CHARACTERIZATION OF NITROGENASE GENE DISTRIBUTION AND ACTIVITY IN WCA-2A PERIPHYTON

By

PUJA JASROTIA

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by
Puja Jasrotia
To my advisor, Dr. Andrew V. Ogram, for always believing in me.
ACKNOWLEDGMENTS

The important thing in science is not so much to obtain new facts
as to discover new ways of thinking about them.

Sir William Bragg (1862 - 1942)
British physicist

These two lines summarize the teachings of Dr. Andrew V. Ogram, my advisor. His guidance and “questions for thought” kept me inquisitive and challenged throughout my academic program. His unconditional support and perseverance helped me wade through the most difficult of situations during research.

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CHARACTERIZATION OF NITROGENASE GENE DISTRIBUTION AND ACTIVITY IN WCA-2A PERiphyton

By
Puja Jasrotia
August 2005

Chair: Andrew V. Ogram
Major Department: Soil and Water Science

Periphyton mats of oligotrophic Everglades are composed of taxonomically and metabolically diverse microbial groups stratified in distinct layers contributing to mat’s internal nutrient cycling. Agricultural activities in south Florida have resulted in changes in the Everglades ecosystem. Effects of eutrophication were evaluated in WCA2A periphyton on N₂ fixing and methanotrophic assemblages along the nutrient gradient. The objectives of this study were addressed in three interdependent sections. Section I focused on compositional analysis of diazotrophs. Phylogenetic analysis indicated that oligotrophic periphyton mats of WCA-2A possess metabolically diverse diazotrophs. The mechanisms responsible from O₂ protections can be spatial and temporal separation. In eutrophic areas, though, the diversity was limited to cyanobacterial sp. However, the groups responsible for actively fixing N₂ cannot be ascertained with DNA based analyses. Section II involved studying the RNA transcripts from the diazotrophic groups actively expressing nitrogenase on a diel basis, so that nitrogen fixation could be attributed to
either phototrophs or heterotrophs in epiphytic mats. The results revealed a diversity of phototrophs responsible for diazotrophy and also identified potential nitrogen fixers. The diel pattern was observed amongst the cyanobacterial groups, which suggests that the mat may comprise predominantly of nonheterocystous bacterial spp., as they were also responsible for daytime expression of nitrogenase. Although the exact mechanism from O₂ protection is unknown, there may be temporal separation involved. These results indicate that oligotrophic epiphytic mats are N-limited, and fix atmospheric N₂ for their growth. Section III of this study analyzed the diazotrophic methanotrophs along the nutrient gradient by studying pmoA. Phylogenetic distribution of diazotrophs revealed methanotrophic sequences as potential N₂ fixers; they are also important for oxidizing methane produced in this marsh. Type I methanotrophs dominated in all three sites typically seen under N-limiting conditions. However the transient site contained type X and type II methanotrophs. Presence of methanotrophs in periphyton mats can be attributed to oxic zones present in these mats. These data provide evidence of genetic diversity of nifH expression in epiphyton over a 24-hr period, and spatial distribution of nifH and pmoA genes in periphyton. However, little is known of the factors that regulate diazotrophic communities in periphyton mats. nifH and pmoA sequences will provide targets for designing quantitative studies on the role of diazotrophs/methanotrophs. Collectively, these data indicate compositional shifts in diazotrophic assemblages from a metabolically diverse community in oligotrophic sites to dominance of cyanobacterial species in nutrient impacted areas. However, active nitrogen fixers in oligotrophic areas are the cyanobacterial species.
CHAPTER 1
INTRODUCTION

Periphyton mats are an integral ecosystem component of the Florida Everglades whose distribution and composition are key indicators of nutrient impact and restoration (McCormick and Stevenson, 1998). Over the past few decades, nutrient enrichment from agricultural runoffs and increased urbanization has affected the hydrology and vegetation in Everglades. WCA2A had the most pronounced nutrient gradient, evident by changes in native vegetation and disappearance of important cohesive calcareous periphyton mats. In oligotrophic sites, periphyton processes are integral to nutrient cycling and primary productivity of this freshwater marsh. Periphyton mats may be a significant source of fixed nitrogen and carbon for these systems, and serve as an important component of the food web. There have been no reports on molecular characterization of the nitrogen fixing assemblages or groups actively expressing nifH the gene encoding nitrogenase reductase. This study documents the genetic potential for nitrogen fixation and identifies potential diazotrophic groups in periphyton mats along the nutrient enrichment gradient of WCA2A. It also provides evidence of active nitrogen fixing groups, some of which have not been described before.

The Florida Everglades

The Florida Everglades, the largest freshwater subtropical wetland in North America, have developed in a low nutrient environment in a limestone depression as a result of organic matter accumulation (Gleason and Stone, 1994). To support agricultural activities and urban development, surface water was controlled by draining more than
10,000 km² of Everglades, which has reduced it to its present day form, which is only 50% of its original extent. The predrainage Everglades ecosystem was predominantly composed of *Cladium jamaicense* (sawgrass), wet prairies, sloughs, tree islands, marl-forming marshes, and short-hydroperiod peripheral marshes (Davis et al., 1994). Construction of an extensive network of canals and dikes was undertaken to drain and compartmentalize the area into multiple hydrologic units: the Everglades Agricultural Area (EAA), the Water Conservation Areas (WCAs; WCA-1, 2A, 2B, 3A, and 3B) (Fig. 1-1) and Everglades National Park (ENP) (South Florida Water Management District, 1999). In WCA-2A, canal waters enriched in phosphorus and other nutrients that drain agricultural lands are discharged slowly southward across the marsh. This has created a complex water quality gradient as described in McCormick et al. (2001). Total phosphorus (TP) concentrations in canal waters range between 100–300 µg L⁻¹ in peripheral areas to 5-10 µg L⁻¹ in the marsh interior (Belanger et al., 1989; McCormick et al., 2001). This has resulted in significant changes in the species composition of aquatic macrophytes and periphyton in the northeast portion of WCA-2A. Phosphorus enrichment has resulted in replacement of over 10,000 ha sawgrass and slough habitat with of cattail in the northern marsh (Davis, 1991; Urban et al., 1993). The Everglades restoration plan aims to restore the biotic integrity by controlling excess nutrient loading and reestablishment of a more natural hydrology (South Florida Water Management District, 1999).

**Floating Periphyton Mats**

Periphyton can be defined as a complex community of bacteria, algae, and fungi that are embedded in a laminated matrix of microbially produced mucilage and calcium carbonate (CaCO₃). Periphyton mats exhibit distinct stratification of the microbial
community into distinct layers. In this regard, periphyton mats are structurally similar to microbial mats. Microbial mats are laminated complex microbial assemblages typically composed of phototrophic and chemotrophic prokaryotes that participate in numerous processes. Since phototrophs are the dominant feature of a microbial mat, they are often referred to as cyanobacterial mats. They are found in many different aquatic habitats, including hydrothermal vents, hot springs, sediments, ponds, and lakes (Jeanthon, 2000; Moorhead et al., 1997; Nold and Ward, 1995; Nubel et al., 2000). Microbial processes in mats include photosynthesis, sulfate reduction, methanogenesis and nitrogen fixation (Bebout et al., 1994; Canfield and Des Marais, 1993; Hoehler et al., 2001). The vertical stratification of the community is representative of the response of organisms to gradients of light, oxygen, sulfide, and pH in relation with their physiological requirements.

Periphyton grows as thick cohesive mats at soil surface (benthic), attached to macrophytes (epiphytic) and at the water surface floating mats start as benthic mats and rise up as a result of trapped gases. Photosynthetic activity of the mat phototrophic microorganisms influences pH changes leading to the precipitation of CaCO₃ within the mat structure (Gleason and Stone, 1994). Marsh water chemistry and nutrient cycling profoundly affected by periphyton processes include: oxygen release and consumption (Belanger et al., 1989); nutrient uptake and storage (McCormick et al., 1998; Wetzel, 1996); and chemical (e.g., calcium) precipitation (Gleason and Stone, 1994). Natural periphyton mats thrive under low nutrient conditions and eutrophication radically altered periphyton structure and composition; the native periphyton composition has been eliminated from areas that receive elevated phosphorus inputs from agricultural drainage canals (McCormick and O’Dell, 1996; Swift and Nicholas, 1987; Vymazal et al., 1994).
Total periphyton phosphorus concentrations in WCA2A are tabulated in Table 1-1. Accompanying the shift from sawgrass to cattail at eutrophic sites are changes in periphyton microbial communities. Calcareous periphyton communities, including cyanobacterial species *Scytonema* and *Schizothrix* and diatoms found in oligotrophic sites of the Everglades are replaced by a noncalcareous periphyton dominated by cyanobacteria (e.g., *Lyngbya* sp. and *Oscillatoria* sp.), filamentous green algae (e.g., *Spirogyra* sp. and *Mougeotia* sp.) and other pollution indicator species at nutrient enriched sites (Browder et al., 1994; McCormick et al., 1998; McCormick and Stevenson, 1998).

Periphyton species composition is controlled by seasonality, sunlight, time available to grow since the last water fluctuation, nutrients (mainly nitrogen and phosphorus) and grazing by invertebrates. Eutrophication has also inversely impacted the productivity of periphyton communities. Bacteria within periphyton mats rely on the autotrophic production of algae, and a decrease in photosynthetic productivity (due to dense shading by *Typha*) leads to decreased heterotrophic activity (Neely and Wetzel, 1995). This reduction of both photosynthetic and heterotrophic microbial productivity in response to *Typha* invasion likely reduces nutrient assimilation and retention capacities of periphyton, and therefore of the entire wetland ecosystem (Grimshaw et al., 1997). Periphyton is also considered base of the food web, and for these reasons periphyton characteristics are considered important indicators of the success strategies for management and restoration of the Everglades (South Florida Water Management District, 1999).
Epiphyton “Sweaters”

Browder et al. (1982) reported dense algal growths on live and dead stems of macrophytes in the Everglades. These epiphytic ‘sweaters’ form a large proportion of the periphytic community. Several lines of evidence indirectly support the hypothesis of a loose nonobligate “ectosymbiotic” nutrient interaction between epiphyton and the plants they grow on. The benefits to epiphyton include provision of an advantageous location for growth (i.e., elevated in the water column, where access to light is greater) and access to secondary nutrient sources from the substratum, as well as the water column. Floating periphyton mats supports a rich diversity of microorganisms including algae, diatoms, cyanobacteria, and eubacteria (Fallon et al., 1985, Green and Edmisten, 1974; Jones, 1980). The habitat for epiphyton colonization suggests closely regulated carbon cycling. Other reports referenced in salt marsh ecosystems have focused on understanding the processes involved in the release of dissolved organic carbon by algal cells associated with the *Spartina* stems and leaves, and its support of bacterial growth. The surfaces of living cells may also provide micro environmental conditions favorable for bacterial processes (e.g., nitrogen fixation) that otherwise could not occur under water conditions (Currin and Paerl, 1998; Green and Edmisten, 1974; Newell et al., 1992). In general, the highest rates of nitrogen fixation are associated with epiphytic communities dominated by cyanobacteria (Green and Edmisten, 1974), although Newell et al. (1992) attributed high rates of epiphytic N₂ fixation to heterotrophic bacteria. However, there are also important differences between these communities. Like floating mats, the epiphytic mats are laminated, however they also include a fungal component not usually found in benthic or floating mat. Epiphytes are subject to periodic desiccation, which may limit the grazing community. The epiphytic community is also further removed from
porewater nutrient fluxes than sedimentary microbial mats. Therefore, it may be expected that species composition and factors controlling rates of N$_2$ fixation may be different between epiphytic and benthic or periphyton communities.

**Nitrogen Fixation in Wetlands**

Nitrogen input sources in wetlands are anthropogenic, precipitation, and/or biological N$_2$ fixation. Nitrogen can be found both in organic and inorganic forms. Organic N exists as detritus and soil organic matter in wetland soils. The dissolved inorganic forms, ammonium (NH$_4^+$), and nitrate (NO$_3^-$), are available for plant and microbial uptake (Howard-Williams and Downes, 1993; Mitsch and Gosselink, 1986). Nitrogen is most frequently found limiting in wetlands because it is continually depleted by microbial denitrification, soil erosion, leaching and chemical volatilization (DeBusk, 1999), and nitrogen fixation is important to the overall N budget of wetlands. Nearly 20% of global N$_2$ fixation occurs in wetlands because of the favorable water and nutrient status for N$_2$ fixing organisms. The habitat for N$_2$ fixers can be the water column, aerobic soil layer, anaerobic soil layer, oxidized rhizosphere of the plants, leaf and stem surfaces of the plants. Higher concentrations of ammonia, nitrate, or oxygen are known to inhibit nitrogen fixation (Hoover, 2000).

**Nitrogen Fixation in Microbial Mats**

Cyanobacterial mats contain diverse cyanobacterial taxa, including heterocystous cyanobacteria, filamentous nonheterocystous cyanobacteria, and unicellular cyanobacteria that exhibit high rates of nitrogen fixation (Bebout et al., 1993; Bebout et al., 1994; Paerl, 1994; Paerl et al., 1996). However, N$_2$ fixation may also be driven by the activities of anoxygenic photoautotrophs, including green and purple bacteria (Pinckney
and Paerl, 1997; Wahlund and Madigan, 1993) or heterotrophic bacteria, fueled by photosynthate released from phototrophs (Paerl et al., 1987; Paerl, 1990).

