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A Comparison of Soils and their Associated Microbial Communities as Affected by Sugarcane Cultivation

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A COMPARISON OF SOILS AND THEIR ASSOCIATED MICROBIAL COMMUNITIES AS AFFECTED BY SUGARCANE CULTIVATION

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I dedicate this work to my parents for their continuous love, support, guidance, and willingness to lose to me in Settlers of Catan.

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Abstract

In Louisiana, sugarcane has been grown in the same soils for over 200 years. A phenomenon wherein soils with a long-term sugarcane cropping history produce decreased yields compared to adjacent land without a recent history of sugarcane cultivation has been documented in multiple sugarcane growing regions. Research in both Louisiana and internationally has shown positive plant growth responses when soils with a long-term cultivation history are sterilized or treated with selective biocides, suggesting there is a biological component to the underlying soil health issue. In this study, soil microbial ecology was compared for paired sites with short and long-term sugarcane cropping histories at six locations in plant cane and two in plant cane and first ratoon. Yield estimates of paired sites revealed crops grown in short-term cultivation soils generally out-yielded their long-term counterparts. Soil properties that can influence microbial ecology, including soil organic matter, macro and micro-nutrients, and soil extracellular enzymes, were generally present at higher levels in short-term cultivation soils, but varied by location. Root staining revealed greater fungal endophyte colonization in long-term cultivation soils. Distance-based redundancy analysis of fatty acid methyl ester biomarkers revealed differences in community structure based primarily on location but also cropping history. Analysis of 16S prokaryotic and ITS fungal amplicon-based metagenomic β-diversity data revealed prokaryotic community structure was also primarily influenced by location, whereas fungal communities differed based on cropping history. This suggests fungi are major contributors to the detrimental effects associated with sugarcane monoculture. Additional α-diversity comparisons of 16S and ITS metagenomes revealed portions of prokaryotic and fungal communities were more commonly associated with short and long-term sugarcane cultivation in both bulk and rhizosphere soils. Candidate microorganisms beneficial to sugarcane growth that were more abundant in soils with a short-term cropping history included 107 prokaryotic genera and 37 fungal genera in bulk soils and 97 prokaryotic genera and 46 fungal genera in rhizosphere soils. Candidate microorganisms detrimental to sugarcane growth that were more abundant in soils with a long-term cropping history included 117 prokaryotic genera and 58 fungal genera in bulk soils and 94 prokaryotic genera and 40 fungal genera in rhizosphere soils.

Chapter 1: Introduction

Sugarcane, *Saccharum* spp. inter-specific hybrids, is a C4-grass cultivated for its ability to store sucrose in its stalks and as a source of biomass feedstock for bioenergy. The plant is vegetatively propagated and grown as a perennial crop with annual harvests of stalks. In 2016, 431,000 acres of land were dedicated to sugarcane production with a total crop value of $820,000,000 (LSU AgCenter Agriculture Summary, 2016).

Sugarcane is grown in tropical to subtropical regions. In the United States, sugarcane is grown in Louisiana, Florida, and Texas. In Louisiana, production is restricted to the southern portion of the state. Planting occurs during August and September, and the first crop, known as “plant cane”, is harvested in the late fall to early winter of the following year. Buds from the stubble remaining after harvest germinate and produce crops known as “ratoons”. In Louisiana, growers typically harvest two ratoons before ploughing out and replanting. Traditionally, sugarcane is planted in the nutrient-rich alluvial soils associated with the banks of Louisiana’s numerous rivers and bayous. Due to the economic benefits of growing sugarcane and climatic constraints affecting other crops, sugarcane has been grown in the same soils for over 200 years (Hilliard, 1979).

Row-crop monoculture can result in yield decline for many crops that has been defined as “the loss of productive capacity of soil caused by biotic and abiotic factors when crops are grown repeatedly on the same land, in short rotation or monoculture, resulting in poor plant growth and development, delayed crop production and reduced yields” (Bennett et al., 2012). In specialized perennial cropping systems, such as tree fruits and nuts, intensive production and repeated long cropping cycles have led to a state of diminished yields characterized by plant stunting, root discoloration, and root tip necrosis (Mazzola and Manici, 2012). This soil condition, often termed as “replant disease”, has been successfully managed using soil fumigation despite a cryptic etiology (Mai and Abawi, 1981). Greenhouse experiments using plants potted in field soils afflicted with apple replant disease showed dramatic increases in seedling growth when soils were sterilized, and plant growth constraints similar to those in untreated field soil could be reintroduced when sterilized soils were amended with as little as 10% non-sterile field soil (Hoestra, 1968). Further greenhouse experiments have shown increased mass in apple seedlings planted in apple replant disease afflicted field soils treated with selective biocides to control for nematodes, oomycetes, or fungi, but these increases in plant growth were not observed in all sites sampled or as dramatic increases in seedling mass as occur following sterilization (Tewoldemedhin et al., 2011). Location specific differences in plant growth response to selective biocides and plant growth differences between selective biocides and sterilization suggest that variable and diverse microorganisms contribute to detrimental soil microbial communities associated with continuous apple cultivation.

In Australia, differences in sugarcane growth in nearby fields of soil with no recent history of sugarcane cultivation or soil with a long-term sugarcane cropping history have been reported as early as 1935 (Magarey, 1996). In the late 1970's and early 1980's, widespread root rot and cane lodging led to coinage of the term "Northern Poor Root Syndrome" to describe poor root development due to biotic and abiotic soil properties (Egan et al. 1984). Further research regarding soil health under continuous sugarcane cultivation lead to the conclusion that Northern Poor Root Syndrome would be more aptly described as a symptomatic response of plants to underlying soil health issues that were better referred to as "yield decline". Sugarcane yield decline has been defined as "the diminishing ability of caneland to produce sugar per harvested hectare" (Magarey, 1994) and "the loss of productive capacity of sugarcane soils under long-term monoculture" (Garside et al., 1997). Visible symptoms of yield decline include a browning of root surfaces, a reduction in the development of fine root systems, and a reduction in overall plant mass (Magarey et al. 1997a). Reports of yield constraints associated with sugarcane monoculture have been reported in Hawaii, Jamaica, and Australia (Innes et al. 1958; Magarey, 1996; Martin et al. 1959) and anecdotally in Louisiana.

The continuous cultivation of sugarcane involves intensive management practices, such as annual fertilizer and pesticide applications, post-harvest burning of crop residues, and the use of heavy machinery for planting and harvesting that may alter soil chemical and physical properties. In general, soils under continuous cultivation of sugarcane exhibit acidification as well as lower cation exchange capacity, less soil organic matter, and increased soil compaction (Bramley et al. 1996; Wood 1985). Site specific differences in the availability of soil micronutrients have also been reported. While iron, copper, and zinc may be more available in soils only recently converted to sugarcane, conflicting results have been obtained in regards to magnesium availability (Bramley et al. 1996; Wood 1985).

A number of studies have implicated a biological component to yield decline in sugarcane. Some of the earliest studies suggesting a biological etiology utilized sterilization of poor yielding field soils as a way to measure their maximum yield potential in the absence of detrimental soil microbiota. When fumigated with methyl bromide, soils under long-term sugarcane out-yielded their non-fumigated counterparts by 20% or more in both field and greenhouse settings (Egan et al. 1984; Garside et al. 1997; Hoy and Schneider 1988a; Magarey and Croft 1995). Similar plant growth responses have occurred in sugarcane fields previously exhibiting yield decline following prolonged heating via solarization (Reghenzani 1988). The yield decline condition was transferred in greenhouse experiments incorporating yield decline afflicted soils to sterile soils at rates as low as 10% resulting in reduced plant growth and increased root rot (Croft et al. 1984).

The contribution of known soilborne sugarcane pathogens to yield decline varies globally. Research in Australia led to the discovery of a previously unidentified endemic oomycete, *Pachymetra chaunorhiza* which has been correlated with the severity of yield decline symptoms and is believed to play a major role in Australian yield decline in high rainfall production areas (Magarey 1984). In Louisiana, various *Pythium* species, most notably *P. arrhenomanes*, are notable soilborne pathogens that, in addition to environmental factors such as freezing or cold temperatures and poor drainage, cause a condition known as “Stubble Decline” that adversely affects sugarcane growth and ratooning ability (Edgerton 1939; Hoy and Schneider 1988b).

Nematodes are also a well-documented group of soilborne sugarcane pathogens. At least 275 species from 48 genera have been associated with sugarcane, but *Pratylenchus*, *Tylenchorhynchus*, *Helicotylenchus*, and *Meloidogyne* are globally the most common parasitic nematodes associated sugarcane (Spaull and Cadet 1990). Surveys of nematode populations in Louisiana soils have also found *Mesocriconema* and *Paratrichodorus* commonly occur in sugarcane (Bond et al. 2000). While studies have yet to specifically compare nematode populations in soils under short and long-term sugarcane cultivation in Louisiana, pasture break, crop rotations to soybean, navy bean, peanut, and maize and bare fallow in Australia have been shown to reduce the numbers of the lesion nematode *Pratylenchus zeae* as compared to continuous sugarcane cultivation (Pankhurst et al., 2005a).

The use of selective biocides suggests the involvement of broad groups of soilborne organisms in limiting sugarcane yields. Soils treated with the fungicide mancozeb (manganese ethylenebis(dithiocarbamate)(polymeric) complex with zinc salt) have shown an intermediate plant growth and root health response compared to total sterilization suggesting fungi are a part of the disease complex (Magarey et al. 1997a). Reduced root colonization by a dematiaceous, sterile fungus following mancozeb treatment was associated with improved plant growth. Nematicides have shown site specific improvement to plant growth when applied alone, but have consistently improved plant growth in combination with mancozeb (Garside et al. 2002). Treatment of soil with metalaxyl (Methyl N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-L-alaninate), an oomycete specific fungicide used to control *Pythium*, provided an intermediate increase in plant growth response between fumigation and an untreated control (Hoy and Schneider 1988a). Differences in plant growth response between selective biocides and total soil sterilization suggest diverse organisms associated with sugarcane roots not recognized as pathogens contribute to the detrimental sugarcane growth associated with monoculture. Deleterious rhizosphere microorganisms have been associated reduced productivity in multiple cropping systems (Barazani and Friedman 2001; Nehl et al. 1996; Schippers et al. 1987; Suslow and Schroth 1982). The biological component of yield decline therefore appears to represent a community level effect.

