MICROBIAL SUCCESSION ASSOCIATED WITH SOIL REDEVELOPMENT ALONG A SHORT-TERM RESTORATION CHRONOSEQUENCE IN THE FLORIDA EVERGLADES

By

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For my mother.

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By

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Chair: Andrew V. Ogram
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The Hole-in-the-Donut (HID) restoration program involves removal of non-native Brazilian pepper (*Schinus terebinthefolius*) from land within Everglades National Park. The restoration approach involves complete clearing of *Schinus* and removal of topsoil down to bedrock. Subsections within the HID are cleared at different times, creating a series of sites at different stages of recovery. As the direct linkage between nutrients retained in parent material and plant roots, soil development in newly cleared sites will be essential to successful reestablishment of plant communities and biogeochemical linkages. Establishment of microbial communities will precede plant colonization. As the primary mediators of biogeochemical cycling of carbon and nitrogen, ecology of microorganisms responsible for key roles in nutrient cycling in developing HID soils may provide insights into the reestablishment of biogeochemical linkages with soil redevelopment, the recovery stage of each site, and whether the direction of recovery is towards that of an undisturbed wetland ecosystem. Methane production potentials

suggest hydrogen as the dominant methanogenic precursor. Further, highest methane production was observed from most recently restored sites; data suggest decreased contribution of methanogenesis to anaerobic mineralization with restoration age. Molecular analyses indicate the presence of all major metabolic groups of methanogens in all sites. Methanogenic communities were stable between seasons; however, both cloning and T-RFLP analyses indicated shifts within the *Methanobacteriales* with restoration age. Denitrifying bacterial communities were active in all study sites. Phylogenetic analyses of genes associated with nitrite reduction indicate the presence of unique lineages in soils from all sites. Iterative statistical analyses of clone libraries suggest different disturbance response regimes of groups harboring different genes encoding for the same enzyme. *nirS* genotypes suggest an approximately linear response of diversity to restoration age, while *nirK* analyses suggest a biomodal response with restoration age. Nitrifying bacterial populations were active in both seasons, although rates decreased significantly in wet season soils. Molecular analyses suggest two genotypes of nitrifiers to dominate restored and undisturbed soils, and each site harbors unique distributions of the genotypes. Pairwise differences in diversity between sites were strongly correlated with soil oxygen demand. Collectively, these data indicate compositional shifts in microbial populations associated with carbon and nitrogen cycling in the context of soil redevelopment and restoration age, and provide significant insights into the response of specific microbial populations to severe disturbance and recovery.

CHAPTER 1
INTRODUCTION

Ecosystem disturbance involves an event occurring over a relatively discrete space and time that alters the physical environment, leading to changes in the structure of populations and communities, density of biomass, spatial distribution of biota, and resource availability (Walker and del Moral, 2003). The types of disturbance imposed upon an ecosystem are grouped into four major categories, as outlined by Walker and Willig (1999); they include earth, air, water, and fire. The four disturbance classes are related to natural processes, such as the movement of tectonic plates, and the interplay of climatic, topographic, and soil factors, for example: hurricanes, wild fires, volcanic eruptions, and land slides (Walker and Willing, 1999). An additional category involves those disturbances imposed upon the environment by the activities of humans, such as agricultural activity, deforestation, and urbanization. The impacts of human activity are apparent in all of the Earth's biomes; it is most often human activity that leads to ecosystem disturbance or restoration (McKibben, 1989). Many of the modern instances of an ecosystem undergoing primary succession are either directly anthropogenic in origin or influenced to some degree by human activities (Walker, 1999).

The Hole-in-the-Donut

The Hole-in-the-Donut (HID) is a 4000-ha region within Everglades National Park (ENP), Florida, USA (Figure 1-1). Once consisting of oligotrophic sawgrass (*Cladium jamaicense* Crantz) prairies and short hydroperiod pinelands, the HID was subjected to agricultural land use practices from 1916 to 1978 (Dalryample et al., 2003).

When farming activity stopped, the HID was left as an abandoned, high nutrient, high oxygen environment (Aziz and Sylvia, 1995). The abandoned farmland within the HID was invaded by *Schinus terebinthifolius* Raddi (Brazilian pepper), an exotic shrub native to Brazil, Argentina and Paraguay (Mytinger and Williamson, 1987). *Schnius terebinthifolius* formed dense thickets of shrubs over the most intensely farmed portions of the HID, which were resistant to common management practices (Dalrymple et al., 2003).

Restoration efforts initiated by ENP began in 1996 and involve complete removal of all plants and much of the soil down to the consolidated oolithic limestone bedrock (Li and Norland, 2001). Following restoration, the most recently restored areas of the HID initiate primary succession, by colonization of bare substrate by plant and microbial communities. Cleared transects are left undisturbed to allow the reestablishment of native wetland plants and microbial communities. HID restoration has been done systematically in specified areas, resulting in regions in different stages of recovery, creating a short-term chronosequence of sites at different stages of recovery.

The disturbance caused by complete soil removal is of the severest nature, resulting in surface denudation and little to no biological legacy of the previous ecosystem (Walker and del Moral, 2003). Thus, each newly cleared site will immediately enter the primary stages of ecological re-development and primary succession. The rates of successional change and the number of states between surface recolonization and stability are not known, and can not be predicted for one particular ecosystem. Changes of state will be controlled through interactions of the biota with the physical environment and thus may not occur in a manner previously observed (Odum, 1969; Walker, 1999).

However, several characteristics of developing ecosystems, as outlined by Odum (1969) may provide insight into how the HID recovery will progress. Initially, newly restored sites will be characterized by open nutrient cycles, low productivity, and communities structured as a result of the random coalescing of individuals. As succession progresses, communities will be predictably structured and stratified by ecological function, and nutrients will be recycled and retained in biomass. Changes in recovery states will be biologically controlled, occur in a predictable manner, and result in a progressively more stable ecosystem. The stable ecosystem will contain maximum biomass and harbor communities that interact symbiotically to sustain function. The relatively short time-span for recovery between restoration sites in the HID provides an excellent opportunity to investigate the application of classical theories of ecosystem development over time periods for which the direction and rate of recovery is not known.

Microbial Indicators

Microorganisms mediate nutrient cycling in terrestrial ecosystems and are an integral part of soil quality. Bacteria maintain the greatest population numbers per gram soil than any other organism. Separated from their environment by little more than their cell membranes, they are very sensitive to environmental conditions (Tate, 1995; Hill et al., 2000). Gross activities of soil microorganisms, such as respiration and biomass, have been commonly used as indicators of soil quality (termed process-level indicators) (D'Angelo and Reddy, 1999; Wander and Bollero, 1999; White and Reddy, 1999; Wright and reddy, 2001; Sjogersten and Wookey, 2002). For instance, observed reductions in soil microbial biomass and respiration have been correlated with soil subjected to nutrient enrichment, loss of organic matter, and heavy metal pollution (Jenkinson, 1988; Frostegard et al, 1993; Arunachalam and Melkania, 1999; Kandler et al., 2000).

Extracellular enzyme activities, such as alkaline and acid phosphatases and β-glucosidase, have been correlated with nutrient cycling and limitation, productivity, and xenobiotic degradation (Tate, 1995; Prenger and Reddy, 2004).

Process-level indicators do not reflect dynamics within the soil microbial community. An understanding of the physiology and population dynamics of certain functional groups of microbes can provide clues about the efficiency of biogeochemical cycling within an ecosystem. Temporal changes in soil microbes and their functions occur in response to changes in biotic and abiotic properties at a site (Walker and del Moral, 2003). Changes in microbial community structure may precede changes in communities of higher organisms. For this reason, studies of the dynamics of functional components of microbial communities under various conditions have attracted considerable attention by ecologists (Kennedy, 1999; Hill et al., 2000).

Schimel and Gulledge (1998) presented cases in which ecosystem function appears to result from differences in microbial community structure. The ability of environmental factors to control species and functional group structure has been demonstrated in the northern Everglades (Castro et al., 2002). One approach to investigating compositions of functional groups of microorganisms is to analyze the distribution of functional genes from genomic DNA extracted from soils. The functional genes maintained by an organism define its interaction with the environment; thus, functional gene ecology provides information on the potential occurrence of the processes associated with these genes. Molecular biological tools applied to study natural assemblages of microorganisms have allowed phylogenetic or functional level

identification of indigenous microbial communities in the context of spatio-temporal variation, land use types, and different environments (Palumbo et al., 2004).

Molecular and biogeochemical approaches have been applied to understand the response and recovery of ecosystems subjected to disturbance. Microbial community structure has been shown to change in response to secondary succession of grassland soils (Kowalchuk et al., 2000), along plant diversity gradients occurring in response to nutrient enrichment (Carney et al., 2004), in wetland soils exposed to varying concentrations of dairy effluent (Ibekwe et al., 2003), and along a phosphorous enrichment gradient in the Florida Everglades (Castro et al., 2002; Castro et al., 2004; Chauhan et al., 2004; Castro et al., 2005; Chauhan and Ogram, 2006). Much work has been done on process-level indicators of ecosystem nitrogen loss during primary succession (Robertson and Vitousek, 1981; Robertson, 1982; Vitousek et al., 1989). However, little is known about the microbial communities mediating these processes, or how they are affected by the various factors imposed upon them during recovery.

The occurrence of primary succession is a relatively uncommon event; few natural or anthropogenic disturbance events are severe enough to completely remove both soil and plant communities, leaving bare substrate. The HID provides a unique opportunity to investigate the dynamics of biogeochemical processes and their microbial mediators in concert with soil formation and accretion. As the direct linkage between nutrients retained in parent material and plant roots, the development of soil in newly cleared sites will be essential to successful reestablishment of plant communities and biogeochemical linkages. The establishment of microbial communities on newly cleared surfaces will likely precede the development of plant communities. Microbial activity

will lead to the destruction of parent material and release of nutrients, as well as a source of new nutrients, through fixation of nitrogen and carbon. As the primary mediators of biogeochemical cycling of carbon and nitrogen, investigation into the ecology of microorganisms responsible for key roles in nutrient cycling in developing HID soils may provide insight into the reestablishment of biogeochemical linkages with soil redevelopment, the recovery stage of each site, and whether the direction of recovery is towards that of an undisturbed wetland ecosystem.

Nutrient recycling and retention within HID restoration sites should grow more efficient with the development of soil and plant communities. Microorganisms are relatively short-term sinks for soil nutrients, and can also play an integral role in ecosystem nutrient loss. Trace gas loss of nutrients due to respiratory activity of soil microbial communities may significantly alter the rate at which biogeochemical linkages and nutrient use efficiencies are reestablished. Carbon loss in gaseous form may occur through heterotrophic respiration or methanogenesis. Nitrogen loss may occur through leaching of nitrates produced during nitrification, or in gaseous forms due to the activity of both ammonia oxidizing bacteria and heterotrophic denitrifying bacteria (Figure 1-2). An understanding of the activity and ecology of the microbial groups mediating ecosystem nutrient loss may provide significant insights into the nature and state of nutrient recycling and retention in developing sites.

Methanogenesis

The concentration of methane (CH_4) in Earth's atmosphere is approximately 1.8 parts per million (ppm) by volume, much less than that of carbon dioxide (CO_2); however, a molecule of CH_4 is approximately 20 times more potent as a green house gas than one of CO_2 (Chapin III et al., 2004). Approximately 80% of atmospheric methane is

derived from freshwater environments, the vast majority from biogenic sources such as plants and methanogenic bacteria. Methane is produced in greatest quantities under anaerobic conditions, such as those present in wetlands. Wetland ecosystems are among the most important natural sources of methane to the atmosphere; they emit approximately 22% of total methane (90×10^6 metric tons per year). Other natural sources, such as rice paddy fields, landfills, and ruminants follow closely behind (Cicerone and Oremland, 1988).

Methanogenic bacteria belong to the Archaeal domain, characterized by extreme phenotypes such as methanogens, halophiles, and thermophiles (De Long, 1992). Methanogens are a specialized group of obligate anaerobes that use a narrow range of electron donors for the reduction of CO_2 to methane, namely H_2, acetate, formate, and a limited range of methyl compounds. The majority of isolated methanogens exhibit the ability to grow on H_2 and CO_2, several species utilize methyl compounds and formate, and a relative few utilize acetate as an electron donor (Garcia et al., 2000). In freshwater and terrestrial ecosystems, methanogenesis occurs through reduction of acetate, CO_2 and formate (Schutz et al., 1989). In these ecosystems, the majority of methane is thought to come from acetoclastic methanogens (Conrad, 1999; Wolfe, 1996). In sulfate-rich marine ecosystems, where methanogens are out competed by sulfate reducing bacteria for resources, methyl compounds are non-competitive precursors of methanogenesis (Madigan et al., 1996).

Methane production has been characterized from a variety of natural sources, including geologic deposits, termites and ruminants, freshwater and oceanic sediments, and wetlands. Major anthropogenic sources are fossil fuel use, waste management

(landfills), animal husbandry, and rice paddy soils. A great amount of work has been done to characterize methane sources and sinks in the natural environments.

Strict nutritional and cultivation requirements and slow growth make the isolation and characterization of methanogens cumbersome. Therefore, most recent research on their ecology has been based on cultivation independent molecular methods. The two most common molecular markers used to study the ecology of methanogens are 16S ribosomal RNA (rRNA) and methyl coenzyme M reductase (MCR) genes. However, primers previously developed to specifically target methanogen 16S rRNA genes by Marchesi et al. (2001) were later determined to be limited in range (Luton et al., 2002). Thus, the most effective way to study methanogens using 16S rRNA genes is to sequence exhaustively or maintain enrichment cultures.

Alternatively, the methanogen-specific *mcr* functional gene has been used as a molecular marker to study the distribution of methanogens in a variety of environments. MCR is an enzyme specific to methanogens that catalyzes the final step in methane production, the reduction of methyl-coenzyme M to methane (Thauer, 1998):

CH_3-S-CoM + HS-CoB $\rightarrow$ CoM-S-S-CoB + CH_4

HS-CoM represent coenzyme M and HS-CoB represents coenzyme B.

The genes *mcrA*, *mcrB*, and *mcrG* are included in the *mcrBDCGA* transcriptional unit, which encodes the α, β, and γ subunits of MCR. The functions of *mcrD* and *mcrC* gene products are not known. Two isozymes of MCR have been identified, and their expression correlated with growth stages. MCRI is synthesized during less active and stationary phase growth and MCRII during exponential growth (Reeve, 1992). Additionally, some methanogens of the orders *Methanobacteriales* and *Methanococcales*

contain an additional isozyme of methyl coenzyme-M reductase, termed Mrt (MRT). The MRT operon is arranged as either *mrtBDA* or *mrtBGA* (Thauer, 1998). The expression of either MCR or MRT is dependent on growth stage or oxidative stress (Ferry, 1999).

There is strong evidence for the evolution of the *mcrA* gene from a single common ancestor (Springer et al., 1995; Garcia et al., 2000; Luton et al., 2002), making phylogenetic approaches to studying methanogens relatively simple. Additionally, the three broad groups of substrate users, the acetotrophs, methylotrophs, and hydrogenotrophs, form distinct phylogenetic clusters associated with their metabolic potential. Thus, genetic data can often be used to infer metabolic capabilities of methanogens inhabiting an environment. However, potential biases of PCR primers targeting *mcrA* have been reported, and must be taken into account upon interpretation of *mcrA* sequence data in an ecological context (Luton et al., 2002; Lueders and Friedrich, 2003). Those designed by Luton et al. (2002) are the most widely spanning, meaning they have shown the ability to amplify genes from all orders of methanogens. However, the affinity of these primers towards hydrogenotrophs of the orders *Methanobacteriales*, *Methanomicrobiales*, and *Methanococcales* may prove problematic when they are employed to investigate the full metabolic potential of methanogens within an environment (Luton et al., 2002; Lueders and Friedrich, 2003; Castro et al., 2004).

Information on the ecology and function of methanogenic assemblages in the developing soils along the HID chronosequence will provide significant insights into the efficiency and state of nutrient cycling within the system. For instance, the occurrence of methanogenesis in environments harboring high concentrations of more energetically

favorable terminal anaerobic electron acceptors was attributed to non-steady state conditions, at which methanogens were able to compete for resources with other functional groups previously shown to preclude their activity (Roy et al., 1997). Further, methanogenesis is the final step in anaerobic carbon mineralization, and generally occurs through two major metabolic pathways; the degree and nature of methanogenic activity through each of these respective pathways may provide insight into the function and efficiency of microbial guilds mediating degradation of higher carbon.

Nitrification

Nitrification is the oxidation of ammonia (NH_3) to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-), most of which is carried out by a restricted group of nitrifying bacteria. The resultant effects of nitrification on ecosystem function are well documented. The initial oxidation of ammonium (NH_4^+) to nitrite produces two moles of H^+ per mole of NH_4^+ consumed, leading to pH shifts in soils and possible losses in quality. Loss of ecosystem nitrogen due to nitrification occurs by three general mechanisms (Chapin III et al., 2004). First, the production of nitrate fuels denitrification, the main loss mechanism of fixed N. Second, cationic nitrate is much more mobile in most soils, and thus presents a greater risk for loss due to leaching. Finally, some evidence exists for abiotic transformation of nitrite to gas, termed chemodenitrification (Reddy and Patrick, 1984; Kowalchuk and Stephen, 2001). Conversely, ammonia oxidizing bacterial (AOB) activity is often harnessed for the benefit of wastewater treatment facilities (Laanbroek and Woldendorp, 1995).

Two classes of bacteria able to produce oxidized N compounds exist in nature, and differ by their carbon source. Heterotrophic nitrifiers gain their energy from the decomposition of organic matter. Many heterotrophic fungi and bacteria, including

actinomyctetes, are able to produce either NO_2^- or NO_3^- from NH_4^+ (Chapin III et al., 2004). While their contribution to nitrate production has been observed in nature, their role in nitrification is negligible in most ecosystems; however, significant rates have been observed in some acidic soils (Schimel et al., 1984; Killham, 1990). Autotrophic ammonia oxidizers (AOB) fix carbon for biomass using energy gained from ammonia oxidation. Most AOB are obligate aerobes, but some strains have demonstrated the ability to proliferate under low oxygen concentrations (Laanbroek and Woldendorp, 1995). Within the AOB are two groups, one that converts ammonia to nitrite and another that converts nitrite to nitrate. These two groups occur together in most ecosystems, NO_2^- does not generally accumulate in soils. Nitrite accumulation has been observed in soils from extremely dry savannahs and heavily fertilized farmlands (Chapin III et al., 2004). Those autotrophic organisms responsible for the conversion of ammonia to nitrite will be the subjects of this review.

Availability of NH_4^+ is the most important determinant of nitrification rates (Robertson, 1989) (Figure 1-3). Concentrations must be high enough for nitrifiers to compete with other soil microbes; this is particularly important to autotrophic nitrifiers, which rely on NH_4^+ as their sole source of energy. Significant nitrification rates have been observed in soils with relatively low NH_4^+ concentrations in bulk soils, perhaps due to spatial heterogeneity. Nitrification is thought to be limited to circumneutral conditions. Bacterial cell membranes are only permeable to ammonia, rather than anionic ammonium, and thus the process is favored in non-acidic conditions (Laanbroek and Woldendorp, 1995). Significant nitrification rates measured in acid soils are attributed to the presence of near-neutral microsites where AOB can thrive. Their existence in acid

soils may be stimulated by urea, which can provide substrate to AMO at low pH (Bothe et al, 2000).

Other factors controlling AOB activity in soils are oxygen concentrations (moisture) and plant communities (Figure 1-3). Soil moisture directly affects O_2 concentrations of the soil atmosphere, as well as microbial metabolism. While some metabolic activity in pure cultures of *Nitrosomonas europaea* was evident under anaerobic conditions, AOB activity is generally thought to cease under highly anaerobic conditions (Stuven et al., 1992). In water-logged soils, AOB abundance is significantly higher in the rhizosphere of plants with aerenchymous root tissue (Reddy and Patrick, 1984; Reddy et al., 1989; Uhel et al., 1989) (Figure 1-4). It is still unknown whether the influence of vegetation on AOB activity is due to allelochemical inhibition (Rice and Pancholy, 1972), decreasing ammonium availability due to immobilization, or factors yet to be determined (Stienstra et al., 1994).

In most ecosystems, AOB constitute a small portion (<<1%) of the total bacterial population. However, AOB play a unique role in global N cycling. Their abundance and distribution in the environment is important to ecologists. The monophyletic nature of AOB in terrestrial and freshwater environments facilitates the use of molecular biological approaches in studying their ecology. Molecular and process level-indicators have been paired in a variety of terrestrial and freshwater environments, such as lakes, forest-to-meadow transects, estuaries, contaminated ground water wells, and waste treatment bioreactors (Rotthauwe et al., 1997; Kowalchuk and Stephen, 2001; Minitie et al., 2003; Araki et al., 2004; Carney et al., 2004; Bernhard et al., 2005).

Taxonomically, we know of three distinct groups of autotrophic AOBs, two monophyletic lineages of obligate aerobes within the beta- and gamma- proteobacteria and anaerobes within the Planctomycetales (not addressed in this review) (Head et al., 1993; Teske et al., 1994; Purkhold et al., 2000). Based on 16S rRNA gene sequence analysis, we know of two AOB lineages within the proteobacteria. AOB of the genus *Nitrosococcus* are found within gamma-proteobacteria, and have been isolated exclusively from marine environments (Alzerreca et al., 1999), and a closely related grouping of the genera *Nitrosomonas* (including *Nitrosococcus mobilis*) and *Nitrosospira* comprise a monophyletic cluster within the beta-proteobacteria, all of which have been isolated from terrestrial or freshwater environments (Purkhold et al., 2000).

Much of the initial knowledge gained about AOB ecology and phylogeny stemmed from isolation of pure cultures. Inherent biases associated with all culture techniques may limit the characterization of *in situ* community diversity (Amann et al., 1995; Klotz and Norton, 1995). Further, the slow growth rates of AOB make them difficult to culture, as cultivation generally selects for faster growing organisms. As a result, early studies of AOB ecology in soils suggested a dominance of *Nitrosospira* populations over *Nitrosomonas* in most terrestrial environments (Belser, 1979).

Not until the development of 16S rRNA gene primers by McCaig et al. (1994) specifically targeting AOB did significant patterns of ecological distribution become apparent. Primers specifically targeting AOB 16S rRNA genes allowed phylogenetic inventories to be constructed in various environments. Further, the degeneracy of these primers permitted the amplification of their target, but also of closely related relatives, allowing for the recovery of novel members of the AOB clades (Kowalchuk and Stephen,

2001). However, such degeneracy has led to the recovery of sequences outside of the target clade, sometimes as great as 70 to 100% of total sequences obtained (Kowalchuk et al., 1999).

Initial phylogenetic characterization of AOB 16S rRNA genes recovered from soils and marine environments divided beta-protebacterial AOB into seven clusters (*Nitrosospira* clusters 1 to 4; *Nitrosomonas* clusters 5 to 7). (Stephen et al., 1996; Purkhold et al., 2000). *Nitrosospira* spp. of clusters 2, 3, and 4 are thought to dominate soils (Kowalchuk et al., 1998; Stephen et al., 1998; Kowalchuck et al., 1999). Patterns of ammonia oxidizer 16S rRNA clones obtained from various soils were strongly correlated with acidity, with cluster 2-type AOB most frequently recovered from acidic soils (Stephen et al., 1998; Kowalchuk et al., 2000). Cluster 3 *Nitrosospira* spp. have recovered from young and early successional soils with high ammonium concentrations (Kowalchuck et al., 2000) and untilled soils (Bruns et al., 1998), while cluster 4 organisms dominated older and late successional soils (Kowalchuck et al., 2000). *Nitrosospira* cluster 3 and *Nitrosomonas* cluster 7 AOB dominated agricultural fields subjected to intense fertilization (Webster et al., 2002; Webster et al., 2005). In general, *Nitrosomonas* spp. are more frequently isolated from high nutrient environments, such as sewage sludge and wastewater (Rotthauwe et al., 1997; Purkhold et al., 2000). *Nitrosmonas* strains have been described as *r* strategists, with low substrate affinities and high maximum activity compared to *K* strategists *Nitrosospira* (Andrews and Harris, 1986; Schramm et al., 1998; Schramm et al., 1999). Phylogenetic surveys of AOB in the environment suggest strong correlations between community structure and environment; to date no study has directly correlated abundance of AOB 16S rRNA sequence types

with nitrification rates (Kowalchuk and Stephen, 2001). Further, the physiological basis for observed difference in AOB sequence types is unknown.

Alternatively, primers developed to target the gene encoding the alpha subunit of the ammonia monooxygenase enzyme (AMO) have been applied for study of AOB ecology. AMO catalyzes that first and rate limiting step, the conversion of ammonia to hydroxylamine (Hollocher et al., 1981):

$NH_3 + O_2 + 2e^- + 2H^+ \rightarrow NH_2OH + H_20$

Hydroxylamine is then oxidized to nitrite in an energy yielding dehydrogenase reaction (McTavish and Hooper, 1993). The *amoCAB* operon is transcribed to form a 3.2 kb RNA (Hommes et al., 2001). *amoA* encodes the 32 kDa acetelyene binding protein of AMO; to date, the functions of the *amoB* and *amoC* genes are unknown (Stein et al., 2000).

The *amoA* gene can serve as a useful target for environmental studies, since it reflects the 16S rRNA phylogeny of beta-subclass AOB very well (Purkhold et al., 2000; Kowalchuk and Stephen, 2001), provides a higher degree of sequence variation and greater phylogenetic resolution of closely related ecotypes (Rotthuwae et al., 1997). In recent years, *amoA* diversity has been investigated in a wide variety of natural environments, including soils, sediments, plant roots, groundwater, marine and fresh waters, and estuaries (Stephen et al., 1996; Rotthuwae et al., 1997; Kowalchuk et al., 1998; Stephen et al., 1998; Kowalchuk et al., 1999; Kowalchuk and Stephen, 2001; Avrahami et al., 2002; Carney et al., 2004).