**Nitrogen Cycle**

Nitrogen cycling is a series of microbially mediated transformations of nitrogen in the environment. Although nitrogen gas accounts for approximately 80% of the earth’s atmosphere, plants and animals are often limited by nitrogen, which is needed for growth. This situation arises because $\text{N}_2$ is a very stable molecule and is not bioavailable to most of the organisms for the formation of biologically crucial, nitrogen-containing compounds such as amino acids, proteins and nucleic acids. Nitrogen limitation within a system can lead to reduction in growth due to the high N demand. Excess N, on the other hand, can lead to eutrophication and effect biodiversity (Flite et al., 2001; Gustafson and Wang, 2002; Howard-Williams, 1985).

The cycling of nitrogen among its many forms is a complex process. Four major microbial processes that dominate the biogeochemical cycling of nitrogen are nitrogen fixation, nitrification, denitrification and nitrogen mineralization (Figure 1-2). Nitrogen gas is incorporated into bacterial and plant tissue as ammonia through nitrogen fixation. Under natural conditions, nitrogen fixation is the main pathway by which new nitrogen enters terrestrial ecosystems. Ammonia can be oxidized to nitrates. Nitrate can be reduced to nitrite and to nitrous oxide, or back to nitrogen gas.

**Nitrogen Fixation**

Nitrogen fixation is a process by which atmospheric nitrogen is made available as ammonia. It can be summarized as follows:

$$\text{N}_2 + 16\text{H}^+ + 8e^- + 16\text{MgATP}^{2-} + 18\text{H}_2\text{O} \rightarrow 2\text{NH}_4^+ + \text{H}_2 + 16\text{MgADP}^- + 2\text{OH}^- + 16\text{H}_2\text{PO}_4^-$$
Nitrogen fixing ability is limited to prokaryotes (Eubacteria, Archaea, and Actinomycetes) (Postgate, 1982; Stewart, 1975; Young, 1992). The amount of nitrogen fixed depends on the interaction between environmental conditions and qualitative and quantitative diversity of the nitrogen fixers (also termed diazotrophs) that can vary from barely detectable to hundreds of kilograms per hectare per year (Hubbel and Kidder, 2003). Although diazotrophs have access to unlimited supply of nitrogen in the form of atmospheric dinitrogen gas (N$_2$), the ability to fix N$_2$ is dependent on the organism’s ability to effectively express, protect, and utilize the enzyme nitrogenase.

**Taxonomic Composition of Diazotrophs**

Diazotrophs are ubiquitous in different environmental, ranging from oceans, fresh waters, soil, root systems of leguminous plants to termite guts. Symbiotic bacteria carry out the most important category of nitrogen fixation, which is limited to a few phylogenetic groups. The most common symbiotic nitrogen fixers are *Rhizobium* species associated with legumes (soybeans, peas, etc.) and *Frankia* species (actinomycete bacteria) associated with alder, *Ceanothus*, and other non-legume woody species. Other symbiotic associations involve *Anabaena azollae*, a nitrogen fixing cyanobacterium, which lives in pores on the fronds of a water fern *Azolla*. However non symbiotic, or free living, N$_2$ fixing bacteria are well known and belong to a wide range of phylogenetic groups including obligate anaerobes (e.g. *Clostridium* sp., *Desulfovibrio* sp.), facultative anerobes (e.g. *Klebsiella pneumoniae*), photosynthetic bacteria (e.g. *Rhodobacter capsulatus*), cyanobacteria (e.g. *Anabaena* sp.), obligate aerobes (e.g. *Azotobacter vinelandii*) and methanogens (e.g. *Methanosarcina barkeri*).

Symbiotic nitrogen fixers have the highest rates of nitrogen fixation, typically 5-20 g m$^{-2}$ yr$^{-1}$. Phototrophic symbionts such as *Nostoc* in association with *Azolla* in rice...
paddies often fix 10 g m\(^{-2}\) yr\(^{-1}\). When *Nostoc* is a free-living phototroph, it fixes about 2.5 g m\(^{-2}\) yr\(^{-1}\). In contrast, free-living heterotrophs fix only 0.1-0.5 g m\(^{-2}\) yr\(^{-1}\).

**Genetics and Regulation of Nitrogenase**

Nitrogenase, which consists of dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein) is a highly oxygen sensitive enzyme that catalyzes the reduction of N\(_2\) to NH\(_4^+\). The electrons are first transferred to dinitrogenase reductase that donates electrons to dinitrogenase. The reduction of N\(_2\) has a high-energy requirement, and therefore occurs only where the bacterium has an abundant carbohydrate supply. As a result, nitrogenase expression is under strict transcriptional control (Robinson et al., 1987). Among nitrogen fixers, energy demands for phototrophs are met through photosynthesis. For heterotrophic and chemolithotrophic nitrogen fixers, oxidation of organic matter or redox reactions provide the reducing power. Nitrogenase consists of two protein fractions: the Mo-Fe containing protein and the Fe containing protein. Neither is active without the other. During reduction, the N\(_2\) molecule is progressively reduced to form partially reduced intermediates, which does not get released from the enzyme, until finally NH\(_3\) is formed and released (Fig. 1-3). Nitrogenase can also catalyze the reduction of many other small triple bonded molecules. Amongst these, acetylene has become a particularly important substrate because its reduction product, ethylene, can be detected readily and quantified with great sensitivity by gas chromatography; this assay is extensively employed as an indirect assay to detect nitrogen fixing activity (Fisher et al., 2000).

**Alternative nitrogenases:** Molybdenum starved *A. vinelandii* expresses vanadium containing *vnf* encoded (vanadium dependent nitrogen fixation) nitrogenase and an alternative only Fe containing *anf* encoded (alternative nitrogen fixation) nitrogenase.
Expression of each nitrogenase is under hierarchical control, dependent on availability of either Mo or V (Jacobson et al., 1986). Whenever Mo is available, expression of the Mo-dependent nitrogenase is stimulated and the expression of others is repressed.

**Nitrogen Fixation (nif) Genes**

At least 20 genes are involved in biological nitrogen fixation (Fig. 1-4). In conditions of nitrogen limitation, genes are regulated positively and nitrogenase is expressed. In oxic conditions and high ammonium concentrations, the genes are regulated negatively, repressing the expression of genes and hence no nitrogen fixation takes place (Hoover, 2000). Interactions between microorganisms, such as the transfer of photosynthate from autotrophs to heterotrophic bacteria, and endogenous rhythms drive the patterns of nif expression in the environment (Zehr et al., 2003). Genetic analysis of the facultative anaerobe *Klebsiella pneumoniae* has served as a model system for analysis of nif genes of other nitrogen-fixing microorganisms, such as *Azotobacter*, *Azospirillum*, *Rhizobium*, *Enterobacter*, cyanobacteria, *Frankia* and other species. The properties and functions of some of the nif gene products of *K. pneumoniae* are electron transfer system and regulatory functions. The Fe protein subunit of nitrogenase is encoded by nifH. Five genes (nif BNEVQ) are involved in formation of the functional Mo-Fe protein. The genes nifMS are believed to be necessary in the processing of the Fe protein. Ammonia represses nif gene product biosynthesis. The genes involved in glutamine synthetase, an enzyme that regulates ammonia assimilation, are referred to as gln, while ntr denotes genes whose products regulate nitrogen assimilation.

In nonheterocystous cyanobacteria such as *Gloeotheca, Cyanothece, Synechococcus, Pleptonema* and *Pseudoanabaena* sp., the nitrogenase structural genes, nifH, nifD and nifK are organized into a single contiguous operon (nifHDK) which is
similar to that found in *Klebsiella, Azotobacter* and *Rhizobium* spp. (Haselkorn, 1986).

Organization of *nif* in heterocystous cyanobacteria such as *Anabaena, Nostoc* and *Calothrix* sp. differs from this pattern. In the vegetative cells, *nifH* is adjacent to *nifD* but *nifK* is separated from *nifD* by an 11 kb open reading frame known as *nifD* element that disrupts the *nifD* gene in the *nifHDK* operon. The *nifD* element contains gene *xisA*, which encodes a site specific recombinase, responsible for excision of the element. The excision of the element from the chromosome takes place during an advanced stage of differentiation, which results in a contiguous *nifHDK* operon and enables simultaneous expression of all three structural genes (Golden et al., 1988). If *xisA* becomes inactivated, it prevents the excision and no nitrogenase is produced.

**Protection from O₂ by Heterocystous, Nonheterocystous and Heterotrophic Diazotrophs**

Diazotrophs have adapted survival strategies due to sensitivity to oxygen and to avoid irreversible damage to nitrogenase. Major characteristics of cyanobacteria are presented in Table 1-2. Since oxygen poisons nitrogenase, daily variations of nitrogen fixation are observed in microbial mat communities. The patterns of these daily variations are also dependent on the type of diazotrophic cyanobacterium and on the dynamics of light, carbon source and oxygen in the mat. These mechanisms include sequestering the nitrogenase into heterocysts, respiratory protection (localized consumption of oxygen), temporal separation of photosynthesis and nitrogen fixation, and protective conformational changes to the enzyme.

For phototrophic heterocystous cyanobacteria, light is the source of ATP generation and electrons are derived from water and transferred to ferredoxin mediated by photosynthetic electron transport. Microbial mats are characterized by daily fluctuations
of oxygen concentration that can be attributed to heterocystous cyanobacteria (Stal, 1995). The heterocysts are formed as response to conditions of nitrogen limitation by many filamentous species, such as *Anabaena, Nostoc, Scytonema* (Fay, 1992) and are photosynthetically inactive. They do not fix CO₂, nor do they produce O₂ (Bergman et al., 1997).

In nonheterocystous cyanobacteria, the daily pattern of nitrogen fixation is less predictable. It depends largely on the type of organism and prevailing conditions in the mat. The daily pattern of nitrogen fixation by nonheterocystous cyanobacteria is the result of the combined effects of O₂, light and in some cases, sulfide (Stal, 1995). Nonheterocystous cyanobacteria must supply nitrogenase with sufficient energy and low potential reducing equivalents. Most nonheterocystous cyanobacteria (*Lyngbya, Plectonema, Phormidium*) are capable of fixing nitrogen only in micro-oxic or anoxic conditions; however, a few strains can fix N₂ in oxic conditions (Bergman et al., 1997).

Non-heterocystous unicellular cyanobacteria (*Cyanothece sp., Gloeocapsa sp.*) exhibit circadian rhythms in temporal separation of photosynthesis and nitrogen fixation. Stored carbohydrates (source of energy), which accumulate as large granules in thylakoids during the daytime, power the nitrogen fixation during the dark. During respiration during dark, as the carbohydrates supply is exhausted, the oxygen within the cell is also used up, hence lowering the oxygen concentrations. This helps protect the nitrogenase from inactivation by oxygen.

Recent research has also suggested consortial interactions (Paerl and Pinckney, 1996) and molecular diffusion within microbial mats as a strategy to protect nitrogenase
from O₂ inactivation by autotrophic and heterotrophic diazotrophs. The consortial association exists between diatoms, cyanobacteria and heterotrophic bacteria.

Heterotrophic bacteria create anoxic zones within the mat by consuming oxygen. This creates reduced conditions for nitrogen fixation. In return, cyanobacteria release carbon compounds during oxygenic photosynthesis, which may serve as carbon source for these heterotrophs. Nitrogen fixation is also impaired when molecular diffusion rates of oxygen exceed the rate of oxygen removal by respiratory processes. The layered structure of the mat reduces the molecular diffusion of oxygen within the mat, hence ensuring maintenance of anoxic zones (Paerl and Pinckney, 1996).

**Methanotrophs**

In recent years, methane has been recognized as one of the most important greenhouse gases. The concentration in the atmosphere increases at a rate of about 1% per year (Cicerone and Oremland, 1988), out of which wetlands contribute an estimated 15–20% of the total methane emitted (Matthews and Fung, 1987). The capability of microorganisms to utilize methane as a substrate for energy generation is ecologically important with respect to the carbon cycle. Hence the activity of methane oxidizers (methanotrophs) is a considerable sink of methane (Reeburgh, 1980; Reeburgh, 1982; Ward et al., 1987; Whalen and Reeburgh, 1990). Methanotrophs are ubiquitous in the environment, and play important roles in the ecology of terrestrial, marine, and fresh water systems (Hanson and Hanson, 1996).

**Taxonomic Composition of Methanotrophs**

Based on physiological and biochemical characteristics, methanotrophs are divided into two main groups: type I methanotrophs, which are members of the class \( \gamma \)-proteobacteria (e.g., *Methylomonas*, *Methyllococcus*, *Methylomicrobium*,...
Methylothermus, Methylohalobium, Methylocaldum, and Methylobacter) and type II methanotrophs, which are in the class α−proteobacteria (e.g., Methylosinus, Methylocella, Methylocapsa, and Methylocystis) (Dedysh et al., 2000; Dedysh et al., 2002; Hanson and Hanson, 1996). A third group (type X) is phylogenetically related to type I methanotrophs, but has some metabolic attributes characteristic of type II. Type X organisms are gram negative, obligate aerobes, catalase and oxidase producers with intracytoplasmic membranes (Topp and Hanson, 1991). Methanotrophic microorganisms are not only aerobic as there is considerable geochemical evidence of anaerobic methane oxidation (Alperin and Reesburg, 1985) (Table 1-3).

