MICROBIAL ECOLOGY OF ANAEROBIC TERMINAL CARBON MINERALIZATION IN EVERGLADES SOILS, WITH EMPHASIS ON SULFATE-REDUCING PROKARYOTIC ASSEMBLAGES

By

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by

Hector F. Castro
For my daughter Matilde.
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In freshwater wetlands, physical and chemical parameters affect the microbial communities responsible for the carbon and sulfur cycles. Due to the metabolic and physiological versatility of sulfate-reducing prokaryotes (SRP), changes in this microbial community would reflect changes at the process level in the Everglades wetlands. This study was conducted in three sites in the Everglades Water Conservation Area 2A, with different levels of eutrophication due to phosphorus loading.

Most-probable-number enumerations showed that SRP were present in higher numbers, more active and more metabolically diverse in eutrophic regions of the marshes. Diversity within dissimilatory sulfite reductase (dsr) genes was found in eutrophic and pristine regions of the marshes. Significantly, *Desulfotomaculum*-like sequences from eutrophic regions were related to those *Desulfotomaculum* able to carry out complete oxidation. In pristine regions, they were related to those unable to carry out
complete oxidation. Molecular techniques revealed that nutrient loading resulted in a selection of different SRP communities.

Culture-independent techniques targeting the archaeal 16S rRNA and methyl coenzyme M reductase (mcr) genes of methanogens revealed a dominance of sequences related to Methanomicrobiales, suggesting that hydrogen plays a major role in methanogenesis.

Everglades WCA-2A is a case where hydrogen is an important methanogenic precursor, and acetate is not the main methanogenic precursor. Hydrogen regulates the fermentation process, shifting the fermentation product proportion, favoring the production of fatty acids and suppressing the production of acetate. The results from dsr terminal restriction fragment length polymorphism (T-RFLP) analyses were useful to distinguish between three sites with different levels of impact, but the mcr T-RFLP may indicate that phosphorus loading is altering the methanogenic community in the transition zones, making them more similar to eutrophic zones. The shift revealed by mcr T-RFLP may be an early indication of phosphorus impact in southern WCA-2A. Clearly, targeting a combination of different microbial communities provides greater insight into the functioning of this ecosystem, as well as providing useful information for planning and implementing ecosystem restoration technologies.
CHAPTER 1
INTRODUCTION

Mineralization of Organic Matter in Wetland Soils

Several microbial processes are responsible for degradation of organic matter in wetland soils. Two major microbial ecological niches, the aerobic and anaerobic, are present in wetland soils (Capone and Kiene, 1988). The dominance of one niche over the other determines the metabolic pathways to be followed in the carbon cycle. In aerobic zones of the soil, aerobic respiration is the predominant process. After oxygen is consumed, anaerobic processes are responsible for the degradation of organic matter (Capone and Kiene, 1988). In the anaerobic zones, in sequential order, denitrification, iron, and manganese respiration, sulfate reduction, and methanogenesis are the prevalent processes (Figure 1-1) (Swift et al., 1979). Several factors determine which processes dominate; among these are the presence, concentrations and types of electron acceptors and donors, carbon sources, redox potential, temperature, and microbial populations (Swift et al., 1979).

Mineralization of organic matter in anaerobic environments is a complex and synchronized chain of events involving several microbial groups (Figure 1-2) (Schink, 1997). In anaerobic environments, polymers are degraded to monomers and most of these monomers are fermented to fatty acids, alcohols, acetate and hydrogen by primary-fermenting bacteria. These bacteria produce extracellular hydrolytic enzymes that degrade complex organic matter. Later, acetate and H₂-CO₂ are converted directly to methane by methanogenic bacteria. Utilization of other fermentation products requires
the concerted activity of secondary fermenting (syntrophic) bacteria, which convert fermentation products to acetate and H$_2$-CO$_2$, and methanogens which use these two products to form methane (Schink, 1997; Cicerone and Oremland, 1988).

In environments where other electron acceptors are found, such as nitrate, iron, manganese, or sulfate, the situation is slightly different (Figure 1-2, e.g. for sulfate reduction). The initial steps of the degradation of complex organic matter are similar; however, fermentation products are utilized and converted to CO$_2$ by microorganisms able to reduce the previously mentioned electron acceptors.

In many wetlands, nitrate is usually present in low concentrations or consumed very quickly, and the electron acceptor forms of iron and manganese are present in low concentrations and in solid phase, thereby reducing their bioavailability (Westermann, 1993). Therefore, the major anaerobic processes governing carbon cycling in wetland ecosystems are likely to be methanogenesis and sulfate reduction. Traditionally, in freshwater wetlands with low sulfate input methanogenesis is the dominant terminal carbon mineralization process, and in marine and salt marshes with a higher sulfate input sulfate reduction is the dominant process (Ward and Winfrey, 1985). However, some reports have demonstrated that sulfate reduction is important in freshwater systems, and a significant amount of the carbon degraded follows this pathway (Bak and Pfennig, 1991a; 1991b; Cappenberg, 1974a; 1974b; Ingvorsen and Brock, 1982; Ingvorsen et al., 1981; Kuivila et al., 1989; Lovley and Klug, 1983).

Very little is known about the microbial communities responsible for the anaerobic terminal carbon mineralization processes in wetland soils, since most of the studies have been conducted at the process level not at the microbial level.
The Everglades Ecosystem

Since the 1920s, the Everglades have been modified by urbanization and agricultural activities, through the construction of levees, canals, and dams to control the water flow. These modifications have caused a loss of half of the ecosystem and waters that used to be part of the ecosystem are diverted to the Gulf of Mexico and the Atlantic Ocean. Several plants and animals have suffered loss of habitat, which combined with contamination caused by human activities, resulted in a decline in their population sizes (Schrope, 2001).

The Everglades was a low-nutrient ecosystem but the discharge of nutrient enriched drainage water from the Everglades Agricultural Area (EAA) has resulted in a well-documented phosphorus gradient in the Florida Everglades Water Conservation Areas (Figure 1-3) (Craft and Richardson, 1993; DeBusk et al., 1994; 2001; Koch and Reddy, 1992; Reddy et al., 1993).

Nutrient inputs resulted in changes in vegetation. Phosphorus-enriched sites are dominated by cattail (Typha spp.), and areas with lower P content are dominated by indigenous sawgrass (Cladium spp.). The vegetation changes resulted in changes in the overall productivity of these marshes and influenced the long-term storage capabilities of the ecosystem, which affected the water quality and ecology of these ecosystems (Reddy et al., 1993).

Areas impacted by agricultural activities have been shown to have high levels of sulfate, the primary electron acceptor for sulfate-reducing prokaryotes (South Florida Water Management District, 2001). Sulfate in the Everglades is derived from natural and human activities. Sulfate can reach the wetlands through the atmosphere, the EAA stormwater runoff, and groundwater rising to the surface in the Everglades (South Florida
Water Management District, 2001). The EAA runoff has high sulfate concentrations due to the use of elemental sulfur to control soil acidity. Two areas in the Everglades with high sulfur concentrations are the EAA and WCA-2A (Bates et al., 1998; 2002).

Previous studies reported the total sulfur content of the impacted site is relatively similar to the non-impacted sites (Bates et al., 1998). However, in the impacted sites, 70-80% of the total sulfur is present as organic sulfur, compared to 50-55% organic sulfur in the non-impacted zones. Further, in the non-impacted zones more sulfur is present as disulfides and sulfate, which may indicate a slower turnover of sulfur due to lower sulfate reduction rate. Sulfate reduction rates have been shown to be greater in the impacted zones compared to non-impacted zones (Gilmour et al., 1998). The vegetation dominating the impacted and non-impacted sites, cattail and sawgrass, did not significantly differ in the amount of organic sulfur in their leaves, 0.14 and 0.11% respectively (Bates et al., 1998).

**Sulfate-Reducing Prokaryotes**

Sulfate-reducing prokaryotes (SRP) constitute a diverse group of prokaryotes that contribute to a variety of essential ecological and biogeochemical functions in many anaerobic environments. In addition to their obvious role in the sulfur cycle, SRP are important regulators of a variety of processes in wetland soils, including organic matter turnover, biodegradation of chlorinated aromatic pollutants in anaerobic soils and sediments, and mercury methylation (Fauque, 1995; Barton and Tomei, 1995). SRP may be an important bacterial group in freshwater wetlands due to several attributes of this microbial group (Widdel, 1988; Widdel, 1992a; Widdel and Bak, 1992; Widdel and Pfennig, 1984):

- versatility in the type of substrate
- versatility in the type of metabolism: sulfate reduction, fermentation, including syntrophism
- the ability of some SRP to form spores allowing survival in environments with fluctuating redox potentials.
- ability of some SRP to survive exposure to oxygen
- lower Kms for acetate and hydrogen than methanogens.

SRP are ubiquitous in these wetlands. Drake et al. (1996) reported $4 \times 10^{11}$ SRP per gram (dry weight) for sediments in P-enriched areas and $5 \times 10^7$ SRP per gram in non-impacted areas. They postulated an enrichment of SRP in the P-enriched site due to a high concentration of sulfate coming from the runoff from EAA. In that study only SRP able to use lactate were enumerated, and it did not provide any notion to the community composition of SRP since several different SRP groups are able to use lactate (Widdel, 1988).

Although it was not the focus of this research, mercury methylation has been shown to be an important environmental problem in this ecosystem. Sulfate-reducing bacteria are one of the principal bacterial groups able to methylate mercury in anaerobic environments (Compeau and Bartha, 1985; Devereux et al., 1996; Hobman et al., 2000; King et al., 2000). Mercury biomagnification in the Everglades resulted in methylmercury concentrations in fishes greater than the FDA concentration approved for human consumption. It is estimated that 1 million acres of the Everglades (half of the whole ecosystem) has large mouth bass with mercury concentrations above the approved FDA limit (Barnett and Vogel, 2001). Moreover, mercury poisoning has been implicated for the deaths of three Florida panthers, and scientists have related mercury to the mortality of wading birds (Kays, 2001). Gilmour et al. (1998) reported that mercury methylation was higher in non-impacted Everglades areas, resulting in a decrease of
methylmercury from north to south, and this opposes the gradient in nutrients, sulfate,
and sulfide concentrations. They suggested that the high nutrient impact in northern sites
resulted in higher sulfate reduction rates and sulfide accumulation, which inhibited
mercury methylation (Gilmour et al., 1998). The opposite occurs in the more pristine
areas in the south zones of the marshes. This observation is in agreement with higher
SRP enumerations for the P-enriched areas reported by Drake et al. (1996).

Although SRP are present in freshwater ecosystems and they play an important role
in elements cycles, we are not aware of any published studies describing the
characterization or ecological role of SRP from these types of freshwater wetlands.

**Phylogeny of Sulfate-Reducing Prokaryotes**

Interest in SRP has increased during the last 10 years because of their importance
to critical processes in ecosystem functioning and environmental remediation. With the
development of rRNA phylogenetic analysis, notable advancements have been made in
the taxonomy and phylogeny of this very diverse group (Stackebrandt et al., 1995).

Phylogenetic classification of SRP by rRNA analysis provided insights into the
evolutionary origins of sulfate reduction in distantly related species, and in facilitated
development of group-specific phylogenetic probes and PCR primers for use in
ecological studies. A comprehensive review of SRP phylogeny is important in order to
assemble recent work from a variety of laboratories and clearly define the SRP genera
belonging to each group. This section will review and compile recent advances in
defining phylogenetic relationships among the various branches of this diverse functional
group of microorganisms.

SRP are a complex physiological bacterial group, and various properties have been
used in traditional classification schemes (Table 1-1). The most important of these
properties were cell shape, motility, GC content of DNA, presence of desulfovirin, cytochromes composition, optimal growth temperature, and complete versus incomplete oxidation of acetate. For classification within a particular genus, different electron donors are tested. Analysis of rRNA sequences has allowed organization of the various SRP species into four distinct groups: gram-negative mesophilic SRP; gram-positive spore forming SRP; thermophilic bacterial SRP; and thermophilic Archaeal SRP.

The assignments of individual species into groups based on rRNA sequence similarity is in general agreement with those obtained by traditional taxonomy, although some exceptions exist and will be discussed below.

**Gram-Negative Mesophilic SRP**

This group of SRP is located within the delta subdivision of the Proteobacteria (Figures. 1-4 and 1-5). At some point in their evolutionary history, the delta subdivision diverged from other Proteobacteria from a common ancestral phototroph, and members of the delta subdivision lost their photosynthetic ability and converted to heterotrophy (Woese, 1987).

The delta subdivision also includes non-SRP such as sulfur-reducing bacteria (*Desulfurella, Desulfuromusa, and Desulfuromonas*), *Myxobacteria* and *Bdellovibrio*, and *Pelobacter* and *Geobacter* (Lonergan et al., 1996). Phylogenetic relationships between SRP and other members of the delta subdivision remain unresolved (Devereux et al., 1989), although it has been suggested that *Myxobacteria* and bdellovibrios may represent aerobic adaptations of an ancestral anaerobic sulfur-metabolizing phenotype (Devereux et al., 1989; Oyaizu and Woese, 1985). Devereux et al. (1990), however, reported that placement of the exact root of *Myxobacteria* (inside or outside the gram-negative mesophilic SRP) is affected by the outgroup sequences used to construct the
tree, suggesting that Myxobacteria may not have originated from within this group of SRP.

*Pelobacter* and *Geobacter* reduce Fe(III) to Fe(II), a metabolic characteristic partially shared with some SRP belonging to delta subdivision. However, to date no known gram-negative SRP that reduce Fe(III) to Fe(II) are capable of conserving energy for growth with Fe(III) as a sole electron acceptor (Lovley et al., 1993).

Two families of SRP have been traditionally proposed within the delta-Proteobacteria: the Desulfovibrionaceae (presented in detail in Figure 1-4) and the Desulfobacteriaceae (presented in detail in Figure 1-5) (Devereux et al., 1990; Widdel and Bak, 1992). However, Garrity et al. (2001) proposed creation of a higher level of classification, the orders Desulfovibrionales and Desulfobacterales, and the creation of new families within these orders. According to the classical SRP classification, the Desulfovibrionaceae family includes the genera *Desulfovibrio* and *Desulfomicrobium*. In Garrity et al. (2001), two families are proposed, the Desulfovibrionaceae and Desulfomicrobiaceae. 16S rRNA sequence analysis allowed reclassification of many species; one example within this group is the reclassification of *Desulfomonas pigra* to *Desulfovibrio piger* (Loubinoux et al., 2002), although *D. piger* differs from other desulfovibrios in its motility (nonmotile) and shape (rod versus vibrioid). Two recently described genera, *Desulfohalobium* (represented by *D. retbaense*; GenBank accession number U48244) and *Desulfonatronum* (represented by *D. lacustre*; GenBank accession number Y14594) have not yet been officially placed within this group, although they fall firmly within this cluster by our analyses. Garrity et al. (2001) has proposed creation of the families Desulfohalobiaceae to include *D. retbaense* and Desulfonatronumaceae to
include *D. lacustre*. Obviously, finding more species related to these SRP would fortify the approval of these proposed families.

The original Desulfobacteriaceae family (Figure 1-5) included all SRP within the delta-Proteobacteria that were not part of the Desulfovibrionaceae (Devereux et al., 1990; Widdel and Bak, 1992). This rather broad definition included species of the genera *Desulfobulbus, Desulfobacter, Desulfobacterium, Desulfococcus, Desulfosarcina, Desulfomonile, Desulfonema, Desulfobotulus, and Desulfoarculus*. Our analyses suggest that several newly proposed genera may fall within the Desulfobacteriaceae on the basis of rRNA sequence analysis. These newly added genera include *Desulfobacula, Desulfospira, Desulfocella, Desulfobacca, Desulfacinum, Thermodesulforhabdus, Desulforhabdus, Desulfocapsa, Desulforhopalus, and Desulfofustis*. Our phylogenetic analyses revealed that the *Desulfobulbus* and *Desulfocapsa/Desulfofustis* clusters might represent a deeply branching group that may constitute a separate family. More physiological characterization and sequence data of related species are required to confirm or reject their placement within the Desulfobacteriaceae. Rooney-Varga et al. (1998) proposed the creation of the family Desulfobulbusaceae to include species of the genus Desulfobulbus. Garrity et al. (2001) proposed the creation of two families within this Desulfobacteriaceae cluster. The Desulfobulbaceae family would include species of the genera *Desulfobulbus, Desulfocapsa, Desulfofustis* and *Desulforhopalus*. The rest of the genera would be included in the family Desulfobacteraceae. Reclassification of *Desulfovibrio sapovorans* as *Desulfobotulus sapovorans*, and *Desulfovibrio baarsii* as *Desulfoarculus baarsii* have recently been proposed (Stackebrandt et al., 1995; Holt, 1993), although no official reclassifications have been published to date concerning these
proposals. Phylogenetic analyses suggest that these two species do not belong to the Desulfovibriionaceae family, a finding consistent with metabolic features (specifically electron donors) of these two species compared with the desulfovibrios (Devereux et al., 1989). Most members of this family are mesophilic; however, Desulfacinum infernum (GenBank accession number L27426) and Thermodesulforhabdus norvegicus (Beeder et al., 1995) are thermophilic. Garrity et al. (2001) proposed the reclassification of genera Desulfobacca, Desulfacinum, Thermodesulforhabdus, Desulforhabdus, and Desulfomonile to the order Syntrophobacterales. This reclassification is in agreement with our phylogenetic analysis, since these genera were a divergent cluster from the Desulfovacteriaceae family (Figure 1-5).

Interesting morphological aspects of the Desulfovacteriaceae family include formation of clumps, as is seen in Desulfosarcina, and the gliding motion of filamentous Desulfonema. Clump formation can provide protection against unfavorable changes in environmental redox potential, and the gliding motility of Desulfonema allows these bacteria to move against chemical gradients to reach areas of favorable nutrient concentration. Moreover, the formation of filaments by this SRP may provide resistance against phagocytosis by ciliates and amoebae (Widdel, 1988).

**Gram-Positive Spore-Forming SRP**

This group is dominated by the genus Desulfotomaculum and is placed within low GC gram positive bacteria (Figure 1-6) that includes other bacteria such as Bacillus and Clostridium. These are the only SRP known to form heat-resistant endospores, a trait shared with many Bacillus and Clostridium species. In contrast with the mesophilic SRP, some species of Desulfotomaculum are thermophilic, although their optimal growth temperatures are lower than those of thermophilic gram-negative and archael sulfate
reducers (Table 1-1). Changes within this gram-positive SRP group are currently being proposed, however, and these changes are supported by our analyses. These proposed changes include reclassification of *Desulfotomaculum guttoideum* to another genus, perhaps *Clostridium* (Stackebrandt et al., 1997). The 16S rDNA analysis suggests that *D. guttoideum* is closely related to a cluster of *Clostridium* and appears on a separate branch from the rest of the *Desulfotomaculum* species. A recently reclassified genus within this group is *Desulfotomaculum orientis*, now *Desulfosporosinus orientis* (Stackebrandt et al., 1997), adding a second genus to this cluster. Garrity et al. (2001) placed the gram-positive SRP in the family Peptococcaceae within the order of the Clostridiales.

Different species within the genus *Desulfotomaculum* exhibit great versatility in the type of electron donors they are capable of using for growth and include acetate, aniline, succinate, catechol, indole, ethanol, nicotinate, phenol, acetone, stearate, and others. Depending on the species, organic substrates are oxidized incompletely to acetate or completely to CO$_2$ (Table 1-1) (Widdel, 1992a). In contrast to delta-Proteobacteria SRP, the ability to use Fe (III) as sole terminal electron acceptor for growth has been described for some gram positive SRP, such as *Desulfotomaculum reducens* (Tebo and Obraztsova, 1998).

Although most spore forming SRP are found in similar environments to delta-Proteobacteria SRP, spore formation allows this group to survive for long periods of desiccation and oxic conditions. For example, *Desulfotomaculum* is the prevalent genus of SRP in rice paddies due to alternating oxic and anoxic conditions as a result of seasonal flooding (Widdel, 1992a).
Bacterial Thermophilic SRP

The two most well characterized species in this group of SRP are *Thermodesulfobacterium commune* (Zeikus et al., 1983) and *Thermodesulfovibrio yellowstonii* (Henry et al., 1994) (Figure 1-7). Both bacteria were isolated from hydrothermal vent waters in Yellowstone National Park, and their optimal growth temperatures are higher than those described for gram-positive spore-forming thermophilic SRP, but lower than those of the archaeal SRP (Table 1-1). Although these two genera have similar physiological and phenotypic characteristics, they differ in shape (vibrio versus rod) and GC content (30% versus 34%) for *T. yellowstonii* and *T. commune*, respectively. Sequence analysis of 16S rRNA also suggests that they are phylogenetically distant (Figure 1-7), confirming their placement in separate genera (Henry et al., 1994). Henry et al. (1994) suggested that categorization of thermophilic SRP (similar physiology but different phylogeny) is similar to the situation with the Desulfovibrio family; a group that shares physiological similarities but is phylogenetically diverse and is grouped within a family. They also suggested that thermophilic SRP and desulfovibrios play similar functional roles in their respective environments. However, Garrity et al. (2001), proposed the placement of *Thermodesulfovibrio* spp. in the proposed family Nitrospiraceae, which includes species of the genera *Nitrospira, Leptospirillum* and *Magnetobacterium*, a cluster that is recovered with high bootstrap support in our phylogenetic analysis. Garrity et al. (2001) also proposed the creation of a new family Thermodesulfobacteriaceae to include species of the genera *Thermodesulfobacterium*. Phylogenies for thermophilic gram-negative bacteria branch deeply in the Bacteria domain, in accordance with the theory of thermophilic origins of the Bacteria (Achenbach-Richter et al., 1987). Both genera...
utilize H$_2$ as an electron acceptor if acetate is present, which in these extreme environments can be derived from thermophilic fermentations or geothermal reactions (Widdel, 1992b). Although these genera have optimal growth temperatures between 65 and 70°C, they can survive at lower temperatures (Nilsen et al., 1996).