The activity of AOB has been studied extensively in the context of primary succession (Rice and Pancholy, 1972; Robertson and Vitousek, 1981; Robertson, 1982;

Robertson, 1989; Vitousek et al., 1989). Nitrification has been implicated as the major source of N loss in developing terrestrial ecosystems (Robertson and Vitousek, 1981; Vitousek et al., 1989). The seasonally inundated nature of the HID may provide enhanced conditions for N loss potential. Ammonium accumulation in the wet season may provide enough substrate for significant nitrification activity during the dry season. High nitrification in the dry season will lead to loss of N due to leaching, and possibly fuel significant denitrification in the wet season. Further, both physiological and molecular responses of AOB to differences in soil parameters have been documented, and the presence or absence of certain genotypes correlated with differences in resource availability. An understanding of the structure and function of AOB in HID soils may provide insight into the potential for N loss at each stage of recovery and the efficiency of N use within the developing soils.

Denitrification

Nitrate respiration can occur through two dissimilatory pathways. The first, reduction of NO_3^- or NO_2^- to NH_4^+, occurs widely but is not considered denitrification in the strict sense. Denitrification is the dissimilatory reduction of nitrate or nitrite to oxidized gaseous forms of nitrogen (either nitric or nitrous oxide), which may be further reduced to dinitrogen gas (N_2). As the major loss mechanism for fixed nitrogen from the biosphere, denitrification plays a crucial role in the balance of the global nitrogen cycle. Significant rates of denitrification may be of consequence in nitrogen limited and agricultural ecosystems. However, it is also a significant source of atmospheric N_2O, a greenhouse gas involved in stratospheric ozone depletion (Chapin III et al., 2004).

Several factors control denitrifying enzyme activity (DEA) in soils (Figure 1-5). Oxygen and moisture levels, temperature, and organic carbon availability are the most

influential factors on DEA (Knowles, 1982). Moisture content indirectly controls the availability of both oxygen and organic carbon; slowed diffusion of O_2 leads to decreased heterotrophic activity. DEA is often highest in facultative soils with renewable supplies of organic carbon, such as periodically inundated wetlands, tidal marshes, and riparian zones.

The ability to denitrify is widespread among bacteria of unrelated systematic affiliation, most likely due to lateral gene transfer events (Zumft, 1997). Although it is a facultative process, the capacity for denitrification is almost exclusively expressed in Eubacterial strains capable of aerobic growth. Prokaryotes constitute the vast majority of organisms capable of denitrification, although a number of fungal isolates have demonstrated the ability, but with minimal cellular energy gained (Kobayashi et al., 1996; Shapleigh, 2000). Many prokaryotes identified as denitrifiers have the ability to couple both O_2 and NO_3^- reduction to ATP synthesis; energy yields from nitrate respiration are similar to those gained by aerobic respiration.

Complete reduction of nitrate to dinitrogen gas requires a suite of four enzymes (Figure 1-6). The second step is the conversion of nitrite to nitric oxide by nitrite reductase (NIR). As the first gas-generating step, it is the defining step of denitrification, and will be the focus of this review (for more details on the molecular basis of denitrification see Zumft, 1997). The NIR reaction is complemented by the activity of two distinct metalloenzymes, one with a copper center (Cu-NIR) and the other with a heme-based cytochrome (Fe-NIR). Both forms of the enzyme occur in the periplasm and appear to be functionally redundant (Coyne et al., 1989; Glockner et al., 1993). Cu-NIR is more widely distributed within prokaryotes, including both archaebacteria and

Eubacteria, while Fe-NIR is more widely spread across environments, but found only in Eubacteria (thus far) (Bothe et al., 2000). Fe-NIR occurs in proteobacteria at a much greater frequency relative to other Eubacterial groups; type strains from four of the five major proteobacteria sub-classes (alpha, beta, delta, epsilon) have been characterized. Currently, no Fe-NIR containing organisms have been identified in the gamma sub-class. To date, there is no apparent agreement in the phylogenetic distribution of the two enzymes types with 16S rRNA gene phylogenies of the harboring organisms (Shapleigh, 2000).

Genes encoding both NIR enzymes are not fully understood. Fe-NIR genes from *Pseudomonas aeruginosa* were adjacent, while those in *Pseudomonas stutzeri* were rearranged into three different transcriptional units (Palmedo et al., 1995). Comparison of four Cu-NIR containing bacteria revealed a single conserved gene. However, quantities of DNA required to encode Fe-NIR and Cu-NIR is significantly different (Shapleigh, 2000).

The phylogenetically diverse nature of denitrifying bacteria makes the design of 16S rRNA group-specific probes impossible. Thus, molecular ecological studies of the distribution of denitrifying bacteria always target functional genes and their products. Highly conserved regions of genes involved in denitrification have allowed for the development of group-specific primers. Fe-NIR and Cu-NIR are encoded by *nirS* and *nirK* genes, respectively. DNA sequences encoding the two enzymes share little sequence homology; thus, probes specific to each gene have been developed (Braker et al., 2000). Because of their role in the gas-producing step of denitrification, *nirK* and

nirS are most often employed in ecological studies of denitrifier distribution (Michotey et al., 2000; Yan et al., 2003; Santoro et al., 2006).

The exact environmental factors affecting the distribution of *nirS* and *nirK* containing organisms in the environment are not fully understood. The ubiquity of the gene, along with its high lateral transfer rate, makes phylogenetic characterization of denitrifying bacterial communities difficult. However, the responses of one or both genotypes to environmental conditions have been reported. A previous characterization of *nirS* and *nirK* diversity in forested upland and wetland ecosystems was only able to recover *nirS* in upland soils, while both were present in wetland soils (Priemé et al., 2001); the actual environmental reasons for the observed patterns were not apparent. More often, both genes are detected within an environment, but the response of the organisms harboring them is different. Liu et al. (2003) reported a greater response of *nirK* than *nirS* containing denitrifiers in marine sediments; *nirK* diversity correlated strongly with nitrate availability, while *nirS* diversity was correlated with oxygen concentrations. Instances of differing responses to the same environmental factor have also been observed. Santoro et al. (2006) reported a greater response of *nirK* to nitrate concentrations in ground waters. *nirS* diversity was still indicative of response, but less pronounced, a greater degree of overlap between genotypes along the nitrate gradient was observed. Thus, investigation into the diversity and population structure of *nirS* and *nirK* in association with shifts in biogeochemical processes in HID soils may provide insight into the state of N cycling, the potential for gaseous N loss, and factors controlling the response of organisms harboring functionally redundant enzymes.

Hypotheses and Objectives

The central hypothesis of this study is that an understanding of microbial assemblages associated with carbon and nitrogen cycling and the measurement of certain forms of carbon and nitrogen can be used to assign value to restoration efforts in the HID, and predict the rates of ecologically important processes regulating availability. Specific hypotheses to be tested include: (i) soil redevelopment will lead to the establishment of methanogen, denitrifying, and ammonia oxidizing microbial communities which will grow more predictably consistent in structure with restoration age; and (ii) seasonal variations in methanogenesis, denitrification, and ammonia oxidation rates will reflect observed differences in community structure and restoration age.

The main objectives of this investigation are to: (i) identify spatial and temporal changes in community structure of microorganisms associated with methanogenesis, denitrification, and ammonia oxidation; and (ii) monitor the relevant biogeochemical processes associated with methanogenesis, denitrification, and ammonia oxidation in restored and undisturbed wetlands. Investigation of the dynamics of these microbial communities may provide insights into the reestablishment of biogeochemical linkages with soil redevelopment, the recovery stage of each site, and whether the direction of recovery is towards that of an undisturbed wetland ecosystem.

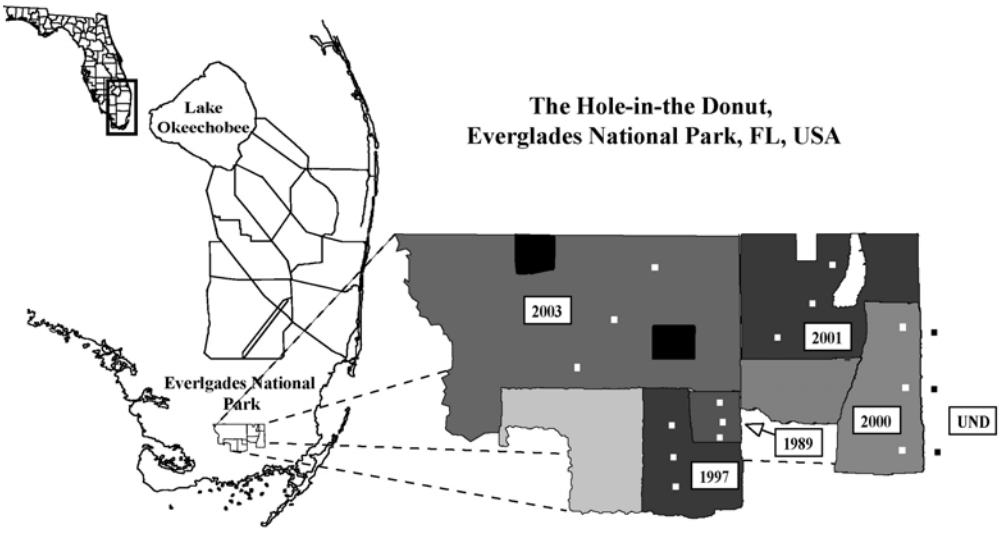


Figure 1-1. The Hole-in-the-Donut restoration area.

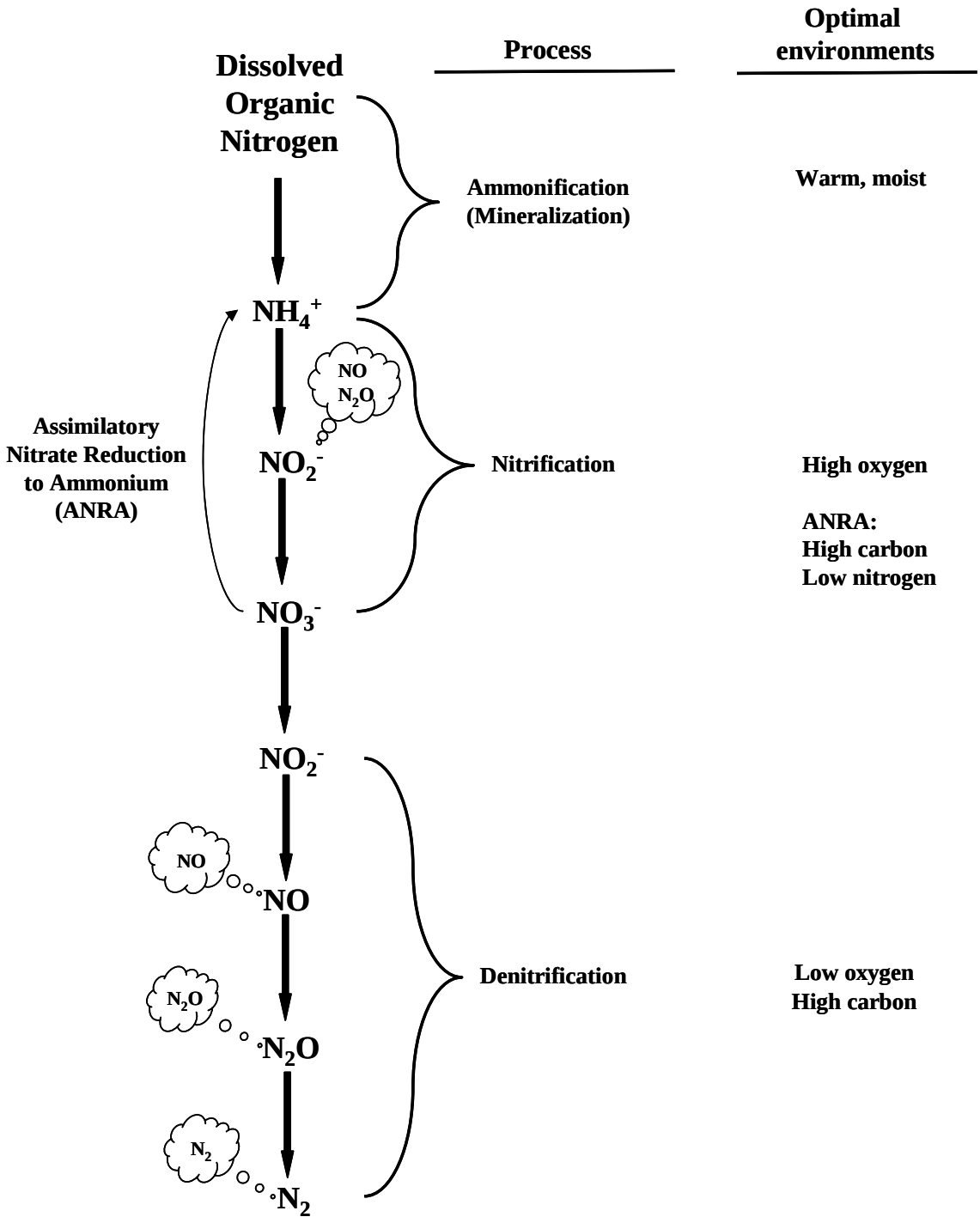


Figure 1-2. Pathways of autotrophic nitrification and of denitrification and the nitrogen trace gases emitted by these pathways. Adapted from Chapin et al. (2004).

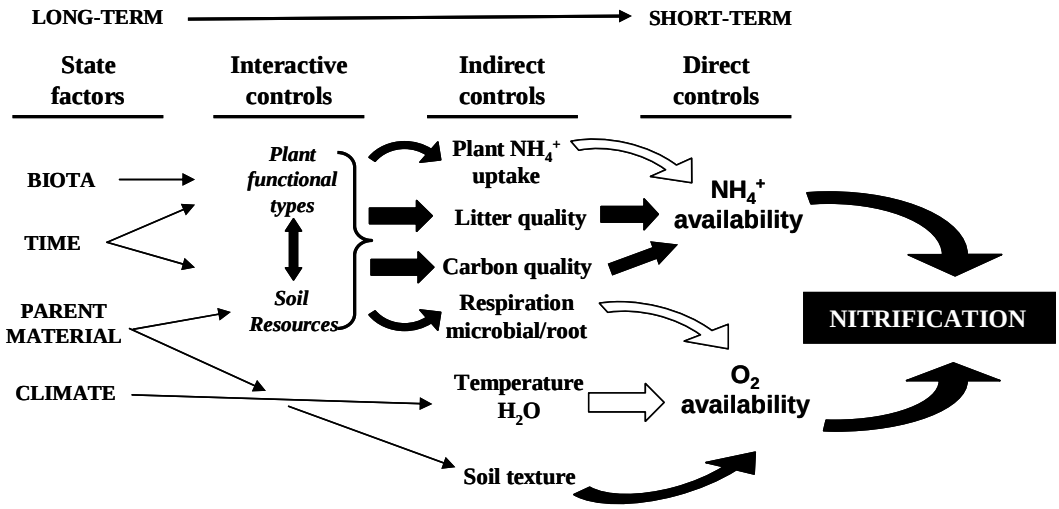


Figure 1-3. The major factors controlling nitrification in soils. Thickness of the arrows represents the strength of effects. Black arrows represent positive influences and white arrows represent negative influences. Adapted from Chapin III et al. (2004).

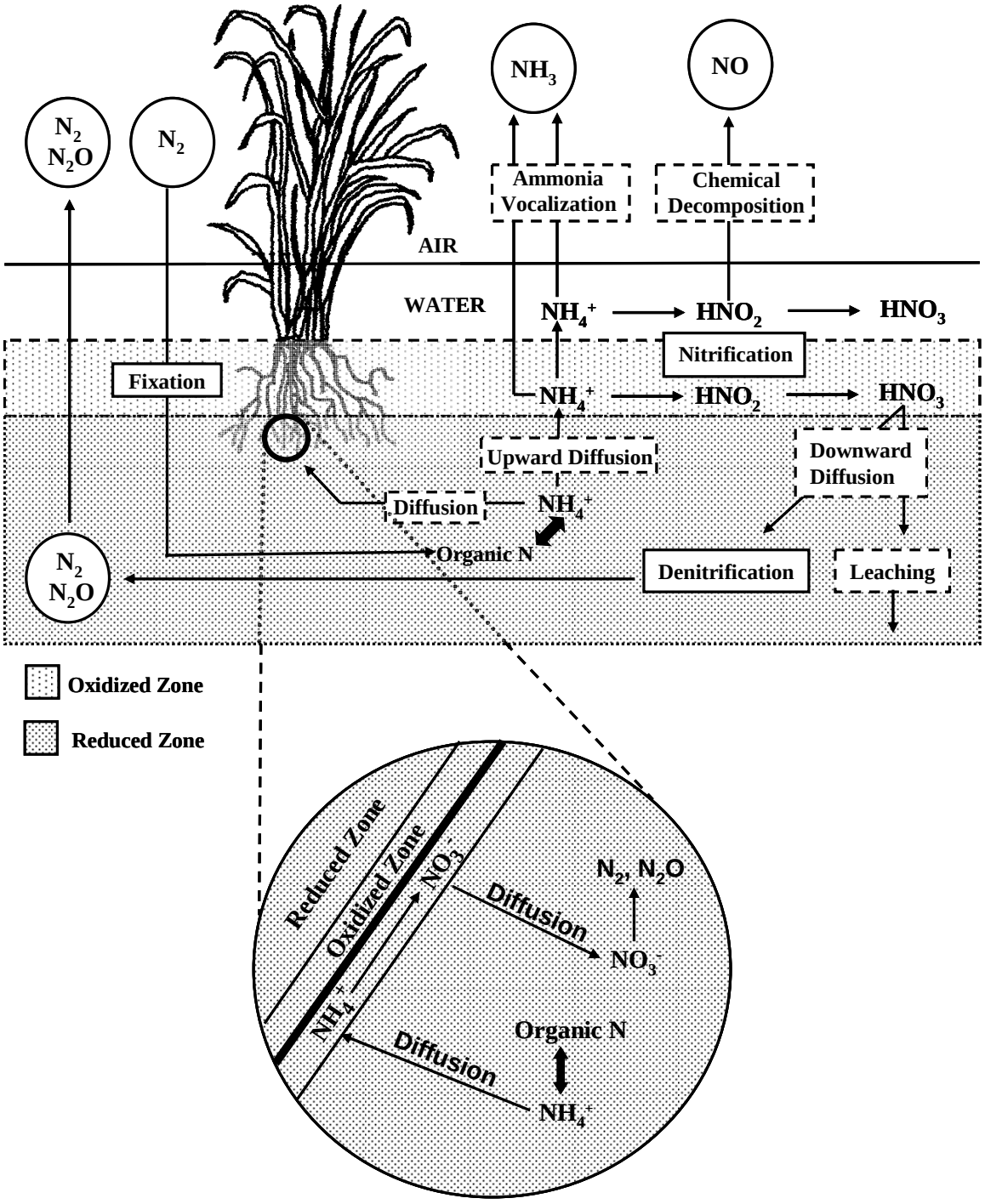


Figure 1-4. Schematic representation of nitrogen cycling in flooded soils and sediments. Inset depicts diffusion processes occurring at the root-soil interface. Adapted from Reddy and Patrick (1984).

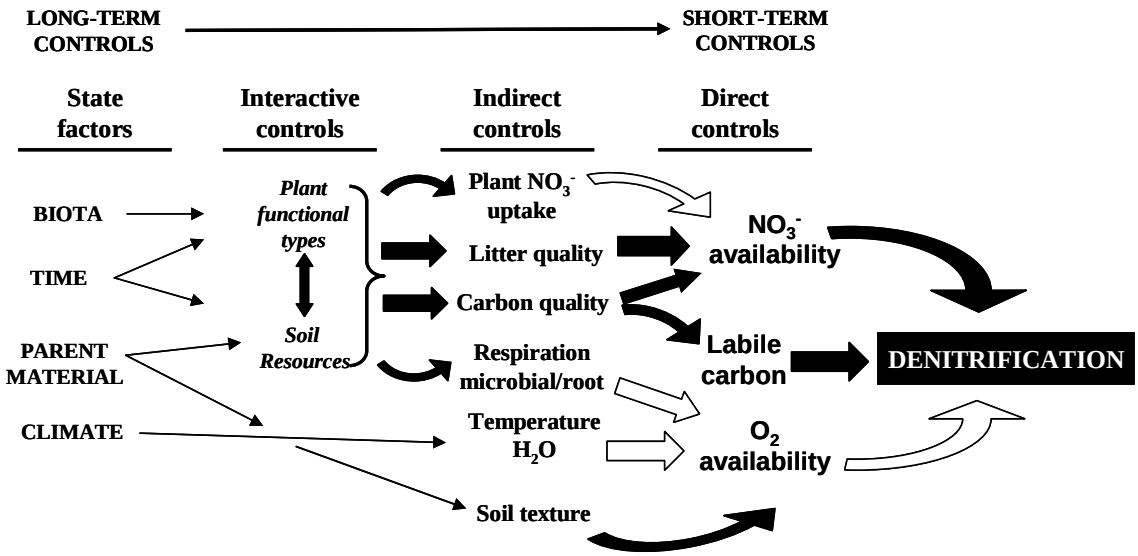


Figure 1-5. The major factors controlling denitrification in soils. Thickness of the arrows represents the strength of effects. Black arrows represent positive influences and white arrows represent negative influences. Adapted from Chapin III et al. (2004).

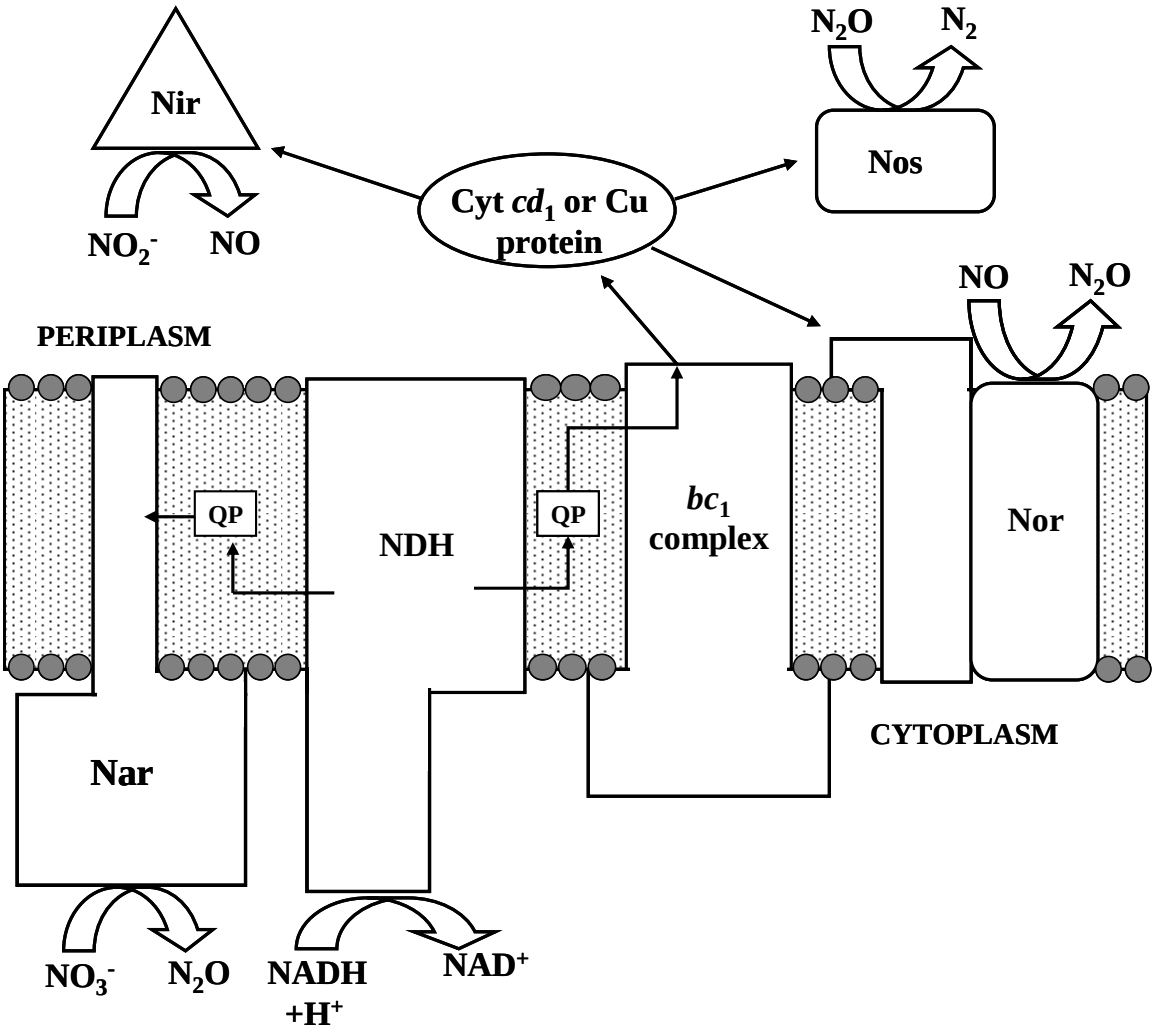


Figure 1-6. The basic arrangement of the nitrogen oxide reductases required for complete denitrification by a single organism. Adapted from Shapleigh (2000).

CHAPTER 2
STRUCTURE AND FUNCTION OF METHANOGENIC ASSEMBLAGES ALONG A SHORT-TERM RESTORATION CHRONOSEQUENCE

Introduction

The Hole-in-the-Donut (HID) is a 4000 ha region within Everglades National Park (ENP), Florida, USA. Once consisting of oligotrophic sawgrass (*Cladium jamaicense* Crantz) prairies and short hydroperiod pinelands, the HID was subjected to agricultural land use practices for 60 years (Loope and Dunevitz, 1981; Dalryample et al., 2003). Pre-agriculture, HID soils were characterized as shallow, poorly drained and low nutrient marls. Intensive rock plowing efforts destroyed underlying limestone bedrock, creating coarsely textured, well drained soil more suitable for vegetable production (Li and Norland, 2001). When farming activity ended, the HID was left as an abandoned, high nutrient, high oxygen environment. Farmland within the HID was invaded by stands of *Schinus terebinthifolius* Raddi (Brazilian pepper), a shrub native to South America, intentionally introduced to Florida as an ornamental in the 1898 (Austin, 1978), and is though to have entered ENP in the 1940's (Bancroft, 1973; Loope and Dunevitz, 1981).

HID restoration plans initiated by ENP in 1996 include complete removal of all plants and much of the soil down to bedrock. Following removal, cleared plots are left undisturbed to allow natural reestablishment of microbial communities and recolonization by native wetlands plants. HID restoration is conducted in specified areas of varying size, such that regions at different stages of recovery are present at one time.

