During the combustion, huge amounts of heat releases with smoke, directly and immediately leading to the volatilization of N and gaseous loss of N as ammonia (NH_3) and nitrogen oxide (NO_x). The transfer of heat to the soil will further increase the soil temperature and affect physical, chemical and biological soil properties (Neary et al. 1999). For example, it can kill soil microbes, decompose organic matter, and change the N forms (Raison 1979). The particulate residues left by fire exist in the forms of ash and charcoal, which are the important pathway for nutrients to return back or redistribute in the ecosystem after the fire and have long-time effects on nutrient cycles

(Qian et al. 2009; Zackrisson et al. 1996). It was reported that fire residues themselves contain a small amount of inorganic and organic N that directly serve as a N source (Qian et al. 2009; Hogue and Inglett 2012). Indirectly, large amounts of P carried by fire residues in particular ash, could stimulate some N processes, such as N₂ fixation, nitrification and N mineralization especially in P-limited ecosystems (Eisele et al. 1989; White and Reddy 1999, 2000). Charcoal with great adsorptive surface on the other hand was found to enhance nitrification and then elevate the nitrate level (Zackrisson et al 1996; Wardle et al. 1998; DeLuca et al. 2002, 2006). Both ash and charcoal can also exert an indirect influence on N availability through modification of the pH and the cation exchange capacity (Raison 1979; Glaser et al. 2002).

Studies on prescribed fire were mostly focused on forests, grasslands and prairies (Ojima et al. 1994; Blair 1997; Grogan et al. 2000; Castaldi and Aragosa 2002; Zackrisson et al. 2004), but comparatively few discussed on wetland ecosystems (Battle and Golladay 2003). However, fire together with water is also important element in the wetland management (Lockwood et al. 2003). In fact, fire has widely been used to control the spread of invasive species in both coastal and inland wetlands for a long time but only been intensively discussed in peatlands (Kirby et al. 1988)

Florida Everglades for example is regarded as a fire-dependent landscape during the long-term and large-scale restoration (Beckage et al. 2005). Besides reestablishing a more natural hydrology, the establishment of natural fire regime is also a guarantee for the successful Everglades restoration (Lockwood et al. 2003). Numerous fire projects have been conducted or been going on in a diversity of landscapes across Everglades, such as the River of Grass Prescribed Fire Plan for the wet prairie and

sawgrass marsh ecosystems, the Pineland burn plan, the mangrove-marsh ecotone fire, and the fire project in Water Conservation Area 2A (WCA 2A) of the northern Everglades (Spier and Snyder 1998; Qian et al. 2009; Beckage et al. 2005). However, most of the projects focused on restoring native plant species, little is known about the fire effects on nutrient biogeochemistry, let alone on the N cycle that has been ignored across the whole Everglades ecosystem (Miao and Sklar 1998; Miao and Carstenn 2006; Qian et al. 2009).

To bridge the gap between the knowledge of fire, N cycle, and wetland restoration, a case study on the N responses to fire at restored and reference wetlands was conducted in the Everglades National Park. Based on the literature aforementioned, the following hypotheses are proposed: 1) fire would increase the N availability; and 2) consequently denitrification rates would increase because of the possible increase of nitrate; 3) fire would increase N₂ fixation because of the possible elevation of available P after fire.

Materials and Methods

Study Site and Sampling Methods

The study site was located in the Hole-in-the-Donut (HID) of Everglades National Park, Florida, USA (Fig. 6-1). This region is subjected to the invasion of Brazilian pepper (*Schinus terebinthifolius*) because of the excess phosphorus introduced by the fertilizer after a long history of farming (Smith et al. 2011). Since 1975, studies to evaluate the effectiveness of fire as a management option for controlling *Schinus* has begun (Doren et al. 1991). Dalrymple et al. (2003) recommended the use of prescribed fire on the restored sites after complete soil removal at three- to five-year intervals to mimic fire frequency in natural vegetation, which would not only reduce coverage by

woody shrubs but would also promote expansion of desirable native plant species such as sawgrass (*Cladium jamaicense*).

Specifically, a restored wetland, cleared in 2000 (Res2000) and a native reference wetland were selected for this study. Soil depth varied between the two sites, with deeper marl soils (Biscayne and Perrine series) in the reference area (10cm) and shallower soils (less than 5 cm) which have developed after site clearing in the restored area (Smith et al. 2011). The primary vegetation in the reference site is a mixture of grasses (*Muhlenbergia* sp., *Andropogon* sp.) and sedges (*Cladium jamaicense* Crantz, *Shoenus* sp.) while the restored sites are dominated by pioneer species such as *Ludwigia* spp., *Baccharis* spp., and *Andropogon* spp. (Dalrymple et al. 2003).

In each site, two sampling stations corresponding to high and low elevation areas were set (Fig. 6-1). At each station, two 30 m x 30 m plots (i.e., one burn and one control) were set side by side with a 2 m buffer strip in-between. Each plot was separated into three 10 m by 30 m subplots which were further divided into seventy-five 2 m x 2 m sampling squares. At each sampling, three squares in each subplot were randomly selected to collect soil and periphyton samples and composited to form one replicate for each subplot (3 replicates for each burn and control treatment plot).

The Res2000 and reference sites were burned on May 4th, 2010. Sampling was conducted before the fire (April 8-10, 2010), then 2 days (May 6-7, 2010), 1 month (June 10-11, 2010) and 1 year (May 25-26, 2011) after the fire. All samples were sealed in plastic bags and kept 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. Fresh periphyton was used to determine nitrogenase activity, while sieved soil samples were used in the determination of all soil microbial and enzyme related parameters. A subsample of sieved soil was oven dried at 105°C for 3 days and ground using a mortar and pestle for moisture content and total nutrient determinations. Periphyton moisture content and nutrients were determined using the sample for nitrogenase activity which was oven dried at 65°C for 3 days and ball milled.

Biogeochemical Analysis

The measurement of the Loss-on-Ignition (LOI), total carbon (TC), total nitrogen (TN), total phosphorus (TP), extractable ammonium ($\text{NH}_4\text{-N}$) and nitrite/nitrate ($\text{NO}_x\text{-N}$) microbial biomass C and N (MBC and MBN), potential mineralizable nitrogen (PMN), the extracellular enzymes related to the N cycle, i.e., N-acetyl- β -D-glucosaminidase (NAG, EC 3.2.1.30) and Leucine aminopeptidase (LAP, EC 3.4.11.1), and the denitrification enzyme activity, followed the same methods described in the Chapter 5. 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).

Nitrogenase activity (N_2 fixation) of periphyton was measured using the acetylene (C_2H_2) reduction (AR) assay described by Inglett et al. (2004). The details were seen in the Chapter 3.

Statistical Analysis

Data were analyzed with JMP v.8[®] statistical software (SAS Institute Inc., Cary, NC). For the pre-fire data, one-way analysis of variant (ANOVA) was used to compared the difference in measured parameters between the restored and reference sites. For the post-fire data, the factors of site and sampling time were fixed and separate one-

way analyses for the different sites and dates were performed. Regressions between different parameters were performed using the linear least-squares method. All results are reported as significant when $P < 0.05$. Data were log transformed when necessary to improve normality.

Results

Fire Effects on Soil Nitrogen Availability

In general, fire did not significantly change the soil total nutrients (Table 6-1). For the reference site, TP content in the burn plots after 2 days of the fire was significantly higher than that in the control plots ($P < 0.05$). Both restored and reference sites showed an increase in extractable $\text{NO}_x\text{-N}$ and $\text{NH}_4\text{-N}$ 2 days after the fire (Fig. 6-2, 6-3). The increase of extractable inorganic N after the fire was greater at the reference site (i.e., approximately twice that of the control plots) compared to the restored site (i.e., only 20 to 40% higher than the control plots); and only at reference site was the difference statistically significant ($P < 0.05$). At longer time scales (1 month to 1 year after the fire), the extractable $\text{NO}_x\text{-N}$ even remained higher in the burn plots than the control plots for the reference site (Fig. 6-2), but the extractable $\text{NH}_4\text{-N}$ at both restored and reference sites dropped to levels below the control plot after 1 month of the fire (Fig. 6-3).