**Archaeal Thermophilic SRP**

The archaeal thermophilic SRP group exhibits optimal growth temperatures above 80°C. Only two species have been described to date both of which were isolated from marine hydrothermal systems: *Archaeoglobus fulgidus* (Stetter, 1988) and *Archaeoglobus profundus* (Burggraf et al., 1990) (Figure 1-8). Major differences between the two species are that *A. fulgidus* possess flagella, are facultative chemolithoautotrophs, and produce small amounts of methane, while *A. profundus* do not possess flagella, are obligate chemolithoheterotrophs, and do not produce methane. *Archaeoglobus veneficus* is a sulfite or thiosulfate reducer, and is not able to reduce sulfate (Huber et al., 1997).

Using 16S and 23S rRNA sequence analysis, Woese et al. (1991) indicated that *A. fulgidus* falls within the Methanomicrobiales and extreme halophiles cluster (kingdom Euryarcheota), as is shown in Figure 1-8. Woese et al. (1991) proposed that sulfate-reducing activity did not arise as early as was proposed by Achenbach-Ritcher et al. (1987), and today *A. fulgidus* is thought to have evolved from methanogenic ancestors. The question of how sulfate reduction in Archaeoglobus was acquired remains unresolved, although Wagner et al. (1998) proposed that either a common ancestor of the Archaea and Bacteria domains possessed the capacity, or the genes were laterally transferred into Archaeoglobus from a member of the Bacteria soon after divergence of the domains.
Existence of Possible Undescribed Groups

Even though SRP are currently divided into four phylogenetic groups, new divisions could be added as more information on the diversity of SRP in extreme environments becomes available. Jorgensen et al. (1992) observed sulfate-reducing activity in sediments from Guaymas Basin in different ranges of temperatures than previously described for the four known groups of SRP. They reported sulfate-reduction between 100-110°C, temperatures from which no SRP have yet been isolated. These authors postulated that SRP may be present in those extreme thermophilic environments and that there may be some hyperthermophilic SRP still to be discovered.

SRP able to degrade complex high molecular weight aromatic hydrocarbons such as naphthalene and phenanthrene have not been isolated to date; however, Coates et al. (1996) reported oxidation of polycyclic aromatic hydrocarbons under sulfate-reducing conditions. Although SRP were not isolated, incubation of $^{14}$C-labeled naphthalene or phenanthrene-spiked harbor sediments under sulfate-reducing conditions resulted in the production of $^{14}$CO$_2$. Moreover, addition of molybdate, a specific inhibitor of sulfate reduction, resulted in a complete inhibition of $^{14}$CO$_2$ evolution, suggesting that undescribed SRP may be present.

The assumption that greater than 99% of bacteria in soils remain uncultivated is another challenging area where phylogeny of SRP may play an important role. Rooney-Varga et al. (1997), using oligonucleotides probes targeting novel uncultivated SRP, found that one of the uncultivated clones played an important role in a salt marsh sediment. Several studies, using phylogenetic analyses of dissimilatory sulfite reductase, the key enzyme in sulfate reduction, found sequences different from previously described sequences, also suggesting the possible presence of undescribed SRP (Chang et al., 2001;
Joulian et al, 2001; Minz et al., 1999; Perez-Jimenez et al., 2001). Therefore, investigation of novel SRP is a relevant topic of research. This is especially true in ecosystems such as the Florida Everglades, where little research has been conducted in the composition and ecology of SRP assemblages.

**Research Hypotheses**

In freshwater wetlands, physical and chemical parameters affect the microbial communities responsible for the carbon and sulfur cycles. Therefore, changes in microbial assemblages would reflect environmental changes. Due to the metabolic and physiological versatility of SRP, changes in assemblage composition would reflect changes at the process level in the Everglades. The Everglades is an appropriate site for this project because several factors can be studied at the same time, including carbon loading, sulfate loading, nutrient impact, and seasonal changes of water table height and hence redox potential.

SRP assemblage dynamics would be intimately related with environmental changes and reflect the effect of nutrient impact on these freshwater ecosystems.

Several specific hypotheses are proposed to gain information on the interactions between sulfate-reducing bacteria and their surrounding environment:

- Nutrient enrichment in impacted areas of the Everglades will result in an increase in total microbial numbers, including SRP and methanogens.

- In areas of the marsh where sulfate and SRP are present in significant amounts, SRP will compete with methanogens for electron donors such as acetate, hydrogen, and/or formate.

- The SRP metabolic shift due to nutrient impact will result in a shift in the composition of SRP microbial assemblages, and molecular techniques could be used to assess impact.
These microbial shifts are related to biogeochemical changes in the ecosystem, and the integration of all these factors are required to develop a comprehensive model for the role of SRP in the Everglades.

**Dissertation Outline**

This research was conducted to understand the role of microorganism in anaerobic terminal carbon mineralization in Everglades soils, with special emphasis on sulfate-reducing prokaryotic assemblages. The study was conducted in the Everglades Water Conservation Area 2A, at three sites having different levels of eutrophication due to phosphorus loading.

Chapter 2 focuses on the determination of sulfate reduction rates and SRP enumerations in eutrophic and pristine areas of the Everglades WCA-2A.

Chapter 3 compares the composition and metabolic diversity of SRP in eutrophic and pristine areas of the Everglades WCA-2A. This study combined culture-based and culture-independent techniques to assess the dynamics of SRP assemblages. The presence of different SRP groups was determined by using molecular techniques targeting the dissimilatory sulfite reductase gene. Traditional enrichment and cultivation techniques using several electron donors combined with 16S rRNA gene sequence analysis of SRP isolates were used to assess the metabolic diversity of SRP in these types of freshwater marshes.

In Chapter 4, methanogenic community composition in eutrophic and pristine areas of the Everglades WCA-2A are described. Culture-independent techniques targeting the archaeal 16S rRNA and methyl coenzyme M reductase genes were used to assess the dynamic of methanogenic assemblages.

Chapter 5 describes the use of the molecular ecology technique terminal restriction fragment length polymorphism (T-RFLP) to explore the dynamics of SRP and
methanogenic assemblages in relation to environmental conditions in Everglades WCA-2A sites having three levels of eutrophication.

Finally, results and implications of this research are summarized in Chapter 6. It is anticipated that the results of this study will contribute to a greater understanding of the factors controlling anaerobic terminal carbon mineralization in Everglades wetland soils.
<table>
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<tr>
<th>Shape</th>
<th>Motility</th>
<th>GC content of DNA (%)</th>
<th>Desulfovirin</th>
<th>Cytochromes</th>
<th>Oxidation</th>
<th>Growth temp. (°C)</th>
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<td>-</td>
<td>b, c</td>
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<td>-</td>
<td>66</td>
<td>+</td>
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<td>+/-</td>
<td>c&lt;sub&gt;3&lt;/sub&gt;, b, c</td>
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<td>C&lt;sup&gt;b&lt;/sup&gt;</td>
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<td><em>Desulfovirin bacterium</em></td>
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<td>-</td>
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<td>C</td>
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<td>+</td>
<td>c&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>-</td>
<td>n.r.&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup> I. Incomplete, <sup>b</sup> C. complete, <sup>c</sup> n.r. not reported
Figure 1-1. Microbial processes responsible for organic matter mineralization in wetland soils according to redox potential.
Figure 1-2. Organic matter mineralization under in freshwater and sulfate-enriched ecosystems.
Figure 1-3. Map of South Florida showing the Everglades Agricultural Areas (EAA), Water Conservation Areas (WCA) and the Everglades National Park.
Figure 1-4. Phylogenetic tree for the gram-negative mesophilic sulfate-reducing prokaryotes, with emphasis on the family Desulfovibrionaceae. Number before branch points represent percentages of bootstrap resampling based on 1000 trees. Bootstrap values below 50% are not presented (adapted from Castro et al., 2000).
Figure 1-5. Phylogenetic tree for gram-negative mesophilic sulfate-reducing prokaryotes, with emphasis on the family Desulfobacteriaceae (adapted from Castro et al., 2000).
Figure 1-6. Phylogenetic tree for the genus *Desulfotomaculum* within the cluster of low G+C content gram-positive bacteria (adapted from Castro et al., 2000).
Figure 1-7. Phylogenetic relationship of gram-negative bacterial thermophilic sulfate-reducing prokaryotes (adapted from Castro et al., 2000).
Figure 1-8. Phylogenetic position of archaeal thermophilic sulfate-reducing prokaryotes (adapted from Castro et al., 2000).
CHAPTER 2
SULFATE REDUCTION AND SULFATE-REDUCING PROKARYOTIC COMMUNITY DYNAMICS IN EUTROPHIC AND PRISTINE AREAS OF THE EVERGLADES WCA-2A

Introduction

Although sulfate-reducing prokaryotes are present in a variety of anaerobic environments, most studies on SRP dynamics have been conducted in marine and salt marsh sediments where sulfate reduction plays a major role in the carbon cycle (Devereux et al., 1992; Devereux et al., 1996, Hines et al., 1999; Rooney-Varga et al., 1997). In freshwater systems with low sulfate input, methanogenesis is considered the major final pathway in the carbon cycle, and very little is known about the role of SRP in freshwater ecosystems (Schink, 1997).

The Everglades is a freshwater ecosystem, which receives high inputs of sulfate due to the use of sulfur in adjacent agricultural areas (Bates at al., 1998; Bates et al., 2002). A major environmental concern in the Everglades related to sulfur loading, is mercury methylation and biomagnification of this toxic compound in the Everglades fauna (Barnett and Vogel, 2001). Mercury methylation in these anaerobic sediments is primarily regulated by SRP environments (Compeau and Bartha, 1985; Devereux et al., 1996; Hobman et al., 2000; King et al., 2000). This is indirect evidence that SRP are present and active in significant amounts in this type of freshwater marshes. To our knowledge, the only study to enumerate SRP in the Everglades was carried out by Drake et al. (1996) in sediments of the WCA-2A along the phosphorus gradient. They reported greater numbers of SRP in the P-enriched regions of the marsh and they ascribed that
enrichment to higher sulfate concentrations coming from EAA runoff. Gilmour et al. (1998) studied factors controlling mercury methylation in the Everglades and showed somewhat high sulfate reduction rates for a freshwater ecosystem. The rates were higher in the northern regions of the Everglades, and they reasoned that this might be due to higher sulfate input and more anaerobic sediments as the result of eutrophication.

The fitness and survival of SRP in the Everglades are likely related to several attributes possessed by members of this microbial functional group. Important characteristics are electron donor and metabolic versatility, lower $K_m$s than methanogens for shared substrates such as acetate and hydrogen, resistance to fluctuating redox potential due to changing water tables, and an ability to survive limited exposure to oxygen (Widdel, 1988; Widdel and Bak, 1992; Widdel and Hansen, 1992; Widdel and Pfennig, 1984). All of these attributes would be important in a system such as the Everglades where the amount and type of electron donors, and redox potential and oxygen exposure, would be affected by factors such as nutrient loading and hydroperiod.

To date, only one published study documented SRP enumeration in samples taken during one sampling trip in August 1994 (Drake et al., 1996), and only one study measured sulfate reduction rates in samples taken in March, July and December 1995 (Gilmour et al., 1998). No information is available for both parameters combined over a period of time including wet and dry seasons in the Everglades. Therefore, information covering an entire hydroperiod would be valuable to understand the dynamics and role of SRP in this ecosystem.

One of the focus of this study was to measured rates of sulfate reduction rates and determined SRP numbers in the well documented eutrophic and pristine regions, F1 and
U3 respectively. These sites represent extremes along the eutrophication gradient. The transition zone, F4, was not included in this portion of the study because it was assumed that the characteristics of this site would be intermediate between the impacted and pristine regions of the marsh. Because F4 was included in studies dealing with the molecular diversity of microbial populations along the eutrophication gradient in WCA-2A (Chapter 5), F4 biogeochemical characterization is presented here in conjunction with the other two sites, F1 and U3.

Specific goals of this study were: (I) to compare sulfate reduction rates; (II) to monitor SRP community sizes during an entire hydroperiod; (III) to investigate the role of different electron donors in sulfate reduction; and (IV) to investigate competition between SRP and methanogens.

**Materials and Methods**

**Site Characteristics, Sampling and Biogeochemical Characterization**

Studies were conducted on samples taken from the Florida Everglades Water Conservation Area 2A (WCA-2A) (Fig. 1-3). Triplicate cores were collected along the phosphorus gradient, from impacted F1 (cattail-dominated sites), and non-impacted zones U3 (sawgrass-dominated sites). The studies were conducted on the 0-10 cm soil layer, which includes nutrient impact greater than 3 years based on peat accretion rates calculated from 137Cs distribution in the soil profile (Reddy et al., 1993; 1999). A thick (5-15 cm) marl/periphyton layer overlays U3. Three soil cores were collected monthly from 4/23/2001 to 9/10/2002. Soil cores were sampled by the staff of the South Florida Water Management District and shipped overnight to the Wetland Biogeochemistry Laboratory, Soil and Water Science Department, Gainesville, FL, where they were sectioned and manually mixed. Subsamples intended for enumeration and measurement
of rates were kept at 4°C until analysis (within 2 to 5 days of sampling). Samples were not processed immediately nor kept under anoxic conditions, such that rates and enumerations reported in this study do not represent field conditions. However, they can be used as reasonable estimators in comparative studies between the two sites. Total phosphorus (TP), total inorganic phosphorus (TPi), ammonium (NH$_4$-N), total Kjeldhal nitrogen (TKN), extractable total organic carbon (TOC), microbial biomass carbon (MBC), microbial biomass nitrogen (MBN) and microbial biomass phosphorus (MBP) were determined according to White and Reddy (1999) and D’Angelo and Reddy (1999) by the Wetland Biogeochemistry Laboratory.

**Microbial Enumerations and Enrichments**

Conventional anaerobic techniques were used throughout the anaerobic microbial work (Balch et al., 1979; Hungate, 1969). Enumerations were conducted using the most-probable-number (MPN) technique, with three tubes per dilution using Basal Carbonate Yeast Extract Trypticase (BCYT) medium (Touzel et al., 1983). Electron donors (20 mM) were added from sterile anoxic stock solutions, and FeSO$_4$(20mM) was used as the sulfate source and reducing agent. Tubes with black precipitate due to sulfide production were considered positive for MPN calculations. Tubes were incubated at room temperature without agitation in the dark for two months.

**Sulfate Analyses**

Sulfate concentrations were measured by ion chromatography using a Dionex Model LC20 equipped with an AS14 column and an ECD50 conductivity detector (Dionex, Sunnyvale, CA), using 3.5mM sodium carbonate/1.0 mM sodium bicarbonate as eluent.
**Sulfate Reduction Measurement**

Sulfate reduction rates were determined as described by Ulrich et al. (1997) with slight modifications according to an extensive comparison study of radiotracer techniques conducted by Meier et al. (2000). Briefly, one g soil subsamples were distributed in 120-ml serum bottles containing 1 ml of reduced BCYT medium under a continuous N2 stream. Test tubes (12 x 75 mm) containing 2.5 ml of anoxic 10% zinc acetate were placed within the bottles as sulfide traps, and the serum bottles were closed with butyl rubber stoppers and aluminum seals (Bellco Glass Inc, Vineland, NJ). Two µCi of carrier-free $^{35}$S-$\text{SO}_4^{2-}$ (ARC, St. Louis, MO) were injected and the radiochemical was diluted with non radiolabeled sulfate to render a final sulfate concentration of 100 µM. The flasks were incubated with shaking at 125 rpm in the dark for two hours at room temperature. After two hours, 8 ml of anoxic 6 M HCl and 8 ml of anoxic 1 M CrCl$_2$ in 0.5 M HCl were added via syringe, and the bottles were shaken at 125 rpm for 48 hours. Trap contents were mixed with scintillation cocktail (Ecoscint A; National Diagnostics, Atlanta, GA) and the radioactivity in the traps and the non reduced $^{35}$SO$_4^{2-}$ radioactivity remaining in the bottles was measured by liquid scintillation counting (LS 5801, Beckman Coulter, Fullerton, CA). Sulfate reduction rates were calculated as described by Fossing and Jørgensen (1989).

**Methanogenesis Rate Measurement**

One g soil was mixed with 2 ml of anoxic BCYT medium under a N$_2$ stream in anaerobic culture tubes that were later closed with butyl rubber stoppers and aluminum seals. The tubes were preincubated for two weeks before substrates were added. Sulfate and acetate (20 mM each) were added from anaerobic sterile concentrated stock solutions. Molybdate (an inhibitor of sulfate reduction) was added at a final
concentration of 2 mM (Oremland and Capone, 1988). The tubes were fitted with three-way Luer stopcocks (Cole-Parmer, Vernon Hills, IL) for gas sampling, and incubated in the dark at 25°C with shaking at 100 rpm. 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°C. The carrier gas was N₂ and the oven temperature was 160°C. All determinations were carried out in triplicate. Headspace pressure was measured using a digital pressure indicator (DPI 705, Druck, New Fairfield, CT).

Results and Discussion

Biogeochemical Characterization

The biogeochemical data provided by the Wetland Biogeochemistry Laboratory are in agreement with historical records previously described for this ecosystem (DeBusk et al., 1998; Reddy et al., 1993; 1999). Biogeochemical parameters for phosphorus, carbon and nitrogen are presented in Figure 2-1, Table 2-1 and Table 2-2, respectively.

Total phosphorus was higher in the impacted zones, followed by transition zones and pristine zones (Figure 2-1). The impacted zone had an annual average of 1347 ± 290 mg TP/kg, with a maximum of 1826 and a minimum of 941 mg TP/kg. For the transition zone, the annual average was 754 ± 209 mg TP/kg with a maximum of 1135 and a minimum of 453. In the pristine soils the TP levels were much lower (303 ± 44 mg/kg), with a maximum of 377 and a minimum of 227 mg/kg. The total inorganic phosphorus followed the same trend as total P, and represented 35% of the total P in the impacted region, 40% in the transition region and 25% in the pristine region. Interestingly microbial biomass P did not follow the same trend, microbial biomass P was higher in the
transition zone (225 ± 114 mg/kg) followed by impacted (154 ± 52 mg/kg) and pristine zones (47 ± 10 mg/kg).

TC and TOC content were basically similar between the three sites (Table 2-1). However, the MBC was higher in the transition region followed by impacted and pristine zones, a similar trend observed for the MBP.

No differences were observed in TN, TKN and NH$_4^-$N (Table 2-2). Once more, microbial biomass followed the same trend as with MBP and MBP, being higher in the transition zone followed by the impacted and pristine regions.

C/N ratio was 15 for impacted zones and 13 for transition and pristine zones. C/P ratio ranged from 1403 in the pristine zones to 310 in the impacted zones, with the transition zones in the middle with a C/P ratio of 482. N/P ratio was higher in the pristine zone (106) followed by transition zones (36) and impacted zones (31). These values are in agreement with Reddy et al. (1999), with the exception of P related ratios. They reported lower C/P and N/P ratios for pristine zones (C/P of 915 and N/P of 60), probably due to the fact that P levels in their study were higher in pristine zones (486 mgTP/kg versus 303 mg TP/Kg in this study).

MBC/TC ratios, a ratio related to carbon availability and the propensity by a soil to accumulate organic carbon, were similar to previously reported values (Reddy et al., 1999). The highest value was found in the transition zone (0.0029), with 0.017 for impacted regions and 0.007 for pristine zones.

MBN/TN followed the same trend, with the highest value in the transition zone (0.06) followed by the impacted (0.03) and the pristine zone (0.008) suggesting high
nitrogen demand, but not nitrogen deficiency since the N/P ratio is higher for the transition zone than the pristine zones.

MBP/TP, an indicator of P assimilation efficiency by microbial populations, was higher in transition zones (0.30) when compared with eutrophic (0.11) and pristine (0.16) regions. These ratios may indicate that the eutrophic zones are N-limited and the pristine regions are P-limited, with no significant limitation of N or P in the transition regions resulting in higher microbial biomass.

**Sulfate Concentrations, Sulfate Reduction Rates and Enumerations of SRP**

The results presented in this section represent the microbial activities of SRP measured between the dates 08/15/01 to 09/10/02. The activity of anaerobic microflora is controlled by redox potential, hence water levels in the marshes would control oxygen penetration in deeper zones of the soil, profile. Water levels for the sampling period versus monthly means from 1996 to 2002 are presented in Table 2-3.

During the rainy season of 2001, water levels were higher than the previously reported averages for 1996-2002. Water levels were lower during the dry season 2001-2002, and higher or similar for the rainy season of 2002 than the reported averages for 1996-2002.

Sulfate concentrations in samples from F1 (1.4 - 0.1 mM) tended to be higher than or similar to samples from U3 (1.2 - 0.1 mM), but were much higher than sulfate in samples from the more pristine zones of the Everglades (0.005 mM or less) (Table 2-4). Most of this sulfate enrichment is due to anthropogenic activities, since the content of sulfate from natural sources is much lower. The average sulfate content for rainfall in these locations is 0.03 mM, and groundwater sulfate concentrations fluctuate between 0.025 to 0.010 mM (Bates et al., 1998). The sulfate concentrations and trends found in
this study are similar to previously reported results. Gilmour et al. (1998), reported values ranging from 0.1 to 0.4 mM for pore water sulfate for the same sampling sites used in this study and they did not find a clear correlation between sites or sampling period. Bates et al. (2002) reported similar sulfate concentrations and found an enrichment of sulfate in the northern regions of the Everglades, but did not find clear trends between eutrophic and pristine sites of the WCA-2A. Bates et al. (1998) speculated that the high variability and lack of a consistent pattern may be due to a myriad of interactive factors such as amount of rainfall, sulfate inputs from the agricultural areas and redox potential which would determine sulfur speciation. In summary, the reported sulfate values for WCA-2A are much higher than those found in the marshes of the southern WCA3A, WCA1 (Loxahatchee National Wildlife Refuge) and in the Everglades National Park, having values of 0.005 mM or less (Bates et al., 2002).

Even though the sulfate reduction rates exhibited high standard deviations typical of those commonly observed with the passive extraction method (Meier et al., 2000), sulfate reduction rates were significantly higher in eutrophic than in pristine sites (p < 0.05). Eutrophic zones have sulfate reduction rates values 5 to 37 times higher than pristine zones (Table 2-4).

Gilmour et al. (1998) reported sulfate reduction rates of 100 to 400 nmol.g$^{-1}$.day$^{-1}$ for F1 and 20 to 600 nmol.g$^{-1}$.day$^{-1}$ for U3, using the intact cores, which is considered to provide a closer approximation to field rates than slurry methods. Therefore, the slurry technique used in this study may have underestimated the sulfate reduction rate by about 3 to 17 times for eutrophic zones and 23 to 50 for pristine zones. However, the results of
this study provide additional evidence that sulfate reduction is greatly enhanced at
impacted sites compared to non-impacted sites.