Physiological Characteristics of Methanotrophs

Methane oxidation

All methanotrophs synthesize methane monooxygenase (MMO), a three component enzyme that catalyses the oxidation of methane to carbon dioxide in methane oxidation (Hanson and Hanson, 1996). Two types of MMO are known, characterized by their distribution within soluble (sMMO) or membrane associated, particulate (pMMO) component of cell extracts. pmoA, which is phylogenetically conserved, encodes for α subunit of pMMO (Costello and Lidstrom, 1999; Murrell et al., 1998). The pmoA phylogeny is generally consistent with the 16S rRNA-based phylogeny of methanotrophs and has been used as a molecular marker in numerous environmental studies to assess methanotrophic diversity (Fjellbirkeland, et al., 2001; Henckel, et al., 1999; Horz, et al., 2001; Murrell, et al., 1998; Ogram, et al., 2005). Type I methanotrophs, including the genera Methylomonas and Methylobacter, are notable as they cannot synthesize sMMO, Type I and Type X utilize the ribulose monophosphate pathway for formaldehyde
assimilation (Fig. 1-5), and have an incomplete tricarboxylic acid (TCA) cycle. Unlike type I, type X methanotrophs can synthesize sMMO under copper limited conditions. *Methylococcus capsulatus* is the best studied member of this group (Hanson and Hanson, 1996). Type II methanotrophs differ from type I and type X methanotrophs in that they possess a complete TCA cycle (Fig. 1-5). *Methylosinus* and *Methylcystis* are the major phylogenetic genera describing type II methanotrophs.

**Factors influencing growth**

Mer and Roger (2001) estimated that 60% to 90% of the methane produced in anaerobic zones of wetlands is re-oxidized by aerobic methanotrophs in the rhizosphere and oxidized soil-water interface. The biochemical pathway for methanotrophy suggests that the process limited to oxygen and methane availability. Type II methanotrophs are known to grow preferentially at lower O$_2$ and higher methane concentrations (Amaral and Knowles, 1995).

**Nitrogen fixation capabilities**

Nitrogen fixation capabilities in methanotrophs have also been thought to distinguish these two groups (Murrell and Dalton, 1983). Type II methanotrophs and members of the type I genus *Methylococcus* have been shown to possess nitrogenase (Murrell and Dalton, 1983). However, studies based on DNA hybridization and enzyme assays suggest that some members of the type I genus *Methylomonas* may also be capable of nitrogen fixation (Oakley and Murrell, 1988).

**Need for Research**

Studies conducted to understand the composition and function of periphyton assemblages in Everglades have focused on either defining biogeochemical characteristics or taxonomic composition based on microscopic studies, enzyme assays
Previous studies documenting nitrogen fixation rates in Periphyton mats have estimated yearly contribution of approximately 10 g N m\(^{-2}\) in unimpacted WCA2A (Inglett et al., 2004). However, no studies have focused on identifying diazotrophic members responsible for the process. Periphyton is considered the base of the food web; changes in nutrient content and taxonomic composition may be used as an early warning indicator of eutrophication. Documenting the compositional changes in diazotrophic assemblages in response to varying nutrient concentrations is important in developing microbial indicators of eutrophication.

Chauhan et al. (2004) reported higher methanogenesis rates at enriched compared to unenriched areas in WCA-2A. This translates into active methanotrophic diversity limited by methane and oxygen concentrations. Periphyton mats are at the surface water/atmosphere interface making it one of the habitats for colonization by methanotrophs. Assessing the diversity of methanotrophic composition will facilitate in defining the interrelated biogeochemical cycling in periphyton mats.

**Objectives**

This study had the following objectives:

- To investigate differences with regard to the diazotrophic assemblages in eutrophic and more pristine regions of the WCA-2A based on \(nifH\) diversity.
- To assay \(nifH\) gene expression and nitrogenase activity during a diel cycle.
- To assess the spatial distribution of genes characteristic of methanotrophic bacteria (the particulate methane monooxygenase gene; \(pmoA\)) in floating periphyton along the nutrient gradient.

**Hypotheses**

- Periphyton \(nifH\) composition differs between the eutrophic and the oligotrophic regions as a result of relative N limitation, although composition may vary with environmental conditions such as seasonal shifts and nutrient concentrations.
• A shift in the most active nitrogen fixing groups will be observed throughout a diel cycle, most likely as a result of the oxygen concentration within the mat during the cycle. Non-heterocystous species will be most active during the night, when oxygenic photosynthesis does not occur. Heterocystous species will be most active during the day during oxygenic photosynthesis. Non-cyanobacterial nitrogen fixers (heterotrophs) will be most active during the night.

• *pmoA* diversity will vary along the nutrient.
Figure 1-1 Location of WCA-2A. The eutrophic zone, F1, is dominated by cattail (*Typha domingensis* Pers), the transient zone, F4, is dominated by a mixture of cattail and sawgrass and the pristine zone, U3, is dominated by sloughs and sawgrass (*Cladium jamaicense* Crantz) (DeBusk et al., 2001).
Table 1-1 Total phosphorus concentrations in Everglades periphyton

<table>
<thead>
<tr>
<th>Location</th>
<th>Habitat</th>
<th>Periphyton P concentrations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>WCA-2A</td>
<td><em>Typha</em></td>
<td>1900 – 3390</td>
<td>Belanger et al., 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2500 – 3750</td>
<td>Craft and Richardson, 1998</td>
</tr>
<tr>
<td></td>
<td><em>Typha/ Cladium</em></td>
<td>500-1750</td>
<td>Craft and Richardson, 1998</td>
</tr>
<tr>
<td></td>
<td><em>Cladium</em></td>
<td>&lt;100</td>
<td>Craft and Richardson, 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;100-640</td>
<td>Belanger et al., 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td>132-385</td>
<td>Davis, 1991</td>
</tr>
</tbody>
</table>

Figure 1-2. The nitrogen cycle
Figure 1-3 Nitrogenase enzyme complex (Taiz and Ziegler, 1998).

Figure 1-4 Organization of *K. pneumoniae* nif genes and functions of their products

*nifJ  nifHDKTY  nifENX  nifUSVWZM  nifF  nifLA  nifBQ*

- Electron transfer
- FeMo-co biosynthesis
- Electron transfer
- Regulation
- FeS & FeMo-co biosynthesis
- FeMo-co biosynthesis
Table 1-2. Types and characteristics of nitrogen fixing cyanobacteria (Stal, 1995)

<table>
<thead>
<tr>
<th>Category</th>
<th>Characteristics</th>
</tr>
</thead>
</table>
| Heterocystous cyanobacteria           | • Exclusively filamentous species; differentiate into heterocysts.  
• Strategy: spatial separation of N₂ fixation and oxygenic photosynthesis and protection of nitrogenase in heterocysts  
• Diazotrophic growth under fully oxic conditions  
• Examples: *Anabaena, Nostoc, Nodularia, Calothrix, Scytonema*  
• Occurrence: freshwater and brackish water blooms, paddy fields, microbial mats; symbiotic with variety of organisms |
| Anaerobic N₂ fixing Non-heterocystous cyanobacteria | • Filamentous and unicellular species  
• Strategy: avoidance (of O₂)  
• Induction and maintenance of nitrogenase only under anoxia or low oxygen  
• Examples: *Plectonema boryanum, Oscillatoria limnetica, Synechococcus* sp., *Phormidium* sp.  
• Occurrence: In different environments |
| Aerobic N₂ fixing Non-heterocystous cyanobacteria | • Filamentous and unicellular species  
• Strategy not precisely known (possibly temporal separation of N₂ fixation and oxygenic photosynthesis in concert with other oxygen protection mechanisms)  
• Diazotrophic growth possible under fully oxic conditions  
• Examples: *Gloeothecae, Oscillatoria, Trichodesmium, Lyngbya, Microcoleus*  
• Occurrence: tropical ocean (*Trichodesmium*), carbonate walls and paddy fields (*Gloeothecae*), microbial mats (*Oscillatoria, Lyngbya, Microcoleus*) |

Table 1-3. Major groups of methanotrophic microorganisms

<table>
<thead>
<tr>
<th>Category</th>
<th>Representative species</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic methanotrophs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td><em>Methylomonas</em> species</td>
<td>Hypersaline and alkali lakes</td>
</tr>
<tr>
<td>Type II</td>
<td><em>Methylosinus</em> species</td>
<td>Acid peat bogs; rice paddies</td>
</tr>
<tr>
<td>Type X</td>
<td><em>Methyllococcus</em> species</td>
<td>Flooded rice fields</td>
</tr>
<tr>
<td>Yeasts</td>
<td><em>Rhodotorula</em> species</td>
<td>Deep sea floor</td>
</tr>
<tr>
<td><strong>Aerobic methanotrophs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANME archaeal groups</td>
<td><em>Methanosarcina</em> species</td>
<td>Hydrothermal vents; marine sediments</td>
</tr>
</tbody>
</table>
Figure 1-5. Pathways for the oxidation of methane Abbreviations: CytC, cytochrome $c$; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase. (Hanson and Hanson. 1996).
CHAPTER 2
SPATIAL VARIABILITY OF nifH DIVERSITY OF DIAZOTROPHS IN PERIPHYTON

Biological nitrogen fixation is the process of conversion of atmospheric dinitrogen (N₂) to ammonium (NH₄⁺). It is an important source of fixed nitrogen in many ecosystems, including freshwater wetlands (Howarth and Marino, 1988; Paerl, 1990; Vitousek and Howarth, 1991) as nitrogen is growth-limiting nutrient in many environments, and also limits the productivity of many ecosystems (Vitousek and Howarth 1991). The ability to fix N₂ is widely distributed among members of the Bacteria and Archaea (Young, 1992), and it is important to characterize the composition of diazotrophic assemblages and to understand the mechanisms employed to protect the oxygen sensitive nitrogenase. Cloning and sequencing of one of the nitrogenase structural genes, nifH (the gene that encodes the highly conserved Fe protein of nitrogenase), has provided a database of sequences from diverse terrestrial and aquatic environments (Lovell et al., 2001; Ueda et al., 1995; Steppe et al., 1996; Young, 1992; Zehr et al., 1998).

Agricultural runoffs and urbanization in south Florida has resulted in changes in the nutrient status of Everglades’s ecosystem components. Periphyton, an important Everglades ecosystem component is composed of taxonomically and metabolically diverse microbial groups that are stratified into distinct layers that may contribute to mat’s internal nutrient cycling. Periphyton microbial assemblages are important to nutrient dynamics in Everglades. Calcareous periphyton mats are influenced by water
quality and hydroperiod, which affects species composition and growth rate (Browder et al., 1994; Swift and Nicholas, 1987). As an important biological indicator, it also represents one of the most sensitive indicators of eutrophication in this ecosystem.

Eutrophication has resulted in a well-defined water phosphorus (P) gradient in WCA2A, which is an impounded wetland in Everglades. The P concentrations range between 5 and 10 µg/L in oligotrophic areas, and the values are 10 to 20 times higher in eutrophic sites (McCormick et al., 1998). Previous studies have reported periphyton structural and functional changes are related to increases in P concentrations (McCormick and O’Dell, 1996), however, nitrogen and iron have also been implicated in affecting changes in periphyton mats (Davis, 1994; McCormick et al., 1998, Swift and Nicholas, 1987).

The phototrophic assemblage of periphyton controls the dissolved oxygen concentrations within the mat and carbon dioxide and calcium concentrations in the water column (Gleason and Stone, 1974), via photosynthesis and respiration. These phototrophic communities are likely responsible for nitrogen fixation. Craft and Richardson (1993) reported that biological nitrogen fixation might account for 75% of the nitrogen inputs in WCA-2A. Inglett et al. (2004) estimated nitrogen fixation rates in periphyton mats of approximately 10 g N m⁻² in unimpacted WCA2A floating periphyton. This indicates the presence of nitrogen fixing microbial assemblages; however, it is unclear which specific groups may be involved in the process. The objectives of this study were to determine the distribution of specific nifH genotypes in floating periphyton in WCA-2A. To our knowledge, this is the first molecular characterization of the periphyton diazotrophic assemblages in the Everglades. We hypothesize that periphyton nifH composition differs between the eutrophic and the
oligotrophic regions as a result of relative N limitation, although composition may vary with environmental conditions such as seasonal shifts and nutrient concentrations.

This study will provide an indication of spatial distribution of nif genes across the nutrient gradient and whether nutrient enrichment selects for species different from those in oligotrophic periphyton.