Alternative cropping practices have also alleviated plant growth constraints associated with long-term sugarcane cultivation, including pasture break, crop breaks, and bare fallow. Each produced increases in the number of millable stalks and overall sucrose yields, when compared to continuous sugarcane cultivation, and restored yields to levels comparable to methyl bromide fumigation (Garside et al., 2002). Organic amendments have also been used to improve plant growth (Dissanayake and Hoy, 1999; Pankhurst et al., 2005a). Sugarcane field soils amended with steam-treated filter-press cake, gin-trash compost, and municipal biosolids had no significant effect, while their non-sterile counterparts improved root health and plant growth (Dissanayake and Hoy, 1999).

One approach to improving the understanding of the biological etiology of yield decline has been to perform extensive isolations from the roots and rhizosphere of yield decline afflicted plants, identify them, and evaluate their pathogenicity to sugarcane by inoculating healthy plants in sterilized soil then examining their effect on plant growth. Due to the positive plant growth response of sugarcane in soils treated with fungicides, fungi have been of particular interest in unraveling the cause of yield decline. An Australian study isolated *Penicillium, Cuvularia, Humicola, Phoma, Gongronella, Mortierella, Chaetomium, Aspergillus, Fusarium, Trichoderma, Cladosporium, Thielaviopsis, Rhizoctonia*, and sterile, dematiacous fungi, but only found that sterile isolates significantly reduced root growth (Magarey and Croft, 1995). A fungal root colonization study from Taiwan consisting of 1,010 isolates found 81% belonged to ten genera: *Fusarium, Trichoderma, Rhizoctonia, Marasmius, Pythium, Monilia, Collectotrichum, Phialophora, Humicola*, and *Thielaviopsis* (Watanabe, 1974). While not all isolated fungi were tested, isolates of *Fusarium, Rhizoctonia, Marasmius, Pythium*, and *Thielaviopsis* were found to have variable degrees of pathogenicity to sugarcane in field or growth chamber experiments. Additional research focusing on the role of frequently isolated *Fusarium* and *Trichoderma* in sugarcane monoculture soils found that culture filtrates reduced sugarcane germination and root growth, suggesting these fungi may produce phytotoxic compounds detrimental to sugarcane growth (Kao and Hsieh, 1986).

Other studies have focused on enumerating and comparing culturable taxa between paired sites with short and long-term sugarcane cropping histories (Pankhurst et al., 2000; Savario and Hoy, 2010). The frequencies of culturable microorganisms compared using various selective media found rhizosphere soils from paired sites under long and short-term sugarcane production differed in the numbers of total bacteria and fungi, fluorescent and total pseudomonad bacteria, actinobacteria, siderophore producing bacteria, and fusarial fungi. However, these differences were not consistent among paired sites suggesting biological factors associated with yield decline may not only vary globally but also locally from site to site as well.

Yield decline research reliant on the culturing of bacteria and fungi may overestimate the importance of those organisms that are readily culturable. *In situ* enumeration of bacterial cells through epiflourescent micrography suggested a single gram of soil may contain as many as 10^{10} bacterial cells distributed amongst 10^4 species, whereas only 10^6 colony forming units were detected by plating (Roesch et al., 2007; Torsvik and Øvreås, 2002). There are roughly 3,150 described and readily culturable species of free-living soil fungi, but estimates as to the total number of species range as high as 5.1 million (Blackwell, 2011; Gams, 2007).

Community level analysis of soil microbiomes provides alternative strategies to elucidate differences in sugarcane microbiomes associated with short and long-term cropping history that experiments focusing on individual plant-microbe interactions may not. Plant species and even genotypes within species mediate rhizosphere microbial community structure through characteristic root exudate profiles creating a dynamic interplay that can affect plant health and productivity (Badri et al., 2009; Berg and Smalla, 2009; Broeckling et al., 2008; Garbeva et al., 2008; Grayston et al., 1998). Methods capable of characterizing the composition of the rhizosphere microbial community offer the potential to unravel root microbiome effects on plant growth and crop yield.

One earlier method compared community level physiological profiles by the pattern of utilization of sole carbon substrates (Garland and Mills, 1991). Community level physiological profiles of rhizosphere soil extracts from short and long-term sugarcane cropping histories found no consistent trends suggesting commonality in rhizosphere prokaryotic communities between short and long-term sugarcane cultivation, but principal component analysis separated locations and individual short and long-term paired sites at some locations (Savario and Hoy, 2010).

Additional methods commonly used in soil microbial ecology have yet to be applied to comparisons between short and long-term sugarcane cultivation in Louisiana. Fatty acid methyl esters (FAME) are the products of fats and methanol that persist in the soil. FAMEs can be extracted and measured using gas chromatography to produce profiles of the relative and absolute abundance of broad taxonomic groups, such as Gram negative bacteria, Gram positive bacteria, actinobacteria, protozoans and nematodes, fungi and arbuscular mychorrhizal fungi (Buyer and Drinkwater, 1997; Cavigelli et al., 1995; Schutter and Dick, 2002). Extracellular soil enzymes catalyze the breakdown of biopolymers, such as cellulose and chitin, and are essential to the cycling of soil nutrients. The activity of these enzymes determined by measuring the colorimetric reactions of cleaved p-nitrophenol tagged substrates can be an informative indicator of the functional diversity of soil samples (Bandick and Dick, 1999).

Handelsman et al. (1998) proposed the use of cloning and DNA sequencing in order to study the putative gene functions and chemistry of culturable and non-culturable

microbes as a way to bridge the gap between genomics and microbial ecology and coined the term “metagenomics” to refer to collective genomes of an ecosystem. Advances in high throughput nucleic acid sequencing technology have allowed researchers to produce a greater number of shorter sequencing reads at a significantly lower cost. As a result, metagenomics studies have transitioned from a cloning and Sanger sequencing approach to using a shotgun sequencing of whole genomes or a targeted amplicon-based approach using an informative marker gene.

In 1977, the genes from 16s rRNA and 18s rRNA for were used for phylogenetic analysis that reclassified life into three major groups: bacteria, archaea, and eukaryotes (Woese and Fox, 1977). These genes, which when transcribed produce the ribosomal RNA necessary for the translation of proteins, contained both conserved and highly variable regions which were taxonomically informative. Herbert et al. (2003) proposed DNA barcoding using databases of similarly informative genes as a reliable and efficient method of species identification. While efforts to discover a reliable universal barcode for fungi have not been as successful as the 16S gene in prokaryotes, the internal transcribed spacer (ITS) has been proposed as the most reliable single locus across the fungal kingdom (Schoch et al., 2012). Revised 16S and ITS PCR primers recommended by the Earth Microbiome Project minimize biases in amplification of specific taxa to better capture the structure of microbial communities (Apprill et al., 2015; Smith and Peay, 2014).

Illumina’s “sequencing by synthesis” approach is increasingly used in amplicon-based metagenomics and offers two platforms that provide different levels of coverage: HiSeq and MiSeq. Hiseq can produce over 50 gigabases of data consisting of up to 1.6 billion 100-base paired-end reads during a 10.8 day run, whereas MiSeq produces up to 2.5 gigabases per run from 5 million 150-base or 251-base paired-end reads in a single day. However, despite large differences in sequencing depth, both platforms produce similar metagenomic profiles (Caporaso et al., 2012).

Operational taxonomic units (OTUs) are empirically classified groups based on datasets of recorded characters (Sokal and Sneath, 1963). In the context of amplicon-based metagenomics, operational taxonomic units are commonly defined as a collection of sequences that are no more than 3% different from each other (Schloss and Handelsman, 2006). The clustering of amplicons into OTUs in a crucial and computationally demanding step in the analysis of amplicon sequencing data. Qiime (quantitative insights into microbial ecology) is an open-source pipeline that allows for processing of large datasets created by Illumina and other next generation sequencing platforms. Qiime can be used for the demultiplexing of libraries, quality filtration of reads, OTU picking, taxonomic assignment, and downstream diversity analysis (Caporaso et al. 2010). Qiime also allows the use of curated third-party databases, such as the SILVA 16S and Unite ITS databases, to aid in reference-based OTU clustering and taxonomic assignment (Abarenkov et al., 2010; Quast et al., 2012).

Qiime's core diversity analysis workflow includes both α-diversity metrics that measure the diversity within samples and β-diversity metrics for comparisons of diversity among samples. One of the most commonly used forms of β-diversity distance-based redundancy analysis uses dissimilarity matrices that transform sets of sequences into a matrix of pairwise comparisons based on the abundance of OTUs, as is the case for Bray-Curtis, or the total phylogenetic distance of communities, as is the case for Unifrac (Bray and Curtis, 1957; Lozupone and Knight, 2005). Distance-based redundancy analysis can then transform these matrices into an ordination based on axes of constrained variance to reveal any broad-scale ecological differences in soil microbial communities associated with short and long-term sugarcane cultivation or other land management metadata. *Vegan*, a community analysis package for the statistical computing environment R, allows for post-analysis environmental interpretation of distance-based redundancy analysis by correlating additional metadata, such as soil nutrient data, with variation in soil microbial community structure to account for both biotic and abiotic differences in samples (Oksanen et al., 2017).

The decline in yields associated with long-term sugarcane cultivation remains an incompletely understood complex soil health issue involving biotic and abiotic factors. The objective of this study was to analyze differences in the soil microbial ecology between paired sites under short and long-term sugarcane cultivation at multiple locations by using culture-independent techniques to identify members and compare structure of microbial communities along with an evaluation of the involvement of soil nutrient availability and soil extracellular enzyme activities to better understand how the continuous cultivation of sugarcane affects soil health and crop productivity.