Soil development is a critical first step in plant colonization on bare substrate. Soil formation results from complex interactions between physical, chemical and biological factors. Subsequently, soil will become the direct link between biotic and abiotic factors that drive primary succession (Walker and del Moral, 2003). Recolonization by microorganisms will precede the establishment of plant communities. Biogeochemical processes mediated by soil microbial communities will contribute both to soil formation and release of nutrients for plants. Significant geochemical differences between undisturbed and cleared sites have been reported (Li and Norland, 2001). Microbe-mediated processes are most sensitive to disturbance, therefore study of microbial communities may be an effective measure to assess the response of soil to perturbation (Nannipieri et al., 2003). Complete soil removal likely destroyed linkages between functional groups of microorganisms. Microorganisms play a central role in carbon and nitrogen cycling, such that development of microbial communities is critical to soil quality and the reestablishment of biogeochemical linkages (Nannipieri et al., 2003). Functional shifts within bacterial groups could potentially alter processes at the ecosystem scale (Schimel and Gulledge, 1998).

Anaerobic microorganisms, such as those found in anoxic soils characteristic of many mature wetlands, mineralize organic carbon through a variety of terminal electron accepting processes. In a developing system, such as the HID, establishment of anaerobic microbial communities may occur in concert with soil profile development. Methanogenesis is a major process responsible for terminal anaerobic carbon mineralization in freshwater wetlands (Schimel and Gulledge, 1998). Methyl coenzyme M reductase, partially encoded for by *mcrA,* is the enzyme responsible for the terminal

step in methane production, the operon and *mcrA* are functionally linked and phylogenetically conserved in methanogens (Leuders et al., 2001; Luton et al., 2002), making *mcrA* a candidate gene for monitoring potential shifts in methanogenic populations in developing HID soils. The objectives of investigating methane and methanogen dynamics in the HID were (i) to assess whether differences in structure and function of methanogenic assemblages may be used as an indicator of soil profile development along the restoration gradient; and (ii) to gain insight into the state of both carbon cycling and anaerobic electron accepting processes in developing soils along the restoration chronosequence.

Materials and Methods

Site Characteristics, Sample Collection, and Biogeochemical Characterization

Samples were collected in April and November 2004. Plots 20 x 20 m^2 were established in sites restored in 1989, 1997, 2000, and 2003 (R89, R97, R00, and R03, respectively), and in an undisturbed site (UND). The range of elevation for the five plots was 0.5 to 0.6 m. Within each sampling area, 2 x 2 m^2 grids were used to establish 81 sampling nodes, which were monitored for soil depth, ground coverage, and elevation. Nine nodes were chosen based on relative range of soil depth within each site, 3 from each depth range (shallow, intermediate, deep). Sampling nodes were color coded and marked for future sampling efforts. Soil samples were taken with a plastic coring device; however, due to non-uniform soil cover in recently restored sites, grab samples were collected where necessary. Individual samples from each depth range were combined to make three representative soil samples, which were used for molecular and geochemical analyses. Soil samples were kept on ice and transported to the laboratory within 72 h of collection, where they were manually mixed and large roots removed. Subsamples for

DNA analysis were stored at -70 °C until analysis. Biogeochemical analyses were conducted at the Wetland Biogeochemistry Laboratory (D'Angelo and Reddy, 1999; White and Reddy, 1999; Wright and Reddy, 2001). Values for select parameters are presented in Table 2-1.

Methane Production Potentials

Two g soil, sampled in November 2004, from UND, R89, R97, R00, and R03 sites were mixed with 25 ml of anoxic modified basal carbonate yeast extract trypticase medium (Touzel and Albagnac, 1983) under an N_2 stream in 50 ml anaerobic culture bottles that were later closed with butyl rubber stoppers and aluminum crimp seals. Tubes were pre-incubated for ten days prior to addition of electron donors. Acetate and formate (20 mM each) were added from N_2 sparged sterile stock solutions. The bottles were fitted with three-way Luer stopcocks (Cole-Parmer, Vernon Hills, IL) for gas sampling, and incubated in the dark at 25^{o}C without shaking. Methane in the headspace was measured by gas chromatography with a Shimadzu 8A GC equipped with a Carboxen 1000 column (Supelco, Bellefonte, PA) and a flame ionization detector operating at 110 ^{o}C. The carrier gas was N_2 and the oven temperature was 160 ^{o}C. All determinations were carried out in triplicate bottles with soil samples from each site (3 bottles per site). Headspace pressure was measured using a digital pressure indicator (DPI 705, Druck, New Fairfield, CT).

Nucleic Acid Extraction and PCR Amplification

Nucleic acids were extracted from 0.25 g of soil with Power Soil DNA Isolation kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions. PCR amplification was conducted using the primer set designed by Luton et al. (2002), and consists of primers mcrA-f (5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-

3') and mcrA-r (5'-TTCATTGCRTAGTTWGGRTAGTT-3') which amplify a fragment of between 465 and 490 bp of *mcrA*. Each 20 µl PCR reaction mixture contained 7 µl of distilled water, 1 µl of each primer (10 pmol μl^{-1}), 10 µl of HotStarTaq Master Mix (Qiagen, Valencia, CA) and one µl of diluted template DNA.

PCR amplification was carried out in a GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems, Norwalk, CT). The initial enzyme activation and DNA denaturation was performed for 15 min at 95°C, followed by 5 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s extension at 72°C, and the temperature ramp rate between the annealing and extension segment was set to 0.1 $^{o}C\ s^{-1}$ because of the degeneracy of the primers (Luton et al., 2002). After this, the ramp rate was set to $1^{o}C\ s^{-1}$ and 30 cycles were performed with the following conditions: 30 s at 95°C, 30 s at 55°C, and 30 s extension at 72°C, and a final extension of 72°C for 7 min. PCR conditions for T-RFLP analysis were identical, except the annealing temperature was decreased to 53°C. PCR products were analyzed by electrophoresis through 2% agarose gels to confirm amplification of expected size product.

Cloning and RFLP Analysis

Fresh PCR amplicons were ligated into pCRII-TOPO cloning vector and transformed into chemically competent *Escherichia coli* TOP10F cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Positive colonies were screened by PCR amplification with the primer set and PCR conditions described above. PCR production from positive clones was digested with *RsaI* restriction enzyme. Each 10 µl reaction consisted of 5U of enzyme, 1x restriction enzyme buffer, 0.6 µg of bovine serum

albumin, 5 μl of PCR amplicon, and water to volume. Digests were analyzed by electrophoresis through 4% agarose gels.

Sequencing and Phylogenetic Analysis

Representative clones from the most frequently occurring restriction patterns in each library were sequenced at the DNA Sequencing Core Laboratory at the University of Florida using internal vector primers. DNA sequences of *mcrA* genes generated from each treatment were translated into putative amino acid sequences and aligned manually in Se-Al version 2.0a11 (Rambaut, 1996). Alignments were then aligned with Clustal version 1.81 (Thompson et al., 1997). Phylogenetic trees were built with a neighbor-joining analysis using a Jukes and Cantor correction method as implemented in the TREECON software package (van de Peer and de Wachter, 1994). Bootstrap analysis was performed with 100 resamplings of the amino acid sequences.

T-RFLP Analysis

Approximately 100 to 150 ng of PCR product was digested with *RsaI*. The enzymatic digestion reaction consisted of 5 units of restriction enzyme (Promega, Madison, WI), 1x restriction enzyme buffer, 0.6 μg bovine serum albumin, and deionized water to a final volume of 10 μl. Enzymatic digestions were incubated at 37 ^{o}C overnight. One and one half μl of digested product were used for terminal restriction fragment (T-RF) detection by the DNA Sequencing Core Laboratory at the University of Florida. Briefly, digested products were mixed with 2.5 μl deionized formamide, 0.5 μl ROX-labeled GeneScan 500-bp internal sized standard (Applied Biosystems, Perkin Elmer Corporation, Norwalk, CT) and 0.5 μl of loading buffer (50 mM EDTA, 50 mg/ml blue dextran). Samples were denatured by heating at 95 ^{o}C for 3 min and subsequently

transferred to ice until loading of the gel. One µl was electrophoresed through a 36 cm, 5% polyacrylamide gel containing 7 M urea at 3 kV on an ABI 377 Genetic Analyzer (Applied Biosystems). T-RFLP profiles were analyzed with GeneScan version 2.1 (Applied Biosystems). T-RF size (bp) was calculated using internal standards. Peak sizes in base pairs and peak areas were exported to Excel 97 SR-1 (Microsoft Corporation, Redmond, WA) for data analysis.

Diversity Indices

Clone libraries were analyzed by analytic rarefaction employing RarefactWin (version 1.3; S. Holland, Stratigraphy Lab, University of Georgia, Athens [http://www.uga.edu/~strata/software/]). Cumulative expected phylotypes were calculated for each clone library according to Castro et al. (2004). Rarefaction curves were fit to a hyperbolic model with the formula $y = ax/(b + x)$ using Datafit software version 8.0.32 (Oakdale engineering, Oakdale, PA), where y represents number of phylotypes, and x is the number of individuals. Coverage values were determined by comparison of obtained versus cumulative expected phylotypes. Shannon-Weaver values were calculated using default parameters of the program by DOTUR (Schloss and Handlesmann, 2005).

Results and Discussion

To our knowledge, this is the first study to monitor the composition and activity of microbial assemblages during the restoration of a freshwater wetland ecosystem. The short-term chronosequence created by complete soil removal allowed us to characterize those communities initially colonizing bare substrate, and monitor their dynamics in concert with soil accretion and changes in geochemical processes. Monitoring the

development and subsequent changes in methanogenic assemblages may provide insight into possible shifts in terminal anaerobic mineralization processes with soil development.

Methane Production in HID Soils

Observed rates of methane production did not correlate with measured geochemical parameters (Table 2-1), or show clear trends associated with time since restoration. Intrinsic methane production rates were highest in R97 and R03 soils (Table 2-2). UND soil produced the least methane, with rates approximately 30 times lower than intrinsic rates reported from oligotrophic soils of the Everglades Water Conservation Area 2A (Castro et al., 2004). Additions of acetate to microcosms lead to slight increases in methane production after 10 d. R03 showed the greatest rate of methane production, but the average rate was only 1.2 fold higher than in unamended soils. Methane production from acetate was two fold higher in R03 soils compared to unamended microcosms. UND soils were unaffected by acetate addition, and rates suggest a general decline in acetoclastic methanogenesis with restoration age. Overall, less than 2% of acetate was converted to methane over the 10 d incubation period. Formate was added to soil microcosms to assess the activity and population sizes of hydrogenotrophic methanogens. Formate is commonly used as an analogue to H_2-CO_2 in anaerobic mineralization studies (Dolfing and Bloemen, 1985). Hydrogen has been shown to be an important electron donor to methanogenesis in other regions of the Everglades (Castro et al, 2004; Chauhan et al., 2004). Methane production potentials in formate-amended soils were 4 to 17 times higher than in unamended soils, and 4 to 20 times higher than in acetate amended soils. Approximately 18 to 50% of added formate was converted to methane over the 10 d incubation period, production rates and total substrate conversion percentage values were strongly correlated. This may indicate the dominance of hydrogenotrophic

methanogenesis in HID soils. Further, this may be an underestimate of actual hydrogenotrophic production potentials, as only 60% of hydrogenotrophic methanogens are able to utilize formate for methane production (Garcia et al., 2000). Hydrogenotrophic methanogens were 1000 to 100 times more abundant than acetoclastic methanogens in other regions of the Everglades (Chauhan et al., 2004).

Methane production potentials in UND soils were lowest of all study sites, regardless of treatment, and data suggest a general decline in methanogenic activity in older sites (Table 2-2). A previous comparison of Everglades soils indicated greatest methane production from marl after addition of acetate and other carbon sources (Bachoon and Jones, 1992). However, our data suggest that methanogenesis may not be an important part of terminal anaerobic carbon cycling in the HID. Currently, factors possibly limiting methane production in HID soils are unknown. However, short hydroperiods and shallow soils may provide conditions conducive to the occurrence of more energetically favorable terminal anaerobic electron accepting processes, such as denitrification.

Phylogenetic Characterization of Methanogenic Assemblages in HID Soils

PCR amplification of *mcrA* from soils sampled in undisturbed and restored sites, in the dry season (April 2004), yielded the expected ca. 465 to 490 bp *mcrA* fragments. Clone libraries constructed from dry season soils indicated the presence of considerable diversity of methanogens in HID soils. The number of obtained and expected phylotypes was highest in UND soils and lowest in R00 soils (Table 2-3) Coverage of expected *mcrA* diversity within each clone library was ascertained by comparison of observed versus expected phylotypes for each library. Values ranged from 45 to 76%, highest in R97 and R00 libraries, and lowest in UND (Table 2-3). Both measures of sampling

coverage indicate that our clone libraries do not fully represent *mcrA* diversity in HID soils.

mcrA sequences obtained from dry season soils formed seven clades encompassing the orders *Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales* and two clades sharing greatest similarity with uncultured organisms (Figure 2-1). MCR-1 sequences share ca. 90% DNA sequence similarity to *Methanosarcina*; these sequences were obtained from UND, R89 and R00 soils, but not in significant quantities. Related sequences were reported from nutrient impacted regions of the Florida Everglades (Castro et al, 2004). MCR-2 sequences were most abundant in UND soils, but comprised a small percentage of R03 and R00 sequences; they shared highest similarity with uncultivated methanogens in rice paddy (Lueders et al., 2001) and Everglades soils (Castro et al., 2004), sharing 87 to 94% similarity with putative hydrogenotrophs in Rice Cluster I (Lueders et al., 2001). Clones in MCR-3, present in R89, R00, and R03 libraries, were most similar to uncultured *Methanosaeta* spp. obtained from permanently flooded riparian soils (Kemnitz et al., 2004). *Methanosaeta* spp. are specialists able to generate methane only from catabolism of acetate (Boone et al., 1993). Cluster MCR-4 branched deeply within cultured *Methanomicrobiales*, and contained sequences obtained from UND, R89, and R03 soils. MCR-4-like sequences have also been obtained from eutrophic Everglades soils (Castro et al., 2004) and a peat bog (Juottonen et al., 2005); our clones share ca. 85% similarity with Fen Cluster methanogens, a potentially novel group of uncertain function (Galand et al., 2002; Galand et al., 2005). Cluster MCR-5 sequences branch deeply within the *Methanococcales.* They were obtained from all study sites; an increase in MCR-5

abundance was observed in more established sites (Figure 2-2). These sequences are closest to those from uncultivated organisms obtained from rice roots (Chin et al., 2004). Previous characterization of methanogenic assemblages in the Florida Everglades did not recover sequences clustering with *Methanococcales* (Castro et al., 2004; Castro et al., 2005). Cluster MCR-6 sequences, present in UND and R97, clustered outside of cultured *Methanococcales*, and shared greatest similarity with clones from other regions of the Everglades (Castro et al., 2004). Clones associated with MCR-7 were found in all sites, and formed a distinct clade within *Methanobacteriales*. Sequence distributions suggest a general decrease in MCR-7 relative abundance as restoration progresses (Figure 2-2). *Methanobacteriales mcrA* comprised a significant portion of clone libraries constructed from other regions of the Everglades (Castro et al., 2004)

T-RFLP Analysis of Methanogenic Assemblage Structure

Composition of methanogenic assemblages in HID soils was monitored with T-RFLP. The possible phylogenetic affiliations of the T-RFs are presented in Table 2-4. *In silico* analyses of *mcrA* clones indicates that some T-RFs may be associated with distinct phylogenetic groups of methanogens. Averages of T-RF relative frequencies for dry and wet season samples for UND, R89, R97, R00, and R03 sites are presented in Figures 2-3 and 2-4, respectively. PCR amplification of *mcrA* in UND samples was generally weak and we did not obtain significant quantities of amplicons in wet soils for T-RFLP analysis. Thus, only dry season T-RF profiles are presented for the UND site. Dominant T-RFs for each site were obtained consistently from replicate soil samples.

Seasonal Structure of Methanogenic Assemblages

Thirteen T-RFs were obtained from both wet and dry season samples. *In silico* digestion indicate T-RFs 85, 186, and 305 are exclusively associated with cluster MCR-7,

associated with *Methanobacteriales*. Seasonally, there were no significant changes in relative abundance of MCR-7 T-RFs within sites. *Methanobacteriales* T-RFs comprised between 35 to 55% of total fluorescence within each site for both wet and dry seasons. T-RFs 65 and 302 were associated exclusively with cluster MCR-5. Sequences comprising MCR-5 branch deeply within *Methanococcales,* and sequences from all study sites are found within this cluster. Increases in T-RF 302 in R00 and R97 between seasons were evident, but comprised only 5 to 20% of total fluorescence in each site. T-RF 65 comprised between 5 to 10% of total fluorescence in all sites and between seasons. T-RFs 48, 80, 96, and 180 corresponded to multiple phylogenetic clusters. For the most part, these T-RFs remained relatively stable between seasons, and were detected in all soils. Because they share affiliation with two metabolically distinct clusters of methanogens, it is impossible to discern which organisms are contributing greatest to shifts in abundance. T-RFs 198, 201, 315, and 317 had no phylogenetic affiliation and were obtained from all samples, but comprised only a small percentage of total fluorescence. These T-RFs may represent methanogens not obtained in our clone libraries.

Our T-RFLP analysis did not identify shifts in composition of methanogenic assemblages between seasons. Significant shifts in soil moisture between seasons may lead to the development of methanogenic activity hot spots in dry soils. Methanogenic activity has been detected in extremely dry soils (Peters and Conrad, 1995). Rewetting events have been correlated to observed shifts in dominant organisms (Nannipieri et al., 2003). However, organisms inhabiting seasonally water stressed soils are thought to be more resistant to moisture fluctuations (Fierer et al., 2002). Further, slow growing

organisms, such as methanogens, may be less affected by dry-wet cycles (VanGestel et al., 1993). Archaeal communities remained relatively stable during rice field rewetting events (Lueders et al., 2000). Within site differences in methanogenic assemblages observed were less than between site differences across seasons in other regions of the Florida Everglades (Castro et al., 2005).

Shifts in Methanogenic Assemblages with Restoration Age

T-RFs associated with MCR-5 and MCR-7 dominated samples from all study sites, as well as clone libraries (Figures 2-2 and 2-3). The relative abundance of MCR-5 T-RFs (65 and 302) differ slightly within sites, with respect to each other, but significant variation between sites was not evident. Their combined abundance suggests that *Methanococcales* populations remain stable in soils from all sites. Relative abundance of MCR-7 T-RFs decreased with successional stage. Interestingly, T-RF 85 was most abundant in UND, R00, and R03 soils, showing an approximately linear decrease with restoration age. Decreased abundance of T-RF 85 in R89 and R97 soils corresponds with increased abundance of T-RF 305. Shifts in MCR-7 T-RFs are evident in both seasons, but more pronounced in dry season profiles. Assuming these T-RFs are exclusively associated with *Methanobacteriales mcrA*, as *in silico* digestion indicates, this suggests a shift within *Methanobacteriales* populations with restoration age. *Methanobacteriales* were also obtained in different proportions along a nutrient impacted gradient of the Everglades (Castro et al., 2004). T-RFLP analysis of *mcrA* obtained from riparian soils also reported shifts in abundance of *Methanobacteriales*; T-RFs differing by approximately 100 bp were obtained in significantly different quantities in soils subjected to differing periods of inundation (Kemnitz et al., 2004). Thus, the apparent association

of T-RF 85 may represent a shift within the *Methanobacteriales* associated with restoration age.

At best, T-RFLP may be employed as a semi-quantitative measure of community structure. Interpretation of shifts in T-RF abundance may not indicate significant changes in assemblage composition. Further, different efficiencies of labeled and unlabelled primers required use of different annealing temperatures during PCR for cloning and T-RFLP analysis, further allowing for discrepancies between T-RFLP profiles and clone libraries. Such discrepancies have been described previously for *mcrA* PCR-cloning and T-RFLP analyses (Leuders et al., 2003; Castro et al., 2005)

Putative hydrogenotrophic *mcrA* genes were most frequently observed in clone libraries and T-RFLP profiles. This is consistent with the highest methane production resulting from formate addition in all sites. However, the exact proportion of *Methanobacteriales* and *Methanococcales* in HID restorations sites is not reflected in T-RFLP results, as the degenerate primers employed in this study do not provide quantitative recovery of all phylogenetic lineages (Lueders et al., 2003). Further, it has been suggested that the primer set employed for this study is biased toward hydrogenotrophic orders of methanogens, and particularly under represent *Methanosaeta* spp. and *Methanosarcina* spp. (Luton et al., 2002; Castro et al., 2004)

Conclusions

Little work has been done to characterize establishment and succession of microbial communities in the context of ecosystem restoration. Clone libraries suggest initial establishment of all major metabolic guilds of methanogens in the most recently restored site. Methanogens have been shown to colonize bare surfaces as members of biofilms (Kussmaul et al., 1998). All T-RFs obtained were present in all sites, and in

approximately similar ratios between seasons. However, there is some evidence of shifts within *Methanobacteriales* (T-RFs 85 and 305) populations associated with restoration age, suggesting that individual groups of methanogens may respond differently to geochemical and environment differences between restoration sites. Interestingly, T-RFLP profiles of methanogenic communities in early sites of a long-term successional bog chronosequence were nearly indistinguishable; however, differences were evident in late succession sites (Merilä et al., 2006). It must be noted that DNA-based assessments of bacterial community composition provides information on the potential metabolic activity, rather than the actual activity. Thus, further studies of gene expression may, in fact, indicate differences in activity within each site.

In summary, our results suggest that a diverse assemblage of methanogenic bacteria colonize recently restored sites. Seasonal T-RFLP profiles indicated methanogenic assemblage structures to remain consistent in composition despite seasonal changes in biogeochemical parameters. This is consistent with previous studies reporting temporal stability of prokaryotic communities (Lueders et al., 2001; Fierer et al., 2003; Castro et al., 2005). Shifts within certain methanogenic groups in association with restoration age were evident. Both molecular and functional assessments suggest hydrogenotrophic methanogens are responsible for most of the methane production observed along the chronosequence.

Table 2-1. Geochemical parameters of dry (April 2004) and wet (November 2004) season soils.

Study Site	Soil Depth (cm)[a]	Moisture (%)[b]	TC (g kg^{-1})	TN (g kg^{-1})	TP (g kg^{-1})	LOI (%)	MBC (mg kg^{-1})
April 2004							
UND	5.9 (1.5-10)	43.7 (10.4)	159.4 (10)	6.6 (0.8)	0.2 (0.0)	17.03	3881.2 (829)
R89	4.5 (3-6.5)	28.3 (7.4)	164.5 (16)	7.7 (1.6)	0.7 (0.2)	23.96	5003.8 (1323)
R97	5.2 (2-8)	39.1 (18.2)	169.4 (8)	8.2 (0.8)	1.0 (0.2)	24.84	4806.3 (1340)
R00	2.7 (1-5.5)	36.8 (12.2)	161.1 (17)	6.7 (0.9)	0.6 (0.1)	20.88	4185.3 (1472)
R03	1.6 (0.5-2.5)	13.5 (9.1)	139.9 (9.7)	4.0 (0.9)	1.0 (0.1)	15.90	2161.3 (579)
November 2004							
UND	10.1 (2-15)	49.6 (4.5)	192.5 (8.8)	7.2 (0.9)	0.1 (0.2)	16.71	1925.1 (516)
R89	5.4 (1-17)	56.7 (8.9)	340.9 (17.2)	9.2 (1.5)	0.8 (0.2)	14.38	3109.9 (1009)
R97	4.6 (3-11)	53.4 (5.8)	323.7 (15.5)	9.0 (1.4)	1.0 (0.2)	14.19	3237.5 (1303)
R00	3.3 (1–4)	54.8 (0.8)	234.3 (11.3)	7.4 (0.8)	0.6 (0.1)	23.90	2343.7 (494)
R03	1.2 (0-3)	52.2 (7.0)	194.1 (7.7)	5.2 (0.6)	0.9 (0.2)	18.86	1752.5 (680.8)

[a]Values in parentheses represent the range of soil depths measured over 81 samples nodes, as described in the Materials and Methods.

[b]Values in parentheses are standard deviations of the mean values for determinations based on three soil samples; TC, total carbon; TN, total nitrogen; TP, total phosphorous, LOI, loss on ignition; MBC, microbial biomass carbon.

Table 2-2. Potential methanogenesis rates and accumulated CH_4 in wet season soils.

	No Addition		Formate		Acetate	
Site	Rate[a]	Total[b]	Rate	Total	Rate	Total
UND	0.1 (0.0)	41 (16)	0.4 (0.1)	198 (37)	0.1 (0.1)	52 (21)
1989	1.1 (0.3)	513 (112)	26.6 (1.6)	8843 (3951)	1.5 (0.1)	700 (34)
1997	11.4 (4.7)	4193 (283)	36.0 (11.1)	17274 (3066)	7.3 (1.0)	3782 (141)
2000	3.4 (1.3)	1619 (425)	22.6 (13.9)	10844 (3843)	6.6 (12.0)	3178 (701)
2003	7.4 (2.2)	1052 (744)	50.4 (14.7)	24183 (4077)	8.8 (3.3)	2998 (926)

[a]Potential methanogenic rates (in nanomoles per gram soil per hour); Standard errors of the means are shown in parentheses for determinations with three replicate soil samples.

[b]Average total methane accumulated in headspace of three replicate samples were site, expressed as nanomoles of methane accumulated.

Table 2-3. Expected and observed phylotypes and diversity indices for dry season *mcrA* clone libraries for HID soils.

Site	Expected Phylotypes[a]	Observed Phylotypes[b]	Coverage (%)[c]	Shannon's H′
UND	35.35 (1.60)	16 (31)	45	2.09
R89	21.41 (0.13)	14 (39)	67	2.01
R97	13.62 (0.53)	10 (39)	76	1.68
R00	13.29 (0.43)	10 (39)	76	2.10
R03	19.20 (1.32)	12 (37)	63	1.76

[a]Value of constant a from equation $y = ax/(b + x)$ (standard error)

[b]Value in parentheses is total number of clones screened

[c]Expressed as percent of expected phylotypes obtained within each library.

Table 2-4. Phylogenetic affiliation of *mcrA* T-RFs for HID soil samples.