Materials and Methods

Site Description and Sample Collection

Floating periphyton samples were collected in WCA 2A, an impounded wetland located in the northern Florida Everglades. Canal waters enriched in phosphorus and other nutrients that drain agricultural lands are discharged slowly southward across the marsh. This created a complex water quality gradient as described in McCormick et al. (2001). Total phosphorus (TP) concentrations in canal waters have ranged between 100-300 µg L⁻¹ in recent decades compared with TP concentrations of ≤10 µg L⁻¹ in the marsh interior (Belanger et al., 1989; McCormick et al., 2001). As a result, nutrient enriched water in the northeast portion of WCA-2A produced significant changes in the species composition of aquatic macrophytes and periphyton. The oligotrophic marsh interior is composed primarily of sawgrass (Cladium jamaicense Crantz) interspersed with spikerush (Eleocharis spp.) prairies and water lily (Nymphaea odorata Ait.) sloughs. Oligotrophic sloughs also have a characteristic feature, the periphyton, which contributes to high rates of primary productivity in these habitats (Browder et al., 1994). Phosphorus enrichment has been implicated in replacement of these communities with over 10,000 ha of cattail (Typha domingensis Pers.) in the northern marsh (Davis, 1991; Urban et al., 1993). Floating periphyton samples were collected at three sites in WCA-2A, which represented the range of vegetation and nutrient conditions along the gradient: F1
(eutrophic; cattail dominated), U3 (oligotrophic; sawgrass dominated) and the transition region, F4. Grab samples of mats were collected on October 9th, 2002 and October 20th, 2003. Samples were stored on ice and transported to the laboratories in Gainesville. Subsamples for DNA analyses were frozen at \(-70^\circ C\) until analyzed.

**Nucleic Acid Extraction**

Nucleic acids (total DNA) were extracted from periphyton samples with UltraClean Plant DNA kit according to the manufacturer's instructions (MoBio, Solana Beach, CA) but with slight modifications. 50 mg periphyton was thoroughly homogenized either manually or grinding under liquid N\(_2\) (for samples with high CaCO\(_3\) content) after treatment with 1N HCl. Homogenized sample was suspended in 1ml of 1N HCl and vortexed for 1 min. the sample was centrifuged for 1 min. and supernatant was decanted. The recovered pellet was resuspended again in 1 ml of 1N HCl, and the steps were repeated again and followed by resuspending the pellet in 1 ml of TE (Tris-EDTA) buffer (Sambrook et al., 1989). After the extraction, genomic DNA was evaluated on a 0.7% (wt/vol.) agarose gel made in Tris-acetate-EDTA (TAE) buffer (Sambrook et al., 1989). DNA samples were stored at \(-20^\circ C\) until further analysis.

**Amplification of nifH by Polymerase Chain Reaction**

A nested PCR protocol was used to amplify an approximately 460-bp segment of nifH. For first round of the nested reaction; primer pair nifH3 and nifH4 were used, and equal quantities nifH1 and nifH2 were used for the second round of the nested reaction. Details of the nucleotide sequences of the primers used are presented in Table 2-1.

The reaction mixture used for PCR amplification contained 7 µl of distilled H\(_2\)O, 1 µl of each primer (10 pmol/µl), 10 µl of HotStarTaq Master Mix (Qiagen, Valencia, CA), and 1 µl of diluted DNA solution. Primary PCR amplification was carried out in an
iCycler thermal cycler (BIORAD, Hercules, CA) with the following conditions: initial enzyme activation and DNA denaturation of 15 min at 95°C, followed by 30 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min of extension at 72°C, with a final extension of 72°C for 7 min. The same cycling and reaction conditions were used for the second nested reaction. The PCR products were analyzed on a 2% (wt/vol.) agarose gel in TAE buffer to confirm expected size product.

**Amplification of Nitrite Reductase by Polymerase Chain Reaction**

The reaction mixture used for PCR amplification contained 7 µl of distilled H₂O, 1 µl of each primer (10 pmol/µl), 10 µl of HotStarTaq Master Mix (Qiagen, Valencia, CA), and 1 µl of diluted DNA solution. Details of the nucleotide sequences of the primers used are presented in Table 2-1. Touchdown PCR amplification was carried out in an iCycler thermal cycler (BIORAD, Hercules, CA) with the following conditions: initial enzyme activation and DNA denaturation of 5 min at 95°C, followed by 30 cycles of 30 sec at 95°C for denaturation, followed by 10 cycles of annealing for 40 sec at 45°C and 20 cycles for 40 sec at 43°C, and 40 sec of extension at 72°C, with a final extension of 72°C for 7 min. The PCR products were analyzed on a 1% (wt/vol.) agarose gel in TAE buffer to confirm expected size product.

**Cloning of PCR Products and RFLP Analyses**

DNA fragments were inserted into pCRII-TOPO cloning vector and were transformed into chemically competent *Escherichia coli* TOP10F' cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Recombinant colonies were screened for inserts of the expected size (360 bp) by PCR amplification, with nifH1 and nifH2 primer set according to the previously described PCR program. Following screening, the PCR products were digested with the restriction endonuclease *HhaI*
overnight at 37°C. Digested PCR products were electrophoresed through a 4% (wt/vol) agarose gel to visualize the RFLP patterns. Clone libraries were analyzed by analytic rarefaction with the software aRarefactWin (version 1.3; S. Holland, Stratigraphy Lab, University of Georgia, Athens [http://www.uga.edu/~strata/software/]) to confirm that sufficient numbers of RFLP groups were selected to represent the clone libraries from periphyton samples.

**DNA Sequencing and Sequence Analysis**

Selected unique and common clones after comparison by RFLP were sequenced at the DNA Sequencing Core Laboratory at the University of Florida with nifH1 primer. Sequences were compared with previously identified sequences in the National Center for Biotechnology Information database with BLAST (Altschul et al. 1990), and sequences were aligned by ClustalX version 1.8 (Thompson et al. 1997). Phylogenetic trees were generated with TREECON (Van de Peer and De Wachter 1994; Van de Peer and De Wachter 1997) using a neighbor joining method. Bootstrap analysis for 100 replicates was performed to estimate the confidence of tree topologies.

**Results**

Altogether, 140 clones from F1, 109 clones from F4 and 109 clones from U3 were selected for digestion with *HhaI*. Clones from F1 were grouped in 16 operational taxonomic units (OTU), from F4 in 11 OTU and U3 in 15 OTU for classification based on RFLP patterns. Rarefaction analysis based on the numbers of clones per OTU showed a saturation curve, indicating that almost all the diversity in the clone library was covered (Fig 2-1). Phylogenetic analysis of partial *nifH* sequences from F1 (eutrophic), F4 (transition) and U3 (oligotrophic) reveal distinct lineages of cyanobacterial species and other free-living diazotrophs (Fig 2-2, 2-3). Total number of sequences showed that the
nifH diversity was higher in F4 and U3, than in F1. Clones clustered in 4 defined clusters from F1 with majority of sequences (61%) clustering within cyanobacterial clades, and 39% within heterotrophic diazotrophs. From F4, the clustering of clones was within 5 clusters and cyanobacterial cluster represented by 86% of nifH sequences. In U3, 8 clusters were recognized and 54% of nifH sequences clustered within cyanobacterial clades. No RFLP patterns obtained with clones from F1 were similar to patterns obtained from U3. Sequence analysis of clones representative of the RFLP groups from F1, F4 and U3 are presented in Fig 2-4, 2-5, 2-6). Frankia sp. CcI3 sequence was used as an outgroup in the sequence analysis.

Sequences from F1 samples with 91% sequence similarity to known sequences in the database and represented by 54% of clones in the library, clustered with heterocystous cyanobacterial cluster consisting of Nostoc sp. and Anabaena sp. The distinct heterocystous cyanobacterial clade consists of Subsection IV and V, and 7% of the sequences clustered within the unicellular cyanobacterial clade associated to Xenococcus sp. This is consistent with Subsection II representatives Xenococcus sp. and Myxosarcina sp., forming a distinct clade. nifH phylogeny is consistent with cyanobacterial 16S rRNA sequences (Wilmotte, 1994; Givoannoni et al. 1988). There were no sequences detected from F4 and U3 sites clustering with the unicellular cyanobacterial clade. No RFLP patterns were representative of non-heterocystous cyanobacterial group were obtained for F1. A fraction represented by 35% of clones clustered close to δ-proteobacteria. α-proteobacteria cluster was represented by 4% of the clones. The clones clustered with Methylocella silvestris, type II methanotrophs possessing sMMO. M. silvestris is gram-negative, aerobic, non-pigmented, non-motile,
rod-shaped, methane-oxidizer capable of growth between the pH 4.5 to 7 (Dunfield et al., 2003).

Sequences from F4 were distributed throughout the phylogenetic tree, with the majority of the sequences representing cyanobacterial clusters. Clones clustering with *Calothrix* sp., nonbranching filamentous heterocystous cyanobacteria was represented by 5% of sequences. Although belonging to same taxonomic group, *Calothrix* sp. does not cluster with other heterocystous cyanobacterial sequences. Clone sequences cluster of non-heterocystous cyanobacteria represented by *Plectonema boryanum* was only 2%. The majority of clones from F4 (79%) clustered in a novel cyanobacterial clade. Previous studies on cyanobacterial phylogenetic analysis have stressed deep and scattered branching within cyanobacterial clades as deduced by *nifH* phylogeny, which is consistent with 16S rRNA cyanobacterial phylogeny (Giovannoni et al., 1988), hence it is impossible to assign any characteristic designation to this clade. Approximately 5% of the sequences clustered in the δ–proteobacteria clade, represented by sulfate reducing bacteria and *Spirochaeta spp. nifH* sequences for α– proteobacteria group represented by type II methanotrophs and *Rhizobium* sp. was 9%.

Sequences from U3 represented three novel clades; two aligning with cyanobacterial sequences with representation of 18% and 12% clones, respectively, and one with proteobacteria with 5% representation. Very deep and scattered branching makes it impossible to ascribe any designations to these groupings. The remaining sequences clustered with heterocystous cyanobacterial sequences (16%) close to *Anabaena* sp., non-heterocystous cyanobacteria (9%) close to *Plectonema boryanum* and *Lyngbya* sp., α– proteobacteria (26%) close to *Methylobacterium* sp., γ–proteobacteria.
(12%) close to type I methanotroph *Methylomonas methanica* and δ–proteobacteria (2%) close to anaerobe *Desulfovibrio* sp. No clone sequences were recovered from site F1 and F4 clustering with γ–proteobacteria clade.

**Discussion**

Cultivation-independent retrieval of *nif* sequences from different habitats has become a widely used approach to analyze the diversity of nitrogen-fixing bacteria in ecosystems (Lovell et al., 2001; Rösch et al., 2002; Ueda et al., 1995; Widmer et al., 1999; Zani et al., 2000; Zehr et al., 1995, 1998). Nitrogenase gene sequences obtained from periphyton mat samples from F1, F4 and U3 were diverse, including representations from several physiologically distinct groups. Some of the phylotypes recovered from F4 and U3 represented new groups; however, the majority of the sequences were closely related to previously sequenced *nifH* genes in microbial mats.

The results from F1 suggest that heterocystous cyanobacteria were the dominant genotypes during sampling time. Although F1 site has low dissolved O$_2$ concentrations, it is possible that high-localized oxygen pockets exist in periphyton where heterocystous cyanobacteria dominate. In F4, a novel cyanobacterial clade comprised the dominant group, whereas in U3, heterotrophic sequences and cyanobacterial sequences were equally represented. This clearly indicates substantial shifts in the nitrogen fixing assemblages along the nutrient gradient. Previous studies conducted in oligotrophic WCA-2A periphyton (coupled with high TN: TP ratios) suggest periphyton is limited by P and not N (McCormick and O’Dell, 1996), and increased P and N inputs have led to eutrophic conditions in F1 and the shift towards pollution tolerant taxa (McCormick and O’Dell 1996). Based on the genetic diversity of *nifH* partial sequences recovered, U3
periphyton comprised of representative taxa from heterocystous and nonheterocystous cyanobacteria belonging to \( \alpha \), \( \delta \) and \( \gamma \) proteobacteria, indicates potential for nitrogen fixation. Additionally, *Anabaena* and *Lyngbya* are both algal bloom-forming genera, which are selected for by eutrophication; however, our data are insufficient to draw any such conclusions in this case. Detection of heterotrophic sequences reflects the possibility of higher nitrogen fixation activity during the nighttime or consortial nitrogen fixation with cyanobacterial species.

The presence of sequences from anaerobic species (e.g., *Desulfovibrio* sp.) suggests the possibility that either the floating mat had recently broken loose from the benthic layer, (where conditions are more reduced for proliferation of sulfate reducers), or sulfate reducing bacteria expressing *nif* genes were part of the periphyton mat, which suggest anaerobic processes occur. Microbial mats structure is dependent upon nutrient enrichment. Well-formed periphyton mats from oligotrophic Everglades display distinct vertical zonations. The top layer is yellowish brown colored, which is an assemblage of dead plant and animal matter and empty cyanobacterial and mucous sheaths. This top layer has a distinct pigment, sytonemin, which protects it from UV. The bottom light green to dark green layer is most physiologically active and site for metabolic processes including nitrogen fixation. \( \text{O}_2 \) profile studies conducted in the laboratory over a diel period in periphyton mats have conclusively demonstrated anaerobic conditions being present under dark conditions (Scot Hagerthey, SFWMD, personal communication). Rapid oxygen dynamics and relatively slow diffusion of oxygen leads to depletion when darkness stops photosynthesis. Respiration as well as other chemical processes rapidly consumes all oxygen and the mat turns anoxic, often within minutes after photosynthesis.
ceases (Stal, 1995). In the beginning of this study, DNA was extracted from individual layers and PCR amplified with \textit{nifH} primers to assess the presence of nitrogen fixers (data not included). Amplification products were obtained only from middle layer samples.