Chapter 2: Soil and Plant Growth Analysis in Short and Long-term Sugarcane Cropping Systems

2.1 Introduction

Sugarcane (*Saccharum* spp. inter-specific hybrids) is a perennial grass grown in tropical to subtropical regions of the world. Sugarcane is harvested for the sucrose stored in its stalks that can be refined into sugar and more recently used as a source of biomass feedstock for bioenergy. In the United States, cultivation is restricted to the southernmost parts of the country, including southern Louisiana, Florida, and Texas.

In portions of Louisiana with climatic conditions favorable for sugarcane production, drainage issues constrain where the crop is grown. Historically, sugarcane has been planted in alluvial soils that can be drained in areas with climatic conditions not favorable for cultivation of other crops. As a result, sugarcane has been continually produced on some of these soils for over 200 years (Hilliard, 1979).

Reports of differences in plant vigor between soils that have continuously been under sugarcane production and lands recently converted to sugarcane cultivation have been reported as early as 1935 (Magarey 1996). Sugarcane yield decline has been defined as “the diminishing ability of caneland to produce sugar per harvested hectare” (Magarey 1994) and “the loss of productive capacity of sugarcane soils under long-term monoculture” (Garside et al. 1997). Visible symptoms of yield decline include a browning of root surfaces, a reduction in the development of fine root systems, and a reduction in overall plant mass (Magarey et al. 1997a). Yield constraints associated with sugarcane monoculture have been reported for Hawaii, Jamaica, and Australia (Magarey, 1996; Martin, et al., 1959; Innes and Manser, 1958) and anecdotally for Louisiana.

Sugarcane growers utilize a number of techniques that may alter the chemical and physical structure of the soil, such as annual fertilizer and pesticide applications, post-harvest burning of crop residues, and the use of heavy machinery for planting and harvesting. Studies examining the agronomic effects of continuous sugarcane cultivation on soil properties have reported acidification of soils, lower cation exchange capacity, decreased soil organic matter, and increased soil compaction (Wood, 1985; Bramley et al., 1996). The availability of specific soil nutrients under short (no recent) and long-term sugarcane production vary by location. Studies comparing sites that differ by sugarcane cropping history found that iron, copper, and zinc may be more available in soils only recently converted to sugarcane, but conflicting results about magnesium availability have been reported (Wood, 1985; Bramley et al., 1996).

A much larger emphasis has been placed on elucidating a biological component to yield decline. The use of soil sterilization techniques, such as methyl bromide fumigation or soil solarization, have increased plant growth in poor yielding soils under continuous sugarcane cultivation by 20% or more in both field and greenhouse settings (Bell 1935, Egan et al. 1984, Magarey and Croft 1995, Garside et al., 1997; Muchow 1994, Hoy and

Schneider, 1988a; Reghenzani et al., 1988). Growth-limiting soil-borne biological agents involved in yield decline have been transferred from afflicted soils to “healthy” soils by incorporation rates as low as 10% (Croft et al., 1984).

Soil treatments with selective biocides for fungi, oomycetes, and nematodes have all improved plant growth, but not to the same extent as methyl bromide (Magarey et al., 1997; Garside et al., 2002; Hoy and Schneider, 1988a). The intermediate plant growth responses to selective biocides compared to fumigation suggest a diverse community of organisms is involved in the monoculture effect.

Poor yielding soils under continuous sugarcane cultivation can also be improved by crop rotation to pasture, crop rotation, and bare fallow (Garside et al., 2002) and by organic amendments (Pankhurst et al., 2005a). Organic amendments, such as steam-treated filter press cake, gin trash compost, and municipal biosolids provided significant plant growth increases, whereas their sterile counterparts did not (Dissanayake and Hoy, 1999).

Community level analyses of soils cropped to sugarcane can contribute to a better understanding of differences in the soil microbial ecology associated with short and long-term cropping histories. Studies comparing culturable taxa detected qualitative differences in rhizosphere bacterial and fungal communities associated with the different cropping histories in Australia (Magarey et al., 1997; Pankhurst et al., 2000) and Louisiana (Savario and Hoy, 2010). Community level physiological profiles comparing the pattern of utilization of various sole carbon substrates also can provide information on qualitative differences in prokaryotic communities (Garland and Mills, 1991). Community level physiological profiles of rhizosphere extracts distinguished individual locations with differences detected between paired sites with short and long-term sugarcane cropping histories at some locations (Savario and Hoy, 2010).

Additional methods commonly used in soil microbial ecology have yet to be applied to comparisons between short and long-term sugarcane cultivation in Louisiana. Extracellular soil enzymes catalyze the breakdown of biopolymers, such as cellulose by β-glucosidase, and chitin by *N*-acetyl-β-glucosaminidase, and are essential to the cycling of soil nutrients. The activity of these enzymes can be an informative indicator of the functional diversity of soil samples by measuring the colorimetric products of cleaved p-nitrophenol tagged substrates (Bandick and Dick 1999).

Fatty acid methyl esters (FAME) are the products of fats and methanol that persist in the soil. Certain FAMEs are unique biomarkers for broad taxa, such as Gram negative bacteria, Gram positive bacteria, actinobacteria, protozoans and nematodes, fungi and arbuscular mychorrhizal fungi (Cavigelli et al. 1995, Buyer and Drinkwater 1997, Schutter and Dick 2002). FAMEs can be extracted and measured using gas chromatography to produce profiles of the relative and absolute abundance of these broad taxonomic groups to profile the soil microbial community. These profiles can be visualized using distance-based redundancy analysis to reveal any broad-scale ecological differences in the of soil microbial communities associated with short and

long- term sugarcane cultivation or other land management metadata. *Vegan*, a community analysis package for the statistical computing environment R, allows for post-analysis environmental interpretation of distance-based redundancy analysis by correlating additional metadata, such as soil nutrient data, with variation in soil microbial community structure to account for both biotic and abiotic differences in samples (Oksanen et al. 2017).

The decline in yields associated with continuous sugarcane cultivation remains a plant and soil health issue involving biotic and abiotic factors. The objectives of this study were to document differences in plant growth between paired sites under short and long-term sugarcane cultivation and analyze abiotic and biotic factors potentially affecting the soil microbial ecology. Culture-independent techniques were used to identify members and compare the structure of microbial communities and examine the roles of soil nutrients and soil extracellular enzymes to better understand how the continuous cultivation of sugarcane affects soil health.

2.2 Materials and Methods

2.2.1 Paired Site Selection and Soil Sampling

Paired sites for soil sampling were identified based on the following criteria: 1) soil with long-term history of sugarcane cultivation and neighboring land newly converted to sugarcane cultivation both in the first year crop described as plant cane, 2) sugarcane of the same cultivar was planted at both sites in the same season, and 3) paired sites had the same or similar soil series (Table 2.1). The soils with no recent history of sugarcane cultivation previously supported mixed plant communities, either pasture or forest.

Three pseudoreplicates were established for each of two soil niches, bulk and rhizosphere soil, per paired site. Each pseudoreplicate was composed of four subsamples for a total of 12 subsamples (250 grams each) of bulk soil and 12 corresponding subsamples (100 grams each) of rhizosphere soil. Sugarcane plants were dug out from row beds to expose root systems with intact rhizosphere soil. Rhizosphere soil was considered to be soil closely adhering to roots that could be removed without damaging the root cortex. Roots and rhizosphere were cut from plants and placed in a cooler until they could be separated in the lab. Roots from sampling in 2015 were preserved in formalin for later use. Large aggregates wholly adhering to the root system were avoided. Bulk soils from an area adjacent to rhizosphere sampling were collected at a minimum depth of 150 mm. Samples were bagged and placed in coolers with icepacks during transportation and subsequently processed.

Three locations with plant cane in soils with short and long-term sugarcane cropping histories were sampled during the summer of 2014. An additional three locations of plant cane were sampled in of 2015, and the Gonsoulin and St. Gabriel sites were resampled during the first ratoon crop in 2016 to examine changes in soil microbial ecology based on crop year (Table 2.1). The St. Gabriel site was sampled a second

time in first ratoon a week later following rainfall to examine variation related to sampling conditions.

Table 2.1. Descriptions of paired sites with short and long-term histories of sugarcane cultivation at six locations. First year sampling dates were in plant cane and second year sampling dates were in first ratoon cane at two locations.

Location	Coordinates	Sampling dates	Cultivar	Soil series	Cropping history	Texture
Airport	30.0192ºN 91.8758ºW	6/9/2014	HoCP 04-838	Jeanerette Silt Loam	Sugarcane	Silt Loam
					Forest	Silt Loam
Belleview	30.2256ºN 91.2561ºW	6/24/2014	HoCP 96-540	Commerce Silt Loam	Sugarcane	Silt Loam
					Pasture	Loam
Jefferson	29.9937ºN 91.9180ºW	7/3/2014	HoCP 96-540	Jeanerette Silt Loam	Sugarcane	Silt Loam
					Pasture	Silt Loam
Gonsoulin	30.0150ºN 91.9164ºW	6/5/2015 6/27/2016	HoCP 96-540	Jeanerette Silt Loam	Sugarcane	Silt Loam
					Forest	Silt Loam
Iberia	29.9430ºN 91.7125º W	6/3/2015	HoCP 96-540	Schriever Clay	Sugarcane	Silty Clay
					Pasture	Clay
St. Gabriel	30.2698ºN 91.1035ºW	6/8/2015 6/23/2016 6/30/2016	L 01-299	Commerce Silty Clay Loam	Sugarcane	Silty Clay Loam
					Pasture	Clay Loam

Bulk soils were sieved to a uniform size of 4.75 mm. Rhizosphere soils were separated from roots and remaining plant debris was removed from samples by hand. The four subsamples from each psuedoreplicate were combined for bulk and rhizosphere for a total of three bulk soil and three rhizosphere samples, respectively, from each site. Six paired sites were sampled for both bulk and rhizosphere soil for a total of 36 bulk and 36 rhizosphere soil samples from the plant cane crop. An additional 18 bulk and rhizosphere samples (six from Gonsoulin and 12 from St. Gabriel) were collected from paired sites in first ratoon. Combined samples were divided and stored at -80ºC for DNA extraction and sequencing or at -20ºC for fatty acid extractions, whereas air-dried bulk soil samples were stored at room temperature for enzyme assays and chemical analyses.