Fire Effects on Microbial Activities

In this study, there were not significant changes in the MBC and MBN (Table 6-2). The responses of N mineralization measured as PMN to the fire did not show discernable differences (Table 6-2). The responses of the two N-related enzymes (i.e., LAP and NAG) to the fire were similar with each other (Fig. 6-5). In the restored site, the LAP and NAG did not change appreciably up to 1 month after the fire, but increased by 20% and 40% relative to the control plots, respectively, after one year of the fire. In

contrast with the restored site, the enzyme activities in the burn plots at the reference site were significantly greater than those in the control plots immediately (2 days) after the fire; but after one month, the enzyme activities returned to or even fell below the control levels. In addition, the LAP:NAG ratio was seen an increase by 80% after 2 days of the fire at the reference site (Fig. 6-5), but did not change significantly at the restored sites.

Fire Effects on Denitrification

Both the restored and reference sites showed the similar response of denitrification (measured as DEA) to the fire (Fig 6-4). Immediately after the fire, approximately 20% higher DEA rates were observed in the burn plots compared to the control plots at the restored site. For the reference site, the DEA rates at the burn plot were up to twice of the values in the control plots. After one year of the fire, the DEA rates in the burn plots returned to the levels in control plots.

Fire Effects on Periphyton N₂ Fixation

The response of periphyton N₂ fixation to the fire differed between the restored and reference sites. At the restored site, N₂ fixation rates slightly decreased immediately after the fire, but then increased to as high as two folds of those in the control plots one year after the fire. In contrast, at the reference site the rates fell below the control levels after one year of the fire (Fig. 6-6).

Discussion

Responses of Nitrogen Availability to the Fire

Previous researches on the effects of fire on N mineralization in prairie and grasslands has resulted in conflicting results, with fire either causing a decrease (Blair 1997; Ojima et al. 1994; Turner et al. 1997), an increase (Aranibar et al. 2003; Boerner

and Brinkman 2003), or having no effect on N mineralization (Raison 1979). The results depend on different ecosystems and fire behavior; accordingly various mechanisms were proposed to explain the observations. For example, Bell and Binkley (1989) pointed out that N mineralization from soil organic matter is enhanced after the fire due to elevated soil temperature; while Vance and Henderson (1984) concluded that N mineralization decreased after burn because of the poor substrate quality. Stock and Lewis (1986) also considered that these conflicting results may be attributed to the use of different methods to determine N mineralization. In this study, soil PMN was estimated using a 10-day anaerobic incubation method. There was no significant response of mineralized N to the fire at both sites.

Most studies suggest a consistent pattern that fire can increase the availability of soil NH_4^+ and NO_3^- (Wan et al. 2001). NH_4^+ is a direct product of the combustion which is adsorbed by the soil, thus increasing the concentration of NH_4^+ . In this study, higher extractable $\text{NH}_4\text{-N}$ was observed two days after the fire in the burn plots at both restored and reference sites. The release of NH_4^+ from organic matter would further oxidation of NH_4^+ to NO_2^- and then NO_3^- by bacteria (Hobbs and Schimel 1984; Blank and Zamudio 1998). Covington et al. (1991) found NO_3^- was not immediately affected, but 1 year after burning concentrations had become dramatically higher than the pre-fire level. In this study, however, both the restored and reference sites saw an immediate increase in $\text{NO}_x\text{-N}$ two days after the fire which in the reference site, the increase of $\text{NO}_x\text{-N}$ even lasted for one year.

The elevation of the N availability may contribute to the ash deposition with high concentration of nutrients. Grogan et al. (2000) investigated the effect of natural ash

deposition on post-fire ecosystem N cycling by removing the surface ash layer from field plots within 1 week of a wildfire in a Californian bishop pine (*Pinus muricata* D. Don) forest. They characterized the influence of ash on plant, soil and microbial N pools during the first growing season after fire and found that ash deposition during wildfires can enhance soil N availability to plants and facilitate ecosystem N retention.

A recent explanation for the increase of the available N in forest ecosystems was the influence of charcoal on soil N dynamics and in particular, nitrification (DeLuca et al. 2006). In their study, DeLuca and Sala (2006) found that charcoal significantly increased nitrification and the NO_3^- concentration in the soils. Despite these studies, there are few reports on wetland ecosystems, Qian et al. (2009) simulated the fire in the Water Conservation Area (WCA)-2A in the Everglades through muffle furnace combustion but focused only on the effects of ash on the phosphorus. Hogue and Inglett (2012) characterized fire residues (ashes) created by both muffle furnace and flame combustion, observing an increase of available N in char residues after the simulated fire.

In addition, burning can increase extractable P through combustion and heating of organic matter, and pH increases caused by ash also can cause the release of ortho-P bound by iron and aluminum (DeBano and Klopatek 1988). Hogue and Inglett (2012) showed that charcoal formed after burning at low temperature in the HID marl prairie ecosystem, contained more bicarbonate extractable P. For the low-P reference site, the addition of P through ash or charcoal would stimulate the N mineralization and further increase N availability. For example, White and Reddy (1999, 2000) set a series of experiment to test the P loading effects on N mineralization in Everglades wetland soil,

and found that P enrichment in the P-limited soils had the stimulatory effect on microbial activity and led to an increased availability of inorganic N.

Fire Effects on Denitrification

Few studies were focused on the influence of fire on the denitrification. My results showed that there was no significant difference in potential DEA rates, which was similar with the results of Castaldi and Aragosa (2002) that DEA did not change significantly immediately after fire (i.e., 7 days after the fire) in a Mediterranean shrubland. They considered that other factors, such as water content, would affect denitrification more than the fire. In this study, the slightly increase of DEA after 2 days of the fire could be caused by the increase of NO_3^- content, especially for the reference site in which significantly positive correlation between $\text{NO}_x\text{-N}$ and DEA was observed (Fig. 6-7). Or, perhaps the microclimate modified by the fire facilitates the denitrification, especially for the restored site in which no significant correlation existed between the NO_x^- and DEA (Fig. 6-7).

Effects of Fire on Microbial Activities

It is important to study the fire effects on the activity of soil microorganisms and enzyme systems because these are responsible for mineralization processes and availability of nutrient (Saa et al. 1993). Moreover, microbial biomass and enzyme activities are affected by management practices and can be used as sensitive indicators of ecological stability (Ajwa et al. 1999). Many studies have focused on physical and chemical changes in the soil after fire, but few have paid attention to the effects on soil microbial communities (Boerner et al. 2000; Fioretto et al. 2005).

In this study, no significant changes in MBC and MBN were observed (Table 6-2), which agreed with the results from the black pine forest in Turkey reported by Kara and

Bolat (2009). The responses of enzyme to the fire were often studied in a comparatively long time with a year interval. For example, Gutknecht et al. (2010) measured the activity of six enzymes for 3 years following wildfire at the Jasper Ridge Global Change Experiment in a California annual grassland. They found that the wildfire slightly decreased enzyme activities (by 10-20%) in year 1 post fire, with a larger decrease (by 25-50%) in the second year after the fire. The response was gone by year 3, suggesting that the microbial community was able to recover by 3 years following wildfire.

The two N-related enzymes measured in this study, LAP and NAG, are inducible enzymes which are synthesized or activated when needed. For example, Chróst (1991) found out that the activities of LAP were induced at limited N availability. NAG involved in the degradation of chitin which is a relevant source of N in soils. In this study, LAP activities were significantly positively related with PMN (Fig. 6-7), indicating that LAP activity can be used as a reliable index of N mineralization in soils. NAG activity in this study decreased a month after the fire in the restored and reference sites. This result agreed with Boerner et al. (2008) who observed reductions in chitinase activity in the fire treatments in North American forest ecosystems and they considered it may have been the result of the deposition of more labile organic matter following fire. The response of N acquisition enzymes could depend on fire intensity or severity other than nutrients (Boerner et al. 2000). For example, with low-severity fires (as in grassland type systems like the HID) little heat is transferred downward, minimizing the direct effect of heat on microbial death, and thus, resulting in no significant short-term change in the enzyme activities.