Detection of \textit{nifH} sequences characteristic of methanotrophs from all three sites is a significant observation, with sequences from F1 and F4 clustering with Type II methanotrophs ($\alpha$-proteobacteria), and U3 cluster with Type I methanotrophs ($\gamma$- proteobacteria) with high sequence similarity. Similar results were obtained with amplification with primers targeting \textit{pmoA}, the gene for pMMO expression (results presented in Chapter 4). This observation is consistent with previous reports documenting methanotrophic \textit{nifH} sequences obtained from freshwater assemblage, oligotrophic oceans, rice roots (Ueda et al., 1995; Zani et al., 2000; Zehr et al., 1998).

Clustering of microorganisms based on \textit{nifH} sequences has important implications for the use of the \textit{nifH} sequence in ecological studies. These results indicate a very tightly regulated nitrogen fixation with little or no buildup of ammonia in the system (laboratory experiments for detection of denitrifiers from all three sites was negative; data not included). The amplified \textit{nifH} fragment provides taxonomic information on organisms from the environment with the genetic potential for N$_2$ fixation and can also be used to determine the taxonomic identity of unknown N$_2$-fixing organisms. This is the first report of mapping \textit{nifH} diversity of periphyton along with assessing the effects of nutrient enrichment in selection of species groups, which is critical for understating the nitrogen cycling within the mat. The results demonstrate that the genetic potential for nitrogen fixation is extremely diverse. DNA based \textit{nifH} assessment is limited in profiling the
community structure and cannot attribute nitrogen fixation potential to either cyanobacterial species, which are primary source of organic carbon in the periphyton mat or the heterotrophic bacteria associated with cyanobacteria. Studying the RNA transcripts, which enabled us to ascribe nitrogenase expression to particular groups of diazotrophs, further assessed this aspect.
### Table 2-1. Nucleotide sequences of primers

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide position</th>
<th>Primer nucleotide sequence (5′-3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>nifH1</td>
<td>639 to 655</td>
<td>5′-TGY GAY CCN AAR GCN GA-3′</td>
<td>Zani et al. 2000</td>
</tr>
<tr>
<td>nifH2</td>
<td>1000 to 984</td>
<td>5′-ADN GCC ATC ATY TCN CC-3′</td>
<td></td>
</tr>
<tr>
<td>nifH3</td>
<td>1018 to 1002</td>
<td>5′-ATR TTR TTN GCN GCR TA-3′</td>
<td></td>
</tr>
<tr>
<td>nifH4</td>
<td>546 to 562</td>
<td>5′-TTY TAY GGN AAR GGN GG-3′</td>
<td></td>
</tr>
</tbody>
</table>

All four of these primers were degenerate (Y = T or C; R = A or G; D = A, G, or T; and N = A, C, G, or T).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide position</th>
<th>Primer nucleotide sequence (5′-3′)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NirS1F</td>
<td>763 to 780</td>
<td>5′-CCT A(C/T)T GGC CGC C(A/C)C A(A/G)T-3′</td>
<td>Braker et al., 1998</td>
</tr>
<tr>
<td>NirS6R</td>
<td>1638 to 1653</td>
<td>5′-CGT TGA ACT T(A/G)C CGG T-3′</td>
<td></td>
</tr>
<tr>
<td>NirK1F</td>
<td>526 to 542</td>
<td>5′-GG(A/C) ATG GT(G/T) CC(C/) TGG CA-3′</td>
<td></td>
</tr>
<tr>
<td>NirK5R</td>
<td>1023 to 1040</td>
<td>5′-GCC TCG ATC AG(A/G) TT(A/G) CA(A/G) T-3′</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2-1 Rarefaction analyses for *nifH* clone libraries for periphyton samples from site F1, F4 and U3.
Figure 2-2. Phylogenetic tree of cyanobacterial nifH clones from F1, F4 and U3. Numbers at branch points refer to bootstrap analysis based on 100 resampling.
Figure 2-3. Phylogenetic tree of proteobacterial nifH clones from F1, F4 and U3. Numbers at branch points refer to bootstrap analysis based on 100 resampling.
Figure 2-4. Clones distribution from eutrophic site F1

Figure 2-5. Clones distribution from transition site F4
Figure 2-6. Clones distribution from oligotrophic site U3
CHAPTER 3
NITROGENASE GENE EXPRESSION IN EPIPHYTON OVER A DIEL PERIOD

Nitrogen fixing capabilities are limited to a few prokaryotes, which fix nitrogen in diverse environments ranging from fresh water ecosystems to oceans, soils, rice roots, guts of termites, sediments (Braun et al., 1999; Ohkuma et al., 1996; Picard et al., 1992; Steppe et al., 1996; Ueda et al., 1995; Widmer et al., 1995; Zehr et al., 1998). Phototrophic cyanobacteria represent an important group responsible for nitrogen fixation in these environments. Cyanobacteria have evolved numerous strategies to protect the oxygen sensitive nitrogenase enzyme from irreversible inactivation from oxygen evolving photosynthesis. These adaptations range from spatial segregation into heterocysts that lack oxygen-evolving photosystem II during the daytime by heterocystous cyanobacteria and temporal separation of photosynthesis during the daytime and nitrogen fixation during the nighttime by members of nonheterocystous cyanobacterial species including unicellular forms. However, these groups are also capable of aerobic nitrogen fixation (Stal, 1995).

Periphyton mats exist as different forms in the oligotrophic Florida Everglades. As floating mats on water surfaces, benthic mats on the sediment bed, as epiphytic mats attached to live or dead stems of macrophytes. Our understanding of periphyton community dynamics and nutrient cycling comes from extensive biogeochemical studies conducted on floating periphyton mats (Gleason and Stone 1994; Inglett et al., 2004; McCormick and O’Dell 1996; McCormick and Stevenson, 1998). Although epiphytic mats have been shown to make significant contributions to marsh primary production
(Lovell et al., 2001) and N₂ fixation (Casselman et al., 1981; Currin and Paerl, 1998; Green and Edmisten, 1974; Newell et al., 1992) in other studies, there is not much known of nutrient dynamics in epiphytic mats in the Everglades. Other ecosystem studies are also limited in elucidating relevant details such as environmental controls on the nutrient cycling within these mats. Epiphyton mats may also contribute to nutrient cycling and primary productivity of this freshwater marsh.

Studies of WCA2A periphyton mats have reported P limitations (McCormick and O’Dell 1996); however, Inglett et al. (2004) reported high rates of nitrogen fixation from WCA-2A periphyton, suggesting possible nitrogen limitation in periphyton.

The first part of the current study indicated that a diverse diazotrophic community is present in these periphyton mats. Molecular detection of nitrogenase genes does not indicate that microorganisms are actively fixing nitrogen. To better understand the relationships between nutrient impact and biogeochemical cycling, it is important to know which nitrogen-fixing microorganisms are present and are expressing the nitrogenase enzyme.

This part of the study was designed to investigate diversity and transcriptional activities of diazotrophic bacteria over a 24-hr period in epiphyton mats in oligotrophic WCA2A. We hypothesized that a shift in the most active nitrogen fixing groups will be observed throughout a diel cycle, most likely as a result of the oxygen concentration within the mat during the cycle. Non-heterocystous species will be most active during the night, when oxygenic photosynthesis does not occur. Heterocystous species will be most active during the day during oxygenic photosynthesis. Non-cyanobacterial nitrogen fixers (heterotrophs) will be most active during the night.
PCR of genomic DNA obtained from epiphytic mat samples was used to identify microbial groups responsible for nitrogen fixation. To monitor expression of genes in the environment, reverse transcriptase PCR (RT-PCR) was employed, which has been used extensively in other studies (Kowalchuk et al. 1999; Noda et al. 1999; Zani et al., 2000). Comparison of sequences obtained by RT-PCR and PCR was used to investigate the diversity of organisms expressing nitrogenase.

Materials and Methods

Site Description and Sample Collection

Epiphyton samples were collected from WCA 2A, an impounded wetland located in the northern Florida Everglades. Canal waters enriched in phosphorus and other nutrients that drain agricultural lands are discharged slowly southward across the marsh. This created a complex water quality gradient as described in McCormick et al. (2001). Total phosphorus (TP) concentrations in canal waters have ranged between 100-300 µg L⁻¹ in recent decades, compared with TP concentrations of ≤10 µg L⁻¹ in the marsh interior (Belanger et al. 1989; McCormick et al. 2001). As a result, nutrient enriched water in the northeast portion of WCA-2A produced significant changes in the species composition of aquatic macrophytes and periphyton. The oligotrophic marsh interior is composed primarily of sawgrass (*Cladium jamaicense* Crantz) interspersed with spikerush (*Eleocharis* spp.) prairies and water lily (*Nymphaea odorata* Ait.) sloughs. Oligotrophic sloughs also have a characteristic feature, the periphyton, which contributes to high rates of primary productivity in these habitats (Browder et al., 1982). Phosphorus enrichment has been implicated in replacement of these communities with over 10,000 ha of cattail (*Typha domingensis* Pers.) in the northern marsh (Davis, 1991; Urban et al.,
1993). Due to environmental conditions, the originally proposed diel gene expression analysis and N fixation rates could not be conducted on floating periphyton samples. Epiphytic “sweaters” collected from U3 (oligotrophic site) in February 2005 were instead used for this part of the study. Epiphyton samples were collected from a slough in oligotrophic U3 in WCA 2A on February 1, 2005. Samples were stored on ice and transported to the laboratories in Gainesville. For mRNA studies, epiphytic samples were taken 5 times over a 24-hr period. Samples were frozen under liquid N₂ and immediately stored at –70°C until analyzed. Genomic DNA extraction and total RNA extraction was conducted from these samples.

**Nucleic Acid Extraction**

**Genomic DNA extraction**

Total DNA was extracted from epiphyton samples with UltraClean Plant DNA kit (MoBio, Solana Beach, CA) according to the manufacturer's instructions, with minor modifications. Approximately 50 mg epiphyton sample were thoroughly homogenized either manually or grinding under liquid N₂ after treatment with 1N HCl (for samples with high CaCO₃ content). Homogenized sample was suspended in 1ml of 1N HCl and vortexed for 1 min. the sample was centrifuged for 1 min. and supernatant was decanted. The recovered pellet was resuspended again in 1 ml of 1N HCl, and the steps was repeated again and was followed by resuspending the pellet in 1 ml of TE (Tris-EDTA) buffer (Sambrook et al. 1989). After extraction, genomic DNA was evaluated on a 0.7% (wt/vol.) agarose gel made in Tris-acetate-EDTA (TAE) buffer (Sambrook et al. 1989). DNA samples were stored at –20°C until further analysis.
Total RNA extraction

Total RNA was extracted from epiphyton using the RNeasy plant minikit (Qiagen, Valencia, CA) according to the manufacturer's protocol with modifications, and resuspended in 30 µl of RNase-free H2O. Approximately 50 mg of epiphyton were thoroughly homogenized either manually or grinding under liquid N2 after treatment with 1N HCl (for samples with high CaCO3 content). Homogenized samples were suspended in 1ml of 1N HCl and vortexed for 1 min. the sample was centrifuged for 1 min. and supernatant was decanted. The recovered pellet was resuspended again in 1 ml of 1N HCl, and the steps were repeated again, followed by resuspending the pellet in 1 ml of TE (Tris-EDTA) buffer (Sambrook et al. 1989). On column DNA digestion was conducted using RNase-Free DNase Set (Qiagen, Valencia, CA) for 15 min at room temperature. After extraction, RNA was evaluated on a 0.7% (wt/vol.) agarose gel made in Tris-acetate-EDTA (TAE) buffer. RNA was either immediately stored at –70°C or was used immediately for reverse transcription reaction (RT-PCR).

Amplification of nifH

Polymerase chain reaction

A nested PCR protocol was used to amplify an approximately 460-bp segment of nifH. For the first round of nested reaction, primer pair nifH3 and nifH4 was used, and equal quantities nifH1 and nifH2 were used for the second round of the nested reaction. Details of the nucleotide sequences of the primers used are highlighted in Table 3-1.