SRP enumerations revealed a considerable number of lactate- and acetate-oxidizing
SRP in the samples (Table 2-4). Lactate was selected as an electron donor for SRP since
lactate can be used by most SRP species, with the exception of *Desulfobacter* spp. and
some *Desulfobacterium* spp. Many SRP capable of complete oxidation of electron
donors utilize acetate, including strains of the genus *Desulfobacter* and some
*Desulfotomaculum.* Therefore, enumerations with lactate and acetate would provide
information on numbers of known SRP groups. MPN-enumerations for lactate were an
order of magnitude higher in samples from F1 than in samples from U3. For acetate,
MPN enumerations were one to two order of magnitude higher in samples from F1 than
in samples from U3. In samples from both sites, MPN estimates for lactate-utilizing SRP
were similar to estimates obtained for acetate-utilizing SRP, with the exception of two
samples in U3 where lactate oxidizers were below the detection limit. Lactate and
acetate MPN enumerations did not vary as the sulfate reduction rates did, indicating that
the rates may change drastically but not the total SRP population. Hines et al. (1999)
reported similar results in rhizosphere of *Spartina alterniflora.*

For samples taken on 7/11/02 and 9/10/02, propionate, butyrate and formate were
included as electron donors in the MPN enumerations (Table 2-5). SRP able to oxidize
these electron donors were present in considerable numbers in eutrophic regions of the
marsh. Only propionate oxidizers were detected in the pristine zones, and their numbers
were one order of magnitude lower than those observed in the eutrophic zone. Butyrate
and formate oxidizers were in concentrations lower than $3.0 \times 10^4$ MPN/gram wet soil.
In conclusion, MPN enumerations showed that SRP were present in considerable numbers and active in both zones of the marsh. F1 contained slightly higher soil sulfate concentrations than most typical freshwater systems (0.1 to 0.3 mM). These relatively high sulfate concentrations correlated with high numbers SRP and with high sulfate reduction rates. In U3, the sulfate concentrations were similar or lower than those found in F1. Lower sulfate reduction rates and SRP numbers were observed at this site. Similar trends were observed in a previous study conducted in eutrophic areas of WCA-2A and pristine regions of WCA–3A (Drake et al., 1996). Our enumerations were lower than those reported by Drake et al. (1996), but they used a combination of lactate and hydrogen as electron donors whereas we used lactate or acetate individually. Lactate can be used as an electron donor by most SRP species, with the exception of Desulfobacter and some Desulfobacterium species. In our samples, the numbers of lactate- and acetate-utilizing SRP were similar, and DSR sequence analysis did not reveal sequences related to Desulfobacter or Desulfobacterium (Chapter 3). It can be expected, therefore, that most of the cultivable SRP enumerated were complete-oxidizers.

**Effect of Common Electron Donors on Sulfate Reduction Rates**

Several electron donors were tested for their effect on sulfate reduction rates. In samples from 09/12/2002. The electron donors tested were lactate, acetate, butyrate, propionate and formate. Molybdate was added as a control resulting in a complete inhibition of sulfate reduction rates. Due to the high standard deviation of the method, no significant differences were observed between the control flasks with no electron donor added and most of the ones with added electron donor. The single exception was the addition of 1mM formate, which resulted in an increase of sulfate reduction rates for the eutrophic sites. In samples from the pristine sites, the rates were much lower, making it
difficult to obtain statically meaningful data, a trend of increase of sulfate reduction rates was observed for 0.1 mM formate (Figure 2-2). Ingvorsen et al. (1981) reported that addition of acetate and lactate did not affect sulfate reduction rates in sediments from the eutrophic Lake Mendota. Stimulation of sulfate reduction rates was observed with hydrogen and other electron donors such as propionate, butyrate, glucose, and ethanol. They explained the lack of stimulation due to the possibility that other microbial groups outcompeted SRP for these electron donors, or that the electron donors were presented in sufficient concentration and addition of more electron donor did not result in stimulation of sulfate reduction rates. In our samples, the lack of response to electron donors may be explained by the presence of sufficient electron donors in the eutrophic samples. In addition, SRP may not be present in pristine zones in significant numbers to yield observable changes upon the addition of electron donors during a four-hour sulfate reaction determination.

**Methanogenesis Versus Sulfate Reduction.**

Potential methanogenesis rates were determined in samples taken on 12/07/01, 01/31/02 and 05/29/02 (Table 2-6). Intrinsic initial methanogenesis rates (without added carbon source) were approximately five to nine times higher for soils samples of F1 (0.015 to 0.026 µmol g⁻¹ h⁻¹) than for U3 (0.003 µmol g⁻¹ h⁻¹). Furthermore, amounts of methane accumulated within six days were four to ten fold higher in F1 than in U3. Addition of sulfate or molybdate did not result in significant changes in either the initial methane production rate or in the methane accumulated in six days, due to the fact that methanogenic rates were very low making it difficult to observe any significant differences. Addition of acetate resulted in an increase in methane production rates for
12/07/01 and 01/31/02 samples, with approximately four times the amount of methane observed for F1, versus two to four times for the pristine sites. No changes were observed for samples from 05/29/02. No significant statistical differences were observed with the combined addition of acetate and sulfate or molybdate (p < 0.05). Due to limited sample availability, these assays were conducted on a small scale (one gram per tube), which may have resulted in high standard deviations due to sample heterogeneity. However, when a p < 0.1 was used some trends were observed for the eutrophic site F1. Sulfate addition in the presence of acetate resulted in an approximately 60% reduction of initial methane production rates for the 12/07/01 and 01/31/02 samples, for 05/29/02 samples only a 15% reduction was observed. The methane accumulated after 6 days tended to be lower than the methane accumulated in flasks receiving only acetate. In U3 samples, no significant changes were observed among treatments.

Hydrogen has been shown to be an important electron donor in eutrophic zones of this marsh (Chauhan et al., 2003). Therefore, in samples for 05/29/2002, formate was tested in the presence and absence of sulfate and molybdate. Formate was tested as an equivalent source of H₂-CO₂, a common practice in anaerobic digestion studies because it is easier to work with and diffusion problems from the gas headspace to the liquid media are avoided (Dolfing and Bloemen, 1985). Also, most SRP able to use hydrogen can use formate, with the exception of some Desulfobulbus, Desulfo bacter and Desulfotomaculum (Widdel, 1988). No significant changes were observed in U3 samples. However, F1 samples showed an increase in the potential methanogenic rates, indicating that hydrogenotrophic methanogens are in higher numbers than acetoclastic
methanogens in this region of the marsh. Addition of sulfate or molybdate did not have an effect on the potential methanogenic rate or methane accumulated after 6 days.

In summary, methanogenesis rates and accumulated methane in six day incubation experiments were higher in F1 than in U3, which is in agreement with previously published reports (Drake et al., 1996; Reddy et al., 1999). Methanogenesis in U3 did not respond to addition of acetate, suggesting that acetoclastic methanogenesis may not be a major process in these regions of the marsh. This finding is in agreement with Drake et al. (1996) who reported six orders of magnitude greater numbers of acetoclastic methanogens in eutrophic site F1 compared to a more pristine site in WCA-3A. In U3, no major changes were observed upon addition of acetate and molybdate or sulfate, suggesting that acetate-utilizing SRP may not be important in this region of the marsh, although they were found in significant numbers in the MPN enumerations. On the contrary, acetate-utilizing SRP may play a role in eutrophic zones of the marsh, as suggested by the observations that addition of sulfate to F1 microcosms inhibited methanogenesis to some extent. The observed lack of response to acetate in the presence of sulfate suggests that SRP present in the pristine zones may be using electron donors other than acetate, or they may be using another type of metabolism such as fermentation.

**Methanogenesis Versus Sulfate Reduction Rate in Everglades Soils**

Degradation of organic matter via sulfate reduction or methanogenesis can be described according to the following equations (Ingvorsen and Brock, 1982):

- **Sulfate Reduction**: \[2(CH_2O) + SO_4^{2-} + H^+ \rightarrow 2CO_2 + HS^- + 2H_2O\]
- **Methanogenesis**: \[2(CH_2O) \rightarrow CO_2 + CH_4\]
Assuming that rates of methane production and sulfate reduction are affected by the same caveats in our experimental settings, we can estimate that the amount of organic matter channeled through sulfate reduction, relative to methanogenesis, ranged from 26% in the fall to 1% in the winter for the eutrophic sites. For the pristine sites, sulfate reduction plays a minor role with 6% of the total carbon in the fall samples, and 0.5 % in the winter samples. Clearly the role of sulfate in this freshwater system is highly variable due to several factors, such as loading from the agricultural areas, rainfall, and redox potential, which would regulate sulfur speciation.
Table 2-1. Carbon parameters for the study sites.

<table>
<thead>
<tr>
<th></th>
<th>TC (g/kg)</th>
<th>TOC (mg/kg)</th>
<th>MBC (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>F1</td>
<td>418 ± 13</td>
<td>431-398</td>
<td>2844 ± 809</td>
</tr>
<tr>
<td>F4</td>
<td>362 ± 52</td>
<td>431-257</td>
<td>3103 ± 860</td>
</tr>
<tr>
<td>U3</td>
<td>421 ± 27</td>
<td>450-355</td>
<td>2598 ± 743</td>
</tr>
</tbody>
</table>

Table 2-2. Nitrogen parameters for study sites.

<table>
<thead>
<tr>
<th></th>
<th>TN (g/kg)</th>
<th>TKN (mg/kg)</th>
<th>NH₄-N (mg/kg)</th>
<th>MBN (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>F1</td>
<td>28 ± 1</td>
<td>30-26</td>
<td>423 ± 116</td>
<td>719-294</td>
</tr>
<tr>
<td>F4</td>
<td>27 ± 5</td>
<td>34-22</td>
<td>495 ± 207</td>
<td>977-261</td>
</tr>
<tr>
<td>U3</td>
<td>31 ± 3</td>
<td>36-26</td>
<td>377 ± 84</td>
<td>436-259</td>
</tr>
</tbody>
</table>

Table 2-3. Water levels for eutrophic and pristine sites.

<table>
<thead>
<tr>
<th></th>
<th>Eutrophic (F1)</th>
<th></th>
<th>Pristine (U3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water level (cm)</td>
<td>Mean</td>
<td>Water level (cm)</td>
</tr>
<tr>
<td>08/15/01</td>
<td>61.3</td>
<td>36.9</td>
<td>↑</td>
</tr>
<tr>
<td>12/07/01</td>
<td>29.3</td>
<td>36.2</td>
<td>↓</td>
</tr>
<tr>
<td>01/31/02</td>
<td>18.6</td>
<td>27.1</td>
<td>↓</td>
</tr>
<tr>
<td>03/12/02</td>
<td>21.9</td>
<td>25.6</td>
<td>↓</td>
</tr>
<tr>
<td>07/11/02</td>
<td>69.5</td>
<td>45.3</td>
<td>↑</td>
</tr>
<tr>
<td>09/10/02</td>
<td>38.7</td>
<td>38.3</td>
<td>≈</td>
</tr>
</tbody>
</table>
Table 2-4. Sulfate concentrations, sulfate reduction rates, and MPN enumerations for eutrophic (F1) and pristine (U3) Everglades WCA-2A soils.

<table>
<thead>
<tr>
<th></th>
<th>F1 (impacted)</th>
<th>U3 (non-impacted)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Summer 01</td>
<td>Fall 01</td>
</tr>
<tr>
<td>Sampling day</td>
<td>08/15/01</td>
<td>12/07/01</td>
</tr>
<tr>
<td>Water table (cm)</td>
<td>61.3</td>
<td>29.3</td>
</tr>
<tr>
<td>Sulfate (mg/g soil)</td>
<td>0.09 (0.03)</td>
<td>0.04 (0.01)</td>
</tr>
<tr>
<td>Sulfate reduction rate (nmol.g(^{-1}).day(^{-1}))</td>
<td>119 (34)(^a)</td>
<td>94 (39)</td>
</tr>
<tr>
<td>Lactate</td>
<td>9.3 x 10(^6)(^b)</td>
<td>1.5 x 10(^6)</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.5 x 10(^6)</td>
<td>2.3 x 10(^6)</td>
</tr>
<tr>
<td>Sulfate (mg/g soil)</td>
<td>0.03 (0.004)</td>
<td>0.01 (0.001)</td>
</tr>
<tr>
<td>Sulfate reduction rate (nmol.g(^{-1}).day(^{-1}))</td>
<td>26 (8)</td>
<td>4.7 (1.4)</td>
</tr>
<tr>
<td>Lactate</td>
<td>9.3 x 10(^5)(^b)</td>
<td>9.2 x 10(^4)</td>
</tr>
<tr>
<td>Acetate</td>
<td>4.3 x 10(^5)</td>
<td>3.6 x 10(^3)</td>
</tr>
<tr>
<td>Marl/periphyton layer (cm)</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\) standard errors are shown in parentheses.

\(^b\) MPN 95 % confidence limits
Table 2-5. MPN enumerations with several electron donors.

<table>
<thead>
<tr>
<th>Eutrophic (F1)</th>
<th>Pristine (U3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sampling day</strong></td>
<td><strong>Sampling day</strong></td>
</tr>
<tr>
<td>07/11/02</td>
<td>07/11/02</td>
</tr>
<tr>
<td><strong>Water table (cm)</strong></td>
<td><strong>Water table (cm)</strong></td>
</tr>
<tr>
<td>69.5</td>
<td>62.2</td>
</tr>
<tr>
<td><strong>Sulfate (mg/g soil)</strong></td>
<td><strong>Sulfate (mg/g soil)</strong></td>
</tr>
<tr>
<td>0.03 (0.001)</td>
<td>0.03 (0.0001)</td>
</tr>
<tr>
<td><strong>Sulfate reduction rate</strong></td>
<td><strong>Sulfate reduction rate</strong></td>
</tr>
<tr>
<td>(nmol.g(^{-1}).day(^{-1}))</td>
<td>(nmol.g(^{-1}).day(^{-1}))</td>
</tr>
<tr>
<td>25.4 (5.8)</td>
<td>0.8 (0.2)</td>
</tr>
<tr>
<td><strong>Enumeration</strong></td>
<td><strong>Enumeration</strong></td>
</tr>
<tr>
<td>(MPN/gram wet soil)</td>
<td>(MPN/gram wet soil)</td>
</tr>
<tr>
<td>Lactate</td>
<td>Lactate</td>
</tr>
<tr>
<td>9.3 x 10(^5) (2.0 - 43 x 10(^5))</td>
<td>9.2 x 10(^3) (2.0 - 43 x 10(^3))</td>
</tr>
<tr>
<td>Acetate</td>
<td>Acetate</td>
</tr>
<tr>
<td>2.3 x 10(^5) (0.5 - 11 x 10(^5))</td>
<td>3.6 x 10(^3) (0.8 - 17 x 10(^3))</td>
</tr>
<tr>
<td>Propionate</td>
<td>Propionate</td>
</tr>
<tr>
<td>4.3 x 10(^5) (1.0 - 20 x 10(^5))</td>
<td>9.2 x 10(^3) (2.0 - 43 x 10(^3))</td>
</tr>
<tr>
<td>Butyrate</td>
<td>Butyrate</td>
</tr>
<tr>
<td>4.3 x 10(^5) (1.0 - 20 x 10(^5))</td>
<td>&lt; 3.0 x 10(^3)</td>
</tr>
<tr>
<td>Formate</td>
<td>Formate</td>
</tr>
<tr>
<td>2.3 x 10(^5) (0.5 - 11 x 10(^5))</td>
<td>&lt; 3.0 x 10(^3)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>69.5</td>
<td>62.2</td>
</tr>
<tr>
<td>38.7</td>
<td>60.6</td>
</tr>
<tr>
<td>0.03 (0.001)</td>
<td>0.01 (0.0002)</td>
</tr>
<tr>
<td>0.01 (0.002)</td>
<td></td>
</tr>
<tr>
<td>25.4 (5.8)</td>
<td>0.8 (0.2)</td>
</tr>
<tr>
<td>2.3 x 10(^5) (0.5 - 11 x 10(^5))</td>
<td>3.6 x 10(^3) (0.8 - 17 x 10(^3))</td>
</tr>
<tr>
<td>15.6 (6.1)</td>
<td>2.5 (0.9)</td>
</tr>
<tr>
<td>2.3 x 10(^5) (0.5 - 11 x 10(^5))</td>
<td>&lt; 3.0 x 10(^3)</td>
</tr>
<tr>
<td></td>
<td>3.6 x 10(^4) (0.8 - 17 x 10(^4))</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.2 x 10(^3) (2.0 - 43 x 10(^3))</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Butyrate</td>
</tr>
<tr>
<td></td>
<td>&lt; 3.0 x 10(^3)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Formate</td>
</tr>
<tr>
<td></td>
<td>&lt; 3.0 x 10(^3)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2-6. Potential methanogenic rates for eutrophic (F1) and pristine (U3) soils.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Rate (^a)</th>
<th>µmoles CH(_4) (^b)</th>
<th>Rate</th>
<th>µmoles CH(_4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>F1 - 12/07/2001</strong></td>
<td></td>
<td></td>
<td><strong>U3 - 12/07/2001</strong></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.015 (0.004)</td>
<td>2.0 (0.8)</td>
<td>0.003 (0.001)</td>
<td>0.2 (0.02)</td>
</tr>
<tr>
<td>SO(_4)^2-</td>
<td>0.029 (0.004)</td>
<td>2.3 (0.6)</td>
<td>0.005 (0.001)</td>
<td>0.2 (0.04)</td>
</tr>
<tr>
<td>MoO(_4)^2-</td>
<td>0.016 (0.009)</td>
<td>2.6 (1.6)</td>
<td>0.004 (0.001)</td>
<td>0.4 (0.3)</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.057 (0.005)</td>
<td>8.8 (0.4)</td>
<td>0.007 (0.002)</td>
<td>0.4 (0.1)</td>
</tr>
<tr>
<td>Acetate + SO(_4)^2-</td>
<td>0.034 (0.014)</td>
<td>7.6 (4.6)</td>
<td>0.007 (0.001)</td>
<td>0.3 (0.03)</td>
</tr>
<tr>
<td>Acetate + MoO(_4)^2-</td>
<td>0.050 (0.003)</td>
<td>10.6 (3.3)</td>
<td>0.006 (0.001)</td>
<td>0.4 (0.1)</td>
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<tr>
<td><strong>F1 - 01/31/2002</strong></td>
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<td></td>
<td><strong>U3 - 01/31/2002</strong></td>
<td></td>
</tr>
<tr>
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<td>1.4 (0.4)</td>
<td>0.004 (0.002)</td>
<td>0.4 (0.3)</td>
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<tr>
<td>SO(_4)^2-</td>
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<td>0.7 (0.3)</td>
<td>0.004 (0.001)</td>
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<tr>
<td>MoO(_4)^2-</td>
<td>0.017 (0.004)</td>
<td>1.4 (0.3)</td>
<td>0.006 (0.002)</td>
<td>0.4 (0.2)</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.049 (0.009)</td>
<td>7.8 (0.7)</td>
<td>0.016 (0.002)</td>
<td>2.9 (2.1)</td>
</tr>
<tr>
<td>Acetate + SO(_4)^2-</td>
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<td>4.0 (1.1)</td>
<td>0.007 (0.002)</td>
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</tr>
<tr>
<td>Acetate + MoO(_4)^2-</td>
<td>0.039 (0.001)</td>
<td>6.9 (2.4)</td>
<td>0.006 (0.001)</td>
<td>0.3 (0.3)</td>
</tr>
<tr>
<td><strong>F1 - 05/29/2002</strong></td>
<td></td>
<td></td>
<td><strong>U3 - 05/29/2002</strong></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.026 (0.004)</td>
<td>3.2 (0.7)</td>
<td>0.003 (0.002)</td>
<td>0.4 (0.3)</td>
</tr>
<tr>
<td>SO(_4)^2-</td>
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<td>2.8 (0.7)</td>
<td>0.002 (0.002)</td>
<td>0.1 (0.1)</td>
</tr>
<tr>
<td>MoO(_4)^2-</td>
<td>0.030 (0.005)</td>
<td>3.5 (0.5)</td>
<td>0.002 (0.001)</td>
<td>0.1 (0.1)</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.036 (0.010)</td>
<td>7.1 (2.6)</td>
<td>0.003 (0.001)</td>
<td>0.2 (0.2)</td>
</tr>
<tr>
<td>Acetate + SO(_4)^2-</td>
<td>0.032 (0.010)</td>
<td>5.1 (2.1)</td>
<td>0.004 (0.003)</td>
<td>0.5 (0.6)</td>
</tr>
<tr>
<td>Acetate + MoO(_4)^2-</td>
<td>0.041 (0.009)</td>
<td>7.0 (1.5)</td>
<td>0.005 (0.003)</td>
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<td>Formate</td>
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<td>0.005 (0.002)</td>
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<td>0.006 (0.003)</td>
<td>0.4 (0.2)</td>
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<tr>
<td>Formate + MoO(_4)^2-</td>
<td>0.086 (0.017)</td>
<td>6.5 (0.9)</td>
<td>0.003 (0.001)</td>
<td>0.4 (0.1)</td>
</tr>
</tbody>
</table>

\(^a\) potential methanogenic rates (µmol.g\(^{-1}\).h\(^{-1}\))

\(^b\) µmoles CH\(_4\) accumulated at the end of 6 days incubation.
Figure 2-1. Total phosphorus (TP), total inorganic phosphorus (Tpi) and microbial biomass phosphorus (MBP) levels (mg/kg) in (A) eutrophic, (B) transition, and (C) pristine sites of the Everglades WCA-2A.
Figure 2-2. Effect of formate on sulfate reduction rates (SRR, nmol.g⁻¹.day⁻¹). F1 and U3 represent control with no electron donor added. The concentrations of formate are represented by 0.1F, 0.1 mM; 1F, 1 mM; 5F, 5 mM; 20F, 20 mM. MoO4 represent controls with addition of molybdate.
CHAPTER 3
COMPOSITION AND METABOLIC DIVERSITY OF SULFATE-REDUCING PROKARYOTES IN EUTROPHIC AND PRISTINE AREAS OF THE EVERGLADES WCA-2A

Introduction

Terminal carbon mineralization in low sulfate freshwater ecosystems is typically controlled by methanogenesis. However, as was shown in Chapter 2, sulfate reduction can be important in the Everglades WCA-2A, and a significant amount of carbon may be mineralized via this pathway. SRP are a very diverse microbial group and possess several attributes, which may be advantageous in areas impacted by agricultural activities with higher levels of sulfate.