2.2.2 Greenhouse Experiment

Single-node-cuttings of the sugarcane cultivar HoCP 96-540 were collected from the Louisiana State University Agricultural Center Sugar Research Station at St. Gabriel, LA. Cuttings were treated in a 50ºC hot water bath for 20 min before planting in Speedling Styrofoam trays with steam-treated soil in a greenhouse at Louisiana State University campus in Baton Rouge, LA. Single node cuttings were allowed to grow for 3 wk before being transplanted to field soils.

Field soil samples were collected from the paired sites sampled in 2015, St. Gabriel, Gonsoulin, and Iberia (Table 2.1), following the methods described above. Field soils were air-dried and sieved to uniform particle size. Each sample was then divided in two: one half steam-treated and the other untreated. Soils for planting were mixed with equal parts field soil and steam-treated sand to improve pot drainage. Each mixed soil-sand sample was then divided in thirds and placed in 3.8 L pots. Sugarcane plants (108 total) were selected and transplanted into sand-soil treatment pots. Plants were then watered daily and treated with fertilizer weekly starting 1 wk after transfer using 100 ml of Miracle-Gro All-Purpose Plant Food Fertilizer solution (Scotts, Marysville, OH).

Plants from all treatments were evaluated and compared for different growth parameters 2 mon after transplanting. Root systems and shoots were separated, and plant roots were washed free of any soil. Plants were measured for fresh root weight, dry root weight, dry shoot weight, height at the top visible dewlap, and shoot number.

2.2.3 Field Crop Yield Component Estimates

Individual and aggregate yield components were determined and compared for paired sites at the three locations in plant cane during 2015 and two locations in first ratoon during 2016. The stalk populations for sugarcane in short and long-term paired sites were estimated by averaging the stalk counts from ten, 3 m row lengths. Five 10-stalk bundles were cut, weighed, and measured to estimate stalk weights and heights. Total recoverable sugar was determined using near-infrared spectroscopy and commercially recoverable sugar (CRS) was estimated as 85% to total recoverable sugar. Aggregate yield components Mg of cane per hectare and Mg of sucrose per hectare were calculated using stalk population, stalk weight, and CRS.

2.2.4 Soil Analysis

2.2.4.1 Particle Size Analysis

Fifty grams of air-dried soil was placed in a shaker bottle with 10 ml of 10% sodium hexametaphosphate and deionized water then samples were placed on a reciprocal shaker for 4 h. The soil suspension was then transferred to a 1 L graduated cylinder and adjusted to a 1 L volume with deionized water and then stirred further. A hydrometer was used to measure density after 40 sec and 24 h of stirring. Sand content was determined by calculating the proportion of the initial soil samples no longer in

suspension. Clay content was determined by calculating the proportion of soil samples still in suspension at the 24 h reading, and the silt content was calculated by subtracting the previously calculated clay and sand content. Soil texture was reported in Table 2.1 using the average sand, silt, and clay content.

2.2.4.2 Soil Moisture and Organic Matter

In order to determine soil moisture, field moist soils were weighed and subsequently oven-dried overnight at 105ºC before being cooled in a desiccator. Dried samples were then re-weighed. Organic matter was measured using a loss-on-ignition method. Moisture-free samples were placed in a muffle furnace at 400ºC for 24 h and weighed a third time. The difference between first and second weights was used to determine soil moisture. The difference between second and third weights was used to determine organic matter.

2.2.4.3 Soil Testing

Bulk soil samples were sent to the LSU AgCenter Soil Testing and Plant Analysis Laboratory for testing. Soil pH (1:1 soil:water) testing was performed as described by Mclean (1982). Plant available phosphorus, potassium, calcium, magnesium, sodium, and sulfur were extracted by Mehlich 3 solution and analysed using atomic absorption sepctroscopy (Mehlich 1984). Copper, zinc, manganese, and iron were quantified using DTPA extractable (Baker and Amacher 1982). Soluble salts were determined by conductivity following the Rhoades (1982) method. Total carbon and total nitrogen were determined using dry combustion.

2.2.4.4 Inorganic Nitrogen

Protocols to quantify inorganic nitrogen were adapted from Hood-Nowonty et al. (2010) and Weatherburn (1967). A gram of air-dried soil and 10 mL of 2 *M* KCl were placed in a 15 mL centrifuge tube and placed on a reciprocal shaker for 1 h. Soil solutions were then filtered through Whatman No. 42 filter paper, and 40 µL of filtrate was placed into a microplate well pre-loaded with 200 µL of saturated vanadium (III) chloride solution (0.5 g VCl_3, 0.2 g sulfanilamide, and 0.01 g N-1-ethylenediamine dihydrochloride dissolved in 200 mL of 0.5 *M* HCl). Samples were incubated at 37 ºC for 1 h. Nitrate-N was quantified by linear regression of potassium nitrate standards measured at an absorbance of 540nm using an Eon Microplate Spectrophotometer (BioTek, Winooski, VT). Nitrate-N was reported in mg/kg. In order to quantify ammonium-N, 40 µL of filtrate was placed into a microplate well pre-loaded with 100 µL of a sodium salicylate solution (6.8 g sodium salicylate, 5.0 g sodium citrate, 5.0 g sodium tartrate, and 0.025 g sodium nitroprusside dissolved in 100 mL of deionized water). In order to induce a colorimetric reaction, 100 µL of 2% bleach in 1.5 *M* NaOH was added to microplate wells. Samples were incubated for 50 min at room temperature. Ammonium-N was quantified by linear regression of ammonium sulfate standards measured at an absorbance of 540 nm using a BioTek Eon Microplate Spectrophotometer. Ammonium-N was reported in mg/kg.

2.2.5 Soil Extracellular Enzyme Activity

The protocol described by Tabatabai (1994) was used to measure the activity of β-glucosidase in bulk soils. Air-dried bulk soil (0.5 g each) was added to three 50-mL flasks serving as two experimental replicates and a control for each soil sample. Modified universal buffer (2 mL - 1 *M* tris(hydroxymethyl)aminomethane (THAM), 1 *M* sodium hydroxide, 0.1 *M* boric acid, 0.1 *M* maleic acid, and 0.07 *M* citric acid) pH 6.0 was added to all samples, and 0.5 mL of 0.05 *M* p-nitrophenyl β-D-glucoside solution was added to the experimental replicates. Samples were then capped with rubber stoppers, mixed, and incubated at 37ºC for 1 h. Following incubation, samples were uncapped, and 0.5 mL of 0.5 *M* calcium chloride was added. Samples were stirred then 2 mL of 0.1 *M* THAM pH 12 was added and mixed well. The p-nitrophenyl ß-D-glucoside solution was then added to control samples.

Standards of p-nitrophenol were prepared from stock 1 g L^{-1} solution of p-nitrophenol by making a 1:100 dilution and subsequently pipetting 0, 1, 2, 3, 4 and 5 mL of diluted p-nitrophenol into clean glass vials. Standards were adjusted to a volume of 5 mL using deionized water for concentrations of 0, 10, 20, 30, 40, and 50 µg mL^{-1} before adding 1 mL of $CaCl_2$ and 4 mL of THAM.

Samples and standards were filtered into clean Erlenmeyer flasks through Whatman No. 2 filter paper. For each sample and standard, three-200 µL aliquots were transferred into wells on a 96-well plate. Colorimetric analysis of p-nitrophenol was measured by absorbance at 420 nm using a BioTek Eon Microplate Spectrophotometer. Linear regression of p-nitophenol standards was performed in Microsoft Excel and used to calculate the µg mL^{-1} concentration of p-nitrophenol in samples. Glucosidase activity was reported in µmol p-nitrophenol g^{-1} h^{-1}.

The protocol described by Tabatabai (1994) was used to measure the activity of *N*-acetyl-β-glucosaminidase in bulk soils. Air-dried bulk soil (0.5 g) was added to three 50-mL flasks serving as two experimental replicates and one control for each soil sample. Sodium acetate buffer (0.1 *M*) was added to each flask followed by 0.5 mL of 10 *mM* p-nitrophenyl-*N*-acetyl- β -D-glucosaminide solution in the experimental replicates only. Samples were capped with rubber stoppers, mixed, and incubated at 37ºC for 1 h. Following incubation, samples were uncapped, and 0.5 mL of 0.5 *M* $CaCl_2$ was added. Samples were stirred prior to adding 2 mL of 0.1 *M* THAM pH 12 and mixed again. The p-nitrophenyl-*N*-acetyl-B-D-glucosaminide solution was then added to control samples.

Standards of p-nitrophenol were prepared as described above. Samples and standards were then analyzed to determine enzyme activity as described above for glucosidase. *N*-acetyl-β-glucosaminidase activity was reported in µmol p-nitrophenol g^{-1} h^{-1}.

2.2.6 Root Staining and Colonization

In order to estimate the extent of root colonization by fungal endophytes, an adaptation of the Barrow and Aaltonen (2011) staining process was used with the roots obtained

from the rhizosphere soil sampling in 2015. Roots were cut into 3 cm segments and placed within three plastic cassettes per sample (Hisosette I Cassettes, Product no. 27159-5, Ted Pella Inc.). Roots were cleared by immersion in a 10% KOH solution brought to a light boil for 20 min. Following clearing, roots were rinsed with tap water and soaked in a 10% hydrogen peroxide solution for 20 min followed by an additional 3 min of soaking in 1% HCl. Cassettes were then placed in a 50% acidic glycerol solution containing 0.05% HCl and 0.5% Trypan Blue brought to a light boil for 20 min to stain roots.

The extent of root colonization by fungal hyphae was quantified by a grid intersection method adapted from Giovannetti and Mosse (1980) wherein roots were arbitrarily placed on a petri dish with a half inch grid. At every intersection of a grid line and root segment an observation was made under a dissection microscope at 70x in order to determine the presence or absence of hyphae. Once every intersection was scored this way, roots were arbitrarily rearranged on the plate and scored again. This was repeated until 100 intersections were scored per cassette and 300 intersections per sample.