The reaction mixture used for PCR amplification contained 7 µl of distilled H2O, 1 µl of each primer (10 pmol/µl), 10 µl of HotStarTaq Master Mix (Qiagen, Valencia, CA), and 1 µl of diluted DNA solution. Primary PCR amplification was carried out in an iCycler thermal cycler (BIORAD, Hercules, CA) with the following conditions: initial
enzyme activation and DNA denaturation of 15 min at 95°C, followed by 30 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min of extension at 72°C, with a final extension of 72°C for 7 min. The same cycling and reaction conditions were used for the second nested reaction. The PCR products were analyzed on a 2% (wt/vol.) agarose gel in TAE buffer to confirm expected size product (Sambrook et al. 1989)

**Reverse transcription**

Reverse transcription reactions were performed with Acess RT-PCR system (Promega, Madison, WI) with minor modifications of the manufacturer’s protocol. Reaction mix contained 14 µl of RNAse free H₂O, 5 µl of 5X avian myeloblastosis virus buffer, 0.5 µl of a deoxynucleoside triphosphate (dNTP) mixture (10 mM of each dNTP), and 10 pmol/µl of primers nifH3 and nifH4. The reaction mixtures were gently mixed and 0.5µl of avian myeloblastosis virus RT, 0.5µl of Tfl DNA polymerase were then added along with 1 µl of RNA. First strand of cDNA synthesis was done as follows: 1 cycle for 45 min at 45°C for the reverse transcription reaction, 1 cycle for 2 min at 94°C for AMV RT inactivation and RNA/cDNA/primer denaturation. Second strand synthesis and PCR amplification was followed with the following conditions: 40 cycles of 1 min at 94°C for denaturation, 1 min at 57°C for annealing, and 1 min of extension at 72°C, and a final extension of 72°C for 7 min. The second round of the nested PCR was performed with 1 µl (As the final yield of amplified product varied in concentrations, 2µl and 3µl of the first round product were also used for the second nested round) of the first-round product in the reaction mixture containing 7 µl of distilled H₂O, 1 µl of primer nifH1 and nifH2(10 pmol/µl) and 10 µl of HotStarTaq Master Mix (Qiagen). The PCR was carried out with initial enzyme activation and DNA denaturation of 15 min at 95°C followed by
30 cycles of denaturation at 95°C (1 min), annealing at 55°C (1 min), and extension at 72°C (1 min). Two types of negative controls confirmed that the RT-PCR results were from RNA and not from contaminating DNA. The first control used RNAse free water instead of RNA sample, and the second consisted of directly using RNA samples and subjecting them to nested PCR without the RT step.

**Cloning of PCR products and RFLP analyses**

DNA fragments were inserted into pCRII-TOPO cloning vector and were transformed into chemically competent *Escherichia coli* TOP10F' cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Recombinant colonies were screened for inserts of the expected size (360 bp) by PCR amplification, with nifH1 and nifH2 primer set according to the previously described PCR program. Following screening, the PCR products were digested with the restriction endonuclease *Hha*I overnight at 37°C. Digested PCR products were electrophoresed through a 4% (wt/vol) agarose gel to visualize the RFLP patterns. Clone libraries were analyzed by analytic rarefaction with the software aRarefactWin (version 1.3; S. Holland, Stratigraphy Lab, University of Georgia, Athens [http://www.uga.edu/~strata/software/]) to confirm that sufficient numbers of RFLP groups were selected to represent the clone libraries from epihyton samples.

**DNA sequencing and sequence analysis**

Selected unique and common clones after comparison by RFLP were sequenced using nifH1 primer by the DNA Sequencing Core Laboratory at the University of Florida. Sequences were compared with previously identified sequences in the National Center for Biotechnology Information database with BLAST (Altschul et al. 1990), and sequences were aligned by ClustalX version 1.8 (Thompson et al. 1997). Phylogenetic trees were
generated with TREECON (Van de Peer and De Wachter 1994; Van de Peer and De Wachter 1997) using a neighbor joining method. Bootstrap analysis for 100 replicates was performed to estimate the confidence of tree topologies.

Results

Phylogenetic Analysis Based on DNA Sequences

Four clone libraries constructed with samples from an epiphytic mat were screened. A total of 29 clones from library 1, 40 clones from library 2, 40 clones from library 3 and 37 clones from library 4 with inserts of the correct size were obtained from RFLP digestion with HhaI enzyme. Clones from library 1 were grouped in 7 operational taxonomic units (OTUs), from library 2 in 8 OTUs, library 3 in 11 OTUs, and library 4 in 7 OTU. In total 146 clones were screened. The objective of this part of the study was to identify the diazotrophic groups present in the epiphytic samples at the time diel study was initiated. So the samples represent time 0, i.e., Time 11.15, when the diel study was initiated. The rarefaction analysis based on the number of clones per OTU approached saturation, suggesting that almost all the diversity in the clone library was detected (Fig 3-1). Phylogenetic analysis of partial nifH sequences reveals that in addition to cyanobacterial nifH sequences, sequences relating to δ-proteobacteria were found in the epiphytic mat (Figure 3-2). Sequence analyses of clones representative of the RFLP groups are presented in Figure 3-3.

The majority of nifH sequences were distributed amongst the cyanobacterial clades. Clones clustering with nonheterocystous unicellular cluster, including Myxosarcina sp., Xenococcus sp., Gloeothecae sp., Dermocarpa sp., comprised 25% of the total sequences (cluster I). Clones clustering with nonheterocystous cyanobacteria clusters were represented by 11% of the sequences (cluster II). Clone clustering with heterocystous
cyanobacteria, including *Anabaena* sp., *Nostoc* sp., *Calothrix* sp., amounted to 8% of the total distribution (cluster III). Two clone clusters (cluster IV and cluster VI) represented by 19% and 25% of the sequences respectively that clustered with uncultured cyanobacterial clones. Clusters V (10% of the clones) and VII (9% of the clones) remained unidentified, however, since both these clusters branched in between the cyanobacterial cluster, and may represent novel groups of epiphytic mats. Sequences were also obtained clustering with δ-proteobacteria clade represented by *Desulfovibrio* sp.

**Phylogenetic Analysis Based on RT-PCR Amplified cDNA**

A total of 410 cDNA sequences were screened by RT-PCR over a 24 hr period. A total of 47 clones each from library 1 and 2 grouped in 5 OTU each were obtained for time point 7.15. 46 clones from library 1 grouped in 4 OTU and 47 clones from library 2 grouped in 5 OTU were obtained at 11.15 time point. For time point 15.15, 46 clones were grouped in 3 OTU. For 19.15 timepoint, 48 sequences from library 1 were grouped in 6 OTU and 42 sequences from library 2 were grouped in 6 OTU. 47 clones from library 1 and 48 clones from library 2 from time point 23.15 were grouped in 5 OTU respectively. The rarefaction analysis based on the number of clones per OTU approached saturation for each timepoint, indicating that almost all the diversity in the clone library was detected (Figs. 3-4, 3-5, 3-6, 3-7, 3-8). Phylogenetic analysis of partial *nifH* sequences reveals nitrogenase expression was limited to cyanobacterial *nifH* sequences in the epiphytic mat (Figure 3-9). Sequence analyses of clones representative of the RFLP groups are presented in Fig. 3-10, 3-11, 3-12, 3-13, 3-14. Distribution of clones in clades should be assessed with caution; due to relatively few *nifH* sequences
available in the database and deep branching within the cyanobacterial groups, confidence is not high in our groupings (Figure 3-9). This limits our confidence in the bootstrap values assigned to major branches. However, as the clone groups form very strong clades, their presence cannot be ignored. The results are presented considering the closest known type of cyanobacterial grouping:

**Unidentified cluster I:** Clones clustering were represented by 2% clones from time 15.15, 22% from time 23.15 and 2% from time 7.15 (Fig. 3-15).

**Unidentified cluster II:** Clones clustering were represented by 3% clones from time 11.15, 55% clones from time 15.15, 18% from time 19.15, 3% from time 23.15 and 2% from time 7.15 (Fig. 3-16).

**Unidentified cluster III:** Clones clustering were represented by 65% clones from time 11.15, 43% clones from time 15.15, 19% from time 19.15, 15% from time 23.15 and 5% from time 7.15 (Fig. 3-17).

**Unidentified cluster IV:** Clones clustering broadly within the non-heterocystous unicellular cyanobacterial cluster close to *Myxosarcina* sp., *Xenococcus* sp., *Gloeotece* sp., *Dermocarpa* sp., were represented by 19% clones from time 23.15 and 14% from time 7.15 (Fig. 3-18).

**Uncultured cyanobacterium clones cluster:** Clones clustering broadly within the uncultured cyanobacterial cluster were represented by 10% clones from time 11.15, 41% from time 19.15, 32% from time 23.15 and 8% from time 7.15 (Fig. 3-19).

**Unidentified cluster V:** Clones clustering as unidentified cyanobacterial cluster were represented by 19% from time 11.15, and 9% clones from time 19.15 and time 23.15 (Fig. 3-20).
**Unidentified cluster VI:** Clones clustering as unidentified cyanobacterial cluster was represented by 3% clones from time 11.15 and 2% from time 7.15 (Fig. 3-21).

**Unidentified cluster VII:** Clones clustering as 13% clones represented unidentified cyanobacterial cluster from time 19.15 (Fig. 3-22).

Both *nifH* PCR sequences obtained from time 0 and RT-PCR generated similar clone clusters from cyanobacterial clades. A remarkable aspect is the number of unidentified clades, which form very tight groupings in both phylogenetic trees. Clusters V, VI and VII can be regarded as novel clades. There were no non-heterocystous sequences clustering with *Phormidium* sp. recovered from the genomic DNA sample. Sequences clustering with δ-proteobacteria that are detected in PCR of genomic DNA are not detected in any of the RT-PCR sequences, indicating the groups to which nitrogenase activity can be attributed in epiphytic mats.

**Discussion**

To better understand the structure – function relationship of diazotrophic assemblages in epiphytic communities, the first step of this study was to elucidate spatial and temporal distribution of the microorganisms. This analysis was done by studying the *nifH* phylogenetic distribution based on genomic DNA. The next step was to correlate these diazotrophic assemblages with the groups that are actively expressing nitrogenase over a diel period in these mats. This was achieved by RT-PCR analysis. Because RT-PCR amplification begins with messenger RNA (mRNA) rather than DNA, this shows that *nifH* genes are not only present but also actively transcribing. Studying the transcriptional activities of microorganisms is one of the proofs of active metabolic processes such as nitrogen fixation.
DNA based molecular characterization of $\text{nifH}$ within the epiphytic “sweater” demonstrated that several potential diazotrophic cyanobacterial genera (e.g., *Anabaena* sp., *Nostoc* sp., *Plectonema* sp., *Myxosarcina* sp., *Cyanothece* sp.) are present. Sequences characteristic of heterotrophic bacteria clustering with $\delta$-proteobacteria represented by *Desulfovibrio* sp. were also observed. Epiphytic mats are attached to dead or live stems of macrophytes. PCR amplification results with *Clostridia* sp. specific primers were positive, indicating that cellulolytic bacteria may be important component of these mats, which have readily available organic carbon source, and also suggests the presence of anoxic zones where these obligate anaerobic microorganisms may be active. These organisms may either be associated with cyanobacteria mucilage or residing in microenvironments with lower $O_2$ concentrations (Olsen et al., 1998). These results demonstrated the presence of an active phototrophic and heterotrophic microbial community and heterotrophic metabolic processes closely coupled to phototrophic activities as reported in other tropical and temperate microbial aggregates, biofilm and mat studies (Paerl and Pinckney, 1996). These results depicted the community structure and were used to compare the results obtained by RT-PCR.

Our results indicate that epiphytic diazotrophic community as detected by RT-PCR is represented by complex assemblage of clones clustering with cyanobacterial groups expressing nitrogenase at different time point during a 24-hr period. Expression of $\text{nifH}$ transcripts indicates that $N_2$ fixation in these cyanobacterial mats occurs on a diurnal pattern with different groups being dominant at different times of the cycle. Furthermore, the results of this study demonstrate that it was difficult to characterize several clades, which although clustered within cyanobacterial groups had low bootstrap values or
clustered as deep branches unrelated to any known sequences. This limits our cluster
designations and the results are hence presented on the basis of clusters, which were
identified, and their diel distribution.

Unidentified cluster I contribute to relatively small percentage of total RNA of the
afternoon time of 15.15. The group’s activity during daytime suggests that this group
protects nitrogenase by spatially separating nitrogen fixation during daytime with
simultaneously occurring oxygenic photosynthesis as documented in other studies for
heterocyst forming cyanobacteria. However, the group is not detected in any other
daytime samples. Surprisingly, it appears to be the second most dominant group at 23.15
and remains until 7.15. There are reports of nighttime nitrogenase activity detected in
*Anabaena variabilis* strain ATCC 29413 (Thiel et al., 1995). *Anabaena variabilis* strain
ATCC 29413, which is homologous to *Anabaena* PCC 7120, has three nitrogenases, the
second nitrogenase being expressed under anaerobic conditions in vegetative cells (Thiel
et al., 1995). This gives *A. variabilis* a selective advantage to fix N₂ during the day in
heterocysts (aerobic) or at night in vegetative cells (anaerobic). This suggests, that under
appropriate conditions, either of the nitrogenase forms could support diazotrophic
growth. The expression of both *nif1* (daytime fixation in heterocysts) and *nif2* (under
anaerobic conditions in vegetative cells) are interdependent and although *nif2* is fully
functional, it serves as a subsidiary system, and does not suppress expression of the *nif1*
system (Thiel et al., 1995). This physiological capacity is advantageous for the
diazotroph under low levels of fixed nitrogen, especially under anaerobic or microaerobic
conditions, which are frequently encountered during nighttime in microbial mats. The
distribution of *nif2* gene among the cyanobacteria genera is still unclear, and whether it is
evolutionarily closely related to _nifH_ genes in nonheterocystous cyanobacteria (Thiel et al., 1995). Hence it is difficult to assign any groupings as the sequences cluster may be from heterocystous cyanobacteria that are related to _Anabaena variabilis_ or to unidentified nonheterocystous cyanobacteria whose nitrogenase is similar to the second nitrogenase expressed in _Anabaena variabilis_.