Observed T-RF (bp)	Theoretical T-RF (bp)	Cluster	Order
48	48	MCR-1	*Methanosarcinales*
		MCR-7	*Methanobacteriales*
65	65	MCR-5	*Methanococcales*
80	80	MCR-1	*Methanosarcinales*
		MCR-5	*Methanococcales*
85	84	MCR-7	*Methanobacteriales*
96	95	MCR-2	M*ethanosarcinales*
		MCR-4	*Methanomicrobiales*
		MCR-6	Uncultured
Not Obtained	175	MCR-6	Uncultured
Not Obtained	177	MCR-3	*Methanosarcinales*
		MCR-4	*Methanomicrobiales*
180	179	MCR-3	*Methanosarcinales*
		MCR-4	*Methanomicrobiales*
186	188	MCR-7	*Methanobacteriales*
198	Unknown phylogenetic affiliation		
201	Unknown phylogenetic affiliation		
302	302	MCR-5	*Methanococcales*
305	305	MCR-7	*Methanobacteriales*
315	Unknown phylogenetic affiliation		
317	Unknown phylogenetic affiliation		
Not Obtained	438	MCR-5	*Methanococcales*
458-487	No Cut	Not Obtained	

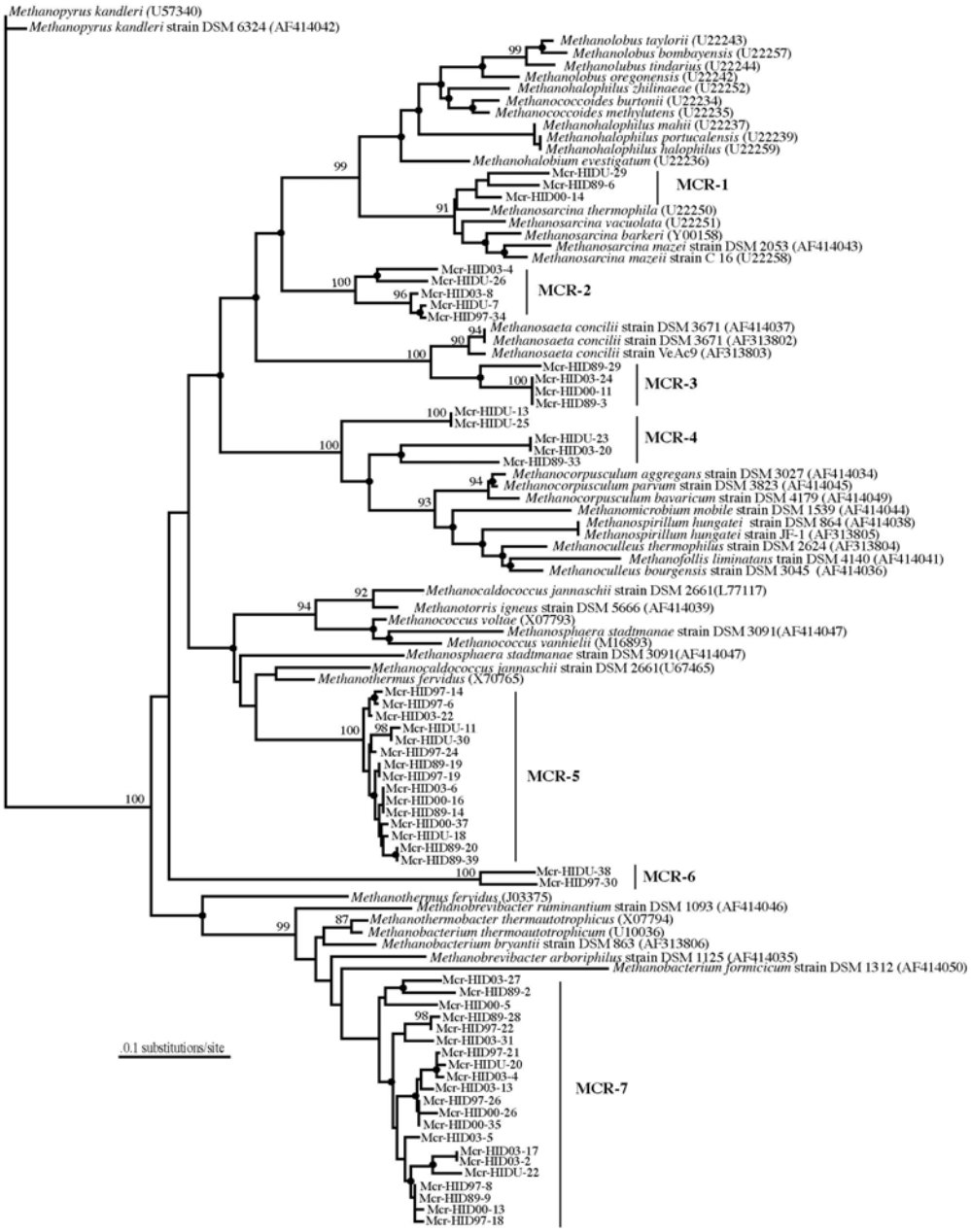


Figure 2-1. Neighbor-joining *mcrA* tree for representative clones from April 2004 soils. Clones are named according to the site of origin; UND, undisturbed site, R, restoration site (followed by year of restoration). Scale bar represents 10% sequence divergence. Numbers at nodes represent percentage of bootstrap resamplings based on 100 replicates. Values greater than 85 are shown, while black dots (●) on nodes represent values between 50 and 85.

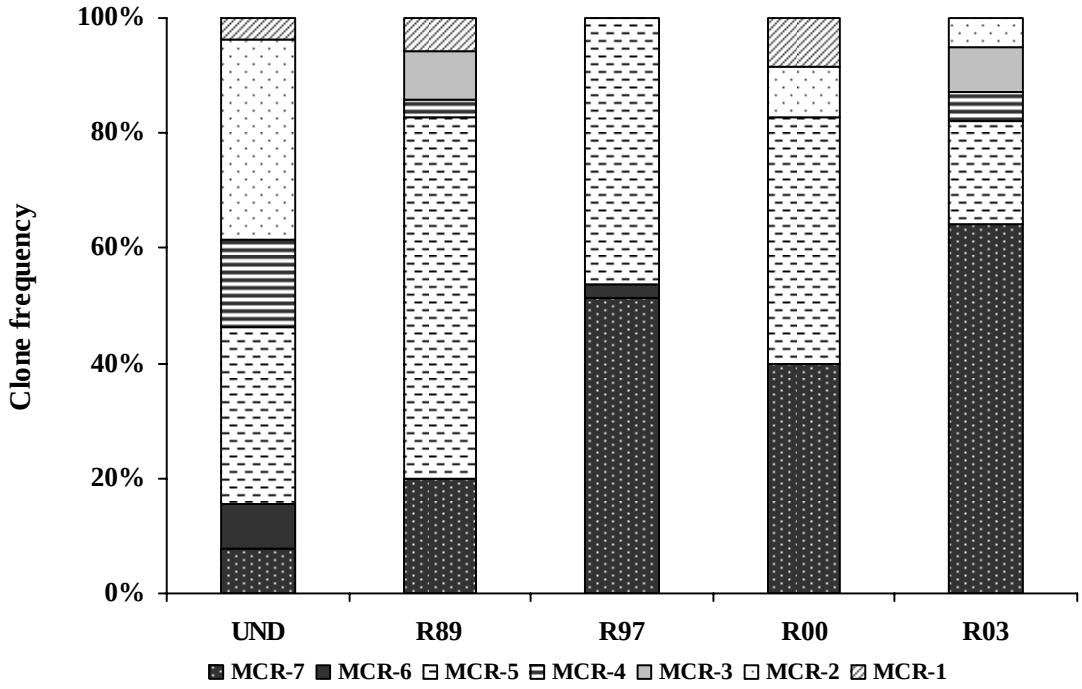


Figure 2-2. Distribution of *mcrA* sequences obtained from dry season soils within designated phylogenetic clusters.

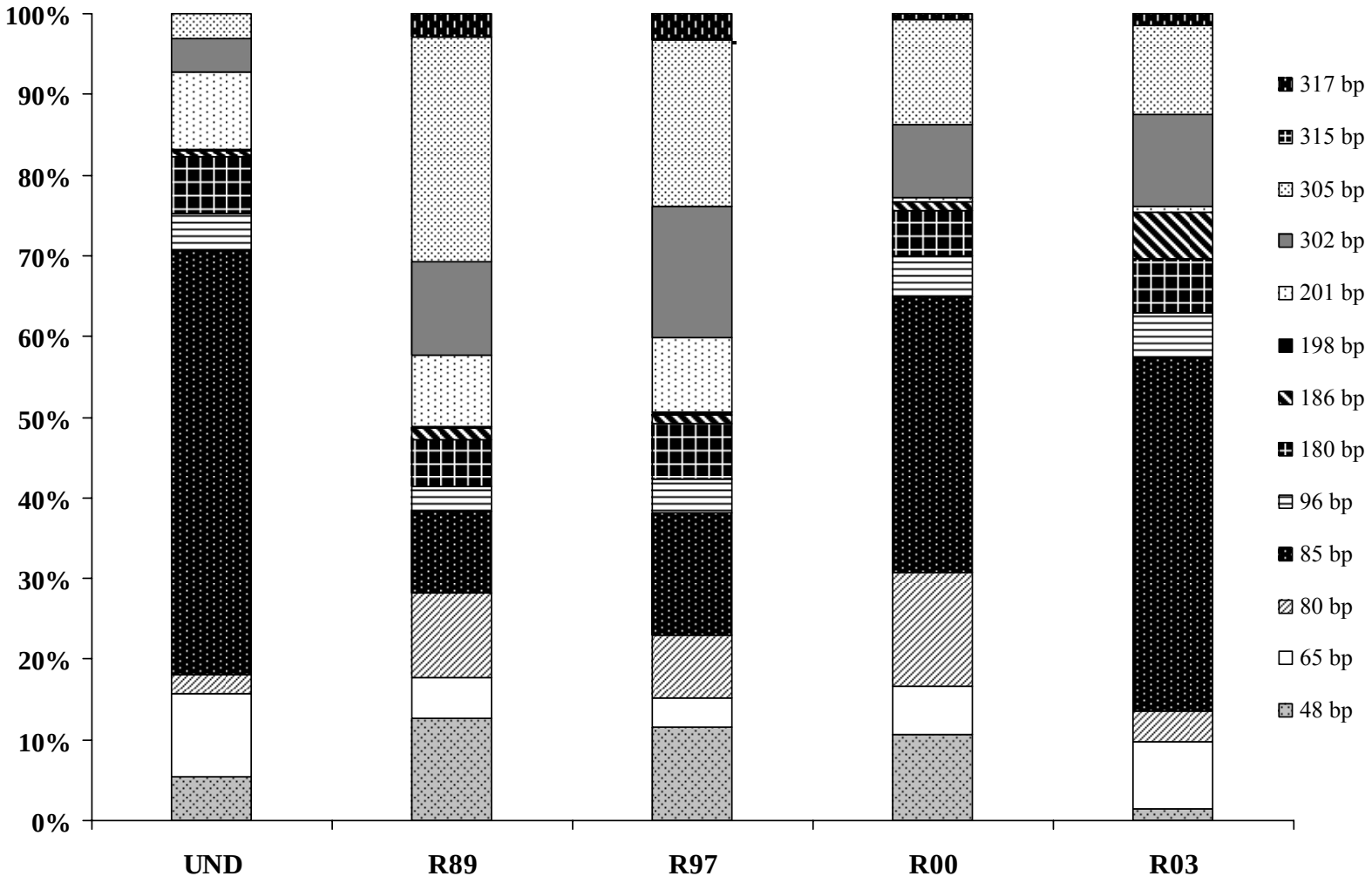


Figure 2-3. Community dynamics for the *mcrA* gene in dry season HID soils determined by T-RFLP analysis. Y-axis values represent percent of total fluorescence.

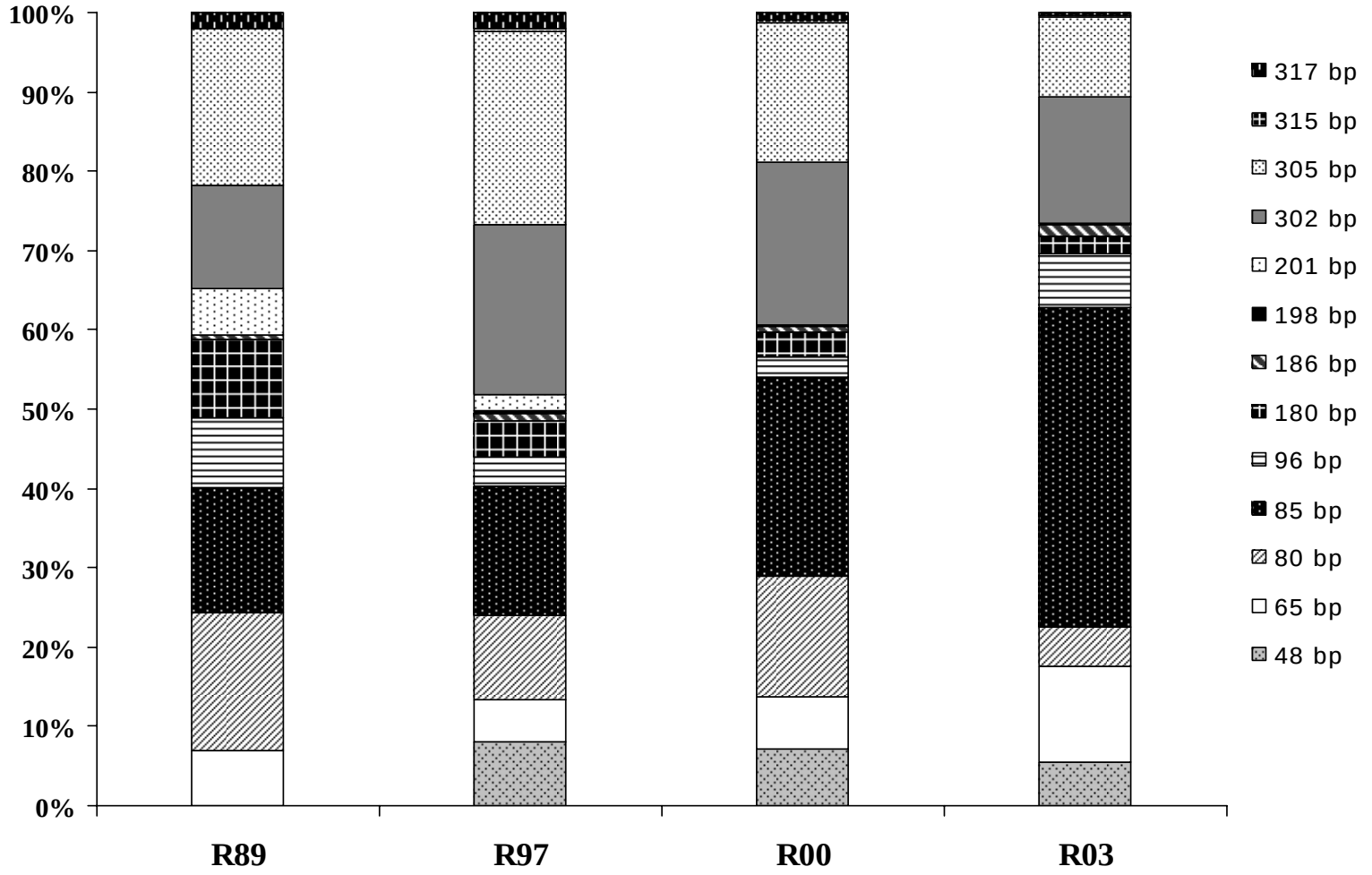


Figure 2-4. Community dynamics for the *mcrA* gene in wet season HID soils determined by T-RFLP analysis. Y-axis values represent percent of total fluorescence.

CHAPTER 3
GENETIC AND FUNCTIONAL VARIATION IN DENITRIFIER POPULATIONS ALONG A SHORT-TERM RESTORATION CHRONOSEQUENCE

Introduction

Nitrogen is the nutrient most likely to limit primary productivity in temperate terrestrial ecosystems. Despite the extraordinary supply of N in the atmosphere, great demand for it by producers and costly energetics of N_2 fixation present the opportunity for supply-side imbalance (Vitousek and Howarth, 1991). Retention of N within terrestrial ecosystems is dependent on the interaction of physical, chemical, and biotic variables. In soils, N can be retained in organic matter, biomass of plants or soil microbiota, or through surface associations with soil particles. The most common N loss mechanisms occur through leaching, denitrification, or disturbances (Payne, 1981).

Denitrification is the microbially mediated dissimilatory reduction of nitrogen oxides to gaseous end products (NO, N_2O, N_2) for energy production (Zumft, 1997). It is the dominant loss mechanism of biologically preferred nitrogen from terrestrial ecosystems, as well as the most prevalent anaerobic respiratory process based on nitrogen (Megonigal et al., 2004). The capability of respiring by denitrification is maintained by a taxonomically diverse group of facultative anaerobic Eubacteria; however, a few Archaea and fungi also exhibit denitrification (Tiedje, 1988; Shoun and Tanimoto, 1991; Usuda et al., 1995). The multi-step process is carried out by a series of membrane bound enzymes. With few exceptions, most bacterial denitrifiers possess the capacity to carry out the entire process (Tiedje, 1988). The defining (first gas forming step) and often rate-

limiting step of denitrification is conversion of nitrite to nitric oxide, and is catalyzed by two distinct but functionally equivalent metalloenzymes (Glockner et al., 1993; Zumft, 1997), i.e., the copper-containing NirK and the cytochrome cd_1 NirS enzymes. The genes encoding the two enzymes, *nirK* and *nirS*, have been used extensively to detect and characterize denitrifiers in activated sludge (You, 2005), marine sediments (Braker et al., 2000; Braker et al., 2001; Liu et al., 2003), forested uplands (Priemè et al., 2002), and freshwater riparian (Schipper et al., 1993; Rich and Myrold, 2004) and wetland ecosystems (Priemè et al., 2002).

High carbon inputs, water column-sediment surface exchange of reduced and oxidized forms of fixed nitrogen, and low oxygen partial pressures may be favorable conditions for the development of robust denitrifying communities in wetland soils (Reddy and Patrick, 1984). In seasonally inundated wetland ecosystems, denitrifying activity may be accelerated, as influx of fresh waters may introduce fresh labile carbon sources and sub-oxic conditions (Bowden, 1987). Activity and dynamics of denitrification in freshwater wetlands have been characterized extensively (Bowden, 1987; Reddy et al., 1989; Hanson et al., 1994; Seitzinger, 1994; White and Reddy, 1999). However, little information exists on the community composition of denitrifiers in these systems (Priemè et al., 2002).

Nitrogen loss due to denitrification in the Florida Everglades has been characterized in permanently flooded, nutrient impacted and oligotrophic regions (D'Angelo and Reddy, 2001; White and Reddy 1999; White and Reddy, 2001), and in both dominant soil types of the ecosystem (marl and peat) (Gordon et al, 1986).

Denitrification has been suggested to be the most important nitrate loss mechanism in these regions (White and Reddy, 1999).

Nitrogen is the most commonly limiting nutrient to primary productivity during primary succession (Vitousek and Howarth, 1989). Thus, its retention within the ecosystem may be crucial to restoration success. Shallow soil depths and periodic inundation of HID restoration sites suggest denitrification as a potentially important mechanism for N loss. Elucidation of the differences in composition and function of denitrifying communities at varying stages of recovery will underpin further interpretation of responses at the physiological and ecological scales. Specific questions to be addressed include the following: (i) what is the phylogenetic composition of denitrifiers in HID soils; (ii) how do communities differ in the context of measured activity and restoration stage; and (iii) are there significant patterns in community composition associated with restoration stage?

Materials and Methods

Site Characteristics, Sampling, and Biogeochemical Characterization

Samples were collected in November 2005. Plots 20 x 20 m^2 were established in sites restored in 1989, 1997, 2000, 2001, and 2003 (R89, R97, R00, R01, and R03), and in an undisturbed site (UND). The range of elevation for the five plots was 0.5 to 0.6 m. Within each sampling area, 2 x 2 m^2 grids were used to establish 81 sampling nodes, which were monitored for soil depth, ground coverage, and elevation. Nine nodes were chosen based on relative range of soil depth within each site, 3 from each depth range (shallow, intermediate, deep). Sampling nodes were color coded and marked for future sampling efforts. Soil samples were taken with a plastic coring device; however, due to non-uniform soil cover in recently restored sites, grab samples were collected at some

nodes. Individual samples from each depth range were combined to make three representative soil samples that were used for molecular and geochemical analyses. Soil samples were kept on ice and transported to the laboratory within 72 h of collection, where they were manually mixed and large roots removed. Subsamples for DNA analysis were stored at -70 °C. Biogeochemical analyses were conducted at the Wetland Biogeochemistry Laboratory (D'Angelo and Reddy, 1999; White and Reddy, 1999). Values for select parameters are presented in Table 3-1.

Denitrifying Enzyme Activity and Gas Analysis

Laboratory denitrifying enzyme activity (DEA) incubations were performed on soils collected in November 2005 according to the method outlined by White and Reddy (1999) with slight modifications. Approximately 15 g of field-moist soil from each site were placed in quadruplet 220 ml serum bottles, which were sealed with butyl rubber septa and aluminum crimp seals. To establish anaerobic conditions in each bottle, the headspace was evacuated to approximately -85 kPa and replaced with O_2-free N_2 gas. Five milliliters of N_2-sparged deionized water were added to each serum bottle to create soil slurries. Approximately 15% of headspace gas was replaced with acetylene (C_2H_2) (Balderston et al., 1976; Yoshinari and Knowles, 1976). Bottles were shaken for 1 h on a longitudinal shaker in the dark to allow C_2H_2 to distribute evenly through the soil slurries. Following pre-incubation, 8 ml of DEA potential solution (56 mg NO_3-N L^{-1}, 288 mg $C_6H_{12}O_6$ L^{-1}, 100 mg L^{-1} chloramphenicol) were added to each bottle, creating a slight over pressure in the head space (Smith and Tiedje, 1979); the original protocol by White and Reddy (1999) employed 1g L^{-1} of chloramphenicol; however, at nearly the same time a report by Murry and Knowles (1999) indicated levels of chloramphenicol greater than

100 to 200 mg L^{-1} may inhibit DEA activity by up to 60%. Samples were incubated in the dark at room temperature (24^{o}C) and continually shaken, and headspace gas was sampled every 1 h for 4 h. A Bunsen absorption coefficient of 0.544 was used to adjust for nitrous oxide dissolved in the aqueous phase (Tiedje, 1982). Potential denitrification rates were determined by the calculated slope of the linear curve produced for cumulative N_2O evolution with time. Two milliliters of headspace gas from each sampling time was stored in N_2-flushed 2 ml serum bottles sealed with butyl rubber stoppers and aluminum crimp seals for 24 to 48 h until determination of N_2O concentrations by gas chromatography.

Gas samples were analyzed for N_2O on a Shimadzu gas chromatograph (GC-14A, Shimadzu Scientific, Kyoto, Japan) fitted with a 3.7 x 10^8 (10 mCi) ^{63}Ni electron capture detector (300 ^{o}C). A 1.8 m by 2 mm i.d. stainless steel column packed with Poropak Q (0.177 to 0.149 mm; 80 to 100 mesh) was used (Supelco, Bellefonte, PA). The carrier gas was 5% methane in argon (v/v) flowing at a rate of 30 ml min^{-1} at 30^{o}C. Working standards consisted of N_2O in He (Scott Specialty Gas, Plumsteadville, PA).

Nucleic Acid Extraction, PCR Amplification, Cloning and Sequencing

Nucleic acids were extracted from 0.25 g of soil with Power Soil DNA Isolation kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's instructions. Purified DNA extracts were used as template in PCR; amplification was conducted using primer sets designed by Yan et al. (2004), consisting of primers 583F (5'-TCA TGG TGC TGC CGC CKG ACG-3') and 909R (5'-GAA CTT GCC GGT KGC CCA GAC-3') which amplify a 326 bp region of *nirK*, and 832 F (5'-TCA CAC CCC GAG CCG CGC GT-3') and 1606R (5'-AGK CGT TGA ACT TKC CGG TCG G-3') which amplify a 774 bp

region of *nirS*. Each 20 µl PCR reaction mixture contained 7 µl of distilled water, 1 µl of each primer (10 pmol μl^{-1}), 10 µl of HotStarTaq Master Mix (Qiagen, Valencia, CA) and one µl of template DNA. PCR amplification was carried out in a GeneAmp PCR system 9700 (Perkin-Elmer Applied Biosystems, Norwalk, CT). PCR conditions for both primer sets were identical and consisted of the following: an initial enzyme activation and DNA denaturation for 15 min at 95°C, followed by 30 s at 94°C, 30 s at 60°C, and 60 s extension at 72°C for 30 cycles, and a final extension of 72°C for 7 min. PCR products were analyzed by electrophoresis on 1.5% TAE agarose gels. To account for the spatial patchiness of soils and attempt to more fully characterize diversity, bulk nucleic acid extracts from all soil samples from within a site were combined.

PCR amplicons were ligated into pCRII-TOPO cloning vector and transformed into chemically competent *Escherichia coli* TOP10F' cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). White colonies were screened for correct inserts by PCR amplification using the protocol and conditions described above. Insert-bearing clones were transferred to 96-well plates containing 200 µl of Luria Bertini broth plus 8% (v/v) glycerol and kanamycin (50 µg ml^{-1}). Plates were incubated for approximately 24 h at 37^{o}C, covered with gas permeable membranes (Breath-easy, Diversified Biotech, USA), and transported to the University of Florida Genome Sequencing Service Laboratory for sequencing with internal vector-specific primers.

Phylogenetic and Diversity Analysis

Nucleotide sequences were manually aligned and translated into putative amino acids in Se-Al v. 2.0 a 11 (Rambaut, 1996) and aligned with Clustal v.1.81 (Thompson et al., 1997). Phylogenetic trees were produced for approximately 100 and 250 amino acid

segments of *nirK* and *nirS*, respectively, using Tejima and Nei corrected distance matrices in the TREECON software package (van de Peer and de Wachter, 1994). Bootstrap analysis (500 resamplings) was used to estimate reproducibility of phylogenies. Similarities of sequences obtained in this study were compared to those obtained from other studies using BLAST queries (http://www.ncbi.nlm.nih.gov) of the nucleotide database.