2.2.7 Fatty Acid Methyl Ester Analysis

Rhizosphere and bulk soil samples were sent to the USDA-ARS laboratory in Lubbock, Texas for methylation, neutralization, and extraction of ester-linked FAMEs. Gas chromatography was used to characterize individual fatty acid methyl esters. FAME biomarkers are classified based on the length of the carbon back-bone chain, the number of double bonds, and the location and positioning of the first double bond. FAME biomarkers represent several distinct taxa: Gram-positive bacteria (i15:0, a15:0, i17:0, a17:0), Gram-negative bacteria (cy17:0, cy19:0), actinobacteria (10Me16:0, 10Me17:0, 10Me18:0), saprophytic fungi (18:1ω9c, 18:2ω6c), and arbuscular mycohrrizal fungi (16:1ω5c) (Buyer and Drinkwater 1997; Cavigelli et al. 1995; Schutter and Dick 2002). Total FAMEs were analyzed using their absolute abundance, and community structure was analyzed using relative FAME abundance.

2.2.8 Statistical Analysis

Soil chemical and biological properties were analyzed by ANOVA using the SAS 9.4 (SAS Institute, Cary, NY) “PROC GLM” test with fixed effects of location and cropping history and pseudoreplicates as random effects when comparing samples from plant cane and fixed effects of location, cropping history, and crop year when comparing samples from plant cane and first ratoon. Treatment means were separated by a least significant difference post-hoc test. FAME results were transformed into a Bray-Curtis distance matrix using the “capscale” function and plotted by distance-based redundancy analysis in R using the *vegan* package (Oksanen et al. 2017). Environmental variables were plotted as vectors based on maximum correlation using the *envfit* function (Oksanen et al., 2017). An alpha level of $\alpha = 0.05$ was used for all analyses.

2.3 Results

2.3.1 Greenhouse Experiment

In a greenhouse experiment using field soils collected from the paired sites of short and long-term sugarcane cultivation sampled in 2015, cropping history and soil sterilization had a significant effect on all plant growth metrics (Table 2.2). Root weight was additionally affected by location. The location x cropping history interaction was significant for stalk and root weight, while location x sterilization interaction was significant for plant height. The three-way interaction was significant for stalk weight.

Table 2.2. ANOVA p-values for factors affecting plant growth traits determined in a greenhouse experiment growing sugarcane in sterile and non-sterile field soils collected from paired sites with short and long-term sugarcane cropping histories at three locations.

	Location	Cropping history	Sterilization
Plant height	NS	0.0001	0.0001
Stalk weight	NS	0.0006	0.0001
Root weight	0.0014	0.0058	0.0003

α = 0.05. NS = not significant.

	Location x Cropping history	Location x Sterilization	Cropping history x Sterilization	Location x Cropping history x Sterilization
Plant height	NS	0.0339	NS	NS
Stalk weight	0.0021	NS	NS	0.0210
Root weight	0.0007	NS	NS	NS

α = 0.05. NS = not significant.

Soil sterilization had a positive or neutral effect on three plant growth metrics, plant height (Figure 2.1), root weight (Figure 2.2), and stalk weight (Figure 2.3), determined for plants grown in pots of field soils from both short and long-term sugarcane cultivation. Plant height was greater in non-sterile short-term cultivation field soils than in non-sterile long-term cultivation field soils for two of three locations (Figure 2.1). However, stalk weight was greater in non-sterile field soils from short-term cultivation for only one of three locations (Figure 2.2), and root weight was not significantly different comparing non-sterile soils from short and long-term cultivation for any of the paired sites (Figure 2.3). In comparisons of plant growth in sterilized short and long-term cultivation soils, stalk height was greater for plants grown in short-term compared to long-term sugarcane cultivation soils for one of three locations; root weight was greater for plants grown in short-term cultivation soil for the same location; and stalk weight was greater for plants grown in short-term cultivation soils from two of three locations.

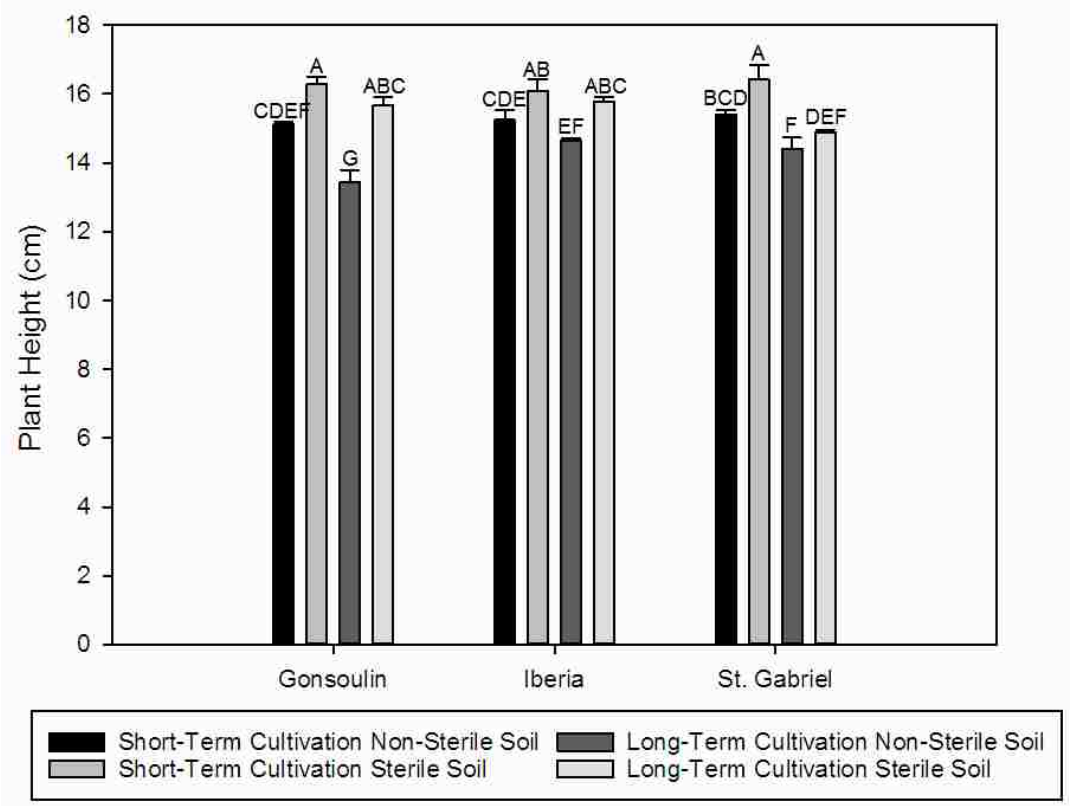

Figure 2.1. Height of sugarcane plants grown in sterilized and untreated field soils from paired sites with short and long-term sugarcane cropping histories at three locations. Letters represent significance class by least significant difference across all treatments. α = 0.05.

2.3.2 Field Crop Yield Component Estimates

All three individual and two aggregate yield components were affected by location and crop year, and all parameters with the exception of CRS were affected by cropping history (Table 2.3). The location x cropping history interaction was significant for CRS and tons of cane per hectare. The location x cropping history interaction was significant for stalk weight and tons of cane, and the cropping history x crop year interaction was significant for CRS.

Yields were generally higher in short compared to long-term sugarcane cultivation soils and in plant cane compared to first ratoon within locations (Table 2.4). The exception was CRS that was higher at two locations in soils under long-term cultivation.

2.3.3 Soil Chemical Properties

All soil chemical properties were affected by sugarcane cropping history and location of the paired sites, except manganese which was not affected by cropping history (Table 2.5). For the two locations evaluated in plant cane and first ratoon, organic matter, total nitrogen, soluble salts, sodium, calcium, magnesium, and manganese were affected by

the crop year. The cropping history x location interaction was significant for most soil chemical properties, whereas the cropping history x crop year and cropping history x location x crop year interactions were not significant for most properties (Table 2.5).

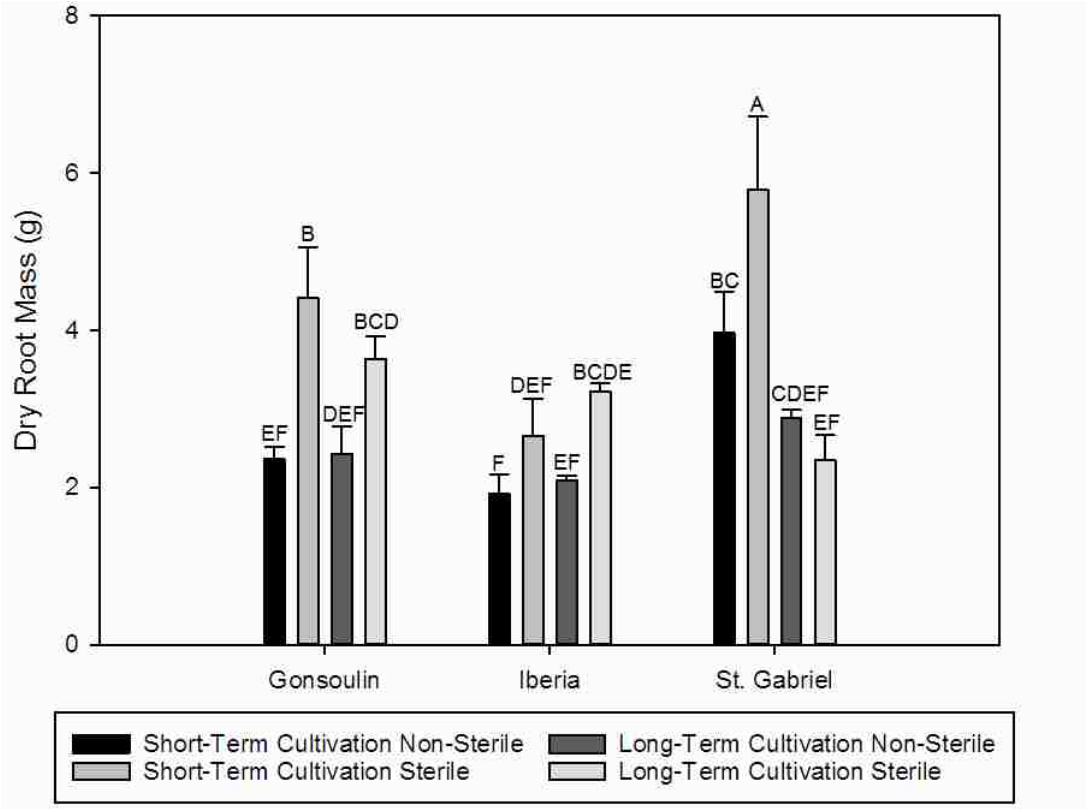

Figure 2.2. Root weight of sugarcane plants grown in sterilized and untreated field soils from paired sites with short and long-term sugarcane cropping histories at three locations. Letters represent significance class by least significant difference across all treatments. α = 0.05.