Most studies conducted on the biogeochemistry of the Everglades focused on processes, but not on the microorganisms that perform the processes, such that very little is known of the microbial communities responsible for the biogeochemical processes and the impacts of nutrient loading on processes within individual biogeochemical cycles. To our knowledge, the only study that has addressed this issue was conducted by Drake et al. (1996), who reported an enrichment of SRP in nutrient impacted zones of WCA-2A. Most characterizations of freshwater SRP populations have been conducted in lakes, rivers, and rice paddies, but to our knowledge no detailed characterization of SRP assemblages has been conducted in freshwater wetlands.

Most studies on the diversity and community dynamics of SRP in ecosystems other than freshwater marshes have utilized techniques targeting 16S RNA (Devereux et al., 1992; 1996; Hines et al., 1999; Purdy et al., 1997; Sahm et al., 1999; Scheid and Stubner,
Since SRP are a diverse phylogenetic group, there is not a single 16S rRNA targeting approach that would cover all the phylogenetic groups, and different approaches targeting the different groups are required to study the complete diversity of SRP. However, some recent studies have exploited the dissimilatory sulfite reductase gene ($\textit{dsr}$) as a target gene in SRP diversity studies (Chang et al., 2001; Joulian et al., 2001; Minz et al., 1999; Perez-Jimenez et al., 2001; Thomsen et al., 2001). DSR catalyzes the conversion of sulfite to sulfide (Figure 3-1). DSR has an $\alpha_2\beta_2\gamma_2$ structure, and PCR primers targeting genes encoding the $\alpha$ and $\beta$ subunits have been designed (Wagner et al., 1998). DSR-targeting approaches encompass all the described phylogenetic SRP groups, making them a suitable tool for studying SRP dynamics in the environment. The first objective of this study was to investigate differences regarding the composition of SRP assemblages between eutrophic and pristine regions of the marsh by targeting dissimilatory sulfite reductase genes.

Molecular techniques based on characterization of nucleic acids directly extracted from environmental sample usually yield a large number of sequences not related to previously described bacteria (Pace et al., 1986). Since MPN enumerations revealed considerable numbers of lactate, butyrate, propionate and acetate-utilizing SRP in eutrophic soils (Chapter 2), we also intended to characterize the culturable fraction of SRP in these soils using the same electron donors as in the MPN-enumerations. These electron donors are common fermentation products in anaerobic soils. SRP have a great metabolic diversity such that a great number of carbon sources can be metabolized by this microbial group. However, our intention was to characterize SRP that utilize the most common fermentation products and not to extensively cover all possible electron donors.
Most described isolates can use lactate, which has been traditionally used as general electron donor to enrich SRP (Widdel, 1988; Widdel and Pfennig, 1984). However, some species of *Desulfobacter, Desulfobacterium,* and *Desulfotomaculum* do not utilize lactate as electron donor (Table 3-1). Complete oxidizer SRP use acetate, a substrate utilized by other bacteria, such as methanogens, but some complete oxidizers such as *Desulfobacterium* spp., *Desulfococcus* spp., *Desulfosarcina* spp., *Desulfonema* spp., and *Desulfoarculus* spp use this carbon source poorly. Moreover, growth in acetate is usually poor and occurs very slowly and some complete oxidizers do not grow in acetate, such as *Desulfomonile* spp. Butyrate is metabolized by species of *Desulfococcus,* *Desulfosarcina, Desulfobacterium,* and some *Desulfotomaculum.* Propionate is used by species of the genera *Desulfobulbus, Desulfococcus, Desulfosarcina, Desulfonema,* one species of *Desulfotomaculum,* and poorly utilized by *Desulfobacterium* spp (Table 3-1).

Since very little is known about culturable SRP in freshwater marshes, the second objective of this study was to characterize culturable SRP that utilize a variety of common fermentation products.

**Materials and Methods**

**Site Characteristics, Sampling and Biogeochemical Characterization**

Studies were conducted in samples taken from the Florida Everglades WCA-2A, previously described in Chapter 2. Samples were collected along the phosphorus gradient and studies were conducted on the 0-10 cm soil layer on selected sampling dates. Soil cores were sampled by staff of the South Florida Water Management District and shipped overnight to the Wetland Biogeochemistry Laboratory, Soil and Water Science Department, Gainesville, FL, where they were sectioned and manually mixed. Subsamples for DNA analysis were kept at -70°C until analysis. Subsamples intended
for enrichment studies were kept at 4°C until analysis within 2 to 5 days of sampling. Pertinent physical and chemical data regarding the two study sites were presented in Chapter 2.

It must be noted that the first clone library was constructed from DNA extracted from samples collected 04/20/2001 (spring 2001), and was not part of the study in Chapter 2. Water tables for samples taken on this date were much lower than the cumulative April mean for 1996-2002; in F1 water levels were –9.1 cm compared to 21.9 cm, and for U3, -0.9 cm compared to 21.9 cm. However, the main biogeochemical parameters were similar to the samples included in Chapter 2. The other clone library was constructed from DNA extracted from samples taken 08/15/01 (summer 2001), and was part of the study described in Chapter 2.

**Nucleic Acid Extraction and PCR Amplification for DSR Diversity Studies**

Nucleic acids were extracted with UltraClean Soil DNA kits (MoBio, Solana Beach, CA) according to the manufacturer's instructions. 0.25 g soil was used for extraction. Nucleic acid extraction was evaluated on 1% agarose gels electrophoresed with TAE buffer. DNA was stored at –20°C until PCR amplification.

PCR amplifications were conducted using the primer set designed by Wagner et al. (1998), which amplifies a 1.9-kb fragment of the DSR gene. This set consists of primers DSR1F (5'-ACSCACTGGAAGCAG-3') and DSR4R (5'-GTGTAGCAGTTACCAGCA-3'). The reaction mixture used for PCR amplifications contained 7 µl of distilled H2O, 1 µl of each primer (10 pmol/µl), 10 µl of HotStarTaq Master Mix (Qiagen, Valencia, CA) and one µl of diluted DNA solution. PCR amplification was carried out in a GeneAmp PCR system 2400 (Perkin-Elmer Applied Biosystems, Norwalk, CT) with the following conditions: initial enzyme activation and DNA denaturation of 15 min at 95°C, followed
by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 90 s extension at 72°C, and a final extension of 72°C for 7 min. The PCR products were electrophoresed on a 1% agarose gel in TAE buffer to confirm amplification of expected size product.

**Cloning of DSR Genes and RFLP Analysis**

Fresh PCR amplicons obtained with primers DSR1F and DSR4R were ligated into the pCRII-TOPO cloning vector and transformed into chemically competent *Escherichia coli* TOP10F’ cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Individual colonies of *E. coli* were screened by direct PCR amplification, with the DSR primers using the previously described PCR programs. RFLP analyses were conducted using the restriction enzyme *Hha*I, and analyzed by 2% agarose gel electrophoresis. Clone libraries were analyzed by analytic rarefaction using the software aRarefactWin (version 1.3; S. Holland, Stratigraphy Lab, University of Georgia, Athens [http://www.uga.edu/~strata/software/]).

**Growth Media and Cultivation**

A basal bicarbonate buffered medium (BCYT) prepared according to Touzel and Albagnac (1983) was used. For enrichment cultures yeast extract and trypctase were not added (BC medium). The BC medium contained (per liter of distilled water): 0.3 g KH$_2$PO$_4$, 0.6 g NaCl, 0.1 g MgCl$_2$.6H$_2$O, 0.08 g CaCl$_2$.2H$_2$O, 1.0 g NH$_4$Cl, 3.0 g KHCO$_3$, 1.0 mg resazurin. The medium was supplemented with 10.0 ml of trace element solution and 10.0 ml of vitamin solution per liter. The pH was adjusted to 6.8.

The composition of the trace element solution (per liter) was: 12.8 g nitrilotriacetic acid (NTA), 1.35 g FeCl$_3$.6H$_2$O, 0.1 g MnCl$_2$.4H$_2$O, 0.024 g CoCl$_2$.6H$_2$O, 0.1 g CaCl$_2$.2H$_2$O, 0.1 g ZnCl$_2$, 0.025 g CuCl$_2$.2H$_2$O, 0.01 g H$_3$BO$_3$, 0.024 g Na$_2$MoO$_4$.2H$_2$O,
1.0 g NaCl, 0.12 g NiCl₂·6H₂O, 0.026 g Na₂SeO₃·5H₂O. NTA was first dissolved in 200 ml of water and pH was adjusted to 6.5 with KOH.

The vitamin solution composition (per liter) was: 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg pyridoxine-HCl, 5.0 mg thiamine-HCl, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg D-Ca-pantothenate, 0.1 mg Vitamin B12, 5.0 mg p-aminobenzoic acid, 5.0 mg lipoic acid.

The gas phase was N₂/CO₂ (80%/20%). Electron donors (20 mM) were added from sterile anoxic 1 M stock solutions. FeSO₄ (20 mM) was used as the sulfate source and reducing agent.

Enrichments were conducted in 120-ml serum bottles containing 45 ml of medium inoculated with 5 g of soil for samples collected on 05/29/2002. Enrichments were set under mesophilic and spore-forming conditions. For spore-forming conditions, the flasks were pre-incubated at 70-80 °C for 20 minutes, to select for spore forming microorganisms. Enrichment cultures were incubated at room temperature until a black precipitate was formed. Colony purification was conducted in serial dilutions of the enrichments using the roll-tube dilution method (Hungate, 1969). Single black colonies were transferred to BC media for further analysis.

**Nucleic Acid Extraction and PCR Amplification for DNA of SRP Isolates**

One milliliter of culture was used for DNA extraction using UltraClean Soil DNA kits (MoBio, Solana Beach, CA) according to the manufacturer's instructions. PCR for the *dsr* gene was conducted using the DSR1F-DSR4R primer set. For 16S rRNA genes, the universal primer set 27F/1492r was used (Lane, 1991). Similar *dsr* PCR reaction conditions as described above were used. For the 16S rRNA gene universal primer set, similar conditions were used with the following modifications: 35 cycles and an
extension for 30 s at 72°C. The PCR products were electrophoresed on 1% agarose gels in TAE buffer to confirm amplification of expected size product. PCR products were screened by enzymatic digestion using HhaI for the 16S rRNA products and RsaI for DSR products. The selected PCR products were purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA). Direct sequencing of dsr and 16S rRNA gene amplification products were conducted using DSR1F and 27F, respectively, at the DNA Sequencing Core Laboratory at the University of Florida.

**Sequencing and Phylogenetic Analysis.**

Deduced amino acid sequences of the DSR α-subunit were aligned and analyzed with ClustalX version 1.81 (Thompson et al., 1997). Only unambiguously aligned amino acid positions from the DSR α-subunit gene found in all cloned sequences were used. The final data set consisted of approximately 180 amino acids. Trees based on aligned sequences were constructed using several phylogenetic methods. Protein distances were estimated with PROTDIST using the JTT replacement model, and trees were inferred using FITCH with global rearrangements in PHYLIP (version 3.6a2; J. L. Felsenstein, Department of Genetics, University of Washington, Seattle, WA). Amino acid alignments were also evaluated with PAUP* version 4.0b8 using parsimony- and distance matrix-based algorithms with default settings (D. L. Swofford, Sinauer Associates, Sunderland, MA). Protein maximum-likelihood trees were obtained with PROML in PHYLIP and with TREE-PUZZLE version 5.0 (Strimmer and von Haeseler, 1996). Bootstrap analyses were performed with 100 resamplings of the amino acid sequences. GenBank accession numbers for partial dsr gene sequences for the diversity studies are AY096038 to AY096074.
For SRP isolates, deduced amino acid sequences of the DSR α-subunit were aligned and analyzed with ClustalX version 1.81. The final data set consisting of approximately 170 amino acids was analyzed by the neighbor-joining method using ClustalX 1.81. For 16S rRNA gene sequences, alignments were also evaluated with PAUP* version 4.0b8 using parsimony-based algorithms. Trees were constructed using heuristic searches with 10 random stepwise additions of taxa and by tree-bisection reconnection branch-swapping. The characters were weighted to give more weight to characters with lower levels of homoplasy. Bootstrap analyses were performed with 100 resamplings in both 16S rRNA gene and DSR protein sequence analyses.

**Results and Discussion**

**Phylogenetic Analysis of Cloned DSR Sequences**

The expected ca. 1.9 kb gene product was obtained from all samples, and RFLP analyses of the clones indicated a high degree of sequence diversity in summer and spring samples. For the spring samples, 27 clones were grouped in 18 RFLP patterns for F1 samples, and 15 clones were grouped in 11 distinct RFLP patterns for samples from U3. For F1 samples collected in summer, 20 clones were grouped in 13 RFLP patterns, and for U3 samples 20 clones were grouped into 12 RFLP patterns. Rarefaction curves approached the plateau of a completed clone library, and no additional clones were screened. All samples revealed a similar degree of diversity as judged by the slope of the rarefaction plots (Figure 3-2). A total of 82 clones with 49 RFLP patterns were observed for the combined cone data. No RFLP pattern obtained with clones from F1 was similar to patterns obtained with U3 clones. Only 3 of 28 patterns and 2 of 21 patterns were shared by samples from F1 and U3, respectively, between spring and summer samples.
A total of 46 clones represented by 40 RFLP patterns were partially sequenced, yielding a deduced amino acid sequence of approximately 180 amino acids of the α-subunit of the DSR protein. Some DNA sequences exhibited different RFLP patterns, but shared a similar deduced initial 180 aa sequence. Therefore, only one representative of each pattern was included in the phylogenetic analysis.

Several phylogenetic approaches were taken to analyze the partial DSR sequences. Most of these approaches yielded similar results, and only minor changes were observed in the placement of some sequences within the major clades (Figure 3-3). Bootstrap values for some clades were not high (only bootstrap values over 50 of 100 replicates are presented), but were not different from previously published bootstrap values for other DSR phylogenetic analyses (Chang et al., 2001; Joulian at al., 2001; Klein et al., 2001; Minz et al., 1999). This is probably due to the fact that partial sequences were used, which would decrease the phylogenetic resolution.

Cloned DSR sequences were distributed in a total of nine clades that encompassed gram-negative mesophilic and thermophilic, gram-positive spore forming, archaeal, and two putative SRP clades composed of sequences from uncultured microorganisms. These clades were stable and consistently recovered by distance, parsimony, and maximum likelihood methods. *Thermodesulfovibrio* DSR sequences were used as the outgroup in the phylogenetic analysis (Klein et al., 2001).

**Cluster DSR-1.** Seven clones were related to *Thermodesulfovibrio* species belonging to the bacterial Nitrospirae phylum. These sequences were present only in the clone library constructed from U3 summer samples. We failed to establish thermophilic enrichments from these samples, suggesting either that these sequences are mesophilic
representatives of this clade, or that we were unable to cultivate thermophilic strains due to unknown growth requirements.

**Cluster DSR-2.** Cluster DSR-2 was uniquely composed of our clones and sequences from uncultured microorganisms. The phylogenetic affiliation of these clones remains uncertain. Clade DSR-2 was deeply divergent and was present in all samples except for the spring samples from U3. Similar DSR sequences from Genbank derived from uncultured sulfate-reducing bacteria from environmental samples, including a marine sediment in Denmark (group II, Thomsen et al., 2001).

**Cluster DSR-3.** Three clones clustered with SRP belonging to the Archaea domain. These clones were present only in clone libraries constructed from samples from Spring U3 samples.

**Cluster DSR-4.** Most clones clustering with the gram-positive *Desulfotomaculum* incomplete oxidizers were found in clone libraries constructed from U3 (10 clones versus one clone from one Spring F1 sample). This clade also included uncultured SRP sequences from group III (Thomsen et al., 2001) and from a sulfidogenic consortium capable of degrading phenanthrene (Perez-Jimenez et al., 2001).

**Cluster DSR-5.** The cluster DSR-5 was composed solely of sequences found in this study, and branched deeply from clades encompassing the gram-negative SRP branches of the tree (clusters DSR-6 and DSR-7) and the complete gram-positive oxidizers (cluster DSR-8). These sequences were present in all samples studied.

**Cluster DSR-6.** This cluster belongs to one of the δ-proteobacteria SRP clusters and is represented by species of the genera *Desulfovirga* and *Thermodesulfotpatlabdus*. This branch was comprised of sequences only found in libraries constructed from U3.
Cluster DSR-7. This cluster also belongs to the δ-proteobacteria SRP, and is represented by species of the genera *Desulfobulbus*, an incomplete oxidizing genus of the Desulfobacteraceae family. These clones were only found in libraries constructed from F1 samples.

Cluster DSR-8. This clade is comprised of gram-positive *Desulfotomaculum* complete oxidizing strains. The eighteen clones in this cluster were found exclusively in libraries constructed from F1, with a uniform distribution observed between spring and summer samples.

Cluster DSR-9. Two clones from libraries constructed from Summer F1 clustered in another clade of δ-proteobacteria SRP encompassing the complete oxidizing genus *Desulfococcus* of the Desulfobacteraceae family.

**Effect of Eutrophication and Season on the Dynamics of DSR Sequences**

F1 samples were dominated by sequences related to cluster DSR-8 of *Desulfotomaculum* complete oxidizers, followed by sequences belonging to non-culturable SRP of clusters DSR-2 and DSR-5 (Figures 3-4A and 3-4B). These samples also included sequences related to *Desulfobulbus* in cluster DSR-7, and minor representation in cluster DSR-4. The seasonal change from spring to summer correlated with an emergence of SRP related to the *Desulfococcus* of cluster DSR-9 and disappearance of sequences of cluster DSR-4, but did not correlated with other major changes of other DSR sequence distributions.

SRP dynamics in U3 were less stable (Figures 3-4C and 3-4D). In spring, no single group was dominant and similar percentages of SRP belonging to clusters DSR-3 (archaeal SRP), DSR-4 (incomplete oxidizing *Desulfotomaculum*), DSR-5, and DSR-6
(Desulfovirga) were found. In summer, representatives of the cluster DSR-1 emerged and dominated the clone libraries with members of the cluster DSR-4. The relative percentages of clones within clusters DSR-5 and DSR-6 decreased. No member of cluster DSR-3 was recovered, and clones representative of cluster DSR-2 emerged.

Changes with regard to the effects of nutrient enrichment on biogeochemical cycling in the Everglades have been well documented (Craft and Richardson, 1993; DeBusk et al., 1994; DeBusk et al., 2001; Koch and Reddy, 1992), although detailed characterization at the microbial level is required to understand the effects of eutrophication on processes governing biogeochemical cycles. This is the first microbial study of which we are aware to characterize the diversity and function of SRP in a freshwater wetland such as the Everglades, and to characterize potential relationships between phylogenetic groups of SRP and the nutrient status of their environment.

Lactate can be used as an electron donor by most SRP species, with the exception of Desulfobacter and some Desulfobacterium species. Many SRP capable of complete oxidation of electron donors utilize acetate, including strains of the genus Desulfobacter and some Desulfotomaculum spp. In our studies, the number of lactate- and acetate-utilizing SRP were similar, and DSR sequence analysis did not reveal sequences related to Desulfobacter or Desulfobacterium. It can be assumed, therefore, that most cultivable SRP in the enumeration medium were complete-oxidizers. This finding contradicts the analysis of DSR sequences from the pristine zones, where a great number of sequences were associated with Desulfotomaculum incomplete oxidizers. However, clones related to Desulfotomaculum incomplete oxidizers may represent SRP that could not be grown under the conditions of this study. Other clones present in U3 are either related to
unculturable SRP or to poorly characterized SRP clades that are currently represented only by a few cultivable SRP (Figure 3-3) (i.e. *Archaeoglobus*, *Desulfovirga*, *Thermodesulfovibrio* species), making it difficult to infer much about their physiology.

Potential methanogenic rates for the pristine soils suggested that acetate-utilizing SRP might not be important in this region of the marsh. On the contrary, acetate-utilizing SRP may play a role in eutrophic zones of the marsh, as suggested by the observations that addition of sulfate to F1 microcosms inhibited methanogenesis to some extent. The observed lack of response to acetate in the presence of sulfate suggests that SRP present in the pristine zones are using electron donors other than acetate, or they may be using another type of metabolism such as fermentation or syntrophic associations.

Incomplete oxidizing *Desulfitomaculum* strains which were found in significant numbers in clone libraries from pristine sites can use electron donors such as hydrogen, formate, and ethanol (Widdel, 1992a). Moreover, incomplete oxidizers would likely outcompete complete oxidizers for substrates used by both groups, such as intermediates of the anaerobic degradation of organic matter such as hydrogen and/or lactate (Widdel, 1988).

DSR sequence analysis revealed a great deal of diversity, and nine phylogenetic clusters were identified in the clone libraries. Samples from F1 exhibited a stable distribution of DSR sequences between spring and summer sampling times. F1 libraries were dominated by sequences related to uncultured SRP and *Desulfitomaculum* complete oxidizers. Relatively few phylotypes belonging to *Desulfobulbus*, *Desulfococcus* and *Desulfitomaculum*-like incomplete oxidizer sequences were cloned. In U3, the distribution was less stable between spring and summer. *Desulfitomaculum* comprised
an important proportion of the SRP population, and *Desulfotomaculum* incomplete oxidizers dominated the distribution in the clone library. A similar dominance of *Desulfotomaculum*-like DSR sequences was reported in groundwater at a uranium mill tailing site (Chang et al., 2001), which may suggest a greater role for gram-positive SRP in the sulfur and carbon cycle than previously ascribed to this group. The Everglades ecosystem is characterized by seasonal water table fluctuations, and the ability of *Desulfotomaculum* to sporulate as a survival mechanism may explain the relatively high number of phylotypes belonging to this group. The selection of *Desulfotomaculum* due to alternating oxic and anoxic conditions has been observed in rice paddies (Stubner and Meuser, 2000; Widdel, 1992a; Wind and Conrad, 1995; Wind et al., 1999).