Fire Effects on Periphyton N₂ Fixation

As an important function of periphyton, N₂ fixation by periphyton at the restored site responded differently from the reference site (Fig. 6-6). For the restored site, periphyton nitrogenase activity became higher in the burn plots compared to the control plots 1 year after the fire. This result was similar to Zackrisson et al. (2004) that in northern boreal forests in Sweden, the N₂ fixation rates increased linearly with time since fire. They attributed the increase to the degree of colonization by cyanobacteria and site factors such as presence of available N. In this study, it is likely that ash deposition from the fire elevated P levels in the restored site (Hogue and Inglett 2012). This pulse of new P may have enhanced the N limitation already present in these sites, thus resulting in stimulated periphyton N₂ fixation.

For the reference site, however, the nitrogenase activities only slightly increase one month after the fire and reduced below their control levels after one year of the fire. DeLuca et al. (2002) pointed out that since fire increased the availability of N and reduces the presence of *P. schreberi* and associated N-fixing symbionts, it is likely that the N₂ fixation rates by *P. schreberi* would decrease following the fire. Another research related to the nitrogenase activities response to the fire was done in Mountain shrub and grassland communities during 2 years following burning (Hobbs and Schimel 1984). They found that nitrogenase activity was depressed by fire 1 year after the burn in the mountain shrub community and they considered that the elevation of the soil inorganic N levels in burn plots may contribute to the depression in nitrogenase activity. The mechanism that excess N input after fire may explain the observed suppression of N₂ fixation in the reference site.

Conclusions

N cycle involves a diversity of input, transformation, and export. Accordingly, lots of uncertainties exist in different ecosystems, such as the factors regulating denitrification, the quantity of the greenhouse gas (i.e., N_2O), and the rates of N_2 fixation. The need to understand N dynamics is further highlighted by the potential impacts of restoration and the action of fire again complicates this understanding. For wetlands, fire and water both shape the restoration and nutrient cycle. Thus it is critical to have a good knowledge of fire effects on nutrient cycle before consideration of using fire as a management tool especially in those ecosystems that limited by certain element. For these reason, a restored wetland with high P and a native wetlands with low P in the HID of Florida Everglades were selected to investigate the impacts of fire on N dynamics. It is the first study of the fire impacts on N cycle in the Everglades.

Different responses of N cycle were observed in an immediate time (2 days), a short-time (approximately 1 month) and a longer time (~ 1 year) following the fire. The increase of N availability agreed with my first hypothesis and other studies in the terrestrial ecosystems. The increase of inorganic N can influence regrowth and seedling establishment of native plant species, invasion of exotic plant species, and ultimately, site recovery potential (Dalrymple et al. 2003; Rau et al. 2007).

One of the most interesting findings in this research is that fire has more stimulant effects on the N availability (i.e., N mineralization, extractable inorganic N, LAP) at the reference site with low P compared to the restored site with high P, indicating fire would exert different influences on N cycle at different P status. Several studies have provided evidence that in P-limited ecosystems, P addition stimulated the N mineralization and nitrification (White and Reddy 1999, 2000). Hogue and Inglett (2012) simulated the

flame and muffle furnace burning after this field fire project and concluded that the fire-produced ash did contain available inorganic P. However, another fire residue, i.e., the charcoal, is also reported to have stimulatory effects on nitrification and N availability in the forest ecosystems (DeLuca et al. 2002, 2006). Little is known about whether the charcoal from a herbaceous ecosystem could work the same way as forests. Since fire intensity decides the fraction of charcoal and ash (Qian et al. 2009; Hogue and Inglett 2012), it will be helpful to know which form contributes more to the increase of N availability before deciding a high or low intensity fire plan.

Moreover, periphyton N_2 fixation increased only in the restored site after one year of the fire but decreased in the reference site. It is well known that in a N-limited ecosystem, P addition can elevate the N_2 fixation (Howarth et al. 1988a; Smith 1990) as a internal N source whereas excess available N (NH_4^+ and NO_3^-) could inhibit N_2 fixation (Howarth et al. 1998b). In this regard, fire could ease the N-limitation that tends to occur in the very young restored sites in the HID which further enhance the restoration through complete soil removal (Dalrymple et al. 2003).

Overall, it is important to know the impacts of fire on N cycle in order to make a balance between N and P that could finally lead to the reestablishment of native wetlands with low nutrients. Practically, our understanding of the mechanisms (ash and charcoal) is also of critical value in determining which fire regime (low or high fire intensity) should be performed.

Table 6-1. Changes of basic soil properties and selected microbial activities 2 days, 1 month and 1 year after the fire in the burn and control plots in the restored (Res.) and reference (Ref.) sites. * denotes the significant difference between the burn and control plots ($P<0.05$).

Site	time after fire	TOC g kg ⁻¹		TN g kg ⁻¹		TP mg kg ⁻¹		TOC:TN molar ratio		TN:TP molar ratio	
		Burn	Control	Burn	Control	Burn	Control	Burn	Control	Burn	Control
Res.	2 days	87 (4)	75 (4)	7.3 (0.3)	6.7 (0.3)	592 (66)	480 (47)	14 (0)	13 (1)	29 (4)	33 (4)
	1 month	110 (13)	134 (19)	7.3 (0.2)	8.2 (0.7)	618 (73)	537 (78)	18 (2)	20 (3)	28 (4)	37 (6)
	1 year	110 (6)	100 (4)	8.2 (0.2)	8.3 (0.3)	646 (67)	609 (51)	16 (1)	14 (0)	30 (3)	31 (3)
Ref.	2 days	69 (6)	66 (7)	6.7 (0.3)	5.7 (0.3)	136 (4)*	102 (4)	12 (1)	14 (2)	136 (4)	102 (4)
	1 month	65 (5)	71 (5)	6.5 (0.2)	6.0 (0.2)	144 (12)	136 (7)	12 (1)	14 (1)	144 (12)	136 (7)
	1 year	65 (2)	63 (3)	6.6 (0.2)	6.6 (0.2)	140 (4)	133 (7)	12 (1)	11 (0)	140 (4)	133 (7)

Table 6-2. Changes of selected microbial activities 2 days, 1 month and 1 year after the fire in the burn and control plots in the restored (Res.) and reference (Ref.) sites. * denotes the significant difference between the burn and control plots ($P < 0.05$).

Site	time after fire	MBC mg kg ⁻¹		MBN mg g ⁻¹		MBC:MBN mass ratio		PMN mg N kg ⁻¹ dw day ⁻¹	
		Burn	Control	Burn	Control	Burn	Control	Burn	Control
Res.	2 days	3773(123)	3710(79)	421(18)	424(15)	9(0.4)	9(0.3)	15(2)	16(1)
	1 month	3510(100)	3936(185)	421(15)	448(36)	8(0.4)	9(0.8)	35(3)	43(3)
	1 year	4291(174)	3815(271)	417(25)	347(38)	10(0.4)	11(0.8)	13(2)	16(2)
Ref.	2 days	2648(153)	2366(175)	234(18)	181(16)	11(0.3)	13(0.6)	7(1)	7(1)
	1 month	2758(115)	2735(104)	221(15)	204(10)	13(0.5)	13(0.4)	20(1)	17(1)
	1 year	2747(418)	2515(127)	211(46)	193(14)	14(1.6)	13(0.6)	5(0)	5(0)