2.3.3.1 Soil Organic Matter

Soil organic matter was affected by cropping history, location, crop year, the cropping history x location interaction, and the three way interaction (Table 2.5). In plant cane, four of six locations had more organic matter in the short-term sugarcane cultivation soils than in the paired soils under long-term cultivation (Table 2.6). Higher organic matter content in soils with a short-term sugarcane cropping history persisted at the two locations that were resampled during the first ratoon crop (Table 2.6).

2.3.3.2 Soil pH

Location, cropping history, and all interactions involving cropping history had a significant effect on soil pH (Table 2.6). At two locations, soils were more acidic under long-term cultivation than short-term, whereas plant cane at one location and first ratoon at another were more acidic under short-term cultivation (Table 2.6)

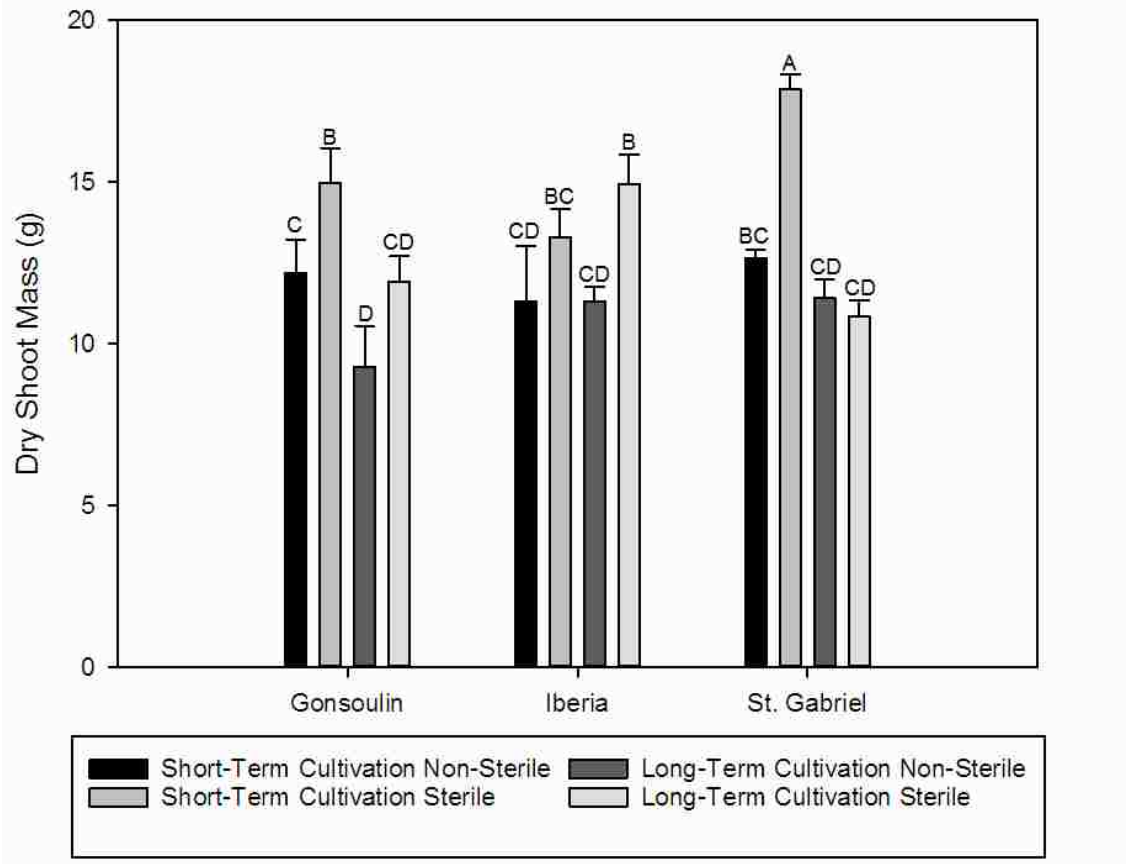

Figure 2.3. Stalk weight of sugarcane plants grown in sterilized and untreated field soils from paired sites with short and long-term sugarcane cropping histories at three locations. Letters represent significance class by least significant difference across all treatments. α = 0.05.

2.3.3.3 Soluble salts

Salts were affected by cropping history, location, crop year, and the cropping history x location interaction (Table 2.5). Greater soluble salts were present in soils under short-term cultivation at three locations, whereas salts were higher in the long-term cultivation soil in plant cane and first ratoon at the Gonsoulin location (Table 2.6).

2.3.3.4 Total Carbon and Nitrogen

Total carbon was affected by cropping history, location, and the cropping history x location, cropping history x crop year, and location x crop year interactions (Table 2.7). For the six locations in plant cane, three paired site soils had greater total carbon under short-term cultivation and three under long-term cultivation (Table 2.7). The two locations with first ratoon sampling exhibited greater total carbon in the soils under long-term cultivation in plant cane but greater total carbon in the soils under short-term sugarcane production in first ratoon (Table 2.7).

Table 2.3. ANOVA p-values for factors affecting five yield component estimates from paired sites with short and long-term sugarcane cropping histories in plant cane at three locations and first ratoon at two locations.

	Location	Cropping history	Location x Cropping history	Crop year	Location x Crop year	Cropping history x Crop year	Location x Cropping history x Crop year
Stalk weight	0.0001	0.0001	NS	0.0001	0.0034	NS	NS
Stalk sucrose content	0.0001	NS	0.0119	0.0001	NS	0.0003	NS
Stalk height	0.0001	0.0001	NS	0.0023	NS	NS	NS
Tons cane per hectare	0.0001	0.0001	0.0079	0.0001	0.0194	NS	NS
Sucrose per hectare	0.0001	0.0001	NS	0.0226	NS	NS	NS

$\alpha = 0.05$. NS = not significant.

Total nitrogen was affected by cropping history, location, crop year, and all two-way interactions (Table 2.7). Among the six locations evaluated in plant cane, total nitrogen was greater in soils under short-term cultivation at two locations but greater in soils under long-term cultivation at at three locations, including the two that were resampled during the first ratoon crop. In contrast, total nitrogen was greater under long-term cultivation in first ratoon crop at these two locations (Table 2.7).

2.3.3.5 Melich-3 Extractable Nutrients

Phosphorus, potassium, sodium, calcium, and magnesium were all affected by cropping history, location, and the location x cropping history interaction (Table 2.5). Calcium, magnesium, and sodium were also affected by crop year, and sulfur was affected by cropping history, location, and the location x crop year interaction (Table 2.5).

Comparisons of Melich-3 extractable nutrients in paired sites at six locations revealed higher levels of nutrients in soils under short-term cultivation when significant differences were detected with the exceptions of calcium at the Jefferson location and magnesium at the Airport and Iberia locations (Tables 2.8 and 2.9). Phosphorus was higher in short-term cultivation soils at two locations, potassium was higher at one location, sodium was higher at three locations, calcium was higher at four locations,

magnesium was higher at four locations, and sulphur was higher at two locations in plant cane and one location in first ratoon (Tables 2.8 and 2.9).

Table 2.4. Yield component estimates from paired sites with short and long-term sugarcane cropping histories in plant cane at three locations and first ratoon at two locations.

Location, crop year, and cropping history	Stalk weight (kg)	Stalk sucrose content (kg/ton)	Stalk height (m)	Cane per hectare (Mg)	Sucrose per hectare (Mg)
Iberia plant cane short-term cultivation	0.72 E	84.78 C	2.17 F	92.55 CDE	7.80 CD
Iberia plant cane long-term cultivation	0.55 F	82.46 C	1.82 G	57.00 G	4.70 E
Gonsoulin plant cane short-term cultivation	1.07 B	84.19 C	2.74 A	101.12 BCD	8.52 BC
Gonsoulin plant cane long-term cultivation	0.80 DE	93.81 B	2.37 DE	83.56 DEF	7.81 BCD
Gonsoulin first ratoon short-term cultivation	1.01 BC	103.62 A	2.58 AB	83.12 DEF	8.62 BC
Gonsoulin first ratoon long-term cultivation	0.76 E	96.76 AB	2.41 CDE	72.10 FG	7.02 CD
St. Gabriel plant cane short-term cultivation	1.26 A	70.53 D	2.65 AB	158.50 A	11.09 A
St. Gabriel plant cane long-term cultivation	0.95 BCD	82.59 C	2.56 ABC	103.61 BC	8.54 BC
St. Gabriel first ratoon short-term cultivation	0.86 CDE	85.80 C	2.51 BCD	111.18 B	9.58 AB
St. Gabriel first ratoon long-term cultivation	0.73 E	84.31 C	2.27 EF	77.06 EF	6.52 D

Letters within a column represent significance class by least significant difference across all locations, cropping history, and crop years. $\alpha = 0.05$.

Table 2.5. ANOVA p-values for soil chemical properties as affected by sugarcane cropping history, location, and crop year of paired sites.