Other groups present in the eutrophic zones with a known phylogenetic association were related to *Desulfobulbus* and *Desulfococcus*, members of the Desulfobacteriaceae family. *Desulfobulbus* is an incomplete oxidizer able to use propionate and to ferment lactate or ethanol in sulfate-free media, and is found in anaerobic freshwater mud. *Desulfococcus* species are complete oxidizers that can ferment lactate and pyruvate, and are found in anaerobic mud and marine habitats (Widdel and Bak, 1992; Widdel and Pfennig, 1984).

The DSR gene has been extensively used as a genetic marker in microbial diversity studies. It has been proposed that the DSR gene was subject to multiple lateral gene transfer events that may confuse the use of this genetic marker in phylogenetic studies (Klein et al., 2001). One such group in question is *Desulfotomaculum*, a genus that is not monophyletic in the DSR phylogeny, but rather is divided between those *Desulfotomaculum* able to perform complete oxidation and those limited to incomplete
oxidation. In our study, this distinction facilitated correlation between two different types of metabolism with two regions of the marsh exposed to different levels of nutrient impact. This metabolic differentiation may be linked to the type of substrates that different Desulfotomaculum spp. utilize. Complete oxidizers are generally more versatile and may utilize a broader range of substrates than do incomplete oxidizers (Widdel and Hansen, 1992). In our findings, Desulfotomaculum complete oxidizers dominated in eutrophic regions of the marsh, and Desulfotomaculum incomplete oxidizers dominated in pristine regions. A general concept in ecology is that specialists outcompete generalists for specific nutrients (Pianka, 1974). Therefore, it may be that the more specialist Desulfotomaculum incomplete oxidizers outcompete generalist complete oxidizers for substrates such as hydrogen and lactate that can be metabolized by both groups of Desulfotomaculum (Widdel, 1988). This would explain the greater abundance of incomplete oxidizers in clone libraries constructed from pristine zones of the marsh (U3), which may have a more narrow range of electron donors than would be found in the eutrophic region (F1).

**Enrichments in Selected Electron Donors**

Common fermentation products were used to establish enrichment cultures under mesophilic and spore-forming conditions to characterize the culturable fraction of SRP in the WCA-2A. Lactate and acetate enrichment culture were transferred seven times, and the propionate and butyrate enrichment culture were transferred five times. SRP were then isolated by the roll tube technique. Positive enrichments were obtained under mesophilic and spore-forming conditions with the enrichment cultures from eutrophic zones (F1) with all the electron donors, with the exception of propionate under spore-forming conditions. This is in agreement with the physiology of previously described
gram-positive spore forming SRP since more known species of *Desulfotomaculum* are able to use butyrate than propionate. For the pristine regions, only enrichments in lactate under both conditions and acetate under mesophilic conditions were positive. This observation is in agreement with results for the clone libraries for these samples, since the clones recovered from pristine soils were related to incomplete oxidizer *Desulfotomaculum* spp. Most roll tubes with acetate as electron donor exhibited very few colonies that grew very slowly. Also, most of the colonies lost the ability to grow in acetate when transferred back to liquid medium for further characterization. It was only possible to recover two colonies from the eutrophic site under mesophilic and spore forming condition in liquid medium.

DNA from selected colonies was PCR amplified using primers targeting 16S rRNA and DSR genes and screened by RFLP analyses, using *Hha*I for 16S rRNA gene amplicons and *Rsa*I for DSR amplicons. Cultures exhibiting unique RFLP patterns for 16S rRNA and DSR genes were sequenced. It must be noted that DNA for several cultures amplified with 16S rRNA gene primers, but did not amplify with DSR primers. This was especially true for cultures grown on lactate from eutrophic sites under spore forming conditions and pristine sites under mesophilic conditions. Moreover, many 16S rRNA PCR amplicons could not be sequenced, but their respective DSR amplicons could be sequenced. This may indicate that those cultures were not pure, or that the removal of 16S rRNA primers with the purification kit was less efficient than for DSR primers, which may have resulted in unsuccessful direct sequencing reactions.

DSR sequences from the cultures fell into two clusters (Figure 3-5). One of the of clusters grouped with SRP gram negative mesophilic species within the δ-Proteobacteria,
and were most closely related to members of the Desulfovibrionaceae family. 

*Desulfovibrio* is an incomplete oxidizer able to use hydrogen, formate, lactate and ethanol, and is commonly enriched from anaerobic environments. Several sequences were obtained from enrichment cultures using lactate, however some of the sequences came from propionate and butyrate enrichment cultures which are electron donors not used by *Desulfovibrio* spp. Unfortunately, no data for the 16S rRNA genes of these cultures were obtained; therefore, these findings require further experimentation to corroborate these unexpected results. Moreover, in the culture-independent DSR analysis (Figure 3-3) no sequences related to Desulfovibrionaceae family were recovered, a possible indication of bias for fast growing organisms in the conditions of our enrichments.

The other group of DSR sequences clustered with the incomplete oxidizers of the genus *Desulfotomaculum* (Figure 3-5). Most sequences were from lactate enrichment cultures under spore-forming conditions from samples of the pristine sites. These sequences were most closely related to *Desulfospirorosinus orientis*, an incomplete oxidizer spore-forming gram positive SRP. Electron donors used by *D. orientis* include hydrogen, lactate, and ethanol. Another unexpected result was observed with culture F1SPA-21, which was recovered from an acetate spore-forming enrichment culture, since this cluster of SRP is generally not thought to contain species able to use acetate.

Cultures related to previously undescribed SRP were not recovered likely indicating cultivation techniques are biased towards previously isolated species. Possibly due to the fact that the required cofactors, environmental factors, interactions with other microorganisms or surface were not provided in the enrichment cultures that bacteria may
find in nature. Similar results were reported by Rooney-Varga et al. (1998) in a study of
the diversity of SRP isolated from a salt marsh sediment.

The 16S rRNA phylogenetic analysis revealed a similar grouping as in the DSR
analysis. One group of cultures was related to gram-negative mesophilic SRP of the
genus *Desulfovibrio*. The other group of SRP as determined by 16S rRNA gene analysis
was related to gram-positive spore-forming SRP. An interesting result was the recovery
of cultures with 16S rRNA genes related to *Clostridium* spp., instead of grouping with
previously described cluster of *Desulfotomaculum* spp. (Figure 3-7). The DSR sequences
from these cultures were related to gram-negative mesophilic SRP. Assuming that these
cultures are pure, which must be subject to further verification, this may indicate the
possibility of a new cluster of *Desulfotomaculum* more closely related to species of
*Clostridium* than previously described *Desulfotomaculum*.

In conclusion, diversity of DSR sequences was found in both zones of the marshes.
Significantly, *Desulfotomaculum*-like sequences from eutrophic regions were related to
*Desulfotomaculum* spp. able to carry out complete oxidation; and in pristine regions they
were related to those unable to carry out complete oxidation. Therefore, molecular
techniques revealed that nutrient loading resulted in selection of different SRP
communities. Classical cultivation techniques provided a different picture of the
diversity of this ecosystem, suggesting that different approaches should be used to be
explore and recover undescribed species. However, molecular techniques revealed a
selection in the type of *Desulfotomaculum* communities present in eutrophic versus
pristine regions of the marsh, suggesting that *Desulfotomaculum* complete-oxidizers are
better adapted to eutrophic conditions than to pristine sites, where a greater number of
Desulfotomaculum incomplete-oxidizing strains were present. This information could be used to design new strategies to enrich and isolate these novel organisms, such as the use of a more complete battery of electron donors related to the ones present in the rhizosphere of eutrophic and pristine sites, different concentration ranges for electron donors which could favor more slowly growing microorganisms used to proliferate in poor nutrient conditions, the presence of surfaces for attachment, and interaction with other microorganisms.
Table 3-1. Physiological properties of selected genera of SRP (adapted from Widdel and Hansen, 1992).

<table>
<thead>
<tr>
<th>Electron donor</th>
<th>Lactate</th>
<th>Acetate</th>
<th>Butyrate</th>
<th>Propionate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-negative mesophilic SRP</strong></td>
<td>Oxidation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfovibrio</td>
<td>I</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Desulfobulbus</td>
<td>I&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Desulfomicrobium</td>
<td>I</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Desulfoarculus</td>
<td>C</td>
<td>-</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Desulfobacter</td>
<td>C&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Desulfoarculus</td>
<td>C</td>
<td>- (/+) / +</td>
<td>(+)</td>
<td>(+) / +</td>
</tr>
<tr>
<td>Desulfococcus</td>
<td>C</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Desulfomonile</td>
<td>C</td>
<td>-</td>
<td>-</td>
<td>n.r.</td>
</tr>
<tr>
<td>Desulfonomema</td>
<td>C</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Desulfosarcina</td>
<td>C</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td><strong>Gram-positive spore-forming SRP</strong></td>
<td>Oxidation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfotomaculum</td>
<td>I/C</td>
<td>+/-</td>
<td>-</td>
<td>(+) / +</td>
</tr>
<tr>
<td><strong>Bacterial thermophilic SRP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermodesulfobacterium</td>
<td>I</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Archaeal thermophilic SRP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Archaeoglobus</td>
<td>I</td>
<td>+</td>
<td>-</td>
<td>n.r.</td>
</tr>
</tbody>
</table>

<sup>a</sup> +, utilized; (+) poorly utilized; - not utilized; n.r., not reported.
<sup>b</sup> I. incomplete
<sup>c</sup> C. complete

Table 3-2. Results of enrichment cultures for eutrophic and pristine soils.

<table>
<thead>
<tr>
<th>Electron donor</th>
<th>Eutrophic (F1)</th>
<th>Pristine (U3)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Acetate</td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>Propionate</td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Butyrate</td>
<td>√</td>
<td>√</td>
<td>-</td>
</tr>
</tbody>
</table>

(√) growth observed; (-) no growth observed
Figure 3-1. Pathway for sulfate reduction illustrating the role of dissimilatory sulfite reductase (A) and *dsr* genes showing the location of primers DSR1F and DSR4R (B). Numbering according to Karkhoff-Schweizer et al. (1995).
Figure 3-2. Rarefaction analysis for clone libraries for spring and summer samples from the eutrophic and pristine regions of the WCA-2A.
Figure 3-3. Neighbor-joining DSR α-subunit tree. The clones are named according to the origin and time of sampling. Scale bar represents 5% change. Numbers at nodes represent percentage of bootstrap resampling based on 100 replicates; only values that are higher than 50 are presented.
Figure 3-4. Spatial and seasonal distribution of DSR clones in eutrophic soils for spring (A) and summer (B) and in pristine soils for spring (C) and summer (D).
Figure 3-5. Neighbor-joining DSR α-subunit tree for sequences from isolates. The cultures are named according to the site and type of enrichment: M, mesophilic; Sp, spore forming; L, lactate; B, butyrate; P, propionate; A, acetate. Numbers at nodes represent percentages of bootstrap resampling based on 100 replicates, only values higher than 50 are presented.
Figure 3-6. Maximum parsimony tree of 16S rRNA gene sequence from isolates related to the gram-negative mesophilic SRP. The cultures are named according to the site and type of enrichment. Scale bar represents 5 nucleotide changes per 100 positions. Numbers at nodes represent percentage of bootstrap resampling based on 100 replicates.
Figure 3-7. Maximum parsimony tree of 16S rRNA gene sequences for isolates related to the gram-positive spore forming SRP. The cultures are named according to the site and type of enrichment. Scale bar represents 5 nucleotide changes per 100 positions. Numbers at nodes represent percentage of bootstrap resampling based on 100 replicates.
CHAPTER 4
PHYLOGENETIC CHARACTERIZATION OF THE METHANOGENIC COMMUNITY IN EUTROPHIC AND PRISTINE AREAS OF THE EVERGLADES WCA-2A

Introduction

Methanogenesis is the main process in the final stages of organic matter degradation in several types of freshwater anaerobic environments. Approximately 80% of atmospheric methane, a greenhouse gas, is derived from biogenic sources. Natural wetlands systems are among the most important sources of methane. They emit approximately 90 x 10^6 metric tons of methane per year, representing 22% of the total methane total emissions, followed by sources such as rice paddy fields, digestive tracts of ruminants and termites, and landfills (Cicerone and Oremland, 1988). Methanogens, the microorganisms responsible for this process, belong to the Archaea domain. The Archaea domain is characterized by extreme phenotypes such as methanogens, extreme halophiles, sulfate-reducing thermophiles, and extreme thermophiles (DeLong, 1992).

Methanogens are a specialized group of anaerobic microorganisms that use a narrow range of substrates, namely acetate, H_2-CO_2, formate, and methyl compounds as electron donors for the terminal reduction of CO_2 to methane. Most species are able to grow on H_2-CO_2, several species can use formate and methyl compounds, and a fewer number of species can grow on acetate (Table 4-1) (Garcia et al., 2000). In freshwater and terrestrial ecosystems H_2-CO_2, acetate, and formate are the main methanogenic precursors (Schutz et al., 1989). Acetate is the main precursor in these types of ecosystems, and approximately 60 to 80% of the methane is produced from acetate.
Methyl compounds are more important methanogenic precursors in marine ecosystems, where methanogens coexist with sulfate reducing prokaryotes in these rich sulfate environments by using these methyl compounds, a class of compounds that can not be metabolized by SRP (Madigan et al., 1997).

Most characterizations of methanogens have been conducted in samples from soils and rhizospheres of rice paddies, and anaerobic biodigesters (Grobkopf et al., 1998a; Lueders et al., 2001; Kudo et al., 1997). Very little is known about methanogens in ecosystems such as freshwater marshes.

Cultivation of methanogens is cumbersome and time consuming due to the slow growth and their nutritional and cultivation requirements. Therefore, most recent research on the ecology of methanogens has been based on non-culture based molecular methods. Most of these studies have targeted the 16S rRNA gene and an extensive number of research papers have been published in this area, mainly focused on rice paddies (Chin et al., 1999; Grobkopf et al., 1998a; 1998b; Joulian et al., 1998; Kudo et al., 1997; Lueders et al., 2000; Lehmann-Ritcher et al., 1999).

An alternative approach to study methanogenic community composition is the use of a functional gene such as the methyl coenzyme M reductase (MCR), an enzyme restricted to methanogens (Thauer, 1998). MCR catalyzes the last step of methanogenesis according to the following reaction:

\[ \text{CH}_3\text{-S-CoM} + \text{HS-CoB} \rightarrow \text{CoM-S-S-CoB} + \text{CH}_4 \]

HS-CoM represents coenzyme M and HS-CoB represents coenzyme B.
MCR has three different subunits $\alpha$ (McrA), $\beta$ (McrB) and $\gamma$ (Mcr G) arranged in a configuration $\alpha_2\beta_2\gamma_2$, and the genes encoding MCR are within the operon $mcrBDCGA$. The activities of the products McrC and McrD remain unknown. Some Methanobacteriales and Methanococcales also contain in addition to $mcr$, an isoenzyme named $mrt$, the genes for the MRT isoenzyme are arranged in a operon $mrtBDGA$ or $mrtBGA$ (Thauer, 1998). The expression of the isoenzyme MCR or MRT is regulated by the growth stage or by oxidative stress (Ferry, 1999).

Several studies have been conducted using this functional gene to assess the diversity of methanogens in several environments such as peat bogs (Hales et al., 1996; Lloyd et al., 1998; Nercessian et al., 1999), marine sediments (Bidle et al., 1999), termite guts (Ohkuma et al., 1995), landfills (Luton et al., 2002), and rice paddies (Lueders et al., 2001; Lueders and Friedrich, 2003; Ramakrishnan et al., 2001).

Extensive biogeochemical research has shown greater rates of methanogenesis in the eutrophic zones of WCA-2A compared to more pristine regions of the marsh, and this was also observed in the studies presented in Chapter 3. Therefore, non-culture based microbial diversity studies may provide valuable information on the effect of eutrophication in archaeal and methanogenic communities.

The objectives of this study were to characterize the archaeal communities using 16S rRNA approaches and the methanogenic communities by targeting the $mcr$ genes.

**Material and Methods**

**Site Characteristics, Sampling and Biogeochemical Characterization**

Studies were conducted on the samples taken from the Florida Everglades WCA-2A, previously described in Chapter 3.
Nucleic Acid Extraction and PCR Amplification

Nucleic acids were extracted with UltraClean Soil DNA kits (MoBio, Solana Beach, CA) according to the manufacturer's instructions.

PCR was conducted using the primer set designed by Luton et. al (2002), which amplifies a ca. 465-490 bp fragment of the mcrA gene; this set consisted of primers mcrA-f (5'-GGTGGTGTMGGATTCACARTRAYGCWASCGC-3') and mcrA-r (5'-TTCATTGCRTAGTTWGGRTAGTT-3'). The reaction mixture used for PCR amplification contained 7 µl of distilled H2O, 1 µl of each primer (10 pmol/µl), 10 µl of HotStarTaq Master Mix (Qiagen, Valencia, CA) and one µl of diluted DNA solution. PCR amplification was carried out in a GeneAmp PCR system 2400 (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 between the annealing and extension segment was decreased to 0.1 °C per second from the default 1°C per second because of the degeneracy of the primers. After this, 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. The PCR products were electrophoresed on 2% agarose gels in TAE buffer to confirm amplification of expected size product.

The primer set combination 23F and 1492R was used for archaeal 16S rRNA gene amplification (Burggraf et al., 1991; and Lane, 1991). The initial enzyme activation and DNA denaturation was performed for 15 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 60 s extension at 72°C, and a final extension of 72°C for 7 min. The PCR products were electrophoresed on 2% agarose gels in TAE buffer to confirm amplification of expected size product.
Cloning of mcr and 16S rRNA Archaeal Genes and RFLP Analysis

Fresh PCR amplicons were ligated into pCRII-TOPO cloning vector and transformed into chemically competent *E. coli* TOP10F’ cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Individual colonies of *E. coli* were screened by direct PCR amplification with the MCR or archaeal 16S rRNA primers using the previously described PCR programs. RFLP analyses were conducted using the restriction enzyme *Taq*I for *mcr* and 16S rRNA amplification products. Digests were analyzed by agarose gel electrophoresis, using 2% and 4% gels for mcr and 16S rRNA digests, respectively. Clone libraries were analyzed by analytic rarefaction using the software aRarefactWin (version 1.3; S. Holland, Stratigraphy Lab, University of Georgia, Athens [http://www.uga.edu/~strata/software/]).

Sequencing and Phylogenetic Analysis

Selected clones were sequenced at the DNA Sequencing Core Laboratory at the University of Florida using the mcrA-f and archaeal 23F primers. Deduced amino acid sequences of the *mcr* α-subunit were aligned and analyzed with ClustalX version 1.81. Since no differences were found with the DSR analysis between different phylogenetic methods, and Luton et al. (2002) did not find any differences between different mcr phylogenetic analysis, the data presented here is a neighbor-joining analysis using ClustalX 1.81. The final data set consisted of approximately 160 amino acids. Bootstrap analysis was performed with 100 resamplings of the amino acid sequences. Archaeal 16S rRNA gene sequence alignments were also evaluated with PAUP* version 4.0b8 using parsimony-based algorithms. Approximately 600 bp per sequence were included in this analysis. Trees were constructed using heuristic searches with 10 random stepwise additions of taxa and by tree-bisection reconnection branch-swapping. The characters
were weighted to give more weight to characters with lower levels of homoplasy.

Bootstrap analysis was performed with 100 resamplings.

**Diversity Index**

The cumulative number of phylotypes was calculated by fitting the rarefaction curves to a hyperbolic model with the formula \( y = \frac{ax}{b + x} \) using Datafit software (v8.0.32, Oakdale Engeeniering, Oakdale, PA) where \( y \) represents the number of phylotypes and \( x \) is the number of individuals. Phylotypes were defined on the basis of unique restriction digestion patterns.

Diversity of the clone libraries was calculated using the Shannon index (Peet, 1974) according to the formula: \( H = -\sum \pi_i \log \pi_i \) from \( i = 1 \) to \( n \), where \( \pi_i \) represents the proportion of a particular phylotype in the clone library and \( n \) is the total number of phylotypes.

**Results and Discussion**

**Phylogenetic Analysis of Cloned Archaeal Sequences**

An expected ca. 1.5 kb 16S rRNA gene product was obtained from all samples, and RFLP patterns of the clones suggested a significant degree of sequence diversity in summer and spring samples. For the spring samples, 32 clones were grouped in 23 RFLP patterns for F1 samples, and 36 clones were grouped in 17 distinct RFLP patterns for samples from U3. Summer F1 samples yielded 38 clones grouped in 20 RFLP patterns, and for U3 samples 38 clones were grouped into 16 RFLP patterns. Rarefaction curves approached the plateau of a completed clone library, and no additional clones were screened. F1 samples exhibited a slightly higher degree of diversity as judged by the slope of the rarefaction curves (Figure 4-1).
A total of 76 16S rRNA gene clones were partially sequenced, yielding sequences of approximately 500 bp in length. Several phylogenetic approaches were taken to analyze the partial archaeal sequences, yielding similar results with only minor changes in the placement of some sequences within the major cluster (Figures 4-2 and 4-3). Bootstrap values for some clades were not high (only bootstrap values over 50 of 100 replicates are presented), probably due to the fact that partial sequences were used which decreases phylogenetic resolution. However, the overall and major clades of the archaeal phylogeny is in very good agreement with previously published archaeal phylogeny (Chin et al., 1999; Garcia et al., 2000; Grobkopf et al., 1998).

Cloned archaeal sequences were distributed in a total of eight clades; one clade in the phylum Crenarchaeota and seven clades in the phylum Euryarchaeota. The seven euryarchaeota clades included methanogens of the orders Methanobacteriales, Methanomicrobiales and Methanosarcinales, and three putative clades composed of sequences from uncultured microorganisms. *Methanopyrus kandleri* sequence was used as the outgroup in the phylogenetic analysis, because this species is the suggested outgroup for the *mcr* analysis (Luton et al., 2002; see below). The nomenclature used for the Archaea domain is according to the Taxonomic Outline of the Prokaryotic Genera, Bergey’s Manual of Systematic Bacteriology (Garrity et al., 2001).