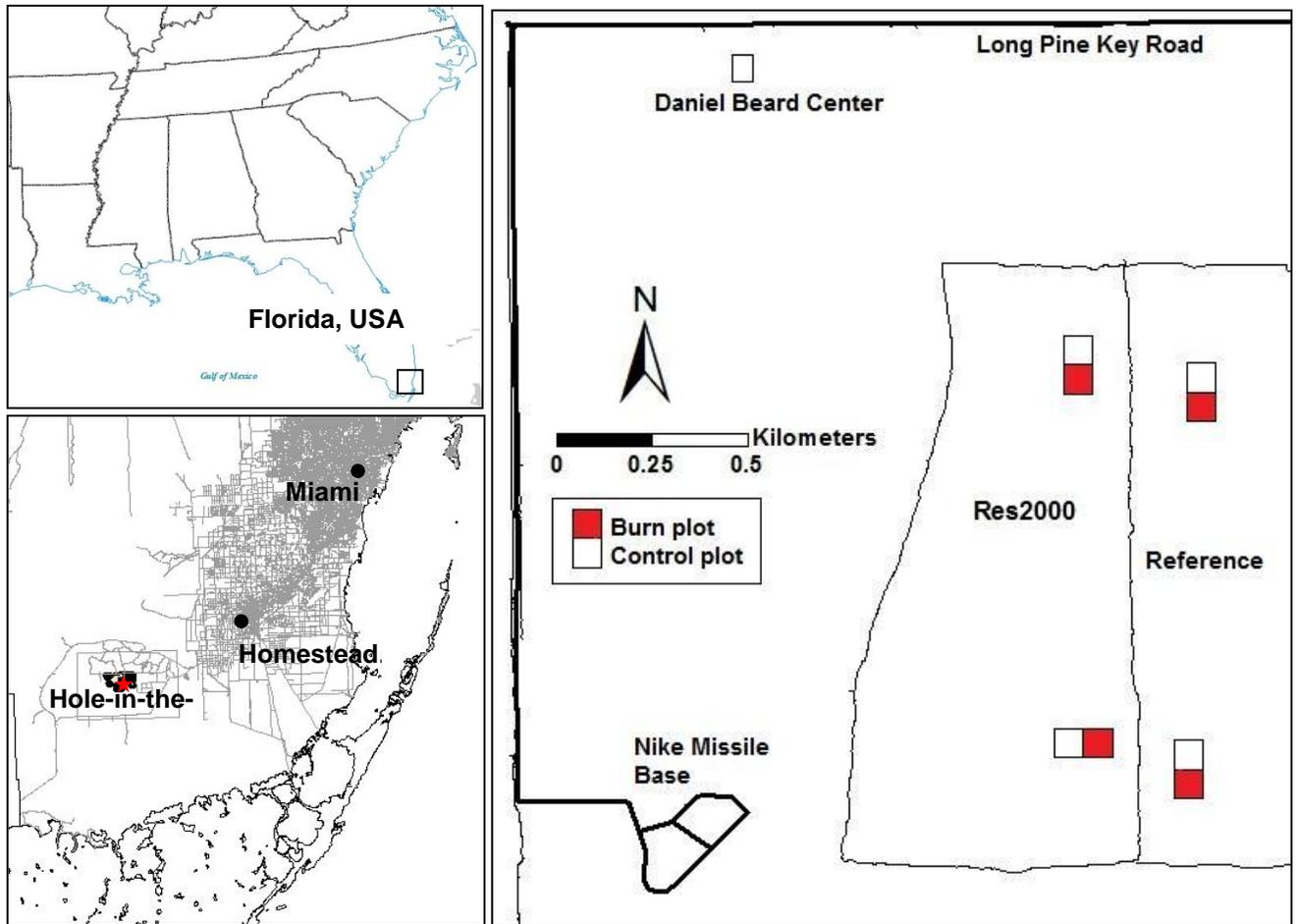


Figure 6-1. Sampling locations in the restored and reference wetland in the Hole-in-the-Donut of Florida Everglades.

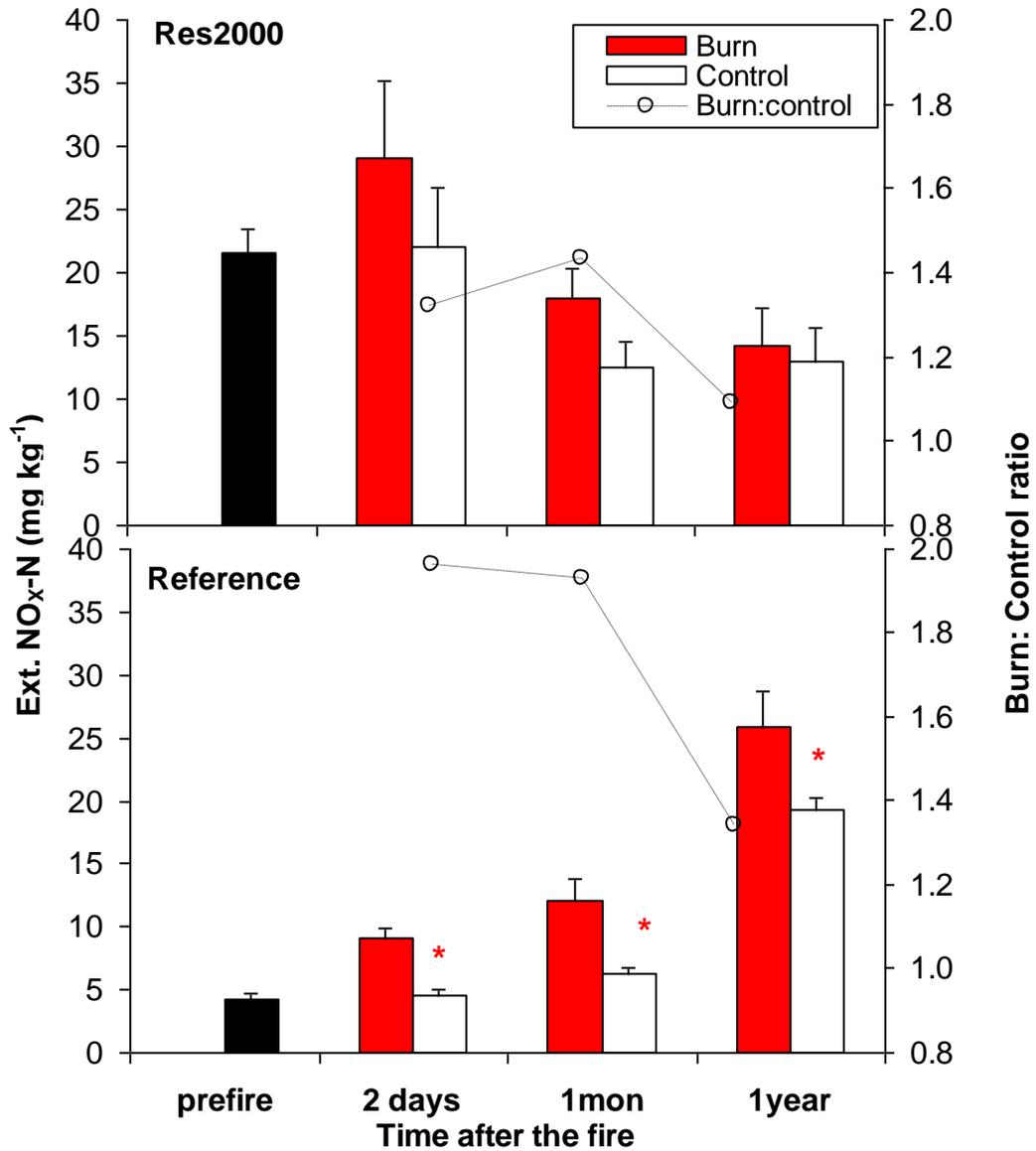


Figure 6-2. Changes of soil extractable NO_x-N in the burn and control plots in the restored (Res2000) and reference wetlands 2 days, 1 month (1mon) and 1year after the fire. The black bar represents the value before fire. The star * denotes significant difference between the burn and control plots (P<0.05).