	Cropping history	Location	Cropping history x Location	Crop year	Cropping history x Crop year	Location x Crop year	Cropping history x Location x Crop year
Soil organic matter	0.0001	0.0001	0.0001	0.0001	NS	NS	0.0093
pH	0.0463	0.0001	0.0001	NS	0.0051	NS	0.0401
Total carbon	0.0001	0.0001	0.0001	NS	0.0001	0.0029	NS
Total nitrogen	0.0001	0.0001	0.0001	0.0002	0.0001	0.0001	NS
Soluble salts	0.0143	0.0001	0.0001	0.0006	NS	NS	NS
P	0.0001	0.0001	0.0001	NS	NS	NS	NS
K	0.0001	0.0001	0.0001	NS	NS	NS	NS
Na	0.0001	0.0001	0.0001	0.0001	NS	NS	NS
Ca	0.0001	0.0001	0.0001	0.0374	NS	NS	NS
Mg	0.0001	0.0001	0.0001	0.0344	NS	NS	NS
S	0.0001	0.0001	NS	NS	NS	0.0449	NS
Cu	0.0139	0.0001	0.0008	NS	NS	NS	NS
Fe	0.0001	0.0001	0.0001	NS	NS	NS	NS
Mn	NS	0.0001	0.0092	0.0001	NS	NS	0.0032
Zn	0.0001	0.0001	0.0001	NS	NS	NS	NS

$\alpha = 0.05$. NS = not significant.

2.3.3.6 DTPA Extractable Nutrients

DTPA extractable micronutrients, copper, iron, and zinc, were affected by cropping history, location and the location x cropping history interaction (Table 2.5). Manganese was affected by crop year and the three-way interaction but not by cropping history (Table 2.10).

DTPA-extractable soil micronutrients were generally more available in short-term cultivation soils at six locations when significant differences were observed, with the exception of manganese, which was greater in long-term cultivation soil for plant cane at one location and the first ratoon of another (Table 2.10). Copper was more available in short-term cultivation soils at three locations; iron was more available at four locations; manganese was more available at one location; and zinc was more available at two locations (Table 2.10).

Table 2.6. Soil pH, organic matter, and soluble salts for bulk soils of paired sites with short and long-term sugarcane cropping histories at six locations.

Location	Crop year	Cropping history	Soil organic matter (g/kg)		Soil pH (1:1)		Soluble salts (g/kg)	
Airport	Plant cane	Short-term	61.5	DE	7.76	A	0.312	DE
		Long-term	50.0	F	7.89	A	0.322	CDE
Belleview	Plant cane	Short-term	58.9	E	7.55	A	0.271	DEF
		Long-term	37.2	G	6.67	B	0.117	GH
Jefferson	Plant cane	Short-term	42.8	FG	4.64	H	0.498	B
		Long-term	37.4	G	5.82	CDE	0.222	DEFG
Iberia	Plant cane	Short-term	92.5	C	5.31	FG	0.330	CD
		Long-term	89.2	C	5.09	GH	0.223	DEFG
Gonsoulin	Plant cane	Short-term	61.0	DE	6.23	BC	0.452	BC
		Long-term	40.7	G	5.68	DEF	0.677	A
	First ratoon	Short-term	86.9	C	6.02	CD	0.334	CD
		Long-term	50.5	F	6.57	B	0.479	B
St. Gabriel	Plant cane	Short-term	104	B	5.65	DEF	0.276	DEF
		Long-term	69.3	D	5.71	DEF	0.116	GH
	First ratoon	Short-term	122	A	5.63	DEF	0.176	FGH
		Long-term	92.0	C	5.74	DEF	0.075	H
	First ratoon, second sampling	Short-term	122	A	5.50	EFG	0.194	EFGH
		Long-term	90.5	C	5.87	CDE	0.082	H

Letters within a column represent significance class by least significant difference across all locations, crop years, and cropping histories. $\alpha = 0.05$.

2.3.3.7 Inorganic Nitrogen

Both nitrate-N and ammonium-N were affected by location and the location x cropping history interaction (Table 2.11). Nitrate-N was greater in short-term than long-term cultivation soils at one of six locations and greater in the long-term soil at one of six locations (Figure 2.4). Similarly, ammonium-N was greater in soil under short-term cultivation at the Jefferson location but greater in the long-term cultivation soil at one location (Figure 2.5).

2.3.4 Soil Extracellular Enzyme Activity

β-glucosidase and *N*-acetyl-β-glucosaminidase extracellular enzyme activities were both affected by cropping history, location and their interaction (Table 2.12). β-glucosidase activity was greater in short-term cultivation soils at two of six locations (Figure 2.6). β-glucosidase activity in the short-term cultivation soil at St. Gabriel was more than twice the activity reported in any other combination of location and cropping

hisotry. *N*-acetyl-β-glucosaminidase activity was greater in soils under short-term cultivation at three of six locations (Figure 2.7).

Table 2.7. Total carbon and nitrogen for bulk soils from paired sites with short and long-term sugarcane cropping histories at six locations in plant cane and two locations in first ratoon.

Location	Crop year	Cropping history	Total carbon (g/kg)		Total nitrogen (g/kg)	
Airport	Plant cane	Short-term	19.19	DEF	1.593	FE
		Long-term	16.33	H	1.407	GF
Belleview	Plant cane	Short-term	20.03	DE	2.033	C
		Long-term	9.087	J	0.967	H
Jefferson	Plant cane	Short-term	17.17	EFG	1.847	CDE
		Long-term	12.07	J	1.223	GH
Iberia	Plant cane	Short-term	10.20	J	1.193	GH
		Long-term	20.45	CD	1.957	CD
Gonsoulin	Plant cane	Short-term	13.81	H	1.583	FE
		Long-term	29.44	A	3.267	A
	First ratoon	Short-term	23.75	B	1.977	CD
		Long-term	15.82	H	1.227	GH
St. Gabriel	Plant cane	Short-term	15.85	H	1.703	DE
		Long-term	23.28	BC	2.500	B
	First ratoon	Short-term	27.45	A	3.137	A
		Long-term	16.42	FGH	1.710	DE
	First ratoon, second sampling	Short-term	28.10	A	3.117	A
		Long-term	16.69	FGH	1.677	FE

Letters within a column represent significance class by least significant difference across all locations, crop years, and cropping histories. α = 0.05.

2.3.5 Root Fungal Endophyte Colonization

Root fungal endophyte colonization was affected by cropping history and the cropping history x location interaction (Table 2.13). Root system colonization was more extensive in soils under long-term cultivation at two of three locations evaluated (Figure 2.8).

2.3.6 Fatty Acid Methyl Ester Analysis

The absolute abundance of total FAMEs in bulk soils was affected by location, cropping and a location x cropping history interaction, whereas rhizosphere soils were only affected by location and a location x cropping history interaction (Table 2.14). The same two locations had greater total FAMEs in short-term sugarcane cultivation and three locations in long-term cultivation (Figure 2.09 and Figure 2.10).

Bray-Curtis distance matrices for relative FAME profiles revealed significant effects on total FAME abundance for cropping history, location, and their interaction for both bulk and rhizosphere soils (Table 2.15).

Table 2.8. Mehlich-3 extractable phosphorus, potassium, and sodium for bulk soils from paired sites with short and long-term sugarcane cropping histories at six locations in plant cane and two locations in first ratoon.

Location	Crop year	Cropping history	Phosphorus (mg/kg)		Potassium (mg/kg)		Sodium (mg/kg)	
Airport	Plant cane	Short-term	13.7	FG	124.1	DEF	11.3	HI
		Long-Term	7.4	G	97.7	EFG	9.1	I
Belleview	Plant cane	Short-term	224.4	A	319.7	BC	86.5	A
		Long-term	9.3	G	86.2	FGH	10.2	I
Jefferson	Plant cane	Short-term	13.8	EFG	50.6	HG	30.6	EF
		Long-term	18.5	DEFG	61.9	HG	8.3	I
Iberia	Plant cane	Short-term	40.0	BCDE	330.2	ABC	26.5	FG
		Long-term	21.2	CDEFG	373.1	A	32.4	DEF
Gonsoulin	Plant cane	Short-term	55.1	B	132.1	DE	48.0	BC
		Long-term	8.9	G	95.0	EFGH	13.8	GHI
	First ratoon	Short-term	65.2	B	149.8	D	60.7	B
		Long-term	9.1	G	108.4	DEF	44.1	CDE
St Gabriel	Plant cane	Short-term	48.9	B	355.2	AB	30.8	EF
		Long-term	53.5	B	325.5	BC	24.7	FGH
	First ratoon	Short-term	39.9	BCDEF	317.1	BC	49.7	BC
		Long-term	43.3	BCD	300.3	C	46.2	CD
	First ratoon, second sampling	Short-term	41.2	BCDE	333.7	ABC	49.3	BC
		Long-term	46.0	BC	341.4	ABC	47.1	BC

Letters within a column represent significance class by least significant difference across all locations, crop years, and cropping histories. α = 0.05.

Bulk and rhizosphere FAME community structures of paired short and long-term cultivation soils at the six locations were visualized using ordination plots. Bulk soil distance-based redundancy analysis captured 37.59% of the constrained variance on axis 1 and 11.80% on axis 2 (Figure 2.11). Clustering was evident for replicate samples from individual sites with variable degrees of separation of paired sites from each location. No larger scale clustering was evident for samples from short-term cultivation sites or long-term cultivation sites across different locations.

Maximum correlation of bulk soil FAME distanace-based redundancy analysis with fungi, fungi:bacteria, arbuscular mycorrhizal fungi, gram negative bacteria, gram positive bacteria, bacteria, actinobacteria, pH, copper, calcium, magnesium, potassium, and gravimetric water content, total carbon, total nitrogen, soil organic matter, silt, clay, β-glucosidase, and *N*-acetyl-β-glucosaminidase were all statistically significant at p =

0.05 suggesting varied possible biological, physical, and nutritional explanations for the observed patterns of clustering (Figures 2.12 and 2.13).

Table 2.9. Mehlich-3 extractable calcium, magnesium, and sulfur for bulk soils from paired sites with short and long-term sugarcane cropping histories at six locations in plant cane and two locations in first ratoon.