Community analyses were performed by generating operational taxonomic units (OTUs) in DOTUR, using the furthest neighbor algorithm and a 3% difference in nucleic acid sequences. Non-parametric estimates of richness and diversity were calculated using DOTUR (Schloss and Handelsman, 2005), including Chao1, Shannon index, and Simpson index.

Statistical Analysis of Phylogenetic Data

To assess whether gross differences observed between denitrifier populations between sites represented statistically different populations, well-aligned subsets of each gene fragment were chosen for analysis using ∫-Libshuff (Schloss et al., 2004) with 1,000,000 randomizations and a distance interval (D) of 0.01 (Santoro et al., 2006) using Jukes-Cantor corrected pairwise distance matrices generated in PAUP (Swofford, 1998). The program employs Monte Carlo methods to calculate the integral form of the Cramér-von Mise statistic by constructing random sub-set populations from the entire data set and comparing the coverage of the generated populations to coverage in the experimentally obtained data set. Populations were considered significantly different with P value below 0.0026 after a Bonferroni correction for multiple pairwise comparison (α=0.05, n = 20).

Analysis of molecular variance (AMOVA), pairwise comparisons of population specific pairwise fixation indices (F_{ST}) (Martin, 2002), and average pairwise sequence similarities were conducted with the program Arlequin (version 3.001, Genetics and Biometry Laboratory, University of Geneva [http://lgb.unige.ch/arlequin]). AMOVA (Excoffier et al., 1992) employs a hierarchically partitioned matrix of Euclidean distances to assess by permutation the significance of variance components at each level of partitioning. All analyses were performed under default parameters, with the following exceptions: analyses were conducted at 90,000 iterations and distance matrices defined haplotype definitions. F_{ST} tests were employed as measures of genetic differentiation between all pairs of samples. The test determines whether samples contain close phylogenetic relatives or more deeply divergent sequences. Mantel tests (Mantel, 1967; Mantel and Valand, 1970) were implemented in Arelquin and used to test correlations between population specific F_{ST} values and geochemical parameters. The method is based on a nonparametric general regression model which employs squared Euclidean distance matrices between variables to test significance of and degree of predictability one variable has on another (Dutilleul et al., 2000).

Parsimony tests (P-test) were implemented in TreeClimber (Schloss and Handelsman, 2006). Clustal X (version 1.83) was used to generate sequence alignments, constructed under default parameters. Trees were constructed by Bayesian analysis as implemented in Mr. Bayes version 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) under default model parameters, with trees sampled every 1000 generations. All Bayesian analyses were run for 1,000,000 generations, of which 10% were discarded to account for initial divergence in log likelihood scores between chains.

The resultant 990 trees were used for analysis in TreeClimber (http://www.plantpath.wisc.edu/fac/joh/treeclimber.html) and compared to 1,000,000 randomly generated trees.

Statistical Analysis of Biogeochemical Data

Environmental parameters were tested for significance across treatment groups (study sites) using one-way ANOVA in JMP version 5.1 (SAS Institute) on both log transformed and raw data. Pairwise comparisons of means were conducted in the same software using Tukey's HSD, which accounts for unequal variances among samples.

Results and Discussion

Soil Biogeochemical Parameters Along the Restoration Gradient

Measured values of nitrate, ammonium, and organic matter content (loss on ignition) in HID soils ranged from 1.8 to 6.8 mg kg^{-1}, 15.2 to 49.5 mg kg^{-1}, and 14.19 to 23.9 %, respectively; values for these parameters did not differ significantly across sites (ANOVA, $p < 0.05$). Potential Denitrifying Enzyme Activity (DEA) ranged from 0.12 to 1.15 mg N_2O-N kg^{-1} hr^{-1}; DEA rates for UND, R89, and R00 sites were significantly different from R97, R01, and R03 sites (ANOVA, $p < 0.05$). There was no evident trend in DEA associated with restoration age or other measured environmental parameters, although there may be disparity between actual and potential activities of denitrifiers in soils. Tiedje (1988) suggested that lab-based determinations overestimate field activity 40 to 100 times. Values reported in this study are within the range of values reported in surface soils from oligotrophic regions of the Everglades using similar methods (White and Reddy, 1999), as well as those reported from riparian (Schipper et al., 1993) and agricultural soils (Pell et al., 1996, Espinoza, 1997). Although there was no clear trend

associated with DEA and recovery stage in HID soils, statistical analysis (randomized complete block design, ANOVA) of site effects on biogeochemical data indicated significant ($\alpha = 0.05$, $P < 0.0001$) within-site effects on DEA. This may indicate that, while there does not seem to be an obvious or homogenously controlling factor on DEA along the restoration gradient, activity may be more strongly controlled by different factors within each restoration site.

Interestingly, relatively high DEA activities were observed in the two most recently restored sites, despite having less than 3 cm of soil and moisture contents similar to all other sites (Table 3-1). DEA activity may be spatially patchy, and is thought to occur in response to hot-spots within soil micro-aggregates (Parkin, 1987). Further, senescing plant material and leaf litter may support anaerobic processes (Kusel and Drake, 1996); Parkin (1987) observed that carbon inputs from a single leaf were sufficient to support 85% of observed denitrification.

A previous investigation of DEA in Everglades soils reported 3 to 30 fold higher activity in marl than peat soils from the Everglades (Gordon et al., 1986). Our results suggest less activity in marl soils compared with values from peat soils reported by White and Reddy (1999) the disparity may be due to lower soil depth at our sites, or that marl sampling sites differed in hydrologic features. Interestingly, potential denitrification from the two marl soils showed identical patterns (Gordon et al., 1986; this study); dentrifying populations in all but R00 soils showed no lag in response following nitrate addition. Nitrate accumulation was approximately linear over the course of the 4h incubation. This indicates denitrifying enzyme systems were fully induced in most sites.

nirS phylogeny

nirS-type denitrification genes were obtained from all HID study sites, and grouped into six (I to VI) distinct phylogenetic clusters (Figure 3-1), the relative abundances of clones from each library are presented in Table 3-2. Seventy-five percent amino acid similarity in DOTUR grouped sequences into phylogenetically distinct clusters. Genes from all clusters shared 78 to 98% similarity of amino acids with previously obtained environmental clones in GenBank. Most notably, of the 117 clones obtained from all study sites, 58% shared greatest similarity with environmental clones obtained from a Michigan wetland; nucleotide sequence similarities ranged from 80 to 98%. Clones of this sequence type may represent a group of uncultivated denitrifying bacteria that are physiologically adapted to wetland ecosystems (Priemé et al, 2002). Thirty percent of all clones were 80 to 87% similar to uncultivated denitrifying bacteria inhabiting high nitrate brackish ground waters in California (Santoro et al., 2006). The remaining 12% of obtained sequences varied in similarity from 76 to 86 % to environmental *nirS* obtained from wetland soils incubated under elevated CO_2 (Lee et al., 2005), activated sludge (Ohsaka et al., 2004), or Baltic Sea cyanobacterial aggregates (Tuomainen et al., 2003).

Clusters I, II, and III shared relatively high average pairwise sequence similarities, ranging from 92 to 97%; these sequence types may represent more abundant NirS-type denitrifiers common to all HID soils. Clusters IV, V, and VI comprised the lower portion of the tree, and consisted of loosely associated divergent lineages, with little apparent redundancy in the library (Figure 3-1, Table 3-2). Of the six designated phylogenetic clusters, only Cluster VI sequences were similar to *nirS* of previously characterized

denitrifiers, and shared 71 to 75% of predicted amino acids with organisms of the alpha-proteobacterial genera *Thauera* and *Azoarcus*, or the beta-proteobacterial *Magnetospirillum* spp. Cluster VI was comprised of sequences from all HID sites. However, sequence similarity is too low to confidently conclude that Cluster VI *nirS* genotypes obtained in this study belong to organisms within these genera. Sequences from all other clusters showed low similarity to cultivated denitrifiers based on both nucleotide and putative amino acid BLAST searches. With the exception of Cluster III sequences, which showed ca. 95% similarity to uncultivated organisms from wetland soils, *nirS* genotypes obtained in this study shared less than 85% similarity with previously reported sequences, and may be indicative of a unique assemblage of denitrifying bacteria in HID soils.

nirK phylogeny

Phylogenetic diversity was apparent from analysis of the 158 obtained *nirK* sequences (Figure 3-2). Clones obtained from all study sites grouped into 12 distinct phylogenetic clusters; within cluster sequence similarities ranged from 85 to 98% (Figure 3-3). Additionally, *nirK* clone libraries, more so than *nirS*, contained a number of deeply branching divergent singletons from all sites: REF (7), R89 (3), R97 (6), R00 (5), R01 (4), and R03 (5). The relative percentages of sequence types within each cluster are presented in Figure 3-3. Of the 158 sequences included in the phylogenetic analysis, 66 (42%) shared 90 to 91% similarity with environmental clones previously obtained from a wetland soil; these sequences comprised clusters A through E, and were obtained from all restoration sites (Figure 3-3). The remaining clusters (F through L) each formed distinct, deeply branching clades. Cluster F sequences were 84 to 92% similar to *nirK* genes

obtained from a potentially novel group of uncultured denitrifiers in fertilized upland soils (Wolsing and Priemè, 2004). Cluster G clones were 82% similar to environmental sequences obtained from peat (Throbäck et al., 2004), and shared 79 to 80% similarity with *nirK* of *Alcaligenes xylosoxidans*. Cluster H sequences shared greatest similarity with uncultivated denitrifiers obtained from forest soils (Priemè, 2002). Sequences comprising Cluster I were 93% similar to those in *Bradyrhizobium japonicum*. Cluster J clones were 92% similar to *nirK* of *Ensifer* sp. 2FB8. Clusters K and L clones were 73 to 84% similar to environmental clones from municipal wastewater (Throbäck et al., 2004). Those comprising Cluster L were also 81 to 87% identical to sequences reported for *Sinorhizobium meliloti*.

Overall, the majority of both *nirS* and *nirK* sequences obtained in this study shared less than 90% sequence similarity with previously reported environmental sequences. While the exact cut-off of sequence similarity to previously reported environmental clones or cultivated isolates for either gene is not known, previous reports have employed a cut off of 75% nucleotide similarity as the threshold for claiming recovery of novel *nir* genes. This is based on the observation of an approximately 75% shared nucleotide identity between the alpha-, beta-, and gamma-proteobacteria (Yan et al., 2003). In consideration of this, and the fact that the majority of sequences obtained in this study shared no significant similarity to cultivated organisms upon BLAST search, it may be likely that our sequences represent several lineages of novel denitrifying organisms. However, the existence of denitfying bacteria from previously characterized lineages within the alpha- and beta-Proteobacteria are evident in both clone libraries (Cluster IV in Figure 3-1; Clusters I and J in Fig 3-2).

While the occurrence of novel lineages of denitrifiers based on studies of both *nirS* and *nirK* have been reported (Yan et al., 2001; Priemé et al., 2002; Liu et al., 2003), the uniqueness of such results may not be uncommon when certain factors are taken into consideration: i) functional gene diversity is generally greater than 16S rRNA diversity (Ward, 2002); and (ii) the ability to denitrify spans all three kingdoms of life (Ward, 2002). The pertinent point regarding novel groups to this study is not their existence, but that soils from each of the HID study sites appear to harbor uniquely divergent populations of denitrifiers.

Richness and Diversity of *nirS* and *nirK* Populations

Rarefaction analysis was used to compare richness of *nir* clone libraries in the context of restoration stage. OTUs were defined by DOTUR using a 97% DNA sequence similarity cutoff. Rarefaction indicated *nirK* and *nirS* richness to be approximately similar between all restoration sites; however, there was a clear difference in OTU richness between populations in UND soils. With the exception of the UND *nirS* curve, which was nearing a plateau, curves for all libraries were steeply sloped at the respective cut off points for sequences obtained from each site, suggesting that our clone libraries do not represent the entire diversity of the denitrifying populations. Coverage values for *nirS* and *nirK* clone libraries for each site are presented in Table 3-3. Coverage for *nirS* libraries ranged from 45 to 90%, R01 and R03 libraries had the highest and lowest coverage values, respectively. *nirK* coverage values ranged from 45 to 100%, and were highest for the UND library and lowest for the R01 library.

Both Simpson and Shannon diversity indices indicate *nirS* libraries to be the most diverse in restoration study sites; UND soils maintain greater *nirK* diversity (Table 3-3).

Greater diversity of *nirS* relative to *nirK* has been previously observed in wetland soils (Prieme et al., 2002), groundwaters (Yan et al., 2001; Santoro et al., 2006), and sediments from a marine oxygen minimum zone (Liu et al., 2003). Differences in diversity are more clearly pronounced in Simpson values versus Shannon values (Table 3-3); this difference may be due to the stronger influence of library eveness on Shannon values (Magurran, 2004). Further, the log-transformed Simpson values presented in Table 3-3 are sample size independent estimates (Magurran, 2004). There is a clear inverse relationship between *nirS* and *nirK* population diversities within sites. This relationship has also been observed in both marine (Braker et al., 2000) and terrestrial environments (Priemè, 2002; Yan et al, 2003), and may suggest that different environmental parameters alter abundances of organisms containing *nirS* or *nirK*, and that community dynamics of each group may alter dynamics of the other (Yan et al., 2003). However, detailed discussion of HID site parameters controlling diversity of *nir* genotypes using the presented diversity and richness estimates must consider that fact that the estimates presented here are based on clone libraries that do not represent the entire diversity of *nir* populations. Attempts to correlate measures of diversity or richness with geochemical parameters or restoration age yielded no significance. Previous studies have also failed to make significant correlations between diversity or richness measures and environmental variables (Yan et al., 2003; Santoro et al., 2006). The inability to correlate statistical measures of community composition with geochemical parameters may be due in part to the relatively poor understanding we have of factors controlling diversity of *nir* genotypes in the environment, or that community structure is controlled by less quantifiable factors (Santoro et al., 2006).

Population-Based Library Compositions

Iterative statistical analyses were employed to assess significant differences in population composition between restoration sites. UND sequences for either *nir* genes were excluded, as the focus of this study was to assess *nir* population dynamics in the context of disturbance recovery. Not only was the UND site never disturbed, it is not at the same successional stage as the restoration study sites.

To assess gross differences in *nir* populations represented by our clone libraries, ∫-Libshuff (Singleton et al., 2001; Schloss et al., 2004) was employed. By comparing random permutations of sequences from two libraries, determination of whether two clone libraries are likely to represent samples drawn from statistically distinct populations can be made. The asymmetrical nature of the test allows for the determination of clone libraries as distinctly different, drawn from the same population, or if one library is the subset of another. If libraries X and Y do not share common ancestry, comparisons of both will result in significant P values (bold in Table 3-4). However, if library X is statistically different from library Y, but Y versus X is not, Y is a subset of X. In cases where homologous (within one library) and heterologous (between two libraries) coverages differ significantly, one can be reasonably certain that the samples are drawn from different populations (Schloss et al., 2004). Advantages of the method are that it operates on an individual sequence level, rather than the arbitrary assignment of OTUs, and does not consider clone frequency (Singleton et al., 2001; Schloss et al., 2004). This method of community differentiation was developed for 16S rRNA gene libraries, but has been used to differentiate functional gene libraries (Horn et al., 2006; Yannarell et al.,

2006;), including *nirS* and *nirK* (Santoro et al., 2006), obtained from sites at different successional stages (Dunfield and King, 2004; Nanba et al., 2004).

Further, to test whether observed phylogenetic structures are the result of random variation, parsimony tests were employed. The test assesses the probability that phylogenetic patterns observed in user-constructed trees varies from randomly constructed trees after multiple iterations (Schloss and Handelsman, 2006). If the observed patterns are due to random variation, than user-generated trees would have similar parsimony scores as randomly generated tress. The null hypothesis of the analysis is that the compared communities share an ancestral community structure and observed patterns are due to accumulation of random variation; significance indicates observed phylogenetic differences between two communities to be the result of selective pressures, such as perturbation, that force differentiation within treatment populations (i.e gain or loss of groups) (Schloss and Handelsman, 2006).

The approach of the two tests differs and must be noted. ∫-Libshuff is based on a continuous statistic, measures community membership (the presence or absence of individuals within a population), and is relatively less sensitive to library size (Schloss et al., 2004). The parsimony (P) test is based on a discrete statistic, measures community structure (the distribution and abundances of individuals within a population), and is more sensitive to library size (Schloss and Handelsman, 2006). However, library sample sizes approximately equal to those employed by this study have been proven effectively large enough for both tests (Singleton et al., 2001; Dunfield and King, 2004; Nanba et al., 2004; Schloss et al., 2004; Schloss and Handelsman, 2006).

Results of the *nirS* analysis indicate that most sequences obtained from each site are site-specific (Table 3-4). Further, the shared similarity between sites most closely related in time since restoration do not differ significantly; this may indicate a succession of shared lineages between the most closely related or all restoration sites. As seen in the phylogenetic analysis, several clusters were comprised of sequences obtained from all sites. However, the deeply divergent taxa appear to be unique to each site, and are likely responsible for much of the difference between restoration sites. Analysis of community covariance with phylogeny, as implemented in TreeClimber, confirms that community structures from each site are significantly different ($P < 0.02$); removal of any site from the analysis did not lead to loss of significance, and pairwise comparisons of all sites were significantly different ($P < 0.02$). Prior studies that removed distinct groups for P test analysis discerned groups responsible for differentiation (Martin, 2002; Schloss and Handelsman, 2006). However, the consistency of P values upon library removal in this study suggests that each site harbors distinct and unique divergent lineages.

Succession of shared sequence types was not as clear when *nirK* clone libraries were analyzed with ∫-Libshuff (Table 3-4). R03 and R01 clone libraries were drawn from the same population, which is a subset of the R00 library. The R00 library differed significantly from R97 and R89 libraries, however, R89 and R97 libraries had shared lineages. A P test including all populations indicated significant differences ($P = 0.032$), and pairwise comparisons for all sites were also significant ($P < 0.02$). Thus, while the "more recovered" sites (R89 and R97) share an underlying community, both harbor unique lineages of denitrifiers, possibly selected for by disturbance recovery stage. A comparison of sites grouped into two data sets consisting of "more" and "less" recovered

clone libraries yield the lowest significance value of any *nirK* library comparisons (P = 0.012). Removal of R97 or R00 libraries from analysis lead to loss of significance between groups (P = 0.08 for R97 and P=0.06 for R00), however when the two libraries were compared they were significantly different. Thus, consistent with ∫-Libshuff analysis, this indicates a divide in population composition between early (< 6 yr) and late (> 6 yr) restoration sites, and suggests that R97 and R00 sites harbor more divergent lineages or that populations differ significantly from R89, R01, or R03.

Statistical analyses of *nirS* suggest shared lineages along the restoration chronosequence. Though it was not addressed in this study, it is likely that Cluster A sequences, which were obtained from all sites represent a group of denitrifiers native to HID soils, regardless of disturbance stage. Interestingly, analysis of *nirK* libraries indicates a bimodal response to recovery stage, with sites closer in disturbance recovery sharing similar, but distinct, communities of denitrifiers. Alternatively, R00 and R97 sites, for which these analyses suggest harbor different populations, may be representative of intermediate states of disturbance. According to the "Intermediate Disturbance Hypothesis", ecosystems at intermediate stages of recovery from disturbance harbor the greatest species diversity (Connell, 1978).

Consistent with *nirK* variation, geochemical data show a similar, but insignificant, trend. Soils in later succession sites share similar related organic matter, nitrate, and ammonium contents (Table 3-1) than sites at early stages of recovery. While it may seem contrived, the variability in the data should not hinder inferences based on geochemical trends. Geochemical analyses were conducted on triplicate composite soil samples; each representative sample was comprised of three soil samples taken at relative

depth intervals (shallow, medium, deep) within each site. Compositing of samples was done in this manner to account for variations in both bedrock surface topography and spatial differences in regions of soil accretion. At successional stages as early as those in the HID, spatial patchiness is inevitable, and likely to overwhelm statistical differentiability. Several previous studies have observed different responses of *nirS*-and *nirK*-type denitrifying communities to environmental gradients; in several cases *nirK* showed greater habitat selectivity (Throbäck et al., 2004; Wolsing and Priemè, 2004; Santoro et al., 2006), however, the opposite has also been reported (Liu et al., 2003, Yan et al., 2003).

Variance within *nirK* Clone Libraries

To further test the observed trends in genetic variation in denitrifier communities among restoration sites, analysis of molecular variance (AMOVA) was implemented (Excoffier et al., 1992); AMOVA has been previously applied for differentiation between community structures based on functional genes (Dunfield and King, 2004; Nanba et al., 2004; Yannarell et al., 2006). Only *nirK* libraries were chosen for this level of analysis, due both to the observed difference in response to recovery and discrepancies in *nirS* phylogenetic analysis in previous studies. Some studies correlating *nirS* response to environmental gradients have included (Braker et al., 2000; Priemé et al., 2002) or excluded (Santoro et al., 2006) regions of insertion or deletion for sequence alignments and phylogenetic analysis. AMOVA estimates the significance of differences in population pairwise fixation indices (F_{ST}). F_{ST} values for a population, or group of populations, are an indication of genetic differentiation; in the case of molecular ecology, it is a representation of within population diversity relative to total population diversity

(in this case, diversity of pooled sequences to diversity of libraries from each site) (Martin, 2002). Pairwise comparisons of F_{ST} values for each site reveal whether genetic diversity between sites differs (Martin, 2002). In relation to total population diversity, low F_{ST} values indicate that diversity of the individual community is similar to that of the two communities combined (Martin, 2002).

Variation of *nirK* populations within sites accounted for approximately 98% of variance, only 2% was due to variation between libraries from each site. The large percentage of variation within population further confirms the uniqueness of *nirK* communities from each site. Although small, variance in diversity between populations differed significantly from pooled populations ($P = 0.013$), consistent with results of the parsimony test, and further confirms the existence of unique lineages of denitrifiers within each restoration site.

F_{ST} values for each site declined with time since restoration; these values are measures of genetic diversity within population compared to the total population. The general decline in values with time since restoration suggests that populations of denitrifiers become more reflective of total observed diversity in HID soils as recovery progresses. Further, F_{ST} values confirm the results of ∫-Libshuff analysis: F_{ST} values between sites nearer in recovery stages are closely related. Values for R89 and R97 range from 0.030 to 0.033 and values for R00, R01, and R03 range from 0.045 to 0.042. This bimodal trend is also evident in average pairwise sequence similarity ($\theta[\pi]$) and nucleotide diversity (Table 3-6).

Pairwise comparison of population F_{ST} and $\theta[\pi]$ values are presented in Table 3-6. Results for comparison of both values between sites are identical, as both are *de facto*

measures of within-population diversity. Significance of both tests implies genetic diversity within sites is less than for sites combined, but that each harbors distinct phylogenetic lineages; this is the case for the R89 community. Insignificance of F_{ST} paired with significant P tests, which is the case for pairwise comparison of R97, R00, R01, and R03, is indicative of high diversity within each of these populations and that each harbors different phylogenetic lineages; this can occur when each population is comprised of many ancient lineages that do no overlap (Martin, 2002).

A Mantel (Mantel, 1967; Mantel and Valand, 1970; Dutilleul et al., 2000) test was used to examine the correlation between pairwise differences in *nirK* population-specific F_{ST} values between sites to matrices of pairwise differences in geochemical parameters. Such analyses have been used previously to test whether observed differences in functional gene diversity correlated with geochemical variables between sampling sites (Francis et al., 2003). The Mantel test judges whether closeness of one set of variables is related to closeness in another set of variables. In the context of this study, the test was employed to determine correlations between observed differences in *nirK* diversity between sites and measured environmental parameters along the chronosequence, and to ultimately gain an understanding of environmental factors most likely controlling the observed differences in *nirK*-type denitrifier populations in HID soils. Pairwise F_{ST} matrices were tested for correlation with environmental factors most likely controlling denitrifier activity: organic matter (loss on ignition), moisture content, and soil oxygen demand. Differences in *nirK* F_{ST} values between sites were strongly correlated with differences in soil moisture content ($r = 0.895$, $P=0.017$), and marginally with differences in organic matter content ($r = 0.61$, $P = 0.05$). The results suggest that soil moisture plays

a strong role on *nirK* population diversity within each site, and may be used to explain differences in populations between sites.

Conclusions

Little work to has been done to characterize denitrifying microbial communities in wetland ecosystems. Further, this is the first study to characterize the development of wetland communities of denitrifying bacteria in response to severe disturbance. While geochemical data in sites of varying stages of recovery since complete soil removal suggest loose trends associated with time since restoration, several lines of evidence indicate the existence of significantly different populations of denitrifying bacterial communities at each of the study sites. *nirS* clone libraries suggest an approximately linear response with time since disturbance, while *nirK* sequences appear to respond bimodally. In either case, this suggests that diversity of functionally redundant enzymes results from adaptation to particular environments. The factors governing community diversity are not entirely clear. However, the most obvious variable is recovery stage, the gradual accumulation of nutrients, soil and associated moisture, and the maturing of plant communities. Further, results of AMOVA indicate population diversity within sites to decline with time since restoration, which may indicate a gradual decrease in species recruitment as conditions within each site converge toward stability. These results highlight the sensitivity of denitrifying bacterial communities to environmental conditions, and provide insight into microbial community dynamics in response to ecosystem recovery.

Table 3-1. Biogeochemical parameters of HID soils as measured in November 2005.

Site	Soil Depth (cm) ‡	Moisture (%)	LOI (%)	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})	Denitrification Potential
UND	10 (2-15)	43.8 (0.6)	16.71	9.9 (0.9)	6.8 (0.5)	0.51 (0.14)
R89	6 (1-17)	59.6 (2.4)	14.38	46.9 (1.9)	3.1 (0.5)	0.48 (0.14)
R97	5 (3-11)	58.8 (4.5)	14.19	49.5 (10.8)	1.8 (0.6)	1.01 (0.27)
R00	3 (1–4)	48.3 (1.4)	23.90	25.1 (7.7)	7.8 (2.8)	0.12 (0.03)
R01	3 (1-7)	41.6 (1.1)	23.58	15.2 (5.1)	6.5 (2.8)	1.15 (0.33)
R03	1 (0-3)	43.5 (10.1)	18.86	26.9 (9.2)	5.1 (2.8)	0.77 (0.11)

‡Values in parentheses are standard deviations of the mean of three replicate samples.

†Potential denitrifying enzyme activity expressed as milligrams of N_2O-N per kilogram soil per hour.