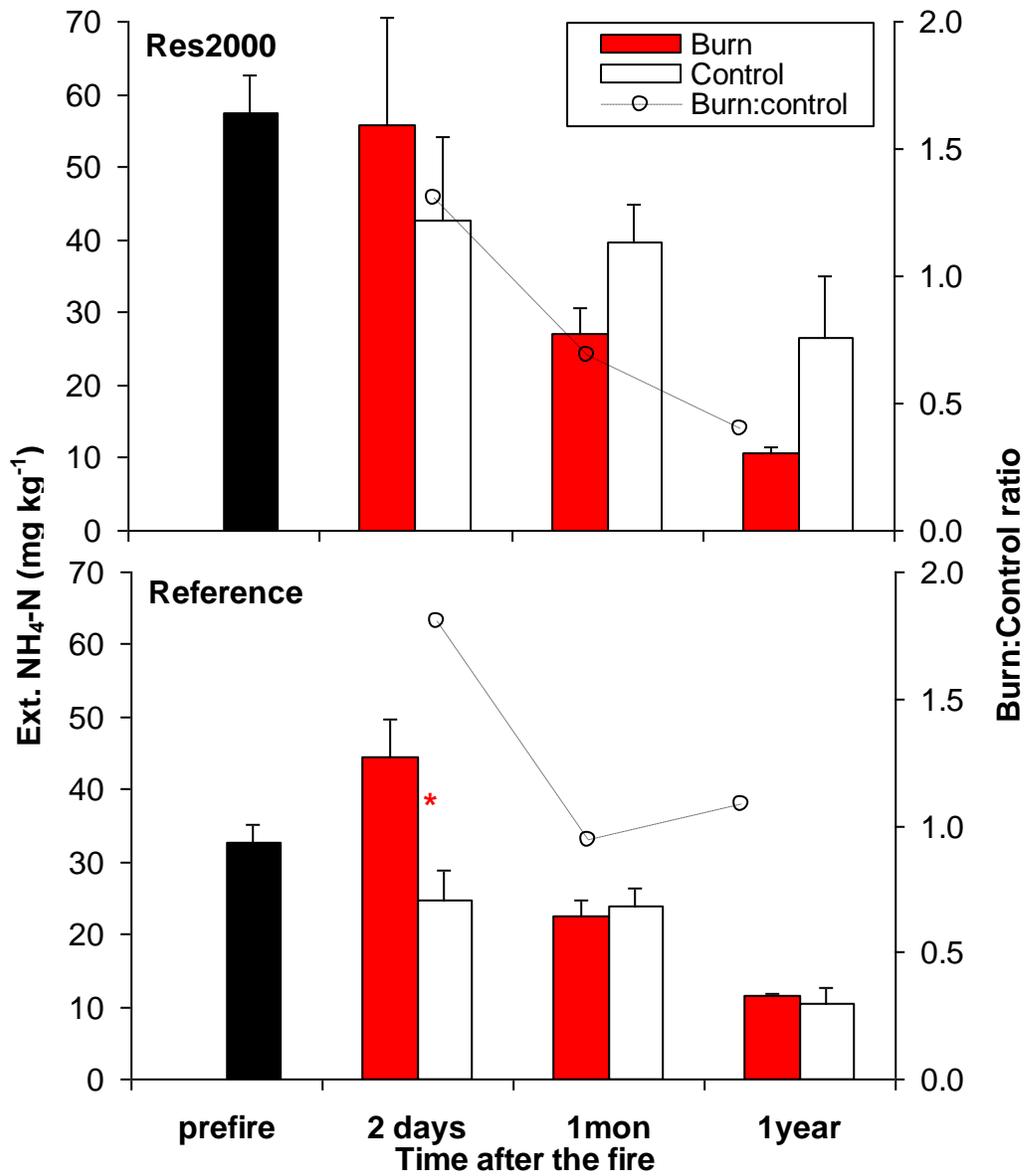


Figure 6-3. Changes of the extractable NH₄-N of the soil in the burn and control plots in the restored (Res2000) and reference wetlands 2 days, 1 month (1mon) and 1year after the fire. The black bar represents the value before fire. The star * denotes significant difference between the burn and control plots ($P < 0.05$).

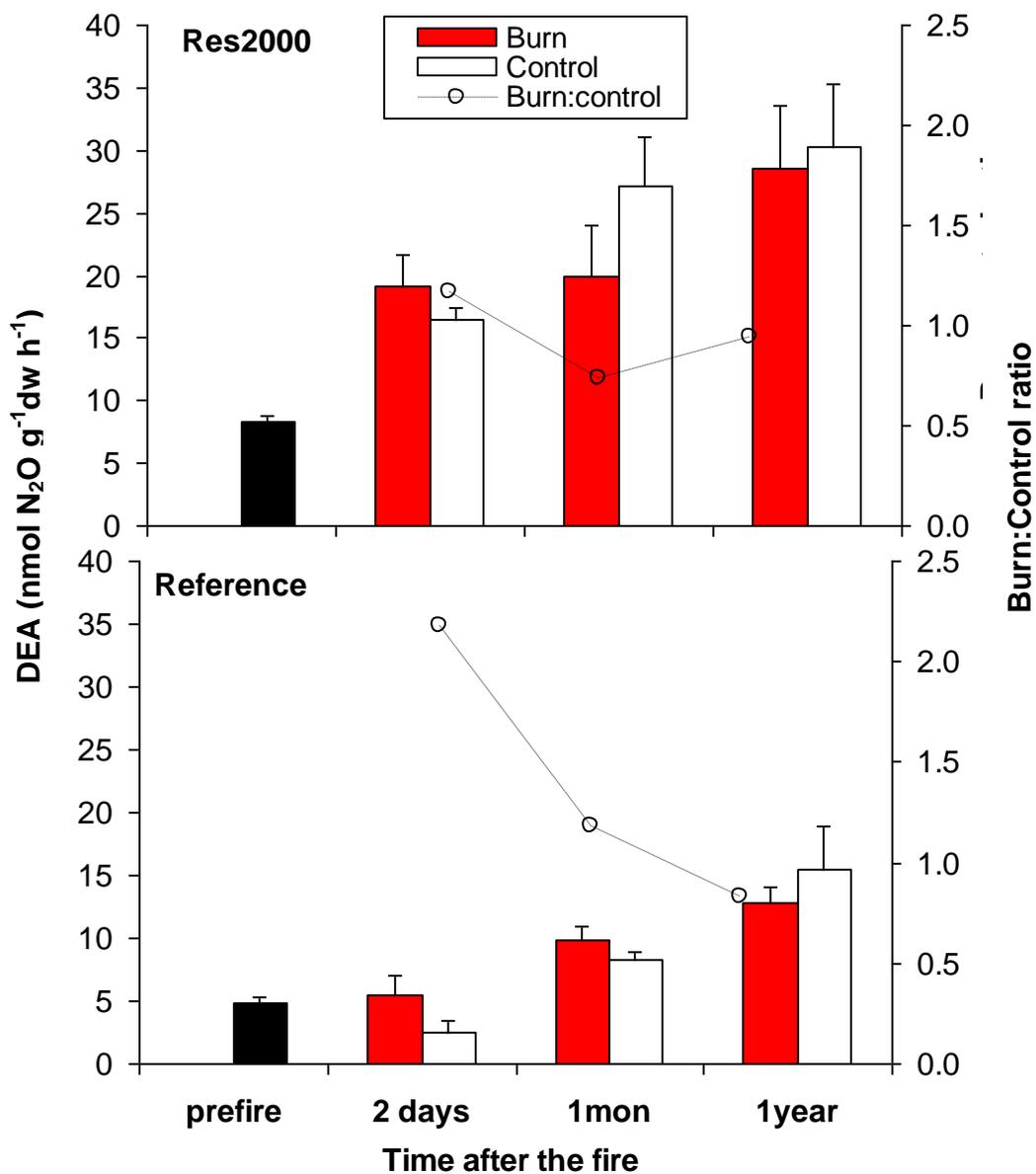


Figure 6-4. Changes of denitrification enzyme activities (DEA) in the burn and control plots at the restored (Res2000) and reference wetlands 2 days, 1 month (1mon) and 1year after the fire. The black bar represents the value before fire.