In unidentified cluster II, nitrogenase expression is not consistent, and maximum expression is found during 15.15 samples during the daytime, which reduces for the rest of the day and night and then exhibits a maximum at 7.15 in the morning, and follows the same cycle of decreasing during the rest of the day. Such differences can be due to sampling, environmental, or extraction efficiency.

Unidentified cluster III shows remarkable patterns of _nifH_ expression, with a maximum activity around 11.15 during daytime, and decreasing nitrogenase expression during the remaining part of the diel cycle. The 7.15 sample exhibited the lowest number of clones. This means that the nitrogen fixation is continuous process during daytime as well as night time by these groups of diazotrophs.

With such a pattern it is difficult to comment on the physiological adaptation by these two groups. However, there are numerous studies of nitrogen fixation by non-heterocystous cyanobacteria including the unicellular forms under fully oxic conditions at the same time oxygenic photosynthesis is carried on (Fay, 1992; Stal, 1995). Though the strategies by which these organisms protect nitrogenase during daytime is still unknown, it is suggested that temporal separation might be the key (Stal, 1995; Fay, 1992).

Nitrogen fixation by nonheterocystous cyanobacteria during dark is dependent on exogenous reserve of carbon that is accumulated during the daytime by photosynthesis.
Dark N₂ fixation is a strategy employed nonheterocystous cyanobacteria to prevent inactivation of their nitrogen-fixing apparatus due to high oxygen concentrations present during daytime. The nighttime nitrogenase activity can also be regulated by circadian rhythm, which has been previously demonstrated in several nonheterocystous cyanobacterial species including unicellular ones (Stal, 1995).

Cluster IV clustered with nonheterocystous unicellular cyanobacterial species, and nitrogenase expression was observed only during nighttime samples of 23.15 and was present till early morning around 7.15. It is interesting to find sequences clustering with unicellular cyanobacterial species. Although this group has been reported from numerous environments (Steppe et al., 2001; Zani et al., 2000; Zehr et al., 1998) as a potential nitrogen fixer, more elucidation is needed on its role. However, results of this study assign unicellular cyanobacteria as an important composition in the nighttime diazotrophic assemblage.

The uncultured cyanobacterium clone cluster, which aligned with uncultured cyanobacterium clone cluster NRE5, appears to be a dominant group contributing to nitrogen fixation during nighttime. The expression of nitrogenase appears to be high in the evening around 19.15, and decreases throughout the night until 11.15. Three more clone clusters were recognized: unidentified cluster V; cluster VI; and cluster VII. All these clusters formed separate clades within the cyanobacterial tree. Positioning of these clusters is also consistent with genomic DNA based characterization, suggesting that they form an important component of the diazotrophic community in Everglades epiphytic mats. Since they still remain uncharacterized, it is not possible to conclude any physiological characteristics based on the distribution of \textit{nifH}.
Cluster V is a feature of dark samples. Its expression is observed from time 19.15 till 11.15, with an exception of 7.15 time point. This might be attributed to spatial heterogeneity in the sample. These clones may be employing the strategy of nitrogen fixation during nighttime by avoiding oxygen under anaerobic conditions. Cluster VI is seen only in 7.15 and 11.15 timepoint samples, and are represented by a small number of clones. This suggests that this group might not be contributing greatly to fixing nitrogen, however, it might represent a very important member responsible for accumulating carbon via photosynthesis during the daytime. Cluster VII was represented only during 19.15-timepoint sample. Number of clone sequences (19% of the distribution) recovered definitely suggests an important member during that time point.

These results indicate that, although nitrogenase activity was only limited to cyanobacterial species, variations were seen on a diel basis. Clones clustering with known sequences definitely suggest that these epiphytic mats are dominated by nonheterocystous cyanobacterial species rather that heterocystous forms which are known to best adapted to diazotrophy by virtue of their capacity to spatially separate oxygenic photosynthesis and nitrogen fixation, but are not tolerant of anoxic conditions which may exist in these epiphytic mats. This assumption supports dominance of nonheterocystous cyanobacterial species. These mats also harbor sequences clustering close to sulfate reducing bacteria that may be involved in sulfate reduction. This possibility also supports dominance of nonheterocystous cyanobacteria (Stal, 1995).

These data also raise some questions regarding nutrient limitation in epiphytic mats in U3, WCA2A. Previous studies (coupled with high TN: TP ratios) suggest oligotrophic periphyton mats are limited by P and not N (McCormick and O’Dell 1996). It is
interesting to note that these microorganisms expressed nitrogenase in a phosphorus-limited environment, suggesting that they may also be limited by nitrogen in this environment. The information base generated from this study can be used in future to efficiently learn about the various environmental factors controlling nitrogenase expression.
Table 3-1. Nucleotide sequences of primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide position</th>
<th>Primer nucleotide sequence (5’-3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>nifH1</td>
<td>639 to 655</td>
<td>5’-TGY GAY CCN AAR GCN GA-3'</td>
<td>Zani et al.</td>
</tr>
<tr>
<td>nifH2</td>
<td>1000 to 984</td>
<td>5’-ADN GCC ATC ATY TCN CC-3'</td>
<td>2000</td>
</tr>
<tr>
<td>nifH3</td>
<td>1018 to 1002</td>
<td>5’-ATR TTR TTN GCN GCR TA-3'</td>
<td></td>
</tr>
<tr>
<td>nifH4</td>
<td>546 to 562</td>
<td>5’-TTY TAY GGN AAR GGN GG-3’</td>
<td></td>
</tr>
</tbody>
</table>

All four of these primers were degenerate (Y = T or C; R = A or G; D = A, G, or T; and N = A, C, G, or T).

Figure 3-1. Rarefaction analyses for nifH clone libraries for epiphyton samples from U3.
Figure 3-2. Phylogenetic tree of genomic DNA nifH clones from U3 epiphytic samples. Numbers at branch points refer to bootstrap analysis based on 100 resampling.
Figure 3-3. Clones distribution from oligotrophic site U3

Figure 3-4. Rarefaction analyses for *nifH* clone libraries for time 11.15 with RT-PCR from epiphyton samples from U3
Figure 3-5. Rarefaction analyses for \textit{nifH} clone libraries for time 15.15 with RT-PCR from epiphyton samples from U3

Figure 3-6. Rarefaction analyses for \textit{nifH} clone libraries for time 19.15 with RT-PCR from epiphyton samples from U3

Figure 3-7. Rarefaction analyses for \textit{nifH} clone libraries for time 23.15 with RT-PCR from epiphyton samples from U3
Figure 3-8. Rarefaction analyses for nifH clone libraries for time 7.15 with RT-PCR from epiphyton samples from U3
Figure 3-9. Phylogenetic tree of RT-PCR nifH clones from U3 epiphytic samples. Numbers at branch points refer to bootstrap analysis based on 100 resampling.
Figure 3-10. Clones distribution for 11.15 time point.

Figure 3-11. Clones distribution for 15.15 time point.
Figure 3-12. Clones distribution for 19.15 time point.

Figure 3-13. Clones distribution for 23.15 time point.
Figure 3-14. Clones distribution for 7.15 time point.

Figure 3-15. Nitrogenase expression at different time points for unidentified cluster I
Figure 3-16. Nitrogenase expression at different time points for unidentified cluster II

Figure 3-17. Nitrogenase expression at different time points for unidentified cluster III
Figure 3-18. Nitrogenase expression at different time points for unidentified cluster IV

Figure 3-19. Nitrogenase expression at different time points for uncultured clone cluster
Figure 3-20. Nitrogenase expression at different time points for unidentified cluster V

Figure 3-21. Nitrogenase expression at different time points for unidentified cluster VI
Figure 3-22. Nitrogenase expression at different time points for unidentified cluster VII
CHAPTER 4
METHANOTROPHIC DIVERSITY ALONG THE NUTRIENT GRADIENT IN PERIPHYTON MATS

Wetlands contribute an estimated 15 to 20% of the total methane emitted on a global basis (Matthews and Fung, 1987). The capability of microorganisms to utilize methane as a substrate for energy generation is ecologically important with respect to the carbon cycle. Most known methanotrophs belong to a group of gram-negative bacteria that can grow on methane as the sole source of carbon and energy. They are widespread in nature and have received increased attention over the past two decades due to their potential role in the global methane cycle (Cicerone and Oremland, 1988) and their ability to cometabolize a number of environmental contaminants (Hanson and Hanson, 1997). The activities of methane oxidizers effects atmospheric methane concentrations (Reeburgh, 1980; Reeburgh, 1982; Ward et al., 1987; Whalen and Reeburgh, 1990). On the basis of phylogeny, physiology, morphology and biochemistry, methanotrophs fall into two major phylogenetic groups, the $\alpha$ subgroup of the class Proteobacteria ($\alpha$-Proteobacteria) (which includes the type II methanotrophs) and the $\gamma$-Proteobacteria (which includes the type I methanotrophs).

Studies on carbon cycling in Everglades have largely focused on composition and activities of sulfate reducing bacteria (Castro et al.2002; Castro et al. 2005), syntrophs (Chauhan et al. 2004), and methanogens (Castro et al. 2004; Chauhan et al. 2004); however, there have been no studies of methane oxidizers in this wetland. The pronounced nutrient gradient in WCA-2A makes it an excellent system in which to
investigate how nutrient concentrations regulate the distribution of methane oxidizers. Chauhan et al. (2004) reported high methanogenesis rates from eutrophic sites, hence it can be hypothesized that nutrient enrichment will select for particular microbial groups.

Distribution of methanotrophs in periphyton mats is of interest because of the fluctuations in oxygen concentrations due to phototrophic activities of resident microorganisms. The mat becomes supersaturated with O₂ during daytime and anoxic during nighttime, suggesting the possibility of a niche for methanotrophs to oxidize methane rising from the soil. Another reason for this investigation stems from a previous study (Chapter 2), which characterized diazotrophic assemblages from F1, F4 and U3 periphyton mats. Type I methanotrophs are selected during conditions of nitrogen availability (Hanson and Hanson, 1996); phylogenetic analysis of nifH sequences reveal sequences from F1 and F4 clustering with Type II methanotrophs (α-proteobacteria), and U3 with Type I methanotrophs (γ-proteobacteria) with high sequence similarity. This suggests that methanotrophs may be an important heterotrophic group involved in nitrogen fixation. The goal of this study was to understand the distribution of methanotrophs in periphyton mats. We hypothesize that pmoA diversity may vary with nutrient concentrations. This information can be used further is assessing their possible contribution to nitrogen fixation and methane oxidation.
Materials and Methods

Site Description and Sample Collection

Floating periphyton samples were collected in WCA 2A, an impounded wetland located in the northern Florida Everglades. Canal waters enriched in phosphorus and other nutrients that drain agricultural lands are discharged slowly southward across the marsh. This created a complex water quality gradient as described in McCormick et al. (2001). Total phosphorus (TP) concentrations in canal waters have ranged between 100-300 µg L⁻¹ in recent decades compared with TP concentrations of ≤10 µg L⁻¹ in the marsh interior (Belanger et al. 1989; McCormick et al. 2001). As a result, nutrient enriched water in the northeast portion of WCA-2A produced significant changes in the species composition of aquatic macrophytes and periphyton. The oligotrophic marsh interior is composed primarily of sawgrass (Cladium jamaicense Crantz) interspersed with spikerush (Eleocharis spp.) prairies and water lily (Nymphaea odorata Ait.) sloughs. Oligotrophic sloughs also have a characteristic feature, the periphyton, which contributes to high rates of primary productivity in these habitats (Browder et al., 1994). Phosphorus enrichment has been implicated in replacement of these communities with over 10,000 ha of cattail (Typha domingensis Pers.) in the northern marsh (Davis, 1991; Urban et al., 1993). Floating periphyton samples were collected in WCA-2A, which represented the range of vegetation and nutrient conditions along the gradient: F1 (eutrophic; cattail dominated), U3 (oligotrophic; sawgrass dominated) and the transition region, F4. Grab samples of mats were collected on October 9th, 2002 and October 20th, 2003.
Samples were stored on ice and transported to the laboratories in Gainesville. Subsamples for DNA analyses were frozen at \(-70^\circ\text{C}\) until analyzed.

**Nucleic acid extraction**

Total DNA was extracted from periphyton samples with slight modifications with UltraClean Plant DNA kit (MoBio, Solana Beach, CA) according to the manufacturer's instructions. Approximately 50 mg periphyton were thoroughly homogenized either manually or grinding under liquid N2 (for samples with high CaCO3 content) after treatment with 1N HCl. Homogenized sample was suspended in 1ml of 1N HCl and vortexed for 1 min. the sample was centrifuged for 1 min. and supernatant was decanted. The recovered pellet was resuspended again in 1 ml of 1N HCl, and the steps was repeated again and was followed by resuspending the pellet in 1 ml of TE (Tris-EDTA) buffer (Sambrook et al. 1989). After extraction, genomic DNA was evaluated on a 0.7% (wt/vol.) agarose gel made in Tris-acetate-EDTA (TAE) buffer (Sambrook et al. 1989). DNA samples were stored at \(-20^\circ\text{C}\) until further analysis.