Table 3-2. Distribution of *nirS* sequences from each study site within designated phylogenetic clusters.

Cluster	Relative abundance of sequences from each clone library (%)						Average similarity (%)[a]	No. of sequences
	UND	R89	R97	R00	R01	R03		
I	32	11	7	27	9	14	97 (4)	44
II	36	0	18	18	0	27	92 (12)	11
III	0	0	16	32	32	21	97 (4)	19
IV	0	13	25	13	25	25	83 (20)	8
V	9	36	14	23	5	14	79 (18)	22
VI	8	23	15	8	15	31	72 (16)	13

[a]Based on pairwise comparison of deduced amino acid sequences within each cluster, values in parenthesis are standard deviations

Table 3-3. Values of *nirS* and *nirK* diversity and richness in HID soils, as estimated by Shannon diversity index, Simpson index, and Chao1 richness calculated using DOTUR (Schloss and Handelsman, 2005).

Site and gene	No. of clones sequenced	No. of OTUs[a]	Shannon index	Diversity[b]	Richness[c]	No. of singletons	Coverage[d] (%)
nirK							
UND	28	13	2.3 (1.9, 2.6)	2.5	20 (13, 54)	6	100
R89	28	12	2.3 (1.8, 2.5)	2.2	22 (14, 66)	7	69
R97	25	11	2.0 (1.7, 2.4)	1.9	21 (13, 65)	7	86
R00	30	16	2.4 (1.9, 2.8)	2.1	38 (21, 102)	12	72
R01	24	12	2.3 (1.9, 2.6)	2.4	19 (13, 48)	7	45
R03	23	12	2.2 (1.9, 2.6)	2.3	26 (15, 79)	8	71
ALL	158						
NirS							
UND	21	8	1.8 (1.4, 2.1)	1.8	11 (8, 31)	4	48
R89	17	10	2.2 (1.8, 2.5)	2.7	15 (11, 39)	6	56
R97	15	16	2.7 (2.4, 3.0)	3.7	32 (20, 80)	12	67
R00	27	16	2.6 (2.2, 2.9)	2.8	82 (38, 212)	12	76
R01	15	12	2.4 (2.1, 2.7)	3.1	16 (13, 34)	7	90
R03	22	18	2.8 (2.5, 3.1)	3.8	53 (27, 144)	15	45
ALL	117						

[a]Estimates of OTUs, Shannon index, diversity and richness are all based on 3% differences in nucleic acid sequence alignments; values in parantheses are upper and lower bounds of 95% confidence intervals as calculated by DOTUR.

[b]Sample size independent estimate of diversity based on negative natural log transformation of Simpson's index values as calculated in DOTUR.

[c]Chao1 values, a non-parametric estimate of species richness.

[d]Coverage values for at distance = 0.01, as calculated by ∫-Libshuff (Schloss et al., 2004).

Table 3-4. Population similarity P values for comparison of nirK and nirS clone libraries determined using Cramer-von Mises test statistic, implemented in ∫-Libshuff (Schloss et al., 2004).

Gene (n)	Site for homologous library (*X*)	P values for comparison of heterologous library (*Y*) with *X*[a]				
		R89	R87	R00	R01	R03
nirK (158)	R89		0.036	**0.000**	**0.000**	**0.000**
	R97	0.400		**0.000**	**0.000**	**0.000**
	R00	**0.000**	**0.000**		***0.000***	**0.000**
	R01	**0.000**	**0.000**	0.040		0.957
	R03	**0.000**	**0.000**	**0.000**	0.304	
nirS (117)						
	R89		0.421	**0.000**	**0.000**	**0.000**
	R97	0.125		0.040	**0.000**	**0.000**
	R00	**0.000**	0.210		0.056	**0.000**
	R01	**0.000**	**0.000**	0.269		**0.000**
	R03	**0.000**	**0.000**	**0.000**	**0.000**	

[a]Values in bold indicate significant P values ($P < 0.0017$)after Bonferroni correction for multiple pairwise comparisons. Libraries are distinct from one another if both comparisons (X versus Y and Y versus X) are significant. Values in italics indicate that library Y is a subset of library X.

Table 3-5. Corrected average pairwise differences (θ[π]), above diagonal) and pairwise fixation indices (FST, below diagonal) for *nirK*.

Site	Result for study site[a]:				
	R89	R97	R00	R01	R03
R89		**4.312**	1.344	**3.201**	**4.564**
R97	**0.060**		**3.021**	1.569	1.825
R00	**0.029**	**0.049**		0.905	0.922
R01	**0.048**	0.028	0.015		-1.031
R03	**0.069**	0.034	0.016	-0.023	

[a]Bold values are significant at $P < 0.05$.

Table 3-6. Fixation indices, average pairwise differences (θ[π]), nucleotide diversity, and shared haplotypes of *nirK* clone libraries as calculated by Arlequin (Excoffier et al., 1992).

Site	F_{ST}	θ[π]	Nucleotide Diversity[⊥]	No. of unique haplotypes	No. of shared haplotypes				
					R89	R97	R00	R01	R03
R89	0.030	89 (44)	0.25 (0.12)[a]	23	-	1	1	2	1
R97	0.033	83 (37)	0.24 (0.11)[a]	21	2	-	4	3	3
R00	0.042	66 (29)	0.19 (0.01)[b]	28	1	4	-	3	4
R01	0.043	64 (27)	0.18 (0.01)[b]	17	2	3	3	-	3
R03	0.045	62 (28)	0.17 (0.09)[b]	18	1	3	4	3	-

[⊥]Values sharing same letter notation are not statistically different, based on pairwise Student's t-test ($P \leq 0.05$).

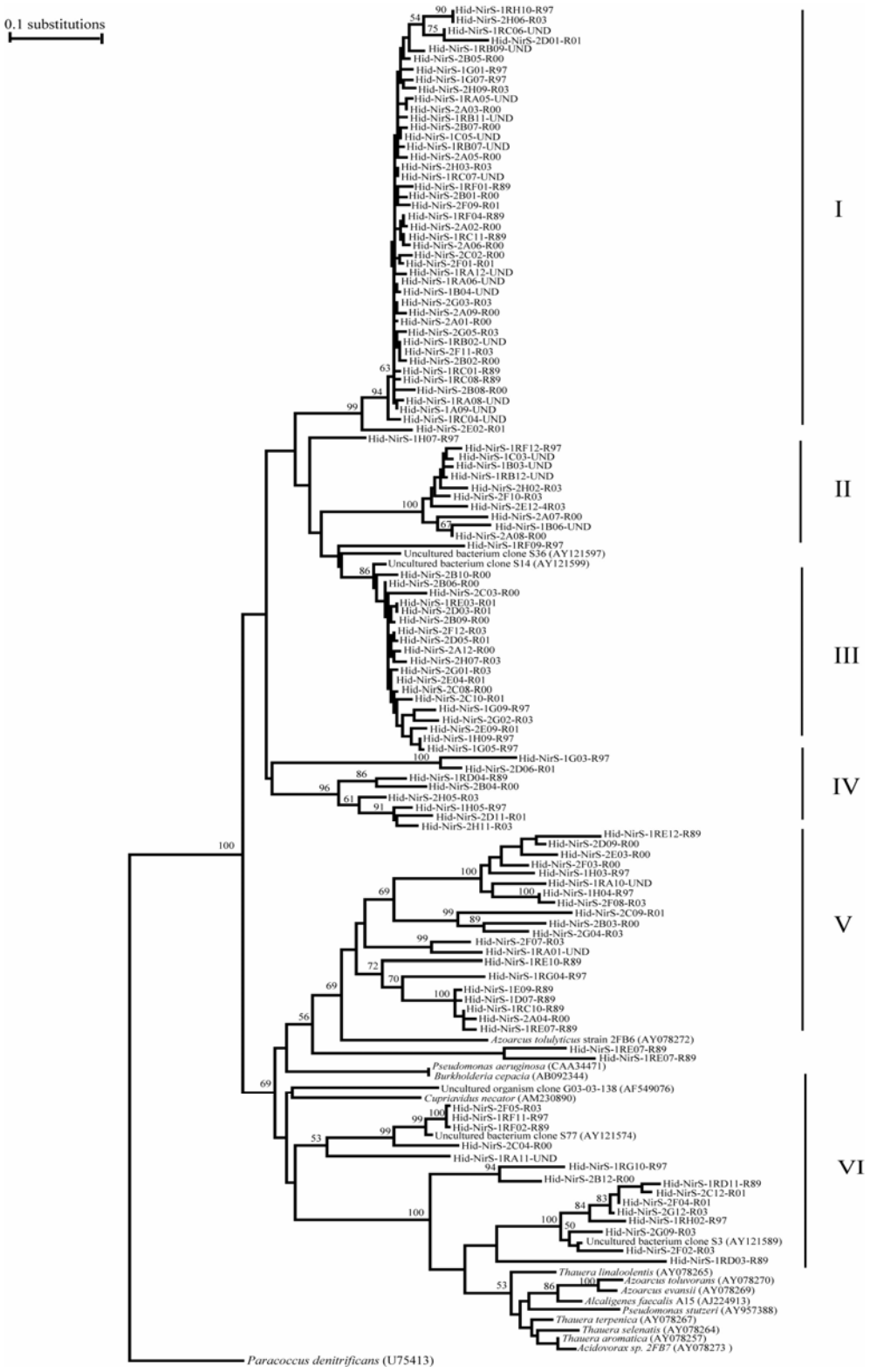


Figure 3-1. Neighbor-joining tree of *nirS* sequences obtained from wet season soils. Values on nodes are bootstrap scores based on percent occurrence of 1000 resamplings.

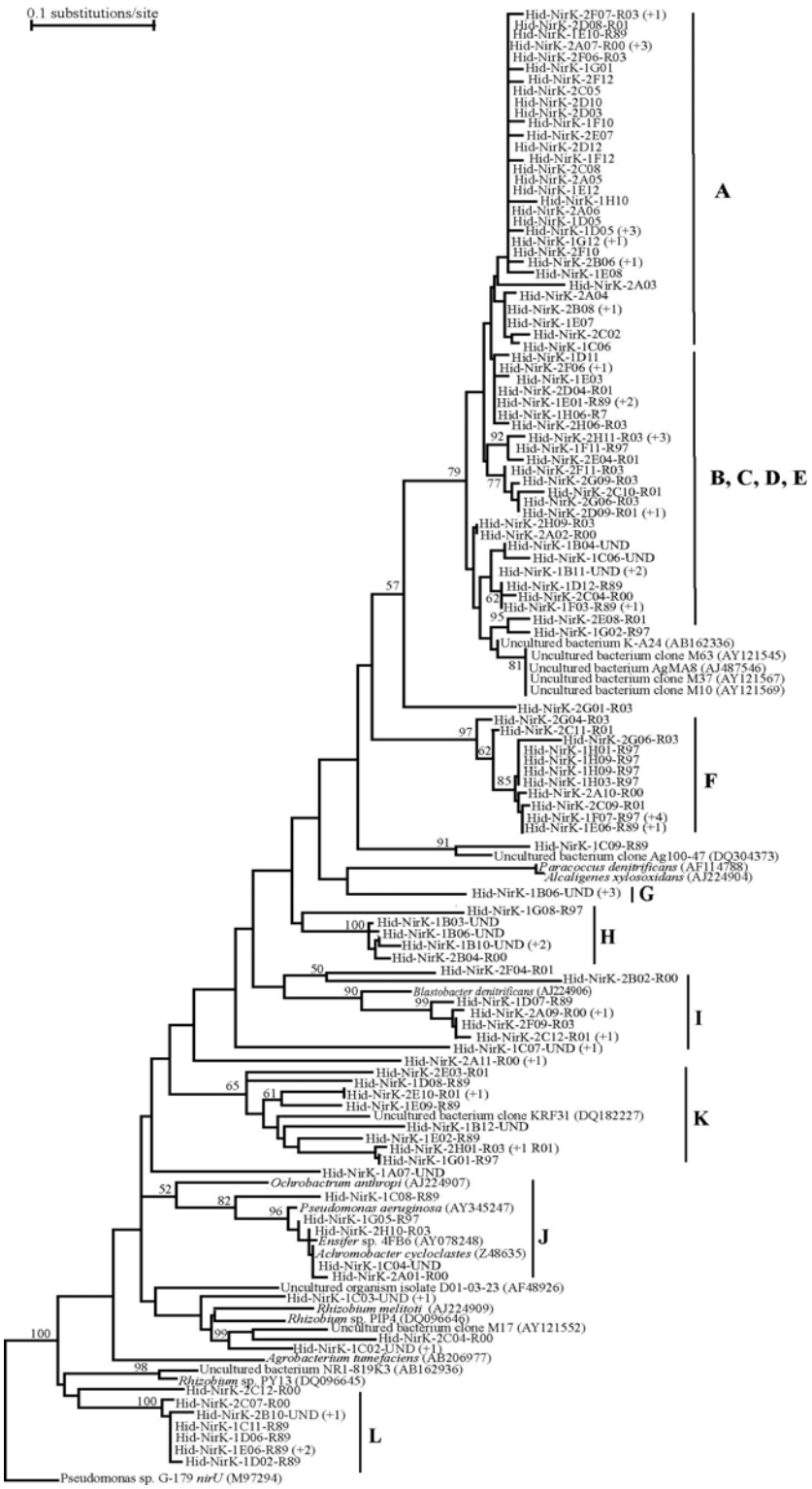
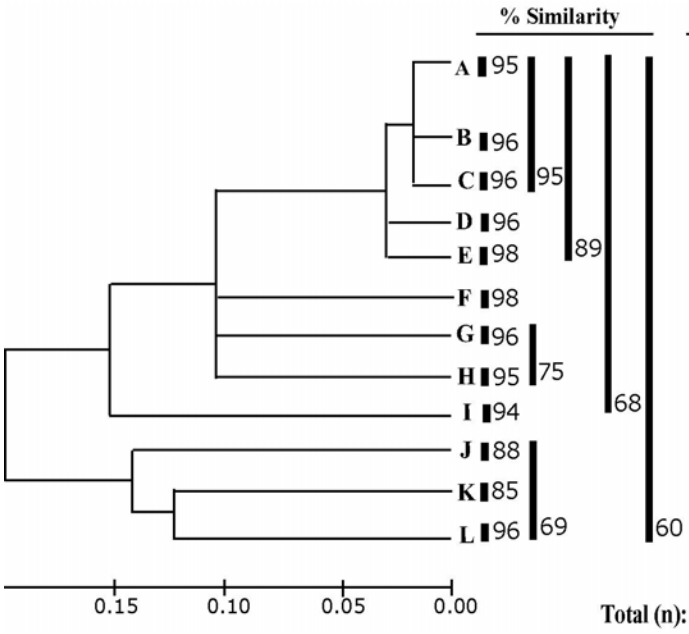


Figure 3-2. Neighbor-joining tree of *nirK* sequences obtained from wet season soils. Values on nodes are bootstrap scores after 1000 resamplings.



	% of each cluster					
	UND	R89	R97	R00	R01	R03
A	10	22	12	29	12	15
B	29	14	-	57	-	-
C	-	22	11	-	44	22
D	-	-	-	-	43	57
E	-	-	50	-	25	25
F	-	13	56	6	13	13
G	100	-	-	-	-	-
H	83	-	-	17	-	-
I	-	17	-	33	33	17
J	40	-	20	20	-	20
K	10	30	10	-	40	10
L	21	50	-	29	-	-
*	7	3	6	5	3	5
Total (n):	28	28	25	30	24	23

Figure 3-3. Sequence analysis of *nirK* clones obtained from wet season soils. Sequences were grouped into clusters (A to L) based on inspection of alignments, distance data, and neighbor-joining trees. Percent similarity is based on comparison of putative amino acids and was determined between all members of each group(s). The percentage of each *nirK* sequence types recovered from each site is listed in the table next to the figure. Asterisk represents sequences that could not be readily assigned to a cluster (singletons).

CHAPTER 4
SEASONAL DIVERSITY AND FUNCTION OF AMMONIA OXIDIZING BACTERIA ALONG A SHORT-TERM RESTORATION CHRONOSEQUENCE

As the linking process between organic nitrogen mineralization and loss of biologically preferable forms of inorganic nitrogen, nitrification is a determinate process in the availability of N within an ecosystem, and an influential factor on productivity of plant and microbial communities. Nitrification is a two-step process involving two distinct groups of bacteria. The first, the conversion of ammonium to nitrite is mediated by ammonia oxidizing bacteria (AOB); the second step is the conversion of nitrite to nitrate, and is mediated by nitrite oxidizing bacteria (NOB). The most common rate-limiting step is the conversion of ammonium to nitrate, carried out by AOB. The first step involves conversion of ammonia to hydroxylamine by ammonia monooxygenase (AMO), while hydroxylamine oxidoreductase converts hydroxylamine to nitrite (Hooper et al., 1997). Nitrate production due to heterotrophic bacterial activity has also been observed, though it is generally limited to conditions of high carbon-to-nitrogen ratios or acidic soils (Pedersen et al., 1999; Bothe et al., 2000; Kowalchuck and Stephen, 2001). Chemolithotrophic AOB are thought to be the major contributors to nitrification in soil, sediment, marine, freshwater, and estuarine environments (Belser, 1979; Bothe et al., 2000).

All AOB possess *amoA*, which codes for the alpha-subunit of AMO. Early characterization of AOB diversity within the environment involved the use of 16S rRNA gene specific primers (Stephen et al., 1996; Kowalkchuk and Stephen, 2001); these

studies revealed significant patterns in phylogenetic clustering of AOB sequences putatively in response to environmental parameters. However, ribosomal DNA does not provide significant evidence of function. Functional genes maintained by an organism define its interaction with the environment. They evolve faster and may provide greater phylogenetic resolution. Recent work by Purkhold et al. (2000) revealed a congruence of phylogenetic clustering between *amoA* and 16S rRNA genes of AOB, allowing for correlation of *amoA* clusters with established 16S rRNA clusters, and subsequently providing further insight into possible mechanisms controlling the ecology of organisms within the established clusters. Since the initial identification of *amoA* as a molecular marker of AOB diversity in the environment (Rotthuwae et al., 1997), diversity and structure of AOB populations have been studied along environmental gradients and correlated with shifts in environmental variables in successional grasslands (Kowalchuk et al., 2000), estuarine sediments (Francis et al., 2003), wastewater bioreactors (Rotthuwae et al., 1997), marine environments (Mullan and Ward, 2005), and in response to global change (Horz et al., 2004).

Despite clear evidence of AOB activity in wetlands and other anoxic systems (Reddy and Patrick, 1984; Laanbroek and Woldendorp, 1995), relatively little work has been done to characterize dynamics of AOB populations or nitrification activity in wetland soils (Duncan and Groffmann, 1994; White and Reddy, 2003). To date, the only study to characterize wetland AOB using molecular approaches was conducted in a manure-impacted treatment wetland (Ibekwe et al., 2003).

Oxygen transport by aerenchymatous plant tissues to saturated soils establishes oxygenated microsites within the rhizosphere conducive to AOB activity (Reddy and

Patrick, 1984; Kowalchuk et al., 1998). Diffusion gradients of reduced and oxidized compounds between aerobic microsites and anoxic bulk soil, or between sediment-surface water column exchange, may provide sufficient supply of resources to maintain nitrifying activity under flooded conditions (Reddy et al., 1989). Further, seasonal inundation provides a unique opportunity to study the response of nitrification and AOB in concert with shifts in availability of regulatory substrates such as oxygen and ammonium.

Primary succession is the development of plant and microbial communities on bare substrate. Parent substrate usually contains sufficient amounts of mineral nutrients, such as phosphorous, but generally harbors negligible amounts of bioavailable N (Vitousek et al., 1989). Thus, nitrogen inputs to developing ecosystems likely originate from exogenous sources, such as atmospheric fixation and rainwater. Successional changes in the availability of N have received much attention, in particular because N most often limits primary production in terrestrial ecosystems (Vitousek and Howarth, 1991). It has been hypothesized that successional changes in nitrate production have substantial effects on ecosystem level N losses. Nitrification has been implicated as the primary mechanism of N loss during succession in upland soils (Robertson and Vitousek, 1982). Further, Rice and Panchloy (1972) hypothesized that nitrification generally decreases with successional stage due to inhibition of nitrifying bacteria by plant allelochemicals in later successional stages. While several studies have proven this hypothesis to be true in the context of primary to secondary succession (Rice and Pancholy, 1972; Robertson, 1982; Robertson, 1989), nitrification rates in ecosystems

undergoing primary succession have indicated the opposite (Robertson and Vitousek, 1981).

Sequential development of microbial and plant communities in concert with soil accretion in HID sites at differing stages of disturbance recovery provides an excellent opportunity to characterize the dynamics of nitrification and AOB during a critical stage of initial ecosystem recovery. Specifically, this study sought to: (i) explore nitrification activity during early stages of primary succession, by assessing the activity of AOB concurrent with the development of soils; and (ii) characterize the activity and population genetic structure of specific genotypes of AOB across seasons and time since restoration, in hopes of elucidating factors that control activity and guild composition within and between seasons both within each site and along the restoration gradient.

Materials and Methods

Site Description, Sampling, and Biogeochemical Characterization

Samples were collected in November 2004 and May 2005. Plots 20 x 20 m^2 were established in sites restored in 1989, 1997, 2000, 2001, and 2003 (R89, R97, R00, R01, and R03, respectively), and in an undisturbed site (UND). The range of elevation for the five plots was 0.5 to 0.6 m. Within each sampling area, 2 x 2 m^2 grids were used to establish 81 sampling nodes, which were monitored for soil depth, ground coverage, and elevation. Nine nodes were chosen based on relative range of soil depth within each site, 3 from each depth range (shallow, intermediate, deep). Sampling nodes were color coded and marked for future sampling efforts. Soil samples were taken with a plastic coring device; however, due to non-uniform soil cover in recently restored sites, grab samples were collected where appropriate. Individual samples from each depth range were combined to make three representative soil samples, which were used for molecular and

geochemical analyses. Soil samples were kept on ice and transported to the laboratory within 72 h of the collection, where they were manually mixed and large roots removed. Subsamples for DNA analysis were stored at -70 °C until analysis. Biogeochemical analyses were conducted at the Wetland Biogeochemistry Laboratory (D'Angelo and Reddy, 1999; White and Reddy, 1999). Values for select parameters are presented in Table 4-1.

Determination of Potential Nitrification Rates

Nitrification potential activities of HID soils were determined using the shaken soil slurry method of Hart et al. (1994). Nine random samples were taken from 20 x 20 m^2 plots within each study site. Samples were sieved through 2 mm mesh, and nine 50 g sub-samples were combined to make a composite for each site. Composite samples were divided into five 15 g replicates. Each soil sample was suspended in 1mM phosphate buffer (pH 7.2) and amended with 1.5mM $(NH_4)_2SO_4$. Samples were shaken in autoclaved, acid rinsed 250 mL Erlenmeyer flasks in the dark for 24 h at 180 rpm and 24^oC. Aliquots (10 mL) of soil slurry were taken for NO_3^- analysis at 5 time points (0, 2, 4, 8, 20, 24 h) and frozen at -80^oC until analysis.

Nitrate concentrations were determined by conversion to nitrite by shaking with cadmium (Jones, 1984). For determination of nitrate, 10 ml samples from each time point and replicates were centrifuged at 5000 rpm for 5 min to separate soil particles from buffer solution. Then, 2 to 2.5 mg of spongy cadmium and 1 ml of 0.7 M ammonium chloride (pH 8.5) were added to 5 ml aliquots of buffer from each sample, in 10% HCl-rinsed 15 ml conical centrifuge tubes (BD Biosciences, San Jose, CA, USA), and shaken on a rotary shaker at 100 rpm for 1.5 h.

Spongy cadmium was generated by reaction of 20% (w/v) cadmium sulfate with one zinc bar (Sigma-Aldrich, St. Louis, MO, USA) for 8 h; spongy cadmium which precipitated on the surface of the zinc bar, was scrapped off into a clean container, acidified with 3 drops of 6N HCl, and washed with 18 MΩ distilled deionized water (DDW) six times. Until use, spongy cadmium was stored under DDW. Activated cadmium was prepared by washing with 6N HCl solution for five minutes, and then rinsed ten times with DDW (at which point the pH of decanted waters was approximately pH 5 or greater).

Colorimetric determination of nitrite concentrations were done by reacting 5 ml of sample solution with 100 µl of combined diazotizing and coloring agents (0.05 g sulfanilamide, 0.05 g N-(1-naphthyl) ethylenediamine, 5 ml of 85% phosphoric acid, and water to final volume of 50 ml) in acid washed 7ml plastic scintillation vials; color was allowed to develop for 15 min, with periodic swirling, prior to analysis. Following color development, 1 ml of sample was transferred to 1.5 ml polystyrene disposable cuvettes (10 mm path length, Fisher Scientific) and nitrite concentrations were determined as a function of absorbance intensity at 540 nm, with a Shimadzu UV 1201 spectrophotometer (Shimadzu, Kyoto, Japan). Nitrate standards prepared by serial dilution of 1000 ppm nitrate solution (Fisher Scientific, Pittsburg, PA, USA) were run during each series of cadmium-reduction reactions. To determine conversion efficiency, nitrite concentrations in standards after shaking were compared to values of nitrite standards; conversion efficiencies for cadmium shaken samples compared to nitrite standards in this study ranged from 95 to 102%, consistent with those reported by Jones (1984). To standardize for differences in initial nitrate concentrations in replicate samples, time zero values were

subtracted from values at each time point, prior to rate determination. Nitrification potentials were determined by the slope of a linear regression of cumulative nitrate concentrations with time (Hart et al., 1994).

Extraction of Nucleic Acids and PCR

Nucleic acids were extracted from approximately 0.25 g soil using the PowerSoil DNA Kit (MoBio, Solana Beach, CA) following the manufacturer's instructions. Extracts were examined by electrophoresis through 1% agarose gels made with tris-acetate-EDTA buffer, staining with ethidium bromide, and visualization under UV light. In an effort to fully characterize communities within HID soils and account for spatial variability, equal volumes of bulk DNA extracts from three replicate soil samples per study site were pooled prior to PCR analysis.