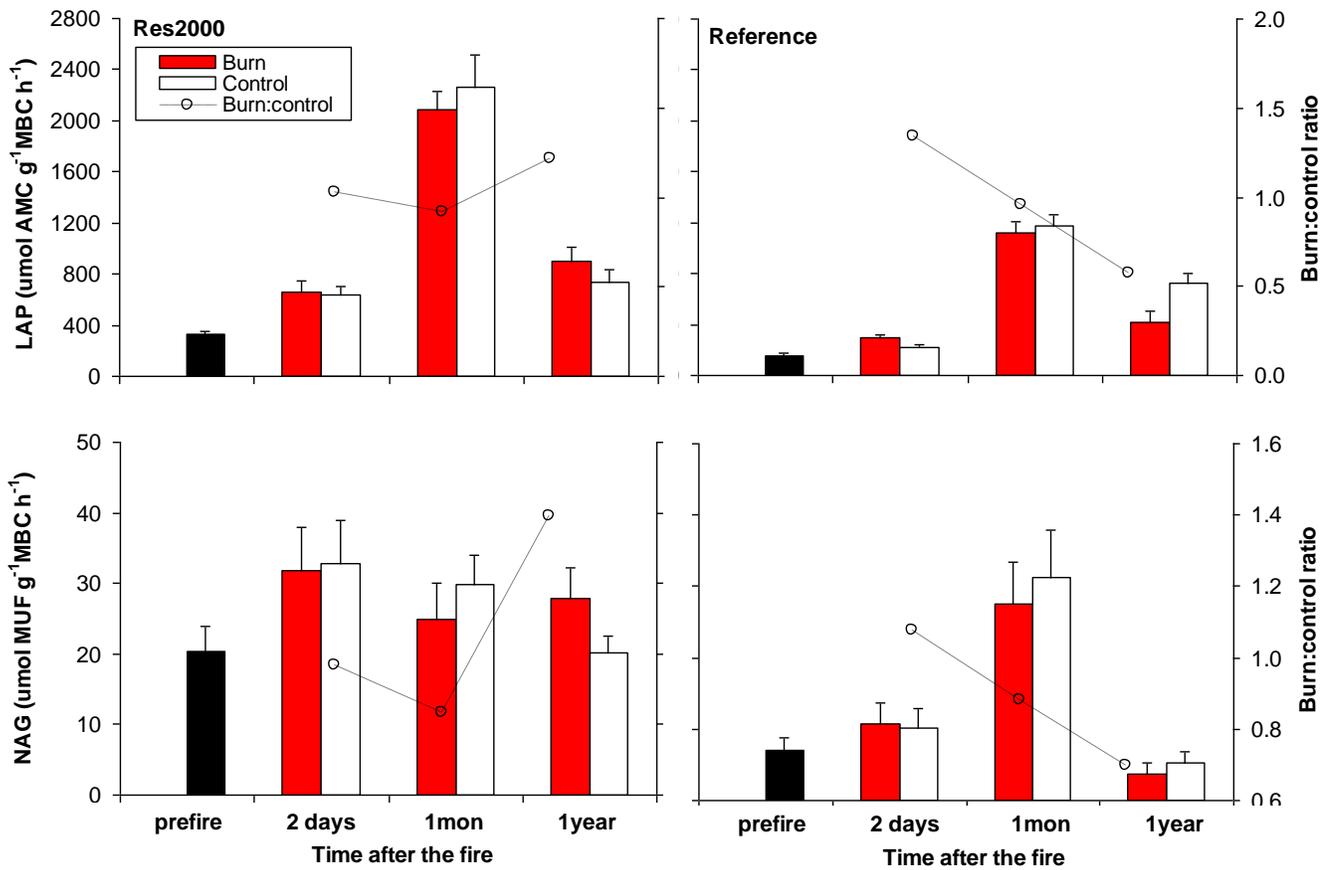


Figure 6-5. Changes of LAP and NAG in the burn and control plots in the restored (Res2000) and reference wetlands 2 days, 1 month (1mon) and 1year after the fire. The black bar represents the value before fire.

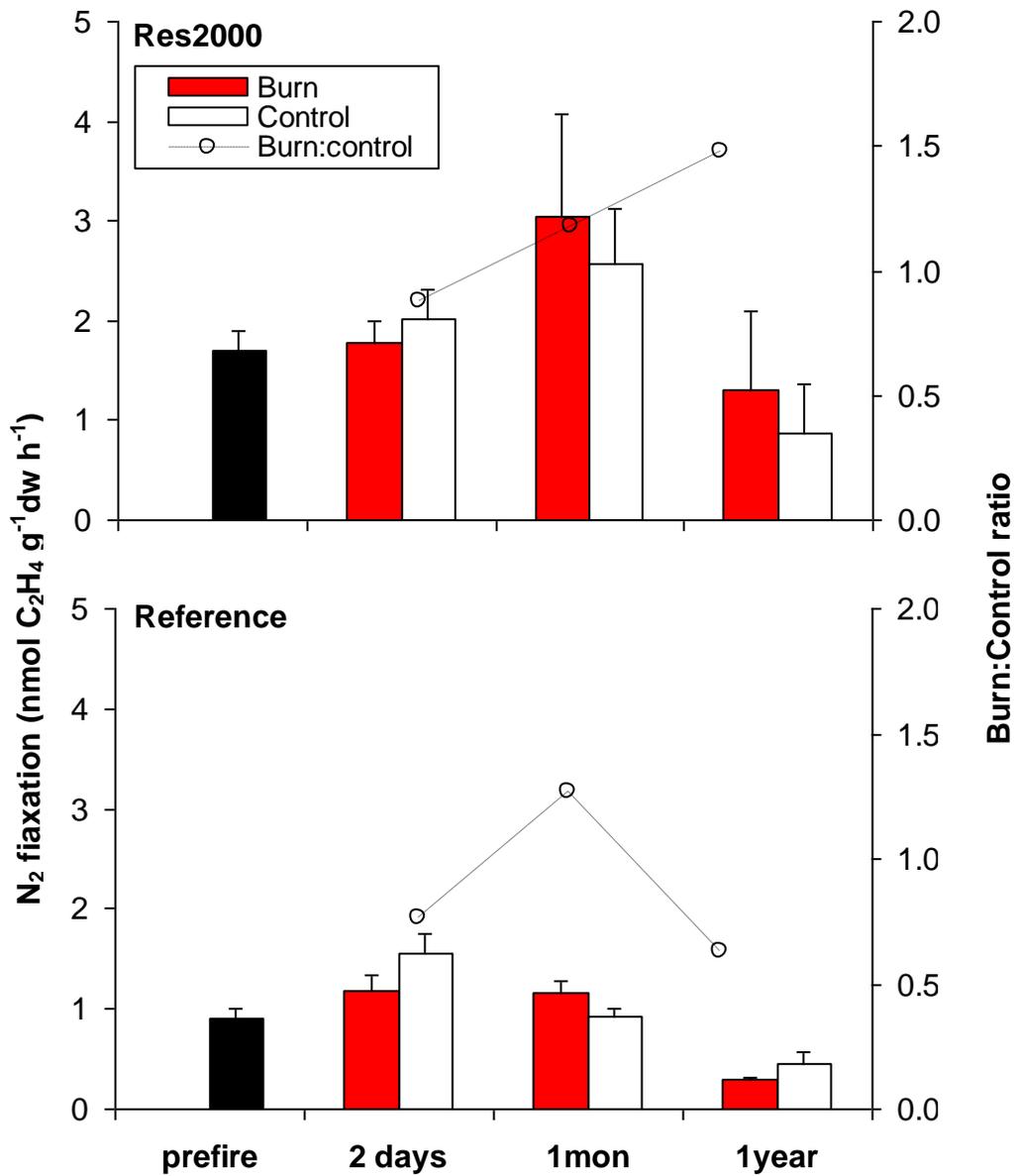


Figure 6-6. Changes of periphyton N₂ fixation under light condition in the burn and control plots in the restored (Res2000) and reference wetlands 2 days, 1 month (1mon) and 1year after the fire. The black bar represents the value before fire.