**Cluster ARC-1.** The ARC-1 cluster is related to the order Methanomicrobiales and was present in all clone libraries. Clones in these clades are ubiquitous in the environment. Methanomicrobiales uncultured sequences with similarities between 94-99% have been recovered from several ecosystems, such as periphyton from freshwater marshes (Prenger et al., 2002), anaerobic bioreactors (McHugh et al., 2003; Wu et al.,
Cluster ARC-2. The ARC-2 clade was composed of our sequences and sequences from uncultured microorganisms. In Genbank, these sequences are referred to as unclassified/uncultured Archaea (environmental samples) or unclassified/uncultured Methanomicrobiales. The phylogenetic affiliation of these clones remains uncertain, but they may represent a deeply divergent branch of the Methanomicrobiales order. These clones were present in all samples except for the summer samples from F1. Similar archaeal sequences deposited with Genbank came from environmental samples, including peatland ecosystem (Basiliko et al., 2002), sediments of a subtropical lake (Nusslein et al., 2001) and landfill leachate and cover soils (Huang et al., 2002; Uz et al., 2001).

Cluster ARC-3. ARC-3 sequences clustered with the genus Methanoseta of the Methanosarcinales order. These clones were present in all samples. This clade included cultured and uncultured archaeal sequences related to Methanoseta spp. present in the previous studies mentioned in cluster ARC-1 and ARC-2 and sequences recovered from rice paddy fields (Grobkopf et al., 1998a, 1998b; Lueders and Friedrich, 2000; Ramakrishnan et al., 2001), municipal wastewater sludge (Williams et al., 2001), bioremediation sites (Dojka et al., 1998) coastal salt marshes (Munson et al., 1997), metal-rich particles from freshwater reservoirs (Stein et al., 2002), water from gold mines
Cluster ARC-4. The ARC-4 clade was related to the genus *Methanosarcina* of the order Methanosarcinales. Only two clones were recovered in clone libraries in this cluster, which were present in summer samples of F1 sites and spring of U3 sites.

Cluster ARC-5. The ARC-5 cluster was related to Methanosarcinales order but this cluster was deeply divergent from other Methanosarcinales. This branch was comprised of sequences only found in libraries constructed from summer samples of U3.

Cluster ARC-6. Two clones were deeply divergent in the Methanosarcinales order in the parsimony analysis. However, these clones were placed as very divergent deep branch grouping with the Methanomicrobiales order in the neighbor-joining analysis. The BLAST report (from Genbank) for these sequences returned representatives of the Methanomicrobiales and Methanosarcinales orders. More information is required to place these clones with a particular order. These clones were present only in clone libraries constructed from samples from summer samples.

Cluster ARC-7. The ARC-7 cluster was deeply divergent from the Methanosarcinales and Methanomicrobiales orders. These clones were found in all clone libraries. Clones related to this group have been reported in marine sediments (Hinrichs et al., 1999; Munson et al., 1997; Stoeck and Epstein, 2003), sediments of a subtropical lake (Nusslein et al., 2001), hypereutrophic lakes (Lanyon et al., 2002), and bacterioplankton of boreal forest lakes (Jurgens et al., 2000).

Cluster ARC-8. The ARC-8 clade is comprised of sequences related to the Crenarchaeota phylum. The clones in this cluster were found in libraries constructed from
F1 summer soils samples and summer and spring samples of U3 soils samples. The Crenarchaeota phylum is composed of extreme hyperthermophilic prokaryotes; although, related sequences have been found in cold and moderate-temperature environments. Our clones were related to clones obtained from moderate temperature environments such as rice paddies (Lueder and Friedrich, 2000), mining wastes (Selenska-Pobell et al., 2002), freshwater reservoirs (Stein et al., 2002) and extreme environments such as hydrothermal vents and sediments (Reysenbach et al., 2000; Teske et al., 2002).

No clone related to the Methanococcales or Methanobacteriales orders were recovered from any season or site in this study.

**Effect of Eutrophication and Season on the Dynamics of Archaeal Sequences**

Clone libraries for F1 sites were dominated by cluster ARC-3 (Methanosaeta cluster), ARC-1 (Methanomicrobiales cluster), and ARC-7 (uncultured cluster), representing almost 80% of the clone library sequences for summer samples and 90% for spring samples (Figure 4-4). The summer F1 clone library also contained ARC-8 (Crenarchaeota cluster), and minor amounts of ARC-4 sequences (*Methanosarcina* cluster) and ARC-6 sequences (uncultured cluster) (Figure 4-4). Seasonal changes between summer and spring for eutrophic F1 sites, resulted in an emergence of clones of cluster ARC-2 (uncultured cluster) and disappearance of sequences of cluster ARC-4, ARC-6, and ARC-8, but still cluster ARC-1, ARC-3 and ARC-7 dominated the clone library.

U3 clone libraries were dominated by clones of the cluster ARC-3, ARC-1, ARC-2, and ARC-8, with minor amounts of cluster ARC-7 (Figure 4-4). Seasonal changes from summer to spring resulted in some changes of the relative amounts of the major cluster,
and in the emergence of cluster ARC-4 and disappearance of cluster ARC-5 (uncultured cluster) and ARC-6.

**Phylogenetic Analysis of Cloned mcr Sequences**

An expected ca. 465-490 bp mcr gene fragment was obtained by PCR from all samples, and RFLP of the clones suggested a significant degree of sequence diversity in summer and spring samples. However, the diversity was lower when compared with the archaeal 16S rRNA gene libraries since the mcr set of primers only targeted methanogenic prokaryotes and not the total archaeal population that was targeted with the archaeal primers. The spring samples yielded 37 clones grouped in 18 RFLP patterns for F1 samples, and 38 clones grouped in 10 distinct RFLP patterns for samples from U3. F1 summer samples yielded 35 clones grouped in 14 RFLP patterns, and for U3 summer samples 29 clones grouped into 9 RFLP patterns. Rarefaction curves approached the plateau of a completed clone library, and no additional clones were screened. F1 samples exhibited a slightly higher degree of diversity as judged by the slope of the rarefaction curves (Figure 4-5).

A total of 52 clones were partially sequenced, yielding sequences of approximately 480 nucleotides in length of the mcrA gene, which translated into a deduced amino acid sequence of approximately 160 amino acids. The overall MCR phylogeny is in very good agreement with previously published MCR phylogeny (Figure 4-6) (Lueders et al., 2001; Luton et al., 2002). It must be mentioned that the primers used by Lueders et al (2001), do not detect all orders of methanogens, in particular Methanomicrobiales; however, the primer set designed by Luton et al. (2002) does seem to cover all the known order of methanogens. Luton et al. (2002) have also has shown that 16S rRNA gene phylogeny and MCR phylogeny are in very good agreement.
Cloned MCR sequences were distributed among a total of seven clades encompassing the orders Methanobacteriales, Methanomicrobiales and Methanosarcinales, and three putative clades composed of sequences from uncultured microorganisms. *Methanopyrus kandleri* sequence was used as outgroup in the phylogenetic analysis (Luton et al., 2002).

**Cluster MCR-1.** Clones in this cluster were related to the order Methanobacteriales. These sequences were present in all clone libraries, with the exception of spring eutrophic samples. Similar clones were found in rice paddies (Lueders et al., 2001) and landfill material (Luton et al., 2002).

**Cluster MCR-2.** The MCR-2 clade was composed exclusively of our clones and sequences from uncultured microorganisms. The phylogenetic affiliation of these clones remains uncertain. The clustering of these clones is in agreement with similar clones recovered from landfill samples by Luton et al. (2002). These clones were present in all samples except for the summer samples from pristine U3 site. Other similar sequences were found in Genbank from environmental samples, including hypereutrophic lakes (Earl et al., 2002) and anaerobic digesters (Hougaard et al., 2000).

**Cluster MCR-3.** These clones clustered with *Methanosarcina* sequences and were found only in samples from eutrophic sites. Luton et al. (2002) and Lueders et al. (2001) also recovered similar sequences from landfill and rice paddy soils samples.

**Cluster MCR-4.** The MCR-4 clade was related to the genus *Methanosaeta* of the order Methanosarcinales, and was recovered from spring eutrophic samples and summer and spring pristine sites. Sequences Mcr-U3SP-18, U3SU-16, and U3SU-33 exhibited similarity of ca. 85% to sequences rice cluster I, outside the *Methanosaeta* cluster.
described by Lueders et al. (2001). However, our bootstrap analysis did not support these as a separate cluster. More sequence information would clarify the assignment of these sequences as an individual cluster.

**Cluster MCR-5.** The MCR-5 cluster was composed of sequences branching deeper in the Methanomicrobiales order, and was found in all samples, with the exception of spring pristine sites. Sequences of this group have been also recovered from landfill soils and have been placed within the Methanomicrobiales order (Luton et al., 2002). However, they are deeply divergent from cultured Methanomicrobiales species. Similar sequences were also recovered from hypereutrophic lakes (Earl et al., 2002) oligotrophic fen (Galand, 2002), and anaerobic digesters (Hougaard et al., 2000). These sequences could be divided into two clusters; however, because there is little information about these groups, they were treated as a single cluster. More information would resolve clustering or dividing these groups.

**Cluster MCR-6.** The MCR-6 cluster was related to Methanomicrobiales and these sequences were found in summer and spring eutrophic samples. Similar sequences were recovered from landfill soils (Luton et al., 2002) and rice paddies (Lueders et al., 2001).

**Cluster MCR-7.** The MCR-7 cluster was related to Methanomicrobiales but in a separate cluster from cultured Methanomicrobiales. These clones were found in all clone libraries.

**Effect of Eutrophication and Season on the Dynamics of MCR Sequences**

Clone libraries for F1 sites were dominated by sequences in clusters MRC-7, MCR-6, and MCR-5 (related to Methanomicrobiales), representing ca. 80% of the clone libraries. The summer F1 clone library also contained MCR-1 (Methanobacteriales cluster), MCR-2 (uncultured cluster), and minor amounts of MCR-3 sequences.
(Methanosarcina cluster) (Figure 4-7). Seasonal changes between summer and spring for eutrophic F1 sites, resulted in an increase of MCR-6 and decrease of MCR-7, an emergence of clones of cluster MCR-4 (Methanosaeta cluster) and disappearance of sequences of cluster MCR-1. Methanobacteriales were not recovered from archaeal libraries; however, these clones represented about 10% of the mcr libraries. Screening of more clones in the archaeal libraries may yield clones within this group.

U3 clone libraries were more dynamic; summer samples were dominated by clones of the cluster MCR-5 and MCR-7, followed by MCR-4 and minor amounts of MCR-1 (Figure 4-7). Seasonal changes from summer to spring resulted in an increase of the relative amount of cluster MCR-7 sequences, the emergence of cluster MCR-2 sequences, the disappearance of cluster MCR-5 sequences (uncultured cluster) and minor changes on the frequency of cluster MCR-1 and MCR-4 sequences. Clearly, the dominance of clones related to the Methanomicrobiales order and the lower representation of Methanosaeta spp., a cluster present in significant numbers in the archaeal 16S rRNA gene libraries, may indicate a PCR bias for the mcr set of primers.

Luton et al. (2002) tested the set of primers with Methanosaeta and Methanosarcina spp., and they obtained amplification for these genera. However, in their libraries, most clones recovered were related to species of the order Methanomicrobiales and Methanobacteriales. Few clones were related to Methanosarcina spp. and none were related to Methanosaeta spp. The authors attributed these observations to PCR biases. Therefore, it seems that this set of mcr primers would be an appropriate primers to study diversity of Methanobacteriales and Methanomicrobiales. The dynamics of these two
methanogenic orders may be a sufficient indicators to assess the effect of eutrophication on microbial populations (see T-RFLP analysis in Chapter 5).

**Diversity Index**

A summary of the indices calculated to assess diversity in archaeal 16S rRNA and \(mcr\) gene clone libraries is presented in Table 4-2. Samples from eutrophic soils were more diverse in both type of libraries when evaluated by using the cumulative expected phylotypes index or the Shannon’s index. Archaeal 16S rRNA gene libraries were more diverse than \(mcr\) clone libraries. MCR clone libraries from spring eutrophic samples were more diverse according to the cumulative expected phylotype. However, no major differences were observed when evaluated using the Shannon’s index.

**Methanogenesis in Everglades Soils**

Although methanogenesis is the main process responsible for terminal anaerobic organic matter mineralization in the Everglades, very little is known about the microbial groups involved in this process (Wright and Reddy, 2001a). This is the first complete report we are aware of that characterized the archaeal and methanogenic communities in pristine and eutrophic zones of the Everglades WCA-2A. The only previous attempt to roughly characterize the methanogenic communities in the Everglades was conducted by Drake et al. (1996) in similar sites as described in Chapter 3. The authors reported an enrichment of almost 6 orders of magnitude in acetoclastic methanogens in eutrophic regions. They also reported an enrichment of acetate-producing microorganisms and \(H_2\)-consuming microorganism in the eutrophic zones of the marsh. The number \(H_2\)-consuming acetogens were similar in eutrophic and pristine sites, which may indicate that the increase of total \(H_2\)-consuming microorganisms is due to hydrogenotrophic methanogens. These differences in microbial enumerations correlate with the lower
methanogenic rates observed for pristine sites when compared to eutrophic sites (Chapter 2). Recently, an enrichment of H2-consuming microorganisms was reported in these soils. Hydrogenotrophic methanogens were 1000 and 100 times higher compared with acetoclastic methanogens in eutrophic and pristine soils respectively, and relative numbers of acetoclastic methanogens were similar between eutrophic and pristine sites (Chauhan et al., 2003).

The methanogenesis rates for samples of eutrophic sites measured using formate were ca 2.5 times higher than methanogenesis rates measured using acetate as substrate. Clearly, all these data combined suggest that hydrogenotrophic methanogens are important in the degradation of organic matter in eutrophic Everglades soils.

Acetate utilization is restricted to two genera in the order Methanosarcinales, *Methanosaeta* and *Methanosarcina*, all other remaining species of methanogens use H2 (Table 4-1). However, it is estimated that 70-80% of the methane produced in nature comes from the conversion of acetate to methane by acetoclastic methanogens (Jetten et al., 1992). Conrad (1999) reported that the contribution to H2 or acetate to methane production is highly variable. There are cases where the hydrogen contribution is higher and the relative proportions are reversed. In certain cases methanogenesis is exclusively driven by hydrogen. Examples include hot spring mats, eutrophic lakes, coastal marine sediments, temperate bogs, Antarctic water bodies and lake sediments. Recently, Horn et al. (2003) reported that hydrogen is the main methanogenic precursor in acidic peat, which is in agreement with other studies conducted in acidic peats (Landsdown et al., 1992; Williams and Crawford, 1984).
There are several reasons for the higher contribution of hydrogen as electron donor for methanogenesis, including additional sinks or loss of acetate by non-methanogenic microorganisms, and additional pools of hydrogen such as geological inputs. Possible reasons for this phenomenon require more research (Conrad, 1999). Acetate can be consumed by non-methanogenic microorganisms using electron acceptors such as oxygen, nitrate, ferric ion and sulfate, if these electron acceptors are available. In Chapter 2, a slight competition for acetate between methanogens and sulfate-reducing prokaryotes was noted in eutrophic soils. Nusslein et al. (2001) also reported the possibility of syntrophic acetate oxidation during methane production in sediment of a subtropical lake, similar to the so called Reversibacter, which is able to reverse homoacetogenesis (Zinder and Koch, 1984; Lee and Zinder, 1988). This process is also described in some species of Clostridium (Schnurer et al., 1996) and Geobacter (Cord-Ruwisch et al., 1988). Therefore, syntrophic acetate oxidation may result in a decrease of the acetate pool and an increase of the hydrogen pool in these environments. Nusslein et al. (2001) failed to detect acetoclastic methanogens in sediments, which exhibited syntrophic acetate oxidation.

In the archaeal 16S rRNA gene libraries, clones related to previously cultured methanogens ARC-1 (Methanobacteriales), ARC-3 (Methanoseta spp.) and ARC-4 (Methanosarcina spp.) might provide some insight into the pathways of anaerobic organic matter mineralization in Everglades WCA-2A soils. Two acetoclastic methanogens were detected in these soils related to Methanosarcina spp. and Methanosaeta spp. Methanosaeta spp are specialists able to grow only on acetate and dominate at low acetate concentration (7-70 uM). Methanosarcina spp are generalists, able to grow also
on hydrogen and methyl compounds, but they grow on acetate at much higher concentrations (0.2-1.2 mM) (Jetten et al., 1992). The dominance of *Methanosaeta*-like sequences strongly suggests that the acetate concentration is low in these ecosystems. Still, *Methanosarcina* were recovered in minor amounts, which may indicate soil niches where the acetate concentration is higher.

Cluster ARC-1 sequences, related to the Methanomicrobiales, were recovered in significant numbers in all clone libraries, indicating that this order may be responsible for hydrogenotrophic methanogenesis. MCR clone libraries were dominated by sequences related to Methanomicrobiales (cluster ARC-5, ARC-6 and ARC-7) and were highly diverse for this particular microbial group. A similar enrichment of Methanomicrobiales and Methanobacteriales, hydrogenotrophic methanogens, was reported for peat soils where hydrogen was an important methanogenic precursor (Horn et al., 2003).

The partial pressure of hydrogen is a main factor controlling the products of fermentation. If the hydrogen partial pressure is kept below $10^{-3}$ atm fermentation to acetate, $H_2$ and $CO_2$ occurs; however, if $H_2$ starts to accumulate the formation of more reduced products such as fatty acids or alcohol is promoted (Zinder, 1984; Schink, 1997).

Clearly, Everglades WCA-2A is not a case where hydrogen is the dominant methanogenic precursor, but it may be a clear case of intermediate hydrogen contribution to methane formation where acetate is not the main precursor of methane formation. Our results clearly support these speculations; however, more research targeting syntrophic hydrogen-producing microorganisms, either as the classic syntrophs using substrates such as short fatty acids and alcohols or the newly described syntrophic acetate oxidation, is needed.
A model for anaerobic mineralization of organic matter for Everglades soils is presented in Figure 4-8. Complex polymers are degraded by primary-fermenting bacteria producing monomers and oligomers that are fermented to fatty acids, acetate, and hydrogen. The produced hydrogen escapes and regulates the fermentation process, shifting the proportion of fermentation products, favoring the production of fatty acids and suppressing the production of acetate. This shift results in an increase in the pool of fatty acids (butyrate or propionate), which are syntrophically degraded to acetate and H$_2$-CO$_2$, increasing the pool of hydrogen for hydrogenotrophic methanogens. Hydrogen is converted to methane by species of the order Methanomicrobiales. Acetate, in lower concentrations than in a typical ecosystem, could be used by sulfate reducing prokaryotes and probably syntrophic acetate oxidizers (which would increase the hydrogen pool). This would result in low acetate concentrations favoring proliferation of the specialist *Methanoseta* spp. instead of the generalist *Methanosarcina* spp.
Table 4-1. Physiological properties of selected genera of methanogens (adapted from Garcia et al., 2000; and Balch et al., 1979).

<table>
<thead>
<tr>
<th>Substrates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>H&lt;sub&gt;2&lt;/sub&gt;+CO&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Formate</th>
<th>Acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methanobacteriales</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanobacterium</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Methanobrevibacter</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Methanotermus</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Methanocococcales</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanococcus</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Methanomicrobiales</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanomicrobium</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Methanocorpusculum</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Methanospirillum</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Methanoculleus</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Methanoplanus</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Methanogenium</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Methanofollis</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Methanosarcinales</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanosarcina</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Methanosaeta</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup>+, utilized; +/- utilized by some; - not utilized
Table 4-2. Expected and recovered phylotypes and diversity index for archaeal 16S rRNA and \textit{mcr} gene clone libraries for eutrophic and pristine soils.

<table>
<thead>
<tr>
<th></th>
<th>Expected phylotypes (^a)</th>
<th>Recovered phylotypes</th>
<th>Shannon’s index</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Archaeal 16S rRNA clone libraries</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1- Summer</td>
<td>36.0 ± 0.8</td>
<td>20</td>
<td>1.25</td>
</tr>
<tr>
<td>F1- Spring</td>
<td>86.7 ± 2.5</td>
<td>23</td>
<td>1.29</td>
</tr>
<tr>
<td>U3- Summer</td>
<td>28.0 ± 0.8</td>
<td>16</td>
<td>1.06</td>
</tr>
<tr>
<td>U3- Spring</td>
<td>32.8 ± 0.8</td>
<td>17</td>
<td>1.10</td>
</tr>
<tr>
<td><strong>mcr clone libraries</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1- Summer</td>
<td>22.7 ± 0.6</td>
<td>14</td>
<td>1.03</td>
</tr>
<tr>
<td>F1- Spring</td>
<td>36.1 ± 0.8</td>
<td>18</td>
<td>1.12</td>
</tr>
<tr>
<td>U3- Summer</td>
<td>13.2 ± 0.1</td>
<td>9</td>
<td>0.83</td>
</tr>
<tr>
<td>U3- Spring</td>
<td>12.9 ± 0.1</td>
<td>10</td>
<td>0.83</td>
</tr>
</tbody>
</table>

\(^a\) Value of constant “\(a\)” from hyperbolic equation \(y = ax/(b + x)\) with the standard error
Figure 4-1. Rarefaction analysis for archaeal 16S rRNA clone libraries for spring and summer samples from eutrophic and pristine regions of the Everglades WCA-2A.
Figure 4-2. Maximum parsimony tree for archaeal 16S rRNA gene sequences from eutrophic F1 sites of the Everglades WCA-2A. The clones are named according to the site and time of sampling. Scale bar represents 1 nucleotide change per 100 sequence position. Numbers at nodes represent percentage of bootstrap resampling based on 100 replicates; only values above 50 are presented.
Figure 4-3. Maximum parsimony tree for archaeal 16S rRNA gene sequences from pristine U3 sites of the Everglades WCA-2A. The clones are named according to the site and time of sampling. Scale bar represents 1 nucleotide change per 100 sequence position. Numbers at nodes represent percentage of bootstrap resampling based on 100 replicates; only values above 50 are presented.
Figure 4-4. Spatial and seasonal distribution of archaeal 16S rRNA clones in eutrophic and pristine soils for spring and summer samples.
Figure 4-5. Rarefaction analysis for mcr clone libraries for spring and summer samples from eutrophic and pristine regions of the Everglades WCA-2A.
Figure 4-6. Neighbor-joining mcr α-subunit tree. The clones are named according to the origin and time of sampling. Scale bar represents 10% sequence divergence. Numbers at nodes represent percentage of bootstrap resampling based on 100 replicates; only values higher than 50 are presented.
Figure 4-7. Spatial and seasonal distribution of \textit{mcr} clones in eutrophic and pristine soils for spring and summer samples.
Figure 4-8. Model explaining the role of hydrogen on anaerobic organic matter mineralization on Everglades WCA-2A soils. SAO, syntrophic acetate oxidizers; SRP, sulfate-reducing prokaryotes.
CHAPTER 5
EFFECT OF EUTROPHICATION ON SRP AND HYDROGENOTROPHIC METHANOGENIC COMMUNITIES IN EVERGLADES WCA-2A SOILS

Introduction

Human activities have resulted in modification of the original Everglades ecosystem (Schrope, 2001). Microbial communities are very sensitive to environmental changes, and may be used to assess the impact of different human activities in different ecosystems (Atlas et al., 1991). Many years of research have shown that loading of phosphorus into the Everglades, a low nutrient ecosystem, has resulted in undesirable changes from the original ecosystem. The ability to correct these problems, to establish recovery goals, and to monitor the success or failure of restoration strategies depend on finding a suitable battery of ecological indicators. Although the influence of phosphorus loading at the process level in the Everglades has received a great deal of attention, most research has targeted macro indicators, such as those based on vegetation or fauna, or chemical and physical parameters involved in biogeochemical cycles (Reddy et al., 1999). Fewer studies have addressed the role of microorganisms and these have mainly targeted gross informative parameters such as microbial biomass, enzymatic activities and microbial enumerations (DeBusk and Reddy, 1998; Drake et al, 1996; Wright and Reddy, 2001). Although valuable information has been obtained from these studies, they provided little insight into the microbial groups responsible for the biogeochemical processes. There are no reports of the effect of eutrophication on the ecology of
microbial populations and the use of microorganisms as possible ecological indicators of nutrient impact in this type of ecosystem.