A 491 bp fragment of *amo*A was amplified using primer set amoA1f (5'-GGGGTTTCTACTGGTGGT-3') and amoA2r (5'-CCCCTCKGSAAAGCCTTCTTC-3') developed by Rottauwe et al. (1997). Each 25 µl reaction contained 12.5 µl of HotStar *Taq* Master Mix (QIAGEN, Valencia, CA, USA), 8.75 µl of distilled water, 1.25 µl of each primer (20 pmol μl^{-1}), and 1 µl of undiluted template DNA. PCR amplification was carried out in GeneAMP PCR system 9600 (Perkin-Elmer, Applied Biosystems, Norwalk, CN, USA). Initial enzyme activation and denaturation were performed at 95 ^{o}C for 15 min, followed by 35 cycles of 95^{o}C for 30s, 55^{o}C for 45s , and 72^{o}C for 45 s, with a final extension step at 72^{o}C for 7 min.

Cloning and Sequencing

Fresh PCR products from all samples were ligated into pCRII-TOPO cloning vector and transformed into chemically competent *Escherichia coli* TOP10F' cells

according to manufacturer's recommendations (Invitrogen, Carlsbad, Calif.). Randomly picked white or light blue clones were inoculated into 96 well plates containing 200 µl of LB broth with kanamycin (50 µg ml^{-1}) and grown overnight at 37°C. Live clones were screened directly for inserts using live cell PCR and SP6 and T7 vector primers. Clones containing the correct insert size were transferred to 96 well plates containing LB broth amended with kanamycin (50ug ml) and 8% (v/v) glycerol and incubated for 24 h at 37°C. Overnight cultures were submitted to the Genome Sequencing Core Laboratory at the University of Florida.

Phylogenetic Analysis

Nucleotide sequences were manually aligned in Se-Al version 2.0a11 (Rambaut, 1996) and aligned with ClustalX version.1.81 (Thompson et al., 1997). Phylogenetic trees were produced from a 450 bp *amoA* fragment, using Jukes and Cantor corrected distance matrices in the TREECON software package (van de Peer and de Wachter, 1994). Bootstrap analysis (1000 resamplings) was used to estimate reproducibility of phylogenies. Bayesian analysis was conducted using ClustalX generated alignments in Mr. Bayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) software under default model parameters for 2.5 million generations. Due to high redundancy in sequence similarity, only sequences sharing less than 97% sequence similarity were included in the final cladogram.

Statistical Analysis of Phylogenetic Data

To assess whether observed AOB clone libraries between sites represented statistically different populations, well-aligned subsets of each gene fragment were chosen for analysis using ∫-Libshuff (Schloss et al., 2004) with 1,000,000 randomizations

and a distance interval (*D*) of 0.01 in PAUP* (Swofford, 1998) employing Jukes-Cantor corrected pairwise distance matrices. The program employs Monte Carlo methods to calculate the integral form of the Cramér-von Mise statistic by constructing random sub-set populations from the entire data set and comparing coverage of the generated populations to coverage of experimentally obtained data sets. Populations were considered significantly different with P value below 0.0017 after a Bonferroni correction for multiple pairwise comparisons (α=0.05, n = 20).

Analysis of molecular variance (AMOVA), pairwise comparisons of population specific pairwise fixation indices (F_{ST}) (Martin, 2002), and average pairwise sequence similarities were conducted with the program Arlequin version 3.001 (Genetics and Biometry Laboratory, University of Geneva [http://lgb.unige.ch/arlequin]). AMOVA (Excoffier et al., 1992) employs a hierarchically partitioned matrix of Euclidean distances to assess by permutation the significance of variance components at each level of partitioning. All analyses were performed under default parameters, with the following exceptions: analyses were conducted at 90,000 iterations and haplotypes were defined by Euclidean distances. F_{ST} tests were employed as measures of genetic differentiation between all pairs of samples. The test determines whether samples contain close phylogenetic relatives or more deeply divergent sequences. Mantel tests (Mantel, 1967; Mantel and Valand, 1970) were implemented in Arelquin and to test correlations between population specific F_{ST} values and geochemical parameters. The method is based on a nonparametric general regression model which employs squared Euclidean distance matrices between variables to test significance of and degree of predictability one variable has on another (Dutilleul et al., 2000).

Parsimony tests (P-test) were implemented in TreeClimber (Schloss and Handelsman, 2006). Clustal X version 1.83 was used to generate sequence alignments, constructed under default parameters. Trees were constructed by Bayesian analysis as implemented in Mr. Bayes version 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) under default model parameters, with trees sampled every 1000 generations. All Bayesian analyses were run for 1,000,000 generations, of which 10% were discarded to account for initial divergence in log likelihood scores between chains. The resultant 990 trees were used for analysis in TreeClimber (http://www.plantpath.wisc.edu/fac/joh/treeclimber.html) and compared to 1,000,000 randomly generated trees.

Statistical Analysis of Biogeochemical Data

Environmental parameters were tested for significance across treatment groups (study sites) using one-way ANOVA in JMP version 5.1 (SAS Institute) on both log transformed and raw data. Pairwise comparisons of means were conducted in the same software using Tukey's HSD, which accounts for unequal variances among samples.

Results and Discussion

Biogeochemical Parameters of Soils Along the Restoration Gradient

Values for select geochemical parameters are presented in Table 4-1. Mean soil depth increased linearly with time since disturbance for both wet ($R^2 = 0.82$, $P = 0.033$) and dry ($R^2 = 0.78$, $P = 0.046$) season measurements. Nitrate concentrations ranged from 4.00 to 36.21 mg N kg^{-1} in the dry season and from 1.79 to 7.83 mg N kg^{-1}in the wet season, R00 soils contained the highest nitrate concentrations in both seasons (Table 4-1). Interestingly, previous studies of soils from other regions of the Everglades have been

unable to extract nitrate, which was attributed to high denitrification rates (White and Reddy, 1999; White and Reddy, 2003). Ammonium concentrations ranged from 5.13 to 16.46 mg N kg^{-1} and 9.86 to 46.96 mg N kg^{-1} in dry and wet season soils, respectively. Ammonimum concentrations in HID soils were approximately 5 to 70 fold lower than in nutrient impacted surface soils of the Everglades (White and Reddy, 2003). There was a significant positive linear increase in ammonium concentrations with time since restoration in both wet ($R^2 = 0.61$, $P = 0.04$) and dry ($R^2 = 0.96$, $P = 0.003$) seasons. Additionally, wet season soil depth correlated well with ammonium concentrations ($R^2 = 0.81$, $P = 0.03$). Initially extractable ammonium concentrations have been used previously as estimates of heterotrophic N mineralization potentials in wetland and upland soils (Williams and Sparling, 1988; Ross et al., 1995, White and Reddy, 2000; White and Reddy, 2003). Thus, the observed linear increase in extractable ammonium with time since restoration may indicate greater mineralization of organic nitrogen, possibly resulting from increased accumulation of labile organic nitrogen (due to greater plant density or carbon accumulation), or a successional increase in carbon limitation of microbial communities.

Dry season nitrification potentials ranged from 0.08 to 0.20 mg NO_3^--N kg^{-1} hr^1, the highest values were observed in R89 and R00 sites and the lowest in UND and R03 soils. In the wet season, nitrification potentials decreased significantly in all sites (ANOVA, $P < 0.05$) and ranged from 0.05 to 0.12 mg NO_3^--N $kg^{-1}hr^{-1}$, highest in R97 and lowest in R03 soils. Dry season values are within the lower range of initial nitrification rates measured in phosphorous impacted Everglades soils, and wet season values are 2 to 4 fold lower that those reported by White and Reddy (2003). There was no

observed correlation between dry season nitrification potentials and other measured biogeochemical parameters, further measurements may elucidate factors controlling AOB activity in dry season soils. Soil oxygen demand (Table 4-1) correlated strongly with wet season nitrification potentials (R^2=0.893, P=0.04), suggesting that *in situ* ammonia oxidation activities may be most limited by oxygen availability in the wet season. Although there was no clear trend associated with nitrification potentials and recovery stage in HID soils, statistical analysis (randomized complete block design, ANOVA) of site effects on biogeochemical data indicated significant (α = 0.05, P = 0.0032) within-site effects on nitrification potentials. This may indicate that, while there does not seem to be an obvious or homogenously controlling factor on AOB activity along the restoration gradient, nitrification activity may be more strongly controlled by different factors within each restoration site, such competition with hetrotrophs for available ammonium, inhabitation by plant allelochemicals, or differences in soil oxygen availability.

Phylogenetic Analysis of *amoA*

An expected 491 bp fragment of *amoA* was amplified from all study sites in both wet and dry season soils. Phylogenetic analysis of the 313 obtained partial *amoA* revealed relatively low phylogenetic diversity of beta-proteobacterial AOB in HID soils. Clone libraries from both seasons were dominated by two sequence types, one corresponding to *Nitrosospira*-like and the other to *Nitrosomonas*-like *amoA* (Figure 4-1, Table 4-2); sequences comprising the two phylogenetic groups were approximately 75% similar on the nucleotide level. Analysis of all clones revealed 181 (58%), 95 sequences from dry and 86 from wet season clone libraries, to be associated with the *Nitrosospira*

(NSP) clade. These sequences were obtained from all soils, and ranged in pairwise DNA sequence similarity from 97 to 100%. NSP clones shared high similarity (98 to 99%, DNA) to database sequences obtained from agricultural soils (Corredor et al., 2005), soils incubated under varying temperature and ammonium regimes (Avrahami et al., 2003), and from rice roots (Rotthuwae et al., 1997). Sequences within the NSP clade were 96 to 97% similar to *amoA* of *Nitrosospira* sp. Nsp 17, isolated from Icelandic soils (Purkhold et al., 2003) and 93% with *Nitrosospira* sp. Nsp 2, isolated from German soils (Purkhold et al., 2003). Little is known about these NSP isolates; they do not cluster tightly within 16S rRNA Cluster 3 *Nitrosospiras* (Purkhold et al., 2000), and may represent a unique group of *Nitrosospiras* or be a divergent member of Cluster 3 (Purkhold et al., 2003). The remaining 132 (42%) sequences were associated with the *Nitrosomonas* (NSM) lineage, sharing ca. 99% sequence similarity with NSM-like clones obtained from activated sludge (Park and Noguera, 2004) and the roots and rhizospheres of different rice cultivars (Briones et al., 2002). HID clones associated with the NSM cluster also shared 98 to 99% DNA sequence similarity with *Nitrosomonas europaea* ATCC 19718. NSP and NSM sequences were obtained from all sites, although abundance varied between sites and seasons (Table 4-2).

The occurrence of both *Nitrosmonas* and *Nitrosospira amoA* have been previously reported from estuarine sediments (Francis et al., 2003; Bernhard et al., 2005), membrane-bound biofilms (Schramm et al., 2000), forest and meadow soils (Mintie et al., 2003), grassland soils (Webster et al., 2002), and in bulk paddy soil and the oxidized rhizospheres of rice plants (Rotthauwe et al., 1997). Several studies have demonstrated the affinity of *Nitrsomonas* spp. for high nutrient environments and *Nitrosospira* spp. to

be generalists; however, current knowledge of *Nitrosomonas* spp. physiologies far out-weighs that of *Nitrsospira* spp. (Kowalchuk and Stephen, 2001). Interestingly, both *amoA* and 16S rRNA genes corresponding to *Nitrosospira* Cluster 3 have been obtained from a young successional grassland (Kowalchuk et al., 2000) and wetland soils (Ibekwe et al., 2003). *Nitrosospiria* of Cluster 3 have been most commonly obtained from undisturbed soils of near neutral pH (Stephen et al., 1996; Kowalchuk and Stephen, 2001). Cluster 3 isolates have shown different responses to commonly regulating factors in pure culture (Webster et al., 2005). The apparent seasonal stability of AOB populations between wet and dry seasons is consistent with previous observations of the presence of both groups in anoxic or sub-oxic environments (Bodelier et al., 1996), and their ability to survive at low ammonium concentrations and prolonged periods of starvation (Bollmann et al., 2002).

Relative abundance of sequence types obtained from dry and wet season soils suggest interesting dynamics between *Nitrosomonas* and *Nitrosospira* type AOB. Dry season clone libraries obtained from R03, R01, R00, and R89 soils suggest an approximately linear decrease in *Nitrosospira* with restoration age. However, this trend is less clear in wet season clone libraries. In both wet and dry seasons, R97 and R03 clone libraries were dominated by *Nitrosmonas*-like and *Nitrosospira*-like *amoA* sequences, respectively. The ecological factors possibly controlling these trends are not clear. Attempts to further investigate the dynamics of *Nitrosomonas* and *Nitrosospira* sequence types in HID soils were unsuccessful. Low gene copy numbers in DNA extracted from individual samples yieled insufficient quantities of *amoA* PCR amplicons for downstream applications such as T-RFLP. Thus, without further data to confirm the

observed trends suggested by the clone libraries, discussion of ecological factors that control AOB diversity in HID soils would be inappropriate.

Statistical Analysis of Clone Libraries

Observed *amoA* sequence diversity was low in HID samples, as evidenced by the rarefaction analysis; clone library coverage, as calculated by ∫-Libshuff (Schloss et al., 2004), ranged from 97 to 100% at evolutionary distances greater than 0.01. Distance matrices of *amoA* from each site and season were subjected to iterative statistical analysis with the software ∫-Libshuff (Schloss et al., 2004) to determine the significance of patterns observed in clone libraries. For the dry season, R89, R97, and R03 clone libraries were significantly different from each other, and R00 and R01 were subsets of R03. For the wet season, R03 was significantly different from R00 and R97, but was drawn from the sample population as R01. Overall, ∫-Libshuff analysis suggests that population membership does not differ significantly in any of the HID sites. Patterns observed in significance between R03 and other clone libraries is likely to due the dominance of NSP sequences in both wet and dry season libraries.

To test the significance of observed population structures between restoration sites based on *amoA* clone libraries, parsimony (P) tests of phylogenetic variation were implemented in TreeClimber (Schloss and Handelsman, 2006). Underlying hypotheses and application of P tests to phylogenetic data sets of this size are explained in detail in Chapter 3. Population structures were significantly different within both wet ($P=0.026$) and dry ($P=0.023$) season clone libraries. Removal of specific groups of sequences or clone libraries from any site did not yield loss of significance, suggesting that each site harbors unique sequence types. Table 4-3 presents the number of unique haplotypes, as

determined by Arlequin (Excoffier et al., 1992); unique haplotypes were determined by pairwise comparison of squared Euclidean distances of sequences from each site. Further, despite high sequence similarity within the NSM and NSP clades, regions of difference may exist, such that subclusters of unique sequence types form within the NSM and NSP clades. A comparison of pooled wet and dry season clone libraries failed to yield significance (P=0.65), suggesting as a whole that community structure did not vary between seasons. Interestingly, individual pairwise comparisons of clone libraries from each restoration site between seasons were significantly different ($P < 0.05$). Thus, while the overall community structure did not shift significantly between seasons, there is clear evidence for within-site shifts in genetic composition of *amoA* between seasons. Further, there were observed shifts in average nucleotide diversity and pairwise sequence similarities between seasons (Table 4-3).

Analysis of molecular variance (AMOVA) was used to assess differences in genetic variation within and between clone libraries from each restoration site. For the dry season, variation among populations accounted for approximately 42% of total variance. For wet season libraries, among population variance accounted for 34% of total variance. Among population variance differed significantly from total population variance ($P < 0.000001$) for both seasons. AMOVA results imply that genetic variation within sites is significantly different from variance when all libraries were pooled. This suggests that each site harbors a unique assemblage of AOB, consistent with the results of P test analyses. The decrease in among population variation in wet season libraries is likely due to the small increases in relative abundance of *Nitrosomonas* sequences in R89, R00, R01, and R03, and *Nitrosospira* in R97 (Table 4 -2). There was no significant

variation (P=0.91) when dry and wet season clone libraries were compared in AMOVA. This is consistent with the recovery of nearly similar distribution of NSM and NSP sequences within each library between seasons.

Dissimilarity indices (F_{ST}) (Reynolds et al., 1983; Martin, 2002) can be used to compare average genetic variation within a group to the variation between groups. By comparing the observed variation with that of randomly generated groups, the analysis evaluates the statistical likelihood that observed variation between two sites is significant. Results of pairwise comparisons of F_{ST} values for dry and wet season clone libraries are presented in Table 4-4 and 4-5, respectively. Pairwise comparisons of F_{ST} and average pairwise sequence similarity values between restoration sites yielded similar patterns for both dry and wet seasons. For both dry and wet seasons, diversity in R89 was significantly higher than R97 and R03 (Tables 4-4 and 4-5), which can likely be attributed to the dominance of *Nitrosomonas* and *Nitrosospira* clones within R97 and R03 libraries, respectively. For dry season libraries, both R97 and R03 F_{ST} values and average pairwise sequence similarities were significantly different from all other sites. The same pattern was observed for R97 in the wet season, however R03 was not significantly different from R00, which may be attributed to the observed increase in *Nitrosomonas* sequences in the R03 wet season clone library.

Population specific F_{ST} values for clone libraries from each restoration site and season are presented in Table 4-3; these values are measures of within population diversity to total population diversity, a low number indicates diversity within a site to be similar to diversity when any two sites are combined (Martin, 2002). Values in Table 4-3 are based on comparison of within-site diversity to total diversity observed within all

sites per season. In both seasons, population specific F_{ST} values were highest in R97 and R03 libraries, indicating that these libraries share least similarity with the total population as represented by our clone libraries. *Nitrosomonas* or *Nitrosospira*-type AOB dominated clone libraries from R97 and R03 sites, respectively.

Correlation of Differences in *amoA* Diversity With Environmental Variables

Mantel tests (Mantel, 1967; Mantel and Valand, 1970; Dutilleul et al., 2000) were implemented to examine correlations between pairwise differences in *amoA* population-specific F_{ST} values between sites and seasons to matrices of pairwise differences in geochemical parameters. A previous study of *amoA* diversity in estuarine sediments correlated differences in *amoA* F_{ST} values between sites to differences in nitrate concentrations and salinity (Francis et al., 2003). The Mantel test examines whether closeness observed in one set of variables is related to closeness in another set of variables. For these data, it was employed to test for correlations between observed differences in *amoA* diversity between sites and seasons, as represented by population-specific F_{ST} values, and factors most likely to affect AOB activity in HID soils: time since restoration, extractable NH_4^+, extractable NO_3^-, potential nitrification rates, soil moisture, and soil oxygen demand. Results of Mantel tests of correlation for differences both within and between season F_{ST} values with biogeochemical parameters are presented in Table 4–6. Pairwise differences in F_{ST} values for dry season clone libraries correlated significantly with soil oxygen demand (SOD) and nitrification potentials. For the wet season, SOD and extractable nitrate concentrations correlated strongly with wet season differences in pairwise F_{ST} values. Interestingly, in both dry and wet season results, one factor is inversely correlated with differences in diversity, and the other positively correlated. In both seasons, pairwise differences in SOD between sites were positively

correlated with diversity, and may indicate oxygen availability as a controlling factor on AOB diversity between sites. Pairwise F_{ST} values were inversely correlated with potential nitrification rates and extractable nitrate concentrations in dry and wet seasons, respectively. Both may be used as indicators of AOB activity in soils, and suggests a decoupling of genetic diversity and activity, as sites with greatest differences in diversity have similar nitrification activities. Pairwise differences between seasons correlated significantly with extractable ammonium, extractable nitrate, nitrification potentials, and soil oxygen demand, and suggest several factors controlling observed difference in diversity between seasons; the magnitude of which each of these correlating factors control diversity between seasons may vary between sties.

Table 4-1. Biogeochemical parameters of dry and wet season HID soils.

Study Site	Soil Depth (cm)[f]	Moisture (%)	SOD ($\mu g\ kg^{-1}$)[†]	Nitrate (mg NO_3^-N kg^{-1})	Ammonium (mg NH_4^+-N kg^{-1})	Nitrification Potential[⊥]
Dry						
UND	8.1 (4-16)	46.2 (1.1)	10.9 (2.5)	36.21	12.81 (1.30)	0.08 (0.02)
R89	3.1 (1-7)	27.8 (8.2)	17.1 (2.9)	7.32 (2.28)	16.46 (2.27)	0.20 (0.02)
R97	2.6 (1-9)	26.3 (14.4)	27.6 (13.7)	8.69 (0.18)	9.26 (2.14)	0.14 (0.03)
R00	1.1 (0-3)	48.5 (1.1)	17.7 (5.2)	14.28	7.94 (2.42)	0.18 (0.03)
R01	1.6 (0-3)	14.8 (2.9)	14.2 (6.3)	9.76 (2.24)	5.13 (0.55)	0.17 (0.02)
R03	0.2 (0 -1)	14.9 (14.3)	20.0(11.5)	4.00 (1.56)	5.54 (1.18)	0.13 (0.05)
Wet						
UND	10 (4-19)	43.8 (0.6)	79.6	6.77 (0.92)	9.86 (1.51)	0.09 (0.02)
R89	5.4 (2-9)	59.6 (2.4)	261.4	3.08 (0.93)	46.96 (3.32)	0.06 (0.01)
R97	4.6 (0-5)	58.8 (4.5)	359.2	1.79 (1.05)	49.48 (18.67)	0.12 (0.02)
R00	3.3 (1-5)	48.3 (1.4)	184.9	7.83 (4.93)	25.06 (13.33)	0.07 (0.02)
R01	2.2 (0-4)	41.6 (1.1)	135.9	6.84 (4.84)	15.21 (8.84)	0.06 (0.02)
R03	1.2 (0-3)	43.5 (10.1)	230.1	5.09 (4.88)	15.89 (5.09)	0.05 (0.01)

[f]Values in parentheses for soil depths represent that range in values from 81 nodes sampled per site; all other values in parentheses represent standard deviations of the mean of three amples per site; SOD, soil oxygen demand; MBN, microbial biomass nitrogen.
[†]Wet season determinations were conducted on a single soil sample

[⊥]Nitrification potential rates are expressed as mg NO_3^--N kg^{-1}soil h^{-1}.

Table4-2. Results of sequence analysis of *amoA* sequences obtained from dry and wet season soils.

Phylogenetic Cluster	Relative percentage (%) of sequence types within each cluster (dry/wet)						Closest cultivated isolate[a]	16S rRNA cluster[b]
	UND	R89	R97	R00	R01	R03		
Nitrosospira	28/12	56/69	0/10	70/77	80/77	100/95	Nsp. sp, 17 (96%)	3
Nitrosomonas	72/88	44/31	100/90	30/23	20/22	0/4	Nsm. europaea (99%)	6
No. of clones	25/25	27/26	26/20	30/26	31/27	27/23		

[a]As determined by BLAST search under default parameters; values in parenthesis are average nucleotide similarity

[b]Based on analyses of 16S rRNA sequences, as outlined by Stephen et al. (1996), and reexamined by Purkhold et al. (2000); Nsm., *Nitrosomonas*; Nsp., *Nitrosospira*

Table 4-3. Fixation indices (F_{ST}), average pairwise differences ($\theta[\pi]$), nucleotide diversity, and unique haplotypes for wet and dry season *amoA* clone libraries as calculated by Arlequin (Excoffier et al., 1992).

Study site	Dry season clone library				Wet season clone libraries			
	F_{ST}	Unique haplotypes	$\theta[\pi]$	Nucleotide diversity	F_{ST}	Unique haplotypes	$\theta[\pi]$	Nucleotide diversity
R89	0.382	21	89.9 (39.9)	0.19 (0.09)	0.336	18	75.5 (33.6)	0.16 (0.08)
R97	0.435	24	5.2 (2.6)	0.01 (0.01)	0.341	17	33.2 (15.1)	0.07 (0.04)
R00	0.412	20	58.0 (25.8)	0.12 (0.06)	0.325	22	64.2 (28.6)	0.13 (0.07)
R01	0.418	19	43.6 (19.4)	0.09 (0.05)	0.325	20	56.3 (25.1)	0.14 (0.07)
R03	0.437	16	2.0 (1.17)	0.004 (0.01)	0.347	20	20.1 (9.2)	0.04 (0.02)

Table 4-4. Corrected average pairwise differences (above diagonal)and pairwise fixation indices (below diagonal) for dry season *amoA* sequences.

	Result for study site[a]:				
Site	R89	R97	R00	R01	R03
R89		**52.91**	-0.11	6.11	**26.34**
R97	**0.38**		**77.73**	**103.92**	**160.35**
R00	0.00	**0.62**		-0.16	**13.20**
R01	0.05	**0.76**	-0.02		**5.07**
R03	**0.24**	**0.97**	**0.25**	**0.14**	

[a]Bold face values are significant at P<0.05.

Table 4-5. Corrected average pairwise differences (above diagonal)and pairwise fixation indices (below diagonal) for wet season *amoA* sequences.

Site	Result for study site[a]:				
	R89	R97	R00	R01	R03
R89		**108.45**	68.37	68.57	**57.73**
R97	**0.48**		**118.43**	**118.43**	**144.07**
R00	-0.02	**0.57**		62.41	46.57
R01	-0.01	**0.57**	-0.02		**47.01**
R03	**0.16**	**0.81**	0.08	**0.09**	

[a]Bold face values are significant at P<0.05.

Table 4-6. Results of Mantel (Mantel, 1967; Mantel and Valand, 1970) correlation tests between pairwise differences of population specific F_{ST} values for wet and dry season *amoA* clone libraries with biogeochemical parameters.

Environmental Parameter	Dry Season		Wet Season		Across Seasons	
	Correlation coefficient	P-value	Correlation coefficient	P value	Correlation coefficient	P value
Time Since Restoration	-0.536	0.158	-0.419	0.300	ND[a]	ND
Extractable NH_4^+	-0.522	0.167	0.147	0.291	-0.756	0.002
Extractable NO_3^-	-0.327	0.258	-0.704	0.017	0.331	0.038
Nitrification Potential	-0.989	0.008	0.600	0.075	0.631	0.008
Soil Moisture	-0.287	0.200	0.399	0.092	-0.279	0.448
Soil Oxygen Demand	0.885	0.017	0.832	0.008	-0.7515	0.008

[a]ND, not determined.