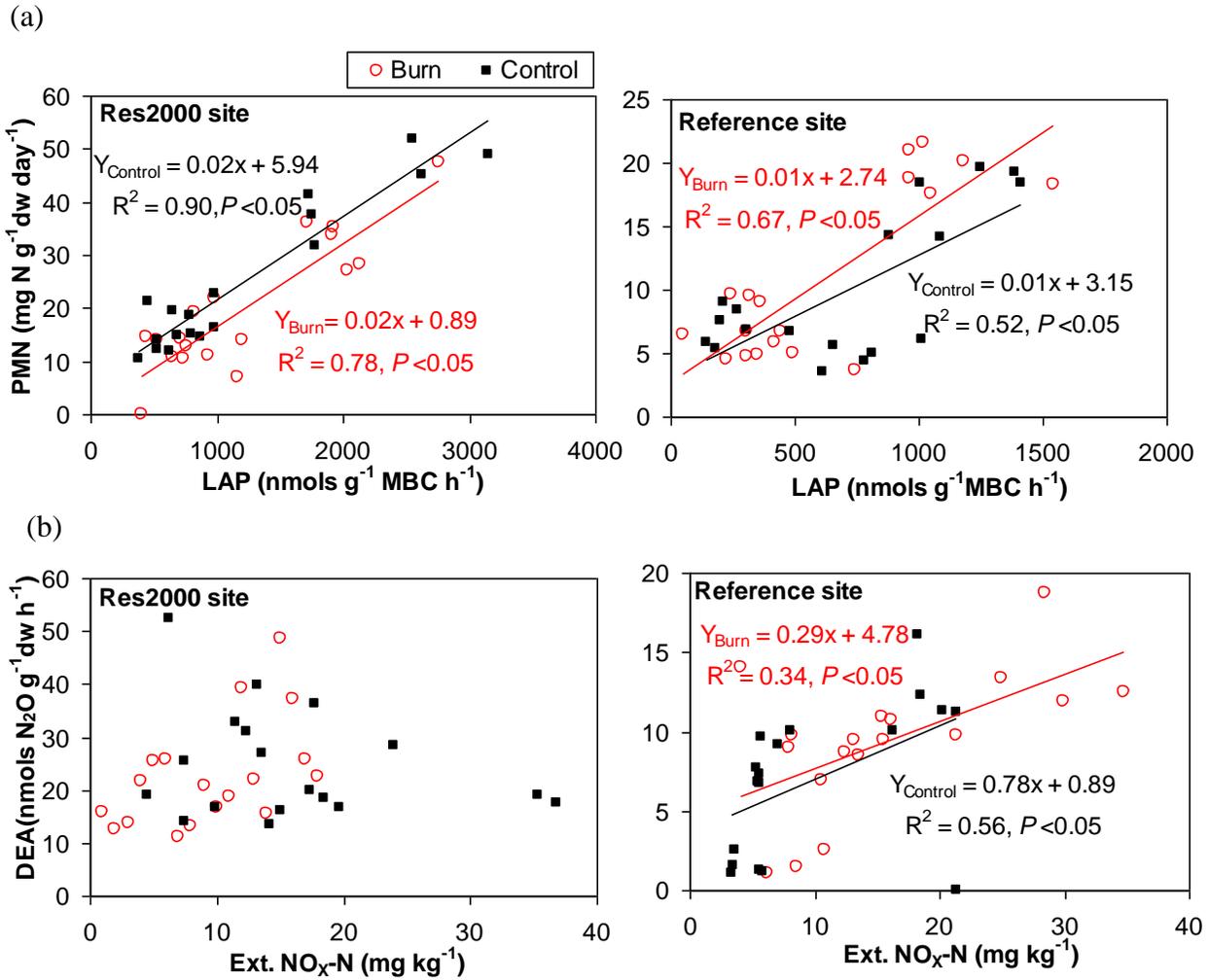


Figure 6-7. Correlation between nitrogen mineralization (measured as potentially mineralizable nitrogen PMN) and Leucine aminopeptidase (LAP) (a) between extractable nitrite/nitrate (Ext. NO_x-N) and denitrification (measured as potentially denitrification rates DEA) (b) in both burn and control plots in the reference and restored sites. Sample numbers N=18.

CHAPTER 7 SUMMARY AND SYNTHESIS

In wetlands, the nitrogen (N) cycle is dynamically controlled by hydrology, fire and phosphorus (P). When a globally increasing likelihood of severe drought has been predicted during this century, it is emergent to investigate the seasonal changes or hydroperiod effects on the N cycle which would further affect the primary production, and the global warming. However, N cycling has received little attention in most of the Everglades system where N could be a limiting element and a serious pollutant. This dissertation investigated the spatial and temporal patterns of N cycling, and their responses to a prescribed fire in the context of ecosystem restoration using the short-hydroperiod, marl prairies of the Hole-in-the-Donut (HID) region of Everglades National Park.

Summary

The nutrient status was different between the restored and reference wetlands using various indicators (Chapter 2). Foliar TN:TP ratio varied with species and seasons, and should be carefully used as an indicator of nutrient limitation. Activities of N-related hydrolytic enzymes (i.e., N-acetyl- β -D glucosaminidase, NAG and Leucine aminopeptidase, LAP) were significantly higher at the restored sites but P-related enzymes (Alkali phosphatase, AP and phosphodiesterase, BisP) were significantly higher at the reference site, suggesting different nutrient availability between the restored and reference wetlands. Periphyton at the restored sites fixed more N_2 compared to that at the reference site, especially in the wet season, consistently indicating that the restored sites were more N limited. Soil and periphyton $\delta^{15}N$ values could be indicators of restoration and N availability, but plant $\delta^{15}N$ patterns during

restoration still requires more explicit testing. These results indicated that the microbial activities could be more sensitive to the nutrient status, and should be paid more attention during the restoration process.

Based on the importance of the N_2 fixation of the periphyton, more detailed exploration on periphyton was done (chapter 3 and 4). Periphyton N_2 fixation in the Hole-in-the-Donut (HID) region of the southern Everglades were quantified. Significantly higher N_2 fixation rates (measured by acetylene reduction) were found in periphyton of the areas cleared in 2000 and 2003 ($3\text{--}10 \text{ nmol g}^{-1} \text{ DW h}^{-1}$) compared to the reference wetland site (less than $1 \text{ nmol g}^{-1} \text{ DW h}^{-1}$). Overall rates were stimulated by light (~ 2 times the measured dark rates), and areal estimates of fixed N were low compared to other areas of Everglades, ranging from $0.1\text{--}0.2 \text{ g N m}^{-2} \text{ yr}^{-1}$ in the restored sites to $0.05 \text{ g N m}^{-2} \text{ yr}^{-1}$ in the reference area. Stable N isotopic ratios (i.e., $\delta^{15}\text{N}$) 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 $\delta^{15}\text{N}$ could serve as indicators of nutrient status and restoration success in these systems.

However, limitation in quantifying N_2 fixation in the window of only two months was obvious that, some interesting signals of periphyton N_2 fixation caused by the fluctuating hydrology could be easily missed, especially in the short-hydroperiod wetland ecosystem. Thus, in Chapter 4 much finer sampling strategy was adopted to better quantify the seasonal pattern of periphyton N_2 fixation. Similar seasonal patterns of N_2 fixation were observed among the natural and restored sites, with higher rates in the wet season. At the native reference area, the seasonal pattern was a gradual change throughout the year with a peak observed in September, while most N_2 fixation occurred

during a brief 2–3 month period at the onset of flooding with a peak in July for the two restored sites. An interesting finding is that the N_2 fixation was controlled by both physical (moisture) and chemical (N and P) parameters. Dry season rates were a function of both moisture contents and P contents, while during flooded months excess N levels appeared to suppress nitrogenase activity. Moreover, seasonal patterns of periphyton $\delta^{15}N$ did not strictly correlate with N_2 fixation. It is suggested that $\delta^{15}N$ could be a good indicator of N_2 fixation in a spatial dimension, but would not be as robust in determining seasonal N_2 fixation since the final $\delta^{15}N$ values of periphyton integrate a variety of N sources and processes (e.g., atmospheric deposition, denitrification).