The advent of molecular microbial ecology resulted in several culture-independent techniques that allow us to fingerprint microbial communities and gain more information about microbial dynamics in the environment (Head et al., 1998, Ogram, 2000). Most of these techniques are based on the pioneering work of Carl Woese on the study of 16S rRNA analysis, and of Norman Pace application to environmental microbial communities (Woese, 1987; Hugenholtz et al., 1998).

One of the methods frequently used to characterize microbial communities is terminal restriction fragment length polymorphism (T-RFLP) analysis (Liu et al., 1997; Marsh, 1999). This method has been applied to several types of environments, including soils, and marine and freshwater ecosystems, and has targeted several phylogenetic and functional microbial groups (Kuske et al., 2002). In this method, target genes are amplified by PCR using one fluorescent dye-labeled primer. The labeled amplicons are digested with restriction enzymes generating a mixture of fluorescent dye-labeled terminal fragments of different sizes. The fragments are separated by polyacrylamide gel electrophoresis or with capillary electrophoresis and a laser reader detects labeled terminal fragments of different sizes. Most studies targeted the 16S rRNA gene; however, due to the microbial diversity present in soils, interpretation of these results may be difficult and many microbial groups can be underestimated. Therefore, the use of primers that target narrow phylogenetic groups or functional groups diminish these methodological limitations (Castro and Ogram, 2002). T-RFLP methodology has all the limitations related to PCR techniques, including template biases and a lack of
quantitation. However, this technique can be valuable for assessing microbial diversity studies where a great number of samples must be processed, and when sequence information is available. Until other microbial ecology techniques are developed, to avoid PCR biases, T-RFLP is a suitable technique for many microbial ecology applications.

The objectives of this study were to characterize the dynamics of sulfate reducing and methanogenic assemblages using T-RFLP targeting \( dsr \) and \( mcr \) genes, respectively, and to relate microbial community dynamics to biogeochemical parameters from the eutrophic, transition and pristine zones of WCA-2A. Principal component analysis was used to correlate T-RFLP data and sample type. Logistic regression was used to assess the influence of soil parameters in the dynamics of T-RFLP profiles.

**Material and Methods**

**Soil Samples**

T-RFLP studies were conducted on samples taken from the WCA-2A, previously described in Chapter 2. The studies were conducted in the 0-10 cm soil layer of triplicate samples taken monthly from 04/01 to 08/02. Soil samples included eutrophic (F1), transition (F4), and pristine (U3) regions of WCA-2A, rendering a total of 36 samples per site. Soil samples were kept at –80°C until DNA was extracted.

**Nucleic Acid Extraction and PCR Amplification**

Nucleic acids were extracted with UltraClean Soil DNA kits (MoBio, Solana Beach, CA) according to the manufacturer's instructions.

PCR was conducted in 50 µl reactions using the primer set designed by Wagner et al. (1998) for the \( dsr \) gene and the primer set designed by Luton et. al. (2002) for the \( mcr \) gene. Primers were fluorescently labeled with 6-FAM (6-carboxyfluorescein) in the 5’
position (Invitrogen, Carlsbad, CA). PCR conditions were similar to those described in Chapter 3 for *dsr* gene, and Chapter 4 for *mcr* gene, with the exception that the annealing temperature was lowered to 54 °C for *dsr* and to 53 °C for *mcr* PCR analysis. PCRs were conducted in 1:10 dilutions for the *dsr* primer set and 1:1 for the *mcr* primer set. The PCR products were electrophoresed on 0.7% and 1.5 % agarose gels, for DSR and MCR amplicons, respectively, to confirm products of the expected sizes. After PCR amplification the products were cleaned and concentrated using QIAquick PCR purification kit (Qiagen, Valencia, CA) to a final volume of 30 µl following manufacturer’s instructions.

**Enzymatic Digestion**

Several restriction enzymes were tested *in silico* using sequence information gained from the clone libraries, and previously published studies of MCR (Luton et al., 2002). The sequences were digested *in silico* using CloneMap version 2.11 (GCG Scientific Inc., Ballwin, MO). The restriction enzyme that produced a greater degree of discrimination for *dsr* analysis was *Rsa*I and for *mcr* was *Sau96*I. Approximately 100-150 ng of PCR product (estimated by visual inspection of gels after PCR kit purification) was digested with the appropriate restriction enzyme. The enzymatic digestion reaction consisted of 5 units of restriction enzyme (Promega, Madison, WI), 1x restriction buffer, 1 µg of bovine serum albumin, and deionized water to a final volume of 10 µl. Enzymatic digestions were incubated at 37°C overnight.

**T-RFLP Analysis**

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, the digested product was 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). The samples were denatured by heating at 95°C for 3 minutes and subsequently transferred to ice until loading of the gel. One µl was electrophoresed in a 36 cm, 5% polyacrylamide gel containing 7 M urea at 3 kV on an ABI 377 Genetic Analyzer (Applied Biosystems). The sizes in base pairs of the T-RF was calculated using the internal standards and GeneScan version 2.1 software (Applied Biosystems). Peak sizes in base pairs and peak fluorescence area were exported to Excel 97 SR-1 (Microsoft Corporation, Redmond, WA) for data analysis. Peaks with heights lower than 50 fluorescent units were filtered out from the final data matrix, a standard practice when analyzing T-RFLP data. Many reports have set these filter value to 25 units (Kuske et al., 2002) or 100 units (Lueders and Friedrich, 2003; Osborn et al., 2000), however under our experimental conditions, 50 units was a reasonable filter value.

T-RFLP Data Analysis

Several problems, also faced by other researchers, were encountered with the final T-RF data matrices. First, some irreproducible peaks with no clear presence pattern were present in only one of the triplicates, and were considered noise and removed from the analysis (Dunbar et al., 2001; Klamer et al., 2002). In most cases, these peaks were minor peaks (area lower than 1% of the total area) and of no known phylogenetic affiliation. Removal of these peaks did not result in any major changes in the final outcome of principal component analysis, but did facilitate data analysis and interpretation. Second, in the case of the dsr T-RFLP, approximately 15 samples were removed from the final set of 108 samples. Those samples resulted in total areas of only 1% of the total area for the majority of the other samples and were dominated by only
one or two peaks. Optimization of PCR conditions for these samples could result in the inclusion of these samples in the data set, but the intention of this study was to evaluate T-RFLP as an efficient tool to assess microbial diversity, more than concentrating on optimizing the conditions of a particular sample. Obviously, this is a drawback of the method as a high-throughput technique. Third, in silico digestions of some mcr sequences revealed the possibility of peaks with differences of 1 base pair. Such peaks were not resolved in this study and were observed as a single broad band in gels. However, several peaks were assigned with the GeneScan software to those broad bands in the range of plus or minus one base pair from the central peak. Therefore, those peaks were considered as a single peak in the data analysis. Principal component analyses that considered these peaks as individual peaks or combined peaks did not alter the final outcome.

Data analysis was conducted: a) qualitatively, scoring the presence or absence of a particular T-RF as 1 or 0 to produce a binary data matrix; and b) quantitatively, using relative abundance of a particular T-RF normalized by the total area of all T-RFs.

Principal component analysis (PCA) was performed using the relative abundance of individual peaks with the Multivariate Statistical Package (MSVP version 3.12d, Kovach Computing Services, Wales, UK). Cluster analysis using the T-RFLP binary data matrix was performed using the genetic distance calculation of Link et al. (1995) combined with dendrogram construction using the unweighted pair group method using arithmetic averages (UPGMA) analysis using the Treecon software version 1.3b (Van de Peer and Wachter, 1994). To assess the influence of soil parameters on the presence or absence (binary data matrix) of a particular T-RF, a logistic regression analysis was
performed using JMP version 4.04 (SAS Institute Inc, Cary, NC). The presence/absence
data were included as dependent variables and the soil parameters as independent
variables.

**Results and Discussion**

**T-RFLP Analysis of the SRP Community**

Typical T-RFLP profiles for DSR amplicons digested with *Rsa*I are presented on
Figure 5-1. The T-RFLP profile was divided into three panels to facilitate visualization
of all relevant T-RFs, because the high relative intensity of T-RF 185 made the
observation of other T-RF present in lower amounts difficult. The possible phylogenetic
affiliations of selected T-RFs are presented in Table 5-1. Although it is expected that a
single peak would represent a group of clones with the same phylogenetic affiliation, it is
clear from Table 5-1 that was not always the case. However, of all the restriction
enzymes tested, digestion with *Rsa*I provided the best possible discrimination between
phylogenetic groups. Certain T-RFs did not have a known phylogenetic affiliation,
which may indicate that the clone libraries did not cover all the diversity of these soils.
Moreover, these T-RFs were in their majority found in samples from the transition zone
(F4), a sample for which we do not have a clone library. Since these T-RFs were present
in significant amounts in all replicates, they were included in the analysis. Further work
is required to characterize these T-RFs.

Averages of the T-RF relative frequencies for the three monthly soil cores for
eutrophic, transition, and pristine regions of WCA-2A are presented in Figures 5-2, 5-3
and 5-4, respectively. For eutrophic zones, the T-RF distribution was uniform and stable
among sampling times. T-RFLP profiles for transition regions were also relatively stable.
However, T-RFLP profiles from pristine regions were more variable. These results are in
good agreement with the seasonal variability found in clone libraries described in Chapter 3. In samples from eutrophic regions, T-RFs present in significant amounts were 41, 64, 140, 165, 185, 188, 211, 234, 245, 327, 330, 364, 411, 435, 445 and 448 bp. T-RF 47, 113, 393, and 464 bp were present in frequencies less than 2%. In samples from transition regions the T-RFs present in considerable amounts were, 41, 64, 140, 185, 218, 245, 276, 292, 315, 327, 330, 364, 411, 435, and 445 bp. T-RF 47, 155, 161, and 464 bp were present in frequencies lower than 2%. In samples from pristine samples, fewer T-RFs were observed. The ones present in significant amounts were 41, 64, 113, 185, 188, 245, 276, 364, 393 and 445 bp. T-RF 140, 165, 411 and 435 bp were present in frequencies lower than 2%.

T-RF 41 bp was recovered from the two clone libraries from eutrophic regions and was related to the cluster DSR-2 (a clade composed of uncultured SRP). This T-RF was present in all the T-RFLP profiles from the eutrophic and transition sites in relatively constant amounts. However, in the pristine zones, the presence of this T-RF was more variable and according to the dsr clone libraries this T-RF may be assigned to DSR-4 (Desulfotomaculum-like sequences incomplete oxidizers) or DSR-1 (clones related to Thermodesulfovibrio species).

T-RF 64 bp was present in most samples from eutrophic, transition, and pristine sites. This T-RF was related to members of the cluster DSR-8 (Desulfotomaculum-like sequences complete oxidizers) and DSR-5 (sequences recovered only in this study). These two clusters were present in the clone library study, but sequences of cluster DSR-8 were present in quantities 3-4 times higher than cluster DSR-5, which may indicate that this T-RF represents cluster DSR-8 in the case of eutrophic sites. However, in clone
libraries of pristine sites, sequences clustering in cluster DSR-5 were present and no sequence from cluster DSR-8 was recovered, indicating this T-RF may be associated to members of the cluster DSR-5.

T-RF 113 bp was found in considerable amounts mainly in the pristine sites. It is related to sequences of the DSR-6 cluster, a cluster only recovered in clone libraries from pristine sites and related to the δ-proteobacteria SRP.

T-RF 140 bp is related to sequences of the DSR-5 cluster (sequences recovered only in this study) and was found in T-RFLP profiles of eutrophic and transition regions, and as a minor component in T-RFLP profiles from pristine zones. This T-RF is shared with cultured gram-negative mesophilic SRP such as a Desulfovibrio spp., a type of SRP that was not recovered in our clone libraries, but was dominant in the enrichment studies.

T-RF 165 bp is related to sequences of the DSR-2 cluster (uncultured SRP) and was present in considerable amounts in all T-RFLP profiles of eutrophic samples and some T-RFLP profiles from pristine sites, but not in profiles from transition zones.

T-RF 185 was present in all the samples in considerable amounts, and was a dominant T-RF in samples from transition regions. This T-RF was related to cluster DSR-8 (Desulfotomaculum-like sequences complete oxidizers). However, no member of this cluster was recovered in clone libraries of pristine sites.

T-RF 188 was observed in high amounts from pristine soil samples and in minor amounts from eutrophic soil samples. This T-RF was not recovered in transition soil samples. T-RF 188 according to the clone libraries is related to cluster DSR-5 (uncultured SRP) and DSR-4 (Desulfotomaculum-like sequences incomplete oxidizers) for pristine sites and DSR-8 for eutrophic sites.
T-RF 211 bp was only recovered in samples from eutrophic sites, and T-RF 218 bp was observed only in profiles of the transition zone; however, their possible phylogenetic associations remains unknown.

T-RF 234 bp was only recovered in T-RFLP profiles from eutrophic soil samples, and it was affiliated with DSR-7 a cluster composed of δ–proteobacteria SRP related to Desulfobulbus spp. and only recovered from eutrophic sites.

T-RF 245 bp dominated T-RFLP profiles of pristine sites and was only present in minor amounts in eutrophic and transition samples. This T-RF is related to members of the cluster DSR-6, a δ–proteobacteria SRP cluster only recovered in clone libraries from pristine sites.

Other groups of T-RFs with unknown phylogenetic affiliation were T-RF 276 bp, recovered from transition and pristine samples, 292 and 315 bp recovered only from transition soil samples, and 327 bp recovered from eutrophic and transition samples.

T-RF 330 bp was a minor component in T-RFLP profiles for eutrophic and transition regions. This T-RF was not relate to any sequence from the clone libraries, although it may be related to cultured SRP, such as Desulfovibrio spp.

T-RF 364 bp was present in significant amounts in all T-RFLP profiles for all the samples, but its phylogenetic affiliation remains unknown.

T-RFs 411 and 435 were present in all samples from eutrophic and transition regions and some pristine soil samples. T-RF 445 in most of the samples from the three sites, but unfortunately, their phylogenetic affiliation remains unknown.
T-RF 448 was only present in samples from eutrophic sites, and was not recovered from clone libraries. However, this T-RF is related to uncultured SRP present in the Genbank database.

T-RF 464 bp is a minor component of T-RFLP profiles of eutrophic and transition soil samples, but its phylogenetic affiliation remains unknown.

Most T-RFLP profiles are in good agreement with results obtained from the clone libraries, with the exception of sequences related to Desulfotomaculum in the pristine regions. T-RFLP profiles from eutrophic and transition regions were dominated by T-RF 185, which is related to Desulfotomaculum-like sequences complete oxidizers, which is on agreement with clone libraries. However, in pristine sites T-RF 185 bp associated with Desulfotomaculum-like sequences (cluster DSR-8), was also present and this cluster was not recovered in the clone libraries. T-RF 188 bp related to cluster DSR-4 (Desulfotomaculum-like sequences, incomplete oxidizers) and cluster DSR-5 (uncultured SRP) which was recovered and in considerable numbers in T-RFLP from pristine sites. Assuming that T-RF 188 represents incomplete oxidizers Desulfotomaculum-like sequences, this may indicate that pristine sites are not dominated by Desulfotomaculum incomplete oxidizers but by a combination of complete and incomplete oxidizers.

Chin et al. (1999) and Lueders and Friedrich (2000) also reported discrepancies between clone library and T-RFLP profile data. They attributed discrepancies to biases in the cloning of the amplification products. Another explanation is the differences in annealing temperatures used in the PCR reaction in the clone libraries and T-RFLP analysis. In order to obtain a PCR product, the annealing temperature was dropped from 58 to 54 °C when the fluorescent labeled primer was used in T-RFLP analysis, a normal
practice for fluorescent labeled primers. This may have resulted in changes in the composition of the PCR amplicon mixture. More studies are required to resolve discrepancies between T-RFLP and clone library data.

**PCA and UPGMA Analysis of SRP Community**

PCA has been used extensively for comparison of complex microbial communities (Dollhopf et al., 2001). In order to apply PCA, the area of an individual peak was normalized to the total area of the peaks. In this study, PCA analysis proved to be a powerful method to discriminate among the three levels of eutrophication (Figure 5-5). PCA axis 1 explained 30.4% of the variability and PCA axis 2 explained 17.5%, with a cumulative percentage of 47.9%. A total of 6 axes were required to explain a cumulative percentage of 72.2%. Although these values may seem low, these are comparable to other T-RFLP studies (Kuske et al., 2002; Klamer et al., 2002).

PCA analysis is based on the area of peaks and it is known that the areas of T-RFs may not be quantitative due to several biases inherent to the T-RFLP techniques. In an attempt to corroborate our PCA analyses, a cluster analysis using the T-RFLP binary data matrix (presence or absence) was performed. The genetic distance (GD) was calculated according to Link et al. (1995) using their formula: \( G_{dxy} = \frac{N_x + N_y}{N_x + N_y + N_{xy}} \), where \( N_x \) is number of T-RF in profile x but not in profile y; \( N_y \) is the number of T-RF in profile y but not in profile x; and \( N_{xy} \) is the number of T-RFs shared by profiles x and y. Dendrograms of the genetic distances were constructed using UPGMA analysis. Dendrograms constructed with UPGMA using binary ordination revealed the same three clusters as in PCA. Three clusters were observed: one for eutrophic; one for transition; and one for pristine soil samples. The UPGMA analysis suggested that eutrophic soil samples were more similar to transition soil samples than to pristine soil samples.
Therefore, the confidence on the clustering of the three types of eutrophication levels is confirmed by two type of analysis, PCA using relative abundance of T-RFs and UPGMA using presence or absence of T-RFs.

**Correlation of Soil Parameters with SRP Communities**

To assess the influence of soil parameters on the presence or absence of a particular T-RF, a logistic regression analysis was performed. The results of the logistic regression are presented in Table 5-2. No logistic regression was performed on T-RFs 41, 64, 185, 245, 364, and 445 bp because these T-RFs were present in all samples. Several parameters correlated with the presence or absence of T-RF with no clear dominance of any soil parameter examined. For the carbon parameters, total carbon and microbial biomass carbon correlated with most of the T-RFs. Extractable total organic carbon did not correlate with any T-RFs. For the nitrogen related parameters, most of the peaks correlated with total nitrogen and microbial biomass nitrogen. However, a smaller number of T-RFs correlated with total Kjeldahl nitrogen and ammonium.

The presence of most T-RFs correlated with the three forms of phosphorus included in this analysis, including total phosphorus, total inorganic phosphorus and microbial biomass phosphorus. A low number of T-RFs correlated with the enzymatic activities determined in these soil samples. Generally, T-RFs correlated with parameters related to total content for carbon and nitrogen, total and inorganic content for phosphorus, and parameters related to microbial biomass composition.

**T-RFLP Analysis of the Methanogenic Community Using the mcr Gene.**

Representative T-RFLP profiles for MCR amplicons digested with *Sau96I* are presented in Figure 5-6 and the possible phylogenetic affiliation of the T-RFs are presented in Table 5-3. As with *dsr* T-RFLP profiles, some T-RFs were represented by
members of different phylogenetic affiliations. However, *Sau96*I in silico digestions provided the best discrimination among sequences from the MCR clone library. Certain T-RFs did not have a known phylogenetic affiliation, and these T-RFs were present in low frequency in the T-RFLP profiles, with the exception of T-RF 392 bp, which was present in most of the samples in considerable amounts.

Averages of the T-RF relative frequencies for the three monthly soil cores for eutrophic, transition, and pristine regions are presented in Figures 5-7, 5-8 and 5-9, respectively. T-RFLP profiles for eutrophic and transition zones were uniform and stable among samples. T-RFLP profiles of samples from pristine regions were more variable. These results are in good agreement with the clone distribution of clone libraries presented in Chapter 4.

In samples from eutrophic regions, T-RFs present in considerable amounts were 130, 229, 372, 375, 392, 398 and 470 bp. The remaining T-RFs were present in minor frequencies (0-5%). In samples from transition regions, the T-RFs present in considerable amounts were 130, 229, 239, 372, 375, 392, 398 and 470 bp. In pristine samples, the T-RFLP profiles were dominated by T-RF 398, with relatively equal frequencies for T-RFs 130, 375, 392 and 398 bp. Moreover, the T-RF that tended to be a minor component of eutrophic and transition T-RFLP profiles were present in higher abundance in U3.