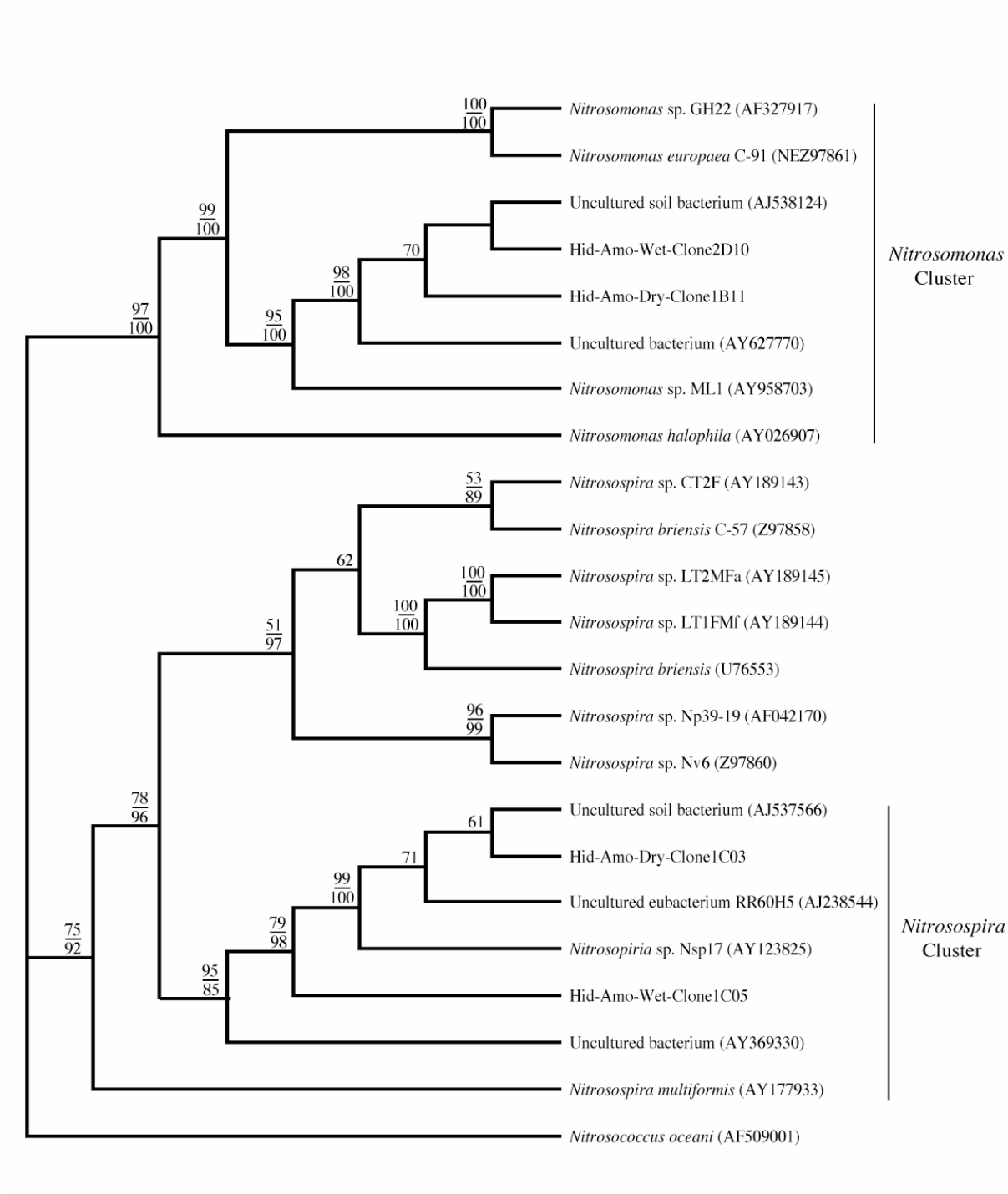


Figure 4-1. Cladogram of representative *amoA* sequences obtained from HID soils. Nodal support values represent percent of 1000 bootstrap resamplings (top) and Bayesian posterior probability after 2.5 million generations (bottom). Nodes with only a single value are unsupported by Bayesian methods.

CHAPTER 5
SUMMARY AND CONCLUSIONS

The severe disturbance imposed upon HID restoration sites due to complete removal of plant communities and much of the remaining soil likely destroyed any vestiges of the previous biogeochemical linkages and the microbial communities which mediate them. The recovery of the HID ecosystem is strongly dependent upon the development of soils. Soil provides a direct linkage between the nutrients retained in parent materials and plant roots, and a medium for the reestablishment of biogeochemical linkages. Nutrient accretion and recycling may play a major role in the rates at which restored sites reach stability. Changes in recovery stage will be largely dependent upon biological activity within each restoration site. The retention of nutrients within restored sites will be strongly dependent upon the activity of plant and microbial communities. Further, imbalances in the cycling of carbon and nitrogen in restored sites may present the opportunity for significant loss of nutrient stores due to microbial processes. Trace gas loss of nutrients due to respiratory activity of soil microbial communities may significantly alter the rate at which biogeochemical linkages and nutrient use efficiencies are restored. Thus, an understanding of the activity and ecology of microbial groups associated with trace gas production, such as methanogenic, ammonia oxidizing, and denitrifying bacteria, maybe provide insights into the state of nutrient recycling and retention in re-developing sites.

The activity and molecular ecology of methanogenic bacteria in HID soils was investigated in Chapter 2. Methanogenic bacteria occupy an exclusive niche in terminal

anaerobic carbon mineralization in most freshwater wetlands; their activity and structure may provide insight into the nature of both carbon cycling and anaerobic electron accepting processes. Methanogenic activity was detected in HID soils from both restored and undisturbed wetlands. Microcosm experiments to determine the most likely methanogenic precursors in soils from each study site strongly suggested hydrogenotrophic methanogenesis as the most favorable pathway of methane formation. Methane production potentials indicated a general decline in methanogenic activity with restoration age and were lowest in soils from undisturbed sites.

Culture independent techniques targeting methyl coenzyme M reductase genes (*mcrA*) were used to assess the dynamics of methanogenic assemblages. *mcrA* clone libraries were dominated by sequences related to hydrogenotrophic methanogens of the orders *Methanobacteriales* and *Methanococcales*, and suggested a general decline in the relative abundance of *Methanobacteriales mcrA* with time since restoration. Terminal restriction fragment length polymorphism (T-RFLP) was employed to monitor the composition of methanogenic assemblages in HID soils between wet and dry seasons, and within restoration sites. Results of T-RFLP analysis indicated methanogeneic assemblages to remain relatively stable between seasons. Interestingly, T-RFLP analysis of soils across the restoration chronosequence indicated a putative shift in *Methanobacteriales* populations with time since restoration, suggesting that factors associated with each site's recovery stage may cause shifts in dominant genotypes. However, further studies into the activity of specific members of *Methanobacteriales* in HID soils are required to determine if these results are due to differences in regulating factors associated with restoration age.

The relatively low activity of methanogenic bacteria in HID soils, as discussed in Chapter 2, suggested the occurrence of more energetically favorable anaerobic electron accepting process in HID soils. Denitrification is the most energetically favorable respiratory pathway in the absence of oxygen. Shallow soil depths and seasonal inundation may provide conditions conducive to the activity of denitrifying bacteria.

Chapter 3 focused on the activity and genetic diversity of denitrifying bacterial populations in restored and undisturbed wet season soils. Denitrifying enzyme activity was detected in soils from both restored and undisturbed sites; however, no trend associated with time since restoration was evident. Factors controlling denitrification activity in HID soils are not clear. A significant difference in activity between sites closely related in time since restoration suggests factors controlling denitrification in HID soils are site specific.

The genetic diversity of denitrifying bacterial populations in HID soils was investigated by construction of clone libraries for genes associated with the enzyme nitrite reductase, which catalyzes the first gas-forming step of denitrification. Two functionally redundant nitrite reductases exist in bacteria and are encoded for by two divergent genes (*nirS* and *nirK*); no bacterium possesses both. Both genes were obtained from soils in all study sites. Phylogenetic analysis of clone libraries constructed from each study site indicated high diversity of both genotypes within HID soils, and suggested the existence of unique lineages of denitrifier populations in soils from each study site. Statistical analysis of *nirS* and *nirK* clone libraries confirmed the existence of unique divergent lineages in soils from each restoration site. Further, these analyses provided evidence of different responses of *nirS* and *nirK* populations to restoration age.

A greater overlap in shared *nirS* phylotypes between all restoration sites suggested a linear response in diversity associated with succession. Interestingly, *nirK* populations from more recently restored sites shared lineages that were statistically distinct from populations recovered for older sites, suggesting a bimodal response with restoration age. Several lines of evidence suggest that existence of unique divergent populations of denitrifying bacteria in soils from each restoration site. This may be in response to different selection factors associated with development of HID soils.

The activity and genetic structure of ammonia oxidizing bacterial populations was the subject of Chapter 4. Nitrification may be a significant source of nitrogen loss in young soils by conversion of biologically preferred ammonium to nitrate which may be subsequently lost from soils due to leaching or denitrification. Nitrification potentials in HID soils were monitored in wet and dry seasons. Rates were highest during the dry season and decreased significantly in the wet season. Nitrification potentials were not correlated significantly with any geochemical parameters or with time since restoration. Statistical analysis suggests control of nitrification activity in HID soils to be site specific.

The genetic structure of ammonia oxidizing bacteria was monitored by cloning and sequencing of *amoA* obtained from HID soils. *amoA* encodes the acetylene binding protein of the ammonia monoxygenase enzyme responsible for conversion of ammonia to hydroxylamine. *amoA* diversity was low relative to other functional genes obtained from HID soils. Of the two *amoA* genotypes obtained in clone libraries, sequences corresponding to *Nitrosospira amoA* were most abundant in samples from recently restored sites. *Nitrosomonas*-like *amoA* were obtained in greater abundance from soils in

older restoration sites. Genetic diversity observed in *amoA* clones libraries was strongly correlated with environmental factors, and suggested *amoA* diversity in dry season soils to be associated with nitrification rates and wet season diversity to be most strongly associated with oxygen availability. While uncertain of *in situ* distributions of the two groups of ammonia oxidizers represented by clone libraries from each site, differences in activities of *Nitrosomonas* and *Nitrosospira* populations in response to environmental factors are well documented. Variations in their abundance in soils of differing restoration age may indicate varying degrees of resource partitioning and niche differentiation. However, quantitative molecular and culture based studies are required for a full understanding of population dynamics of ammonia oxidizing bacteria in developing soils of the HID

APPENDIX A
SUPPLEMENTAL TABLES

Chapter 4

Table A-1. Population similarity P values for comparison of *amoA* dry and wet season clone libraries determined using Cramer-von Mises test statistic, implemented in ∫-Libshuff (Schloss et al., 2004)

Season (n)	Site for homologous library (*X*)	P values for comparison of heterologous library (*Y*) with *X*[a]				
		R89	R97	R00	R01	R03
Dry (166)	R89		0.963	9.963	0.013	**0.000**
	R97	1.000		0.885	0.846	**0.000**
	R00	1.000	1.000		0.900	***0.000***
	R01	0.163	0.129	0.129		1.000
	R03	**0.000**	**0.000**	0.926	0.926	
Wet (147)						
	R89		0.923	0.923	0.962	0.923
	R97	0.650		0.900	0.700	**0.000**
	R00	0.423	0.210		0.577	**0.000**
	R01	0.111	0.148	0.222		0.593
	R03	***0.000***	**0.000**	**0.000**	0.913	

[a]Values in bold indicate significant P values ($P < 0.0017$) after Bonferroni correction for multiple pairwise comparisons. Libraries are distinct from one another if both comparisons (*X* versus *Y* and *Y* versus *X*) are significant. Values in italics indicate that library *Y* is a subset of library *X*.

APPENDIX B
SUPPLEMENTAL FIGURES

Chapter 2

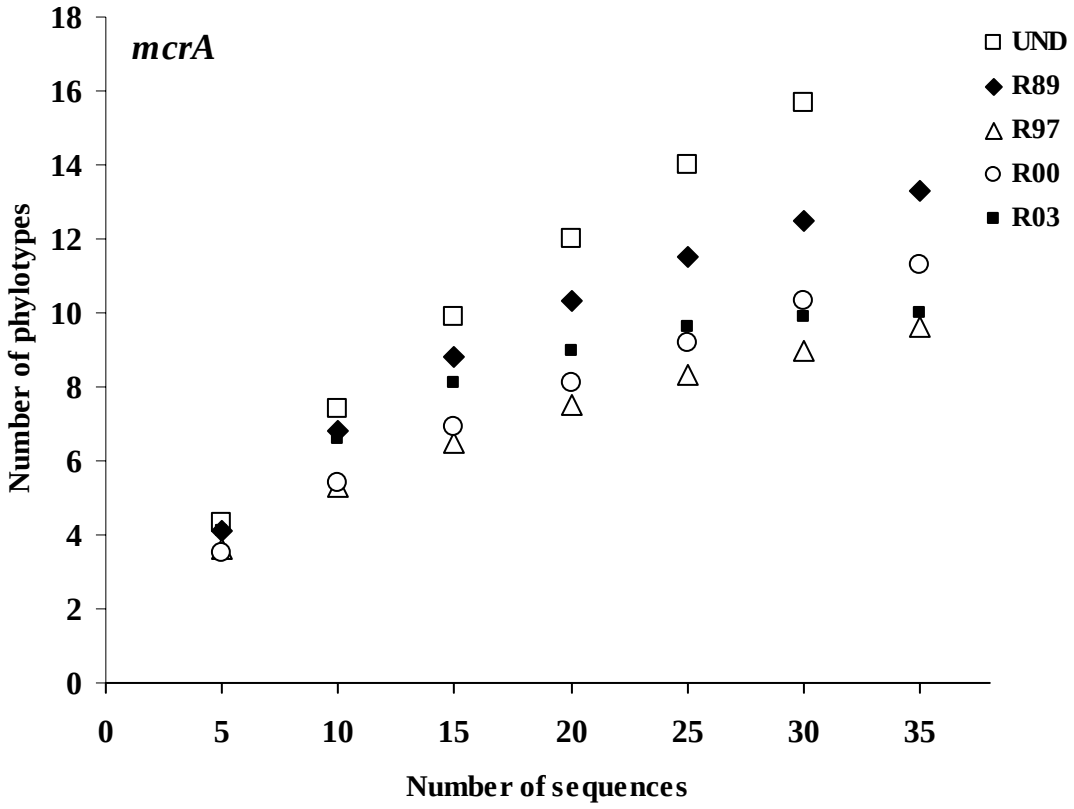


Figure B-1. Rarefaction curves for *mcrA* clone libraries constructed from dry season soils.

Chapter 3

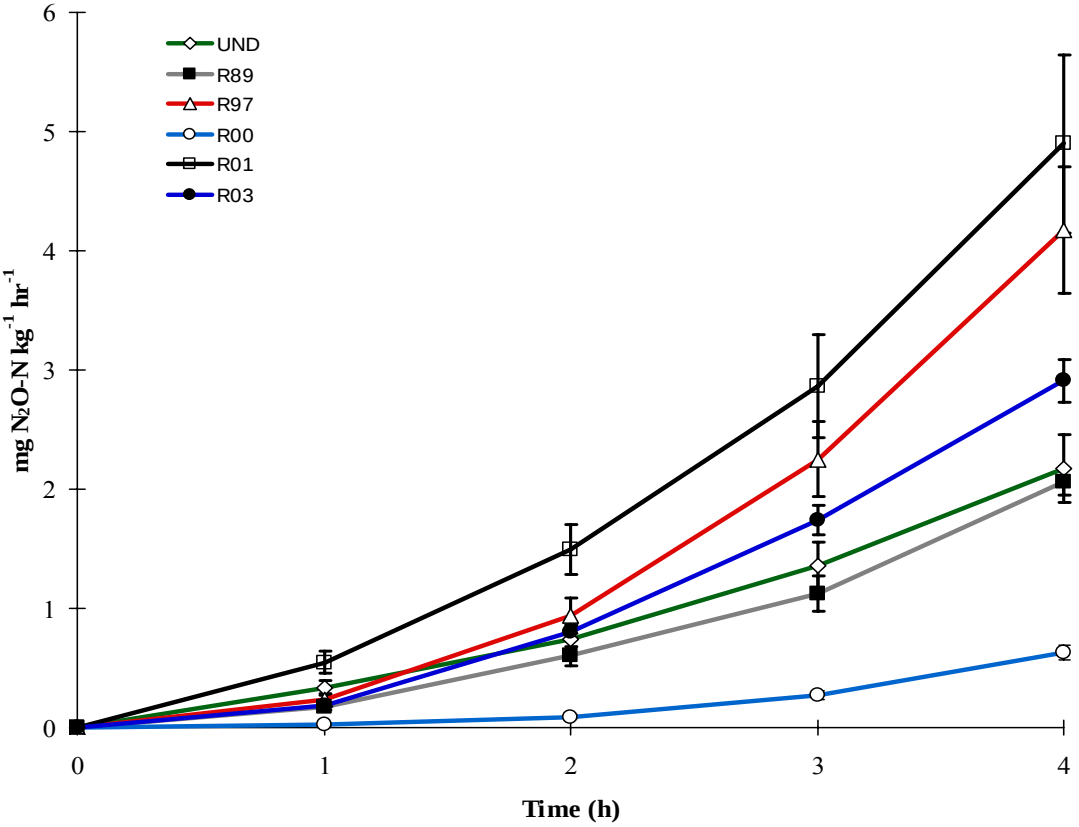


Figure B-2. Potential denitrification rates as a function of N_2O-N production with time. Error bars represent standard error of five replicate determinations.

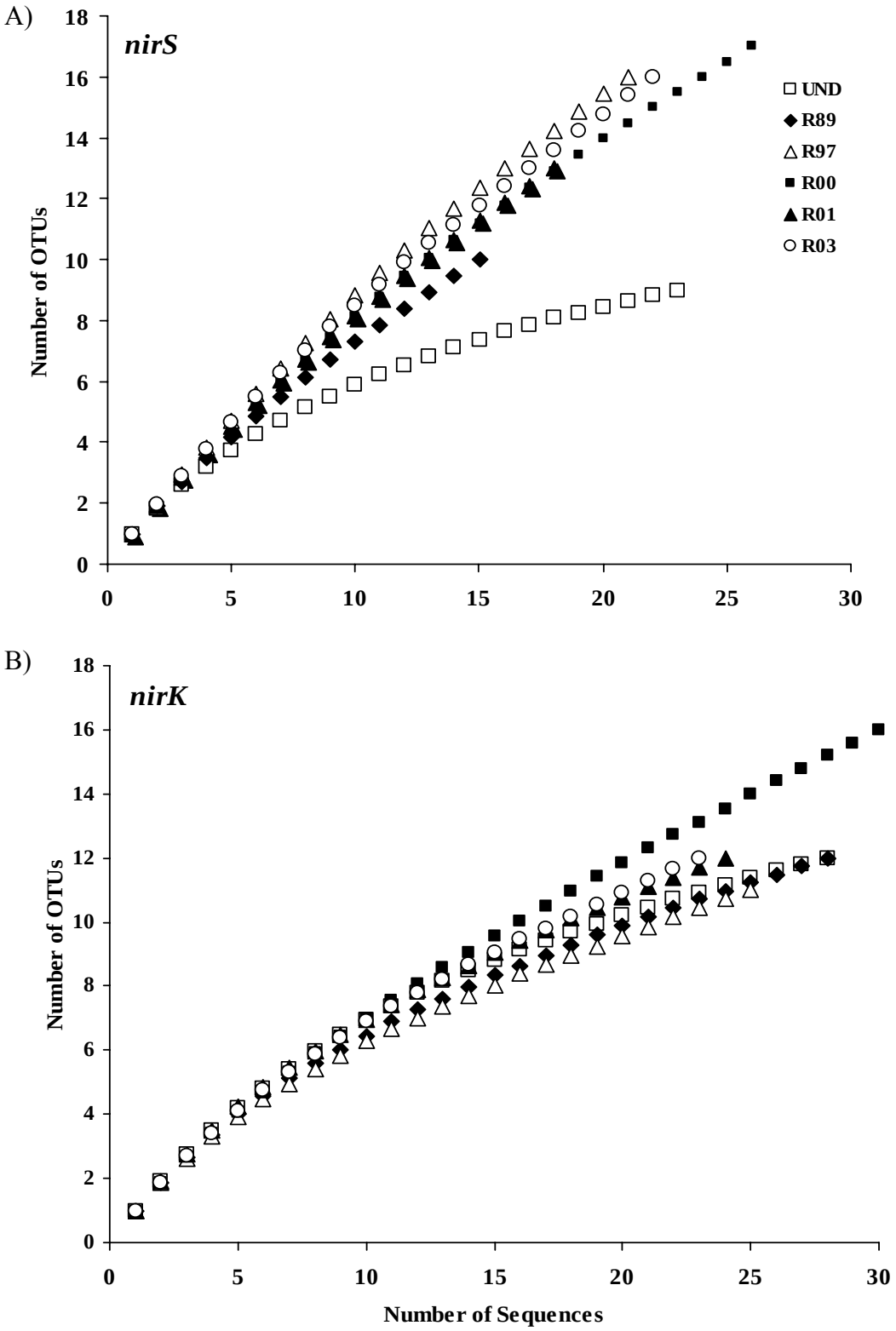


Figure B-3. Rarefaction curves for A) *nirS* and B) *nirK* clone libraries determined in DOTUR (Schloss and Handelsman, 2005) employing a 97% nucleotide sequence similarity cut-off.

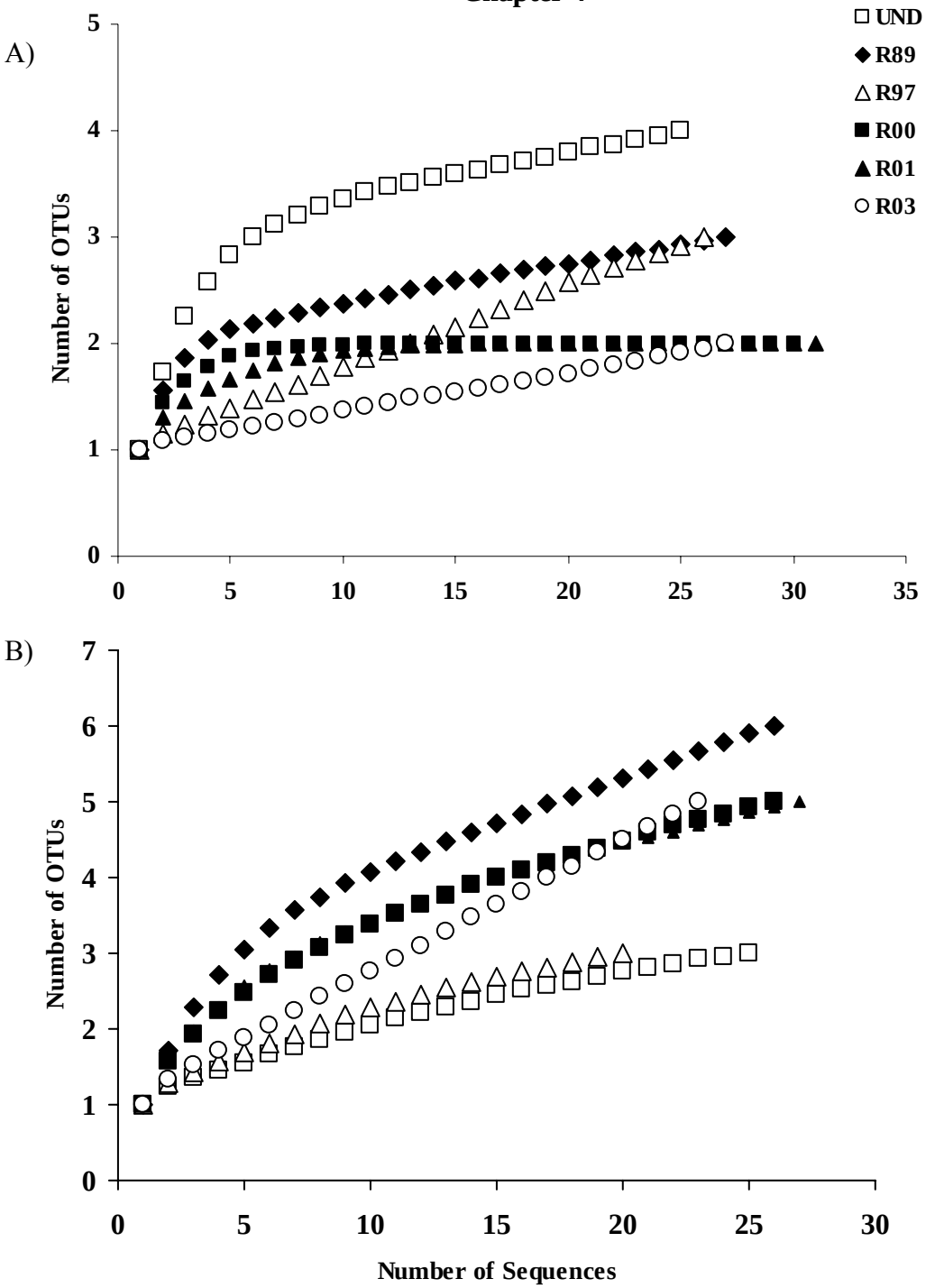


Figure B-4. Rarefaction curves for *amoA* clone libraries from A) dry season (April 2004) and B) wet season (November 2004) soils determined in DOTUR (Schloss and Handelsman, 2005) employing a 97% nucleotide sequence similarity cut-off

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BIOGRAPHICAL SKETCH

Jason M. Smith was born in Rochester, New York, on the 7th of March, 1981. Having spent his entire life in the suburbs growing up with the same people, the abrupt move to Orlando, Florida, in 1997 came as a shock. Warned by his previous teachers about the dregs of southern educational ideals, he entered Lake Mary High School with some skepticism. After finishing high school in 1999, he entered the University of Florida believing he was cut out to be a lawyer.

His initial exposure to science came as a result of University of Florida course-tracking requirements. After taking his first lab-based chemistry course, he realized the excitement of research. He graduated *cum laude* from the University of Florida, Gainesville, Florida, in 2003 with a B.S. in microbiology and cell science.

He joined the Soil and Water Science Department during the final year of his undergraduate career as a student volunteer, studying the ecology of naphthalene biodegradation under Dr. Andrew Ogram. His involvement in the Soil Molecular Ecology Laboratory was integral in the initial development of his fascination with environmental systems. He decided to stay involved, and in 2004 he began pursuing a Master of Science degree under Dr. Andrew Ogram.

Following graduation Jason plans to move to San Francisco, California, to continue working in molecular ecology in the Microbial Ecology and Biogeochemistry Laboratory at NASA Ames Research Center. Eventually, he hopes to obtain a doctorate and join the professoriate.