Periphyton as one component of the ecosystem has its own limitation in characterizing N dynamics; therefore the chapter focusing on soil properties followed. Chapter 5 presented the temporal patterns of soil N based on seven samplings across the wet and dry seasons. NH_4^+ and NO_3^- dominated the wet and dry seasons, respectively, and accumulated NO_3^- levels were quickly consumed by denitrification at the onset of the wet season. The enzymes LAP and NAG showed different seasonal patterns, suggesting these enzymes were regulated by different factors (e.g., moisture content and substrate availability). Moreover, the decreasing MBC:MBN ratio from spring to summer would tell us the changes of microbial composition with the time where in the summer, more root exudates would favor the growth of bacteria.

As in many other systems, in the HID restoration, fire is both a natural process and is considered a technique to further enhance the restoration after the complete soil removal. Therefore it was important to investigate the effects of fire on the N cycle at the restored and reference sites. Different responses were observed immediately (2 days

after the fire), after a short term (1 month after the fire), and a long time (~1 year later) following the fire. Available inorganic N ($\text{NH}_4\text{-N}$ and $\text{NO}_x\text{-N}$) was elevated immediately after the fire in all the three sites. The N-mineralization related parameters in the P-limited reference site showed a greater response to the fire compared to the restored site.

Synthesis

Various indicators were used in this work to demonstrate the nutrient status of restored and native reference wetlands. Though, foliar TN:TP ratio has commonly suggested as a useful indicator of nutrient limitation, this study showed it temporally and spatially varied, and should be used with caution. In contrast, microbial activities, especially the enzyme activities, appear to be more sensitive to changes of nutrients, and thus, would serve as more robust indicators of nutrient impacts.

N_2 fixation, as an important function of periphyton, is a major N source for the restored sites. Based on the rough N budget, it would take approximately 40 years to accumulate the equivalent amount of N as the reference site. The temporal patterns of periphyton N_2 fixation confirmed the importance of the hydrology. It is also the first time that periphyton N_2 fixation has been investigated in detail in the short-hydroperiod, marsh prairie wetlands ecosystems in southern Everglades. Further study is undoubtedly needed to better clarify the relationship of periphyton species composition and its function of N_2 fixation, which would explain the different seasonal pattern of N_2 fixation between the restored and reference sites.

The temporal pattern of soil N processes reflects the response to a variety of factors like temperature, precipitation, and plant growth. This study showed opposite seasonal trends for the extractable NH_4^+ and NO_x^+ , which would correlate with the two

interconnected processes of denitrification and nitrification. It should be highlighted that NO_3^- was accumulated in the dry season and consumed by denitrification at the onset of the wet season, indicating the potential pulse of N_2O once re-wetting after the dry period and its significance in the greenhouse gas emission. The unexpected peak of N_2O in the extreme dry season (i.e., May 2012) should also be paid attention together with the microbial composition inferred from high MBC:MBN, which would potentially suggest the fungi-dominated denitrification pathway.

When integrating the temporal patterns of periphyton N_2 fixation and soil N dynamics, we could get more in depth of the N budget. At the restored sites, both periphyton N_2 fixation and denitrification reached their peaks in July, if soil microbes could also fix N_2 and showed same peak in July, then addition and loss of N could potentially cancel one another, it is uncertain what the net effect of the N budget. Moreover, unlike the long hydroperiod wetlands, the dry and wet period alternate more frequently in short-hydroperiod ecosystems. Accordingly, the seasonal patterns would display differently as shown in this study. It is interesting to know how important the peaks of N_2 fixation and N_2O emission that narrowed only within 2-3 month in the annual N budget.

The results of the fire effects on the N cycle are not conclusive, and many questions remain to be answered with future research. For example, little is known about the mechanisms of the increase of available N after fire in wetland ecosystems. It could be the stimulatory effects of either P addition through ash residue or the adsorptive ability of charcoal on the N cycle. The understanding of the mechanism will facilitate prescribed fire plan since the fire intensity decides the fraction of ash and

charcoal. Moreover, the different effects of fire on the periphyton N_2 fixation between the restored site with high P and the reference site with low P further provide a clue for the management that different frequency and intensity fire would be performed differently in the P-limited reference site and N-limited restored sites (Hogue and Inglett 2012).

Chapter 4 and 5 focused on the temporal patterns of N cycle highlighting the importance of hydrology in regulating the N cycle especially in the short-hydroperiod HID; whereas Chapter 6 emphasized that fire as another management tool also shape the Everglades restoration. After we know the N processes remarkably vary with season, the interaction between fire and hydrology should further be considered. For example, the responses of N cycle to a fire that occurs in a dry season would differ from the responses to a fire that occurs in a wet season.

Overall, this study tried to investigate the spatial and temporal patterns of N cycle in the midst of the large-scale and long-term restoration. A unique ecosystem of HID was selected which simultaneously integrates all the elements of restoration, nutrient limitation, short-hydroperiod, and fire together. These important features further make the study of N cycle more complex but never less interesting. It is the first time to document the N dynamics in detail in the southern Everglades. The periphyton N_2 fixation rates were measured, expanding our knowledge of periphyton that was previously discussed mostly in the northern Everglades and in the biomass and species composition. The seasonal patterns of periphyton N_2 fixation and soil N dynamics require a keen awareness of the different impacts of short hydroperiod on N cycle. The standing-out peaks of N_2 fixation and denitrification rates in a 2–3 month window will give us more thoughts on the annual N budget. Fire together with hydrology is the other

important management tools for wetland restoration. The disturbance of fire makes the N cycle more complex but in the HID it highlighted the spread impacts of P on the N-cycle, such as N availability and N₂ fixation. To restore the P-enrichment wetlands to the original oligotrophic status, it is as important to understand the N cycle as well as the P.

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BIOGRAPHICAL SKETCH

Xiaolin Liao was born in a small city of Jingmen, Hubei Province in China. She had her happiest childhood there enjoying the peaceful life and got the basic knowledge from schools.

After successfully passing the competitive entrance exam to college in 2002, she chose the University of Science and Technology in Beijing China, far away from her hometown, to do her undergraduate study. It was her first time to leave home and stay with peers from different places of the country. However, she likes to make friends with diverse background and it came to her that she built a very solid and helpful friendship during the four undergraduate years. At that time, her major was Environmental Engineering, learning a wide range of knowledge on waste water treatment, air pollution control, and waste disposal, and visiting real factories that put those knowledge into reality. She was impressed by a constructed wetland for waste water treatment once visiting in a petrochemical company, and since then her journey for the wetlands began.

Based on the well-rounded performance during the undergraduate, Xiaolin was recommended for admission without exam to the graduate school in the Beijing Normal University in 2006. She chose to study on the wetland ecology in the top-ten Environmental School in China. During the three years of graduate studies, she got chances to know more about wetlands, e.g., the coastal wetlands in the Yellow River Delta in China, the urban wetlands in Jinnan city of Shandong Province, and the constructed wetland in Beijing. She spent the humid summer and chilly winter in the Yellow River Delta with other colleagues from different institutes studying the biodiversity conservation of endangered birds. Her passion with wetlands increased which further pushed her to go further to know the wetlands around the world.

With the dream of wetlands, Xiaolin was luckily accepted by the Soil and Water Science Department in the University of Florida where she later realized is a center of wetlands studies and for wetlands scientists. She followed her advisor-Dr. Patrick Inglett's study on the biogeochemistry of wetlands in the Everglades. For a foreigner who needs to jump the language barrier, it is really a tough experience to get through. However, Xiaolin finally survived and received his Ph.D. from the University of Florida in the summer of 2012.