T-RF 130 bp was recovered from the two clone libraries from eutrophic regions and was related to the cluster MCR-4 (*Methanosaeta* spp.) and MCR-6 and MCR-7 (Methanomicrobiales). This T-RF was present in relatively constant and considerable
amounts in all T-RFLP profiles from the eutrophic and transition sites. However, in the pristine zones, this T-RF was present in lower and more variable amounts.

T-RF 229 bp was present in T-RFLP profiles for samples from eutrophic and transition sites, and was a minor component of T-RFLP profiles for pristine sites. This T-RF was related to members of the cluster MCR-5 and MCR-7, two clades affiliated with the Methanomicrobiales order, and was only represented by sequences for clone libraries from eutrophic soil samples.

T-RF 239 bp was a significant component in T-RFLP profiles of the transition and pristine sites. It was related to members of the cluster MCR-5 (deeply divergent clade of Methanomicrobiales) and MCR-3 (*Methanosaeta* spp.). The phylogenetic affiliation of this T-RF remains unclear.

T-RF 372 bp, of unknown phylogenetic affiliation, was recovered in greater frequencies in eutrophic sites, followed by transition and pristine sites.

T-RF 375 bp was more important in pristine sites, however this T-RF was related to clones from cluster MCR-1 (Methanobacteriales) and MCR-2 (uncultured representatives) for clone libraries from eutrophic sites and MCR-4 (*Methanosaeta* spp.) for clone libraries from pristine sites.

T-RF 398 was present in all the samples in considerable amounts, being a dominant T-RF in samples from transition and pristine regions. This T-RF was related to cluster MCR-5 and MCR-7, both clusters were related to Methanomicrobiales order.

T-RF 470 was recovered in minor amounts from all the samples. T-RF 470 is related to sequences from cluster MCR-2 (uncultured) recovered in eutrophic clone libraries.
In summary, T-RFLP profiles from eutrophic (and transition) regions were dominated by T-RF 129 bp (MCR-4, -6 and -7), 229 bp (MCR-5 and-7) and 398 bp (MCR-5 and -7), which is in relatively good agreement with the clone libraries results.

The T-RFLP profiles from pristine sites were in good agreement with clone library results, and T-RFLP profiles were dominated by T-RF 129 bp (MCR-4, -6 and -7), 375 bp (MCR-1, -2 and -4), 392 (unknown phylogenetic affiliation) and 398 bp (MCR-5 and -7).

**PCA and UPGMA Analysis of Methanogenic Community**

PCA analysis proved to be a good method to discriminate pristine sites from the other two levels of eutrophication (Figure 5-10). Although two possible different clusters can be observed for eutrophic and transition zones, categorical discrimination between eutrophic and pristine sites was not achieved. PCA axis 1 explained 29.5% of the variability and PCA axis 2 explained 11.1%, with a cumulative percentage of 40.6%. A total of 7 axes were required to explain a cumulative percentage of 72.0%.

Cluster analysis of the T-RFLP binary data matrix using UPGMA confirmed the PCA results. Three clusters were observed, but a fourth cluster composed of eutrophic and transition T-RFLP profiles was also observed. This correlated with the overlap seen for samples of these two sites in the PCA analysis.

Once again, the confidence on the clustering of the three types of eutrophication levels was confirmed by PCA analysis using T-RFs relative abundance and UPGMA using T-RFs presence or absence.

**Correlation of Soil Parameters with Methanogenic Communities**

The results of the logistic regression of soil parameters with the presence or absence of a particular T-RF are presented in Table 5-4. No logistic regression was
performed with T-RFs 72, 88, 130, 141, 209, 362, 372, 375, 392, 398, or 470 bp because these T-RFs were present in most samples. As was the case with \textit{dsr} T-RFLP profiles, several parameters correlated with the presence or absence of a T-RF, with no clear dominance of any particular soil parameter. For the carbon parameters, total carbon and microbial biomass carbon correlated with most of the T-RF, and extractable total organic carbon did not correlate with any T-RF. For the nitrogen related parameters, some peaks correlated with total nitrogen and microbial biomass nitrogen. However, fewer T-RF correlated with total Kjeldahl nitrogen and ammonium.

Most T-RFs correlated with the three phosphorus parameters. A minor number of T-RFs correlated with the three enzymatic activities determined in these soil samples. In summary, T-RFs were correlated to parameters related to total carbon and nitrogen content, total and inorganic phosphorus content, and parameters related to microbial biomass composition.

Clearly, T-RFLP was a powerful technique to discriminate between soils with different eutrophication levels. \textit{dsr} T-RFLP provided a higher level of discrimination between the three types of samples. The \textit{mcr} gene was a weaker system to distinguish among samples since it could not categorically discriminate between eutrophic and transition soil samples.

**Effect of Eutrophication on SRP and Methanogenic Communities**

Sulfate concentration and electron donor concentration and type control the activity of SRP in freshwater marshes (Widdel, 1988). Sulfate concentrations in these marshes were similar between eutrophic and pristine sites (see Chapter 2). Total sulfur concentrations in the 0 to 10 cm soil layer soils is higher than total sulfur content in lower soils layers in eutrophic and pristine sites, indicating sulfur accumulation in the
ecosystem (Bates et al., 2002). The total sulfur content is relatively similar in the 0-10 cm soil layer of eutrophic soils (1.26-1.36%) and pristine soils (1.57-1.74%). However, higher amounts of sulfur is present as organic sulfur (70-80%) in eutrophic sites compared to pristine sites, which have lower organic sulfur content (50-55%). Wright and Reddy (2001b) did not observe any differences in arylsulfatase activities along the eutrophication gradient. This may indicate that the sulfate entering the northern regions of the Everglades is partially fixed in the organic fraction soil of the eutrophic soils and partially moving southward to more pristine sites. Accumulation of organic sulfur in the eutrophic regions can be due to higher assimilation of sulfate due to higher microbial activity in the impacted regions. If sulfate is not driving the differences between the SRP populations along the phosphorus gradient, the greater number and activity of SRP could be explained by differences in the amount and type of electron donor. Our results with the enrichment cultures and MPN enumerations suggested a greater metabolic diversity in the eutrophic zones. Moreover, results from the clone libraries, and partially from the T-RFLP data, suggested differences in the type of metabolism, complete versus incomplete oxidizers for the Desulfotomaculum community.

T-RFLP data using mcr genes clearly discriminated between methanogenic populations of pristine sites from the other two type of sites, but it was not sufficient to discriminate between eutrophic and transition sites. Since methanogens are able to use only a limited number of substrates in freshwater ecosystems (mainly acetate, H$_2$-CO$_2$ and formate), it was not expected to see much discrimination targeting the methanogenic community. However, since the mcr primers targeted mainly hydrogenotrophic methanogens (see Chapter 4), it is clear that the hydrogenotrophic methanogens are
responding in different ways to environmental conditions. Ward and Winfrey (1985) reported different $K_m$ values for growth on hydrogen for different species of hydrogenotrophic methanogens. This may explain the difference between eutrophic and transition zones methanogenic communities with methanogenic communities in more pristine sites. It may be speculated that hydrogen levels in the eutrophic and transition zones are higher due to greater microbial activity that selects for a population with higher $K_m$, low affinity for hydrogen that could proliferate in environments with a higher hydrogen concentration. Alternatively, the methanogenic population in pristine regions may be characterized by hydrogenotrophic methanogens with lower $K_m$ and a higher affinity for hydrogen, a product accumulated in lower concentrations due to lower microbial activity. This is may be an equivalent situation to the one observed with acetate metabolism by *Methanothrix* and *Methanosarcina* spp (Jetten et al., 1982).

Further research, such as cultivation of these microorganisms, is required to verify the possibility of physiological difference among methanogenic populations inhabiting sites with different levels of eutrophication levels.

The results from *dsr* T-RFLP were useful to distinguish the three sites with different levels of impact, but the results of the *mcr* T-RFLP may indicate that the phosphorus loading is altering the methanogenic population in the transition zones, making it more similar to eutrophic zones, an early indication of the expansion of the phosphorus impact in southern WCA-2A. Clearly, targeting a combination of different microbial populations provides greater insight into the functioning of this ecosystem, and, provides useful information for planning and implementation of ecosystem restoration technologies.
Table 5-1. Phylogenetic affiliation of selected *dsr* T-RFs for eutrophic (F1) and pristine (U3) soil samples.

<table>
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<th>T-RF (bp)</th>
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<th>Phylogenetic affiliation</th>
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</tr>
<tr>
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<td>F1SU-14</td>
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</tr>
<tr>
<td></td>
<td>U3SP-09</td>
<td>DSR-4</td>
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</tr>
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Table 5-2. Influence of soil parameters on the presence and absence of selected T-RFs from *dsr* T-RFLP profiles.

<table>
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<td>S</td>
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<td></td>
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<td>S</td>
</tr>
<tr>
<td>NH₄-N</td>
<td>VS</td>
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<td>MBC</td>
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<td>TN</td>
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<td>VS</td>
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<tr>
<td>TPI</td>
<td>VS</td>
</tr>
<tr>
<td>MBP</td>
<td>VS</td>
</tr>
<tr>
<td>APA</td>
<td>VS</td>
</tr>
<tr>
<td>B-glucosidase</td>
<td>S</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>VS</td>
</tr>
</tbody>
</table>

<sup>a</sup> TC, total carbon; TOC, extractable total organic carbon; MBC, microbial carbon biomass; TN, total nitrogen; TKN, total Kjeldahl nitrogen; NH₄-N, ammonium; MBN, microbial biomass nitrogen; TP, total phosphorus; TPI, total inorganic phosphorus; MBP, microbial biomass phosphorus; APA, alkaline phosphatase activity.

<sup>b</sup> VS, very significant P<0.01; S, significant 0.01<P<0.05
Table 5-3. Phylogenetic affiliation of \( mcr \) T-RFs for eutrophic (F1) and pristine (U3) soil samples.

<table>
<thead>
<tr>
<th>Observed T-RF (bp)</th>
<th>Theoretical T-RF (bp)</th>
<th>Phylotype</th>
<th>Phylogenetic affiliation</th>
</tr>
</thead>
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<tr>
<td>72</td>
<td>72</td>
<td>Mcr-F1SP-26</td>
<td>MCR-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mcr-F1SP-38</td>
<td>MCR-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mcr-F1SU-12</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Mcr-U3SP-11</td>
<td>MCR-2</td>
</tr>
<tr>
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<td>Mcr-U3SP-06</td>
<td>MCR-7</td>
</tr>
<tr>
<td>88</td>
<td></td>
<td>Unknown phylogenic affiliation</td>
<td></td>
</tr>
<tr>
<td>No recovered</td>
<td>109</td>
<td>Mcr-F1SU-02</td>
<td>MCR-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mcr-U3SU-15</td>
<td></td>
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<td>115</td>
<td>115</td>
<td>Mcr-U3SU-27</td>
<td>MCR-5</td>
</tr>
<tr>
<td>130</td>
<td>129</td>
<td>Mcr-F1SP-09</td>
<td>MCR-4</td>
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<td></td>
<td></td>
<td>Mcr-F1SU-03</td>
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<td>MCR-6</td>
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<td>MCR-5</td>
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<td>239</td>
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<td>Mcr-U3SU-35</td>
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<td>Mcr-F1SP-15</td>
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<td></td>
</tr>
<tr>
<td>No recovered</td>
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<td>Mcr-F1SU-01</td>
<td>MCR-5</td>
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<tr>
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<td>362</td>
<td>Mcr-F1SU-25</td>
<td>MCR-3</td>
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<td>375</td>
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<td>MCR-1</td>
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<td>Mcr-U3SP-18</td>
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<td>Mcr-U3SU-05</td>
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<td>Mcr-U3SP-12</td>
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<td>MCR-1</td>
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<td>MCR-2</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Mcr-U3SU-33</td>
<td>MCR-4</td>
</tr>
</tbody>
</table>
Table 5-4. Influence of soil parameters on the presence and absence of selected T-RFs from *mcr* T-RFLP profiles.

<table>
<thead>
<tr>
<th>Soil parameter&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Presence/absence&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>115</td>
</tr>
<tr>
<td>TC</td>
<td></td>
</tr>
<tr>
<td>TOC</td>
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</tr>
<tr>
<td>MBC</td>
<td>VS</td>
</tr>
<tr>
<td>TN</td>
<td></td>
</tr>
<tr>
<td>TKN</td>
<td></td>
</tr>
<tr>
<td>NH&lt;sub&gt;4&lt;/sub&gt;-N</td>
<td>VS</td>
</tr>
<tr>
<td>MBN</td>
<td>VS</td>
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<td>VS</td>
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<td>TPi</td>
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<td>MBP</td>
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</tr>
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<td>APA</td>
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</tr>
<tr>
<td>B-glucosidase</td>
<td>S</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Abbreviations are as defined in the footnote of Table 5-2.

<sup>b</sup> VS, very significant P<0.01; S, significant 0.01<P<0.05
Figure 5-1. T-RFLP profiles for *dsr* gene of (A) eutrophic, (B) transition and (C) pristine soils.
Figure 5-2. Community dynamics for the dsr gene in eutrophic soils determined by T-RFLP analysis. Samples are labeled according to the season, month, year that were taken. SP, spring; SU, summer; FA, fall, WI, winter.
Figure 5-3. Community dynamics for the \textit{dsr} gene in transition soils determined by T-RFLP analysis (labels according to Figure 5-2).
Figure 5-4. Community dynamics for the *dsr* gene in pristine soils determined by T-RFLP analysis (labels according to Figure 5-2).
Figure 5-5. PCA ordering generated from T-RFLP profiles for *dsr* gene of eutrophic (F1), transition (F4) and pristine (U3) soils.
Figure 5-6. T-RFLP profiles for mcr gene of (A) eutrophic, (B) transition and (C) pristine soils.
Figure 5-7. Community dynamics for the mcr gene in eutrophic soils determined by T-RFLP analysis (labels according to Figure 5-2).
Figure 5-8. Community dynamics for the mcr gene in transition soils determined by T-RFLP analysis (labels according to Figure 5-2).
Figure 5-9. Community dynamics for the mcr gene in pristine soils determined by T-RFLP analysis (labels according to Figure 5-2).
Figure 5-10. PCA ordering generated from T-RFLP profiles for mcr gene of eutrophic (F1), transition (F4) and pristine (U3) soils.
CHAPTER 6
SUMMARY AND CONCLUSIONS

In freshwater wetlands, physical and chemical parameters affect microbial communities responsible for the carbon and sulfur cycles. Therefore, changes in the composition of microbial assemblages likely reflect environmental changes. Due to the metabolic and physiological versatility of SRP, changes in this microbial community would reflect changes at the process level in the Everglades wetlands. The Everglades is an ideal ecosystem for this type of study because several factors can be studied at the same time, including carbon loading, sulfate loading, nutrient impact, and seasonal changes of water table height and redox potential. SRP community dynamics would be intimately related to, and reflect the effect of nutrient impact on these freshwater ecosystems.

This research was conducted to understand the role of microorganism in anaerobic terminal carbon mineralization in Everglades soils, with special emphasis on sulfate-reducing prokaryotic assemblages. The study was conducted in three sites in the Everglades Water Conservation Area 2A, with different levels of eutrophication due to phosphorus loading.

Chapter 2 focused on the determination of sulfate reduction and SRP enumerations in eutrophic and pristine areas of WCA-2A. The possible competition for shared electron donors between methanogens and SRP was also investigated. MPN enumerations showed that SRP were present in considerable numbers and active in both zones of the marsh. F1 contained somewhat higher soil sulfate concentrations than most typical
freshwater systems. These relatively high sulfate concentrations correlated with high numbers of SRP and with high sulfate reduction rates. In U3, the sulfate concentrations were similar to or lower than those found in F1. Lower sulfate reduction rates and SRP numbers were observed at this site. Methanogenesis rates and accumulated methane in six day incubations were higher in F1 than in U3, which is in agreement with previously published data. Methanogenesis in U3 did not respond to addition of acetate, suggesting that acetoclastic methanogenesis may not be a major process in these regions of the marsh. In U3, no major changes were observed upon addition of acetate and molybdate or sulfate, suggesting that acetate-utilizing SRP may not be important in this region of the marsh, although they were found in significant numbers in MPN enumerations. On the contrary, acetate-utilizing SRP may play a role in eutrophic zones of the marsh, as suggested by the observations that addition of sulfate to F1 microcosms partially inhibited methanogenesis. The observed lack of response to acetate in the presence of sulfate suggests that SRP present in the pristine zones may be using electron donors other than acetate, or they may be using another type of metabolism such as fermentation.

Chapter 3 described the composition and metabolic diversity of SRP in eutrophic and pristine areas of WCA-2A. This study combined culture-based and culture-independent techniques to assess the dynamic of SRP assemblages. The presence of different SRP groups was determined by using molecular techniques targeting the dissimilatory sulfite reductase gene. Traditional enrichment and cultivation techniques using several electron donors combined with 16S rRNA gene analysis of SRP isolates were used to assess the metabolic diversity of SRP in these types of freshwater marshes. Diversity within DSR sequences was found in both zones of the marshes. Significantly,
Desulfotomaculum-like sequences from eutrophic regions were related to those Desulfotomaculum able to carry out complete oxidation of electron donors; in pristine regions they were related to those unable to carry out complete oxidation of electron donors. Molecular techniques revealed that nutrient loading resulted in a selection of different SRP populations. Classical cultivation techniques misrepresented the diversity of this ecosystem, suggesting that different approaches should be used to explore and recover undescribed species. Molecular techniques revealed a selection in the type of Desulfotomaculum populations present in eutrophic versus pristine regions of the marsh, suggesting that Desulfotomaculum complete-oxidizers are better adapted to eutrophic conditions than to pristine sites, where a greater number of Desulfotomaculum incomplete-oxidizing strains are present. Moreover, SRP diversity on eutrophic and pristine sites may be due to degradation of the accumulated carbon like phenolic compounds found in organic matter, which SRP are able to metabolize.

In Chapter 4, phylogenetic characterization and methanogenic community structures in eutrophic and pristine areas of WCA-2A was described. Culture-independent techniques targeting the archaeal 16S rRNA and methyl coenzyme M reductase genes were used to assess the dynamics of methanogenic assemblages. mcr clone libraries were dominated by sequences related to Methanomicrobiales (clusters ARC-5, ARC-6 and ARC-7) and were highly diverse for this particular microbial group. A similar enrichment of Methanomicrobiales and Methanobacteriales, hydrogenotrophic methanogens, was previously reported for peat soils where hydrogen was an important methanogenic precursor.
The partial pressure of hydrogen is a main factor controlling the products of fermentation. If the hydrogen partial pressure is kept low, fermentation to acetate, H\textsubscript{2} and CO\textsubscript{2} occurs; however, if H\textsubscript{2} starts to accumulate the formation of more reduced products such as fatty acids or alcohol is promoted.

Clearly, WCA-2A is not a case where hydrogen is the dominant methanogenic precursor, but it may be a case where intermediate hydrogen contribution to methane formation, and acetate is not the main precursor of methane formation. Our results clearly support these speculations; however, more research targeting syntrophic hydrogen producing microorganisms either as classic syntrophs that use substrates such as short fatty acids and alcohols, or possibly the newly described syntrophic acetate oxidizers, is required. Complex polymers are degraded in the top layer of soils, and the monomers and oligomers are fermented to fatty acids, acetate, and hydrogen. The hydrogen produced regulates the fermentation process, shifting the proportion of fermentation products, favoring the production of fatty acids and suppressing the production of acetate. This shift results in an increase in the pool of fatty acids (butyrate or propionate), which are syntrophically degraded to acetate and H\textsubscript{2}-CO\textsubscript{2}, increasing the pool of hydrogen for hydrogenotrophic methanogens. Hydrogen is converted to methane by species of the order Methanomicrobiales. Acetate, in lower concentrations than in a typical ecosystem could be used by sulfate reducing prokaryotes and possibly syntrophic acetate oxidizers (which would increase the hydrogen pool), resulting in acetate concentrations in the lower range which are favorable for proliferation of the specialist \textit{Methanosaeta} spp. instead of the generalist \textit{Methanosarcina} spp.
Chapter 5, using the molecular ecology technique terminal restriction fragment length polymorphism analysis, explored the dynamics of SRP and methanogenic assemblages in relationship to environmental conditions in Everglades WCA-2A soil with three levels of eutrophication. Principal component analysis results from $d_{sr}$ T-RFLP distinguished three sites with different levels of nutrient impact, but the results of the MCR T-RFLP may indicate that phosphorus loading may be altering the methanogenic population in the transition zones, making it more similar to eutrophic zones. This is an early indication of the extent of phosphorus impact in southern WCA-2A. Clearly, targeting a combination of different microbial populations provides greater insight into the functioning of this ecosystem, and provides useful information for planning and implementation of ecosystem restoration technologies. The present study contributed to a greater understanding of the factors controlling anaerobic terminal carbon mineralization in Everglades wetland soils.
LIST OF REFERENCES


Tebo, B. M. and A. Y. Obraztsova. 1998. Sulfate-reducing bacterium grows with Cr(VI), U(VI), Mn(IV), and Fe(III) as electron acceptors. FEMS Microbiol. Lett. 162:193-198.


BIOGRAPHICAL SKETCH

Hector F. Castro was born in San Jose de Mayo, Uruguay, in 1967. He received his B.S. in chemistry in 1988, and his pharmaceutical chemist degree in 1992, from the School of Chemistry, Universidad de la Republica, Montevideo, Uruguay. He worked in the Microbiology Department, School of Chemistry, from 1989 until he came to study in the United States in 1994. He joined the Soil and Water Science Department at the University of Florida, and in 1996 he completed his Master of Science degree. In 1997 he joined Dr. Andrew Ogram’s research team. He started his Ph.D. studies in the area of molecular biology of pesticide degradation. In the summer of 1997, his daughter Matilde was born in Gainesville, FL. In 1998 he attended the Microbial Diversity Course at the Marine Biological Laboratory, Woods Hole, MA. After this great educational experience and long deliberations, he decided to change his Ph.D. research topic. In 2000 he started working on the microbial ecology of the Everglades. Upon completion of his Ph.D., Hector plans to continue working in microbial ecology as a postdoctoral fellow in Dr. Ogram’s laboratory.