**Amplification of pmoA by polymerase chain reaction**

A189f primer was used in conjunction with the A650r primer to amplify a 478-bp internal section of the *pmoA* gene. Details of the nucleotide sequences of the primers used are highlighted in Table 4-1. The reaction mixture used for PCR amplification contained 7 µl of distilled H2O, 1 µl of each primer (10 pmol/µl), 10 µl of HotStarTaq Master Mix (Qiagen, Valencia, CA), and 1 µl of diluted DNA solution. Primary PCR amplification was carried out in a iCycler thermal cycler (BIORAD, Hercules, CA) with the following conditions: Initial enzyme activation and DNA denaturation of 15 min at 95°C, followed by 30 cycles of 1 min at 94°C for
denaturation, 1 min at 56°C for annealing, and 1 min of extension at 72°C, with a final extension of 72°C for 7 min. The same cycling and reaction conditions were used for the second nested reaction. The PCR products were analyzed on a 2% (wt/vol.) agarose gel in TAE buffer to confirm expected size product.

**Cloning of PCR products and RFLP analyses**

DNA fragments were inserted into pCRII-TOPO cloning vector and were transformed into chemically competent *Escherichia coli* TOP10F' cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Recombinant colonies were screened for inserts of the expected size (478 bp) by PCR amplification, with A189f and A650r primer set according to the previously described PCR program. Following screening, the PCR products were digested with the restriction endonuclease HhaI overnight at 37°C. Digested PCR products were electrophoresed through a 4% (wt/vol) agarose gel to visualize the RFLP patterns. Clone libraries were analyzed by analytic rarefaction with the software aRarefactWin (version 1.3; S. Holland, Stratigraphy Lab, University of Georgia, Athens [http://www.uga.edu/~strata/software/]) to confirm that sufficient numbers of RFLP groups were selected to represent the clone libraries from periphyton samples.

**DNA sequencing and sequence analysis**

Selected unique and common clones after comparison by RFLP were sequenced at the DNA Sequencing Core Laboratory at the University of Florida with A189f primers. Sequences were compared with previously identified sequences in the National Center for Biotechnology Information database with BLAST (Altschul et al. 1990), and sequences were aligned by ClustalX version 1.8 (Thompson et al. 1997). Phylogenetic trees were generated with TREECON (Van de Peer and De Wachter
1994; Van de Peer and De Wachter 1997) using a neighbor joining method. Bootstrap analysis for 100 replicates was performed to estimate the confidence of tree topologies.

**Results**

Altogether, 63 clones from F1, 33 clones from F4 and 31 clones from U3 containing inserts of the expected size were obtained. Clones were classified in Operational Taxonomic Units (OTU) by RFLP digestion with *Hha*I enzyme. Clones from F1 were grouped in 4 OTUs, from F4 in 5 OTUs, and U3 in 1 OTU. Rarefaction analysis based on the number of clones per OTU showed a saturation curve, suggesting that almost all the diversity in the clone library was accounted for (Fig 4-1). Total number of clone sequences as per rarefaction analysis showed that the *pmoA* diversity was high in F1 and F4 in comparison to U3. No RFLP patterns obtained with clones from F1 and F4 were similar to patterns obtained from U3. Phylogenetic analysis of partial *pmoA* sequences from F1, F4, and U3 reveal distinct lineages of type I and type II methanotrophs (Fig 4-2) with clone sequences distributed across known and novel unidentified clades. Sequence analysis of clones representative of the RFLP groups from F1 and F4 are presented in Figs. 4-3, 4-4 and 4-5.

No *pmoA* sequences from F1 samples clustered with known methanotrophic sequence, but formed three separate clades: unidentified cluster I (represented by 34% clones); Unidentified cluster II (represented by 55% clones); and unidentified cluster III (represented by 11% clones) in the γ-proteobacterial cluster represented by type I and type X methanotrophs (fig 4-2). Sequences obtained from U3 exhibited similar RFLP patterns, and grouped together as separate clade in the γ-proteobacterial cluster.
represented by type I and type X methanotrophs (Figure 4-2). Sequences from transient site were distributed throughout the phylogenetic tree. Clones clustering within type II methanotrophic cluster close to *Methylocystis* sp. were represented by 20% of the clones. Clones clustering within type X methanotrophs represented by *Methylococcus* sp. accounted for 6% of the total number. Three unidentified clusters were recognized. Unidentified cluster I included 20% of the clones, and included sequences from F1. Unidentified cluster III was comprised of 31% clones, which also included sequences from site F1. Unidentified cluster IV included 23% of the clones. Since the database of known cultured methanotrophic sequences based on *pmoA* diversity is still not exhaustive, it was difficult to assign any groupings.

**Discussion**

This study provides new information regarding the physiological diversity of methanotrophs recovered from periphyton samples from eutrophic, transient and oligotrophic sites in Everglades. Significant methanotrophic diversity has been reported from other studies involving lake sediments (Auman et al., 2000), rice fields (Henckel et al., 1999), landfills (Wise et al., 1999), soils and peat bogs (McDonald et al., 1999) using *pmoA* as the genetic marker. However, little is known about methanotrophic assemblages in freshwater microbial mats. To our knowledge, this is the first molecular characterization of methane oxidizers in periphyton assemblages in WCA2A, Northern Everglades.

We observed that sites F1, F4 and U3 exhibited a diversity of known and novel *pmoA* gene types. There was a distinct selection for type I methanotrophs in F1 and U3, whereas F4 clones aligned with type I, type X and type II methanotrophs; growth of type II methanotrophs is known to be favored by high CH$_4$ concentrations and
nitrogen limiting conditions and type I methanotrophs dominate out type II methanotrophs under low methane concentrations (Hanson and Hanson, 1996). Sequences from U3 cluster as separate clade with type I methanotrophs. All three types have been found in nutrient rich environments; however, previous studies demonstrate type I methanotrophs are more dominant than type II methanotrophs in eutrophic environments (Hanson and Hanson, 1996). Further, novel clades were also obtained from all 3 sites, which could not be assigned to any particular group. 15 sequences (indicated as novel clades in fig 4-2) were found to be 90-95% homologous to previously known methanotrophs. They clustered with the Type I methanotrophs but separated out as distinct clades. We believe, that these are novel methanotrophs, which are widely distributed in the periphyton mats from the Florida Everglades.

Detection of methanotrophic sequences from periphyton mats from all three sites is significant, as active populations of methanotrophs are generally found at the anoxic-oxic interface of the soil or sediments and rhizospheres. Dominance of single clone cluster in U3 indicates low rates of methanogenesis. Presence of diverse composition of methanotrophic assemblage from F1 and F4 suggests high methanogenesis rates, which according to Chauhan et al. (2004) also are higher in F1 and F4 in comparison to U3. Periphyton mats are extensive intact mats formed as benthic mats and rise up to water-atmosphere interface level as a result of buoyancy created by gases. Methane escaping from benthic sediments might become entrapped in the mats, where it may be oxidized by methanotrophs. Although characterization of
floc layer was not undertaken in this study that is the site where one might expect to find diverse active methanotrophic assemblages.
Table 4-1. Nucleotide sequences of primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer nucleotide sequence (5'-3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A189f</td>
<td>5' GG(AGCT)GACTGGGACCTCTGG 3'</td>
<td></td>
</tr>
<tr>
<td>A650r</td>
<td>5' ACGTCCTTACCGAAGGT 3'</td>
<td>Bourne et al. 2001</td>
</tr>
</tbody>
</table>

Figure 4-1. Rarefaction analyses for *pmoA* clone libraries for periphyton samples from site F1 and F4.
Figure 4-2 Phylogenetic tree of *pmoA* clones from F1, F4 and U3. Numbers at branch points refer to bootstrap analysis based on 100 resampling.
Figure 4-3. Clones distribution from eutrophic site F1

Figure 4-4. Clones distribution from transient site F4
Figure 4-5. Clones distribution from oligotrophic site U3
CHAPTER 5
SUMMARY AND CONCLUSION

Nitrogen fixation contributes substantially to the nitrogen requirements of the primary productivity in the Everglades periphyton mat communities (Inglett. et al., 2004; Craft and Richardson, 1993). Most studies on nitrogen fixation in the Everglades have been limited in their assessment due to differences in assaying methodologies that do not correlate rates of nitrogenase activity to the diazotrophic groups present and actively expressing nif. Molecular techniques are helpful in mapping the genetic diversity of functional genes responsible for biological processes. nifH, one of the functional genes of nitrogenase enzyme complex, was used as a biomarker in this thesis to assess diazotrophic diversity in periphyton mats in WCA2A, showing a diverse array of diazotrophs involved in nitrogen fixation, including cyanobacterial, α-proteobacteria, γ-proteobacteria and δ-proteobacteria.

Nutrient enrichment and the resultant impacts on water quality and other ecosystem components in Everglades have also resulted in a need to identify early warning indicators, which can detect ecosystem changes before the damage is done (Newman. et al., 2003). Microbial indicators are of interest as they may be sensitive to small nutrient changes and respond rapidly and in a predictable manner. Periphyton, by virtue of the presence of a diverse microbial community composition, is an excellent candidate for an early warning indicator. Understanding periphyton microbial composition is key to determining impacts of eutrophication. Results of this study indicated that oligotrophic periphyton mat communities were represented by diverse diazotrophic representatives,
including cyanobacterial, α-proteobacteria, γ-proteobacteria and δ-proteobacteria; however, in eutrophic areas, the diversity was limited to cyanobacterial species, indicating a noticeable shift to bloom forming genera. However, the nitrogenase activity could not be attributed to the primary source of organic carbon in the mat, whether cyanobacteria, or with heterotrophic bacteria associated with the cyanobacteria. Studying the RNA transcripts, and identifying the diazotrophic groups actively expressing nitrogenase at different time points of day and night in epiphytic mats provided this information.

A diel pattern was observed amongst the cyanobacterial groups, suggesting non-heterocystous species were dominant in the mat. This section of cyanobacteria was also responsible for daytime expression of nitrogenase. Although the exact mechanism of O₂ protection is not known, temporal separation may be employed. These results indicate that oligotrophic epiphytic mats actively fix atmospheric N₂, suggesting nitrogen limitation.

This study also characterized the spatial distribution of methane oxidizers along the nutrient gradient. Methanotrophs may also have a potential for nitrogen fixation. Phylogenetic distribution of diazotrophs revealed methanotrophs as important groups of nitrogen fixers. Characterization of the methanotrophs specific gene encoding particulate methane monooxygenase (pmoA) revealed type I methanotrophs as the dominant methanotroph type in the three sites studied. The transition (F4) site also harbored type X and type II methanotrophs. Dominance of type I methanotrophs has been reported under N-limiting conditions. Their presence in periphyton mats can be attributed to oxic microzones present in these mats. The presence of methanotrophs in periphyton mats indicated
their role for methane oxidation. These mats may serve as potential sieves to oxidize methane after ebullition from underlying soil and water column, and are crucial components of this marsh ecosystem. Little is known of the factors that regulate diazotrophic community structure in periphyton mats, therefore, \textit{nifH} and \textit{pmoA} sequences may be useful for designing studies aimed at quantifying the role of diazotrophs and methanotrophs within periphyton mats.

The results of this study demonstrated an active nitrogen fixing assemblage in periphyton mats, and distinct shifts with nutrient enrichment. Periphyton diazotrophs have the potential to be used as assessment indicators for nutrient enrichment; however, due to limitation of the database of nitrogen fixing prokaryotes, it was not possible to confidently relate specific species with \textit{nifH} sequence. Expression of nitrogenase by these microorganisms in this phosphorus-limited environment suggests different nutrient may be limiting in different compartments of the ecosystem. Though gene expression data have been a key to mapping the nitrogenase gene diversity, environmental regulatory mechanisms should be assessed. By the virtue of such a tight independent nutrient cycling in periphyton, flow of nitrogen should be mapped to determine the details of nitrogen cycling within the mats. The most important implication of this research has been identification of specific groups expressing nitrogenase as a function of time. Complete genome sequencing of these diazotrophic groups would provide insight into their genetic regulatory machinery.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Puja Jasrotia was born in New Delhi, India, in 1976. She received her B.S. in home science in 1997 from the University of Delhi, India. She went on to pursue an M.A. in social work from the same University and graduated in 1999. Puja worked as a professional social worker in the development sector in New Delhi, India. She traveled extensively across the country conducting training programs with grassroots level non-governmental organizations (NGOs). She came to the United States in 2001 with her husband and was determined to work for the social sector. While looking for suitable jobs, little did she realize that discussions with her husband on his interesting projects on Florida Everglades would awaken her scientific pursuits. She enrolled in the Soil and Water Science Department at the University of Florida in 2002 to pursue an MS under the guidance of Dr. Andrew Ogram, who also immensely encouraged her to pursue studies in a totally different field of study. Upon completion of her MS, Puja plans to pursue a doctoral program.