Location	Crop year	Cropping history	Calcium (mg/kg)	Magnesium (mg/kg)	Sulfur (mg/kg)
Airport	Plant cane	Short-term	4277.5 A	530.1 F	5.3 FGH
		Long-term	4019.5 ABC	655.8 DE	1.9 IJ
Belleview	Plant cane	Short-term	3732.0 CDE	584.2 EF	4.2 GHIJ
		Long-term	2481.6 FG	339.1 G	2.7 HIJ
Jefferson	Plant cane	Short-term	988.1 J	165.6 I	1.4 J
		Long-term	1479.1 I	232.2 HI	2.6 HIJ
Iberia	Plant cane	Short-term	3499.8 E	746.2 CD	10.0 ABC
		Long-term	3434.5 E	955.9 A	7.0 CDEFG
Gonsoulin	Plant cane	Short-term	2536.1 FG	618.9 EF	8.2 ABCDE
		Long-term	1977.8 H	284.4 GH	6.6 DEFG
	First ratoon	Short-term	2704.8 F	638.0 E	11.4 A
		Long-term	2270.3 GH	320.9 GH	6.3 EFG
St Gabriel	Plant cane	Short-term	3889.1 BCD	876.0 AB	10.3 AB
		Long-term	3401.3 E	749.4 C	6.4 DEFG
	First ratoon	Short-term	4032.6 ABC	934.3 A	9.2 ABCDE
		Long-term	3403.0 E	795.5 BC	4.6 GHI
	First ratoon, second sampling	Short-term	4128.3 AB	947.6 A	9.4 ABCD
		Long-term	3630.4 DE	821.2 BC	4.9 GH

Letters within a column represent significance class by least significant difference across all locations, crop years, and cropping histories. α = 0.05.

Rhizosphere soil distance-based redundancy analysis captured 53.05% of the constrained variance on axis 1 and 21.78% on axis 2, and clustering was evident for individual site replicate samples with variable degrees of separation from the paired site at the same location (Figure 2.14). Four of six sites exhibited separation suggesting significant differences between short and long-term cultivation soils. Maximum correlation of fungi, fungi:bacteria, arbuscular mycorrhizal fungi, gram negative bacteria, gram positive bacteria, bacteria, actinobacteria were all statistically significant at p = 0.05 (Figure 2.15). Long-term cultivation sites from three locations showed a similar pattern of separation related to greater relative abundance of various fungal biomarkers, while the other three long-term cultivation sites showed a similar pattern of separation from short-term cultivation sites related to bacterial biomarkers. The short-term cultivation sites from four of six locations were grouped together suggesting possible similarity in microbial community composition.

Table 2.10. DTPA extractable copper, iron, manganese, and zinc for bulk soils from paired sites with short and long-term sugarcane cropping histories at six locations in plant cane and two locations in first ratoon.

Location	Crop year	Cropping history	Copper (mg/kg)	Iron (mg/kg)	Manganese (mg/kg)	Zinc (mg/kg)
Airport	Plant cane	Short-term	1.179 D	25.87 G	9.652 G	0.557 EF
		Long-term	1.757 D	18.85 G	10.55 G	0.67 DEF
Belleview	Plant cane	Short-term	8.012 A	82.32 EF	9.977 G	19.76 A
		Long-term	2.475 CD	64.07 EFG	17.81 EFG	1.189 DEF
Jefferson	Plant cane	Short-term	1.505 D	272.2 A	53.25 BC	1.749 DEF
		Long-term	1.797 D	115.1 DE	30 E	1.161 DEF
Iberia	Plant cane	Short-term	4.8 B	314.3 A	18.53 EFG	3.879 CDEF
		Long-term	2.75 CD	140.8 CD	12.08 FG	0.969 DEF
Gonsoulin	Plant cane	Short-term	3.995 BC	101.7 DE	25.49 EF	8.749 B
		Long-term	1.855 D	46.41 FG	44.78 CD	0.349 F
	First ratoon	Short-term	2.109 CD	104.5 DE	54.54 ABC	6.76 BC
		Long-term	2.198 CD	87.11 EF	48.3 C	1.1 DEF
St Gabriel	Plant cane	Short-term	5.452 B	290.5 A	29.89 E	4.902 BCD
		Long-term	5.454 B	206.5 B	30.67 DE	2.529 CDEF
	First ratoon	Short-term	5.113 B	287.1 A	49.03 C	4.848 CD
		Long-term	5.042 B	196.8 B	66.53 AB	2.662 CDEF
	First ratoon, second sampling	Short-term	4.937 B	282.6 A	53.99 ABC	4.713 BCDE
		Long-term	5.027 B	190.4 BC	67.99 A	2.822 CDEF

Letters within a column represent significance class by least significant difference across all locations, crop years, and cropping histories. α = 0.05.

Table 2.11. ANOVA p-values for soil nitrate-N and ammonium-N as affected by location of paired sites and sugarcane cropping history. α = 0.05.

Nitrogen form	Cropping history	Location	Location x Cropping history
Nitrate-N	NS	0.0001	0.0001
Ammonium-N	NS	0.0001	0.0001

α = 0.05. NS = not significant.

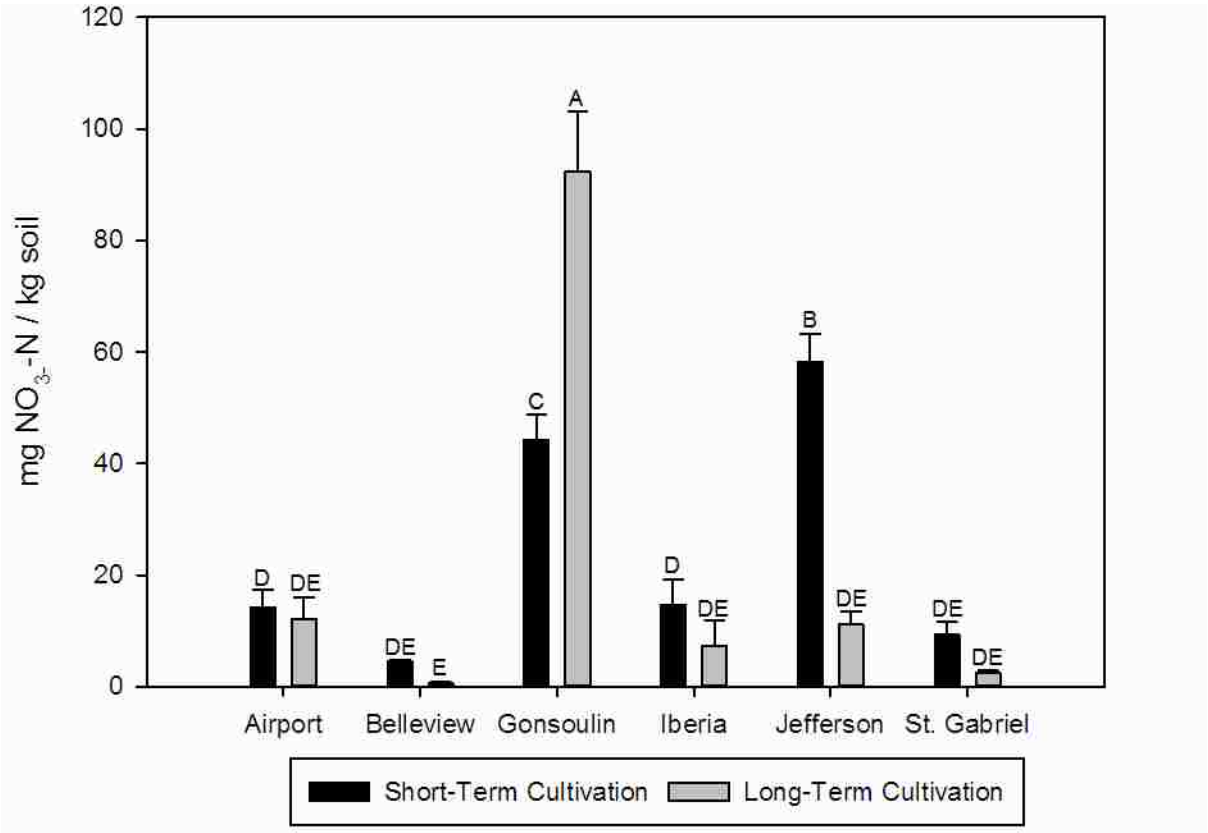

Figure 2.4. Nitrate-N comparison in bulk soils from paired sites with short and long-term sugarcane cropping histories at six locations. Letters represent significance class by least significant difference across all treatments. α = 0.05.

2.4 Discussion

Yield decline associated with monoculture is an issue for many crops (Bennett et al., 2012). Previous sugarcane research described yield decline as a “loss of productive capacity of sugarcane soils under long-term monoculture” (Garside et al. 1997). This condition has been observed in sugarcane producing countries across the globe (Magarey, 1996; Martin, et al, 1959; Innes et al., 1958), and lower yields were detected for crops produced in the long-term cultivation compared to short-term cultivation soils in this study, confirming this issue also occurs in Louisiana where sugarcane has been grown on the same land for over 200 years (Hillard, 1979).

Results from etiological investigations utilizing soil sterilization or partial elimination of the microbial community with selective biocides have suggested there is a biological component involved in the declining yields associated with sugarcane monoculture (Egan et al., 1984; Garside et al., 2002; Hoy and Schneider, 1988; Pankhurst et al., 2005b; Reghenzani et al., 1988). Differences in plant growth responses resulting from application of selective biocides and complete soil sterilization suggested diverse organisms associated with sugarcane roots contribute to the detrimental growth

associated with continuous monoculture and that the biological component of yield decline is therefore a community level effect. Additional studies comparing culturable microbial taxa in the sugarcane rhizosphere demonstrated differences between microbial communities in short and long-term cultivation soils (Pankhurst et al. 2000; Savario and Hoy 2010). Qualitative differences in the composition of the rhizosphere bacterial and fungal communities between short and long-term cultivation soils were detected in Australia and Louisiana. Root colonization by a dematiaceous, sterile fungus was associated with poor plant growth in long-term sugarcane cultivation soils in Australia (Magarey et al. 1997a). A similar result was obtained in this study with more extensive endophytic fungal colonization in roots from plant cane growing in long-term cultivation soils. This result, in conjunction with generally lower stalk height and weight observed in long-term cultivation soils suggests that the changes in the soil fungal community may have a deleterious effect on plant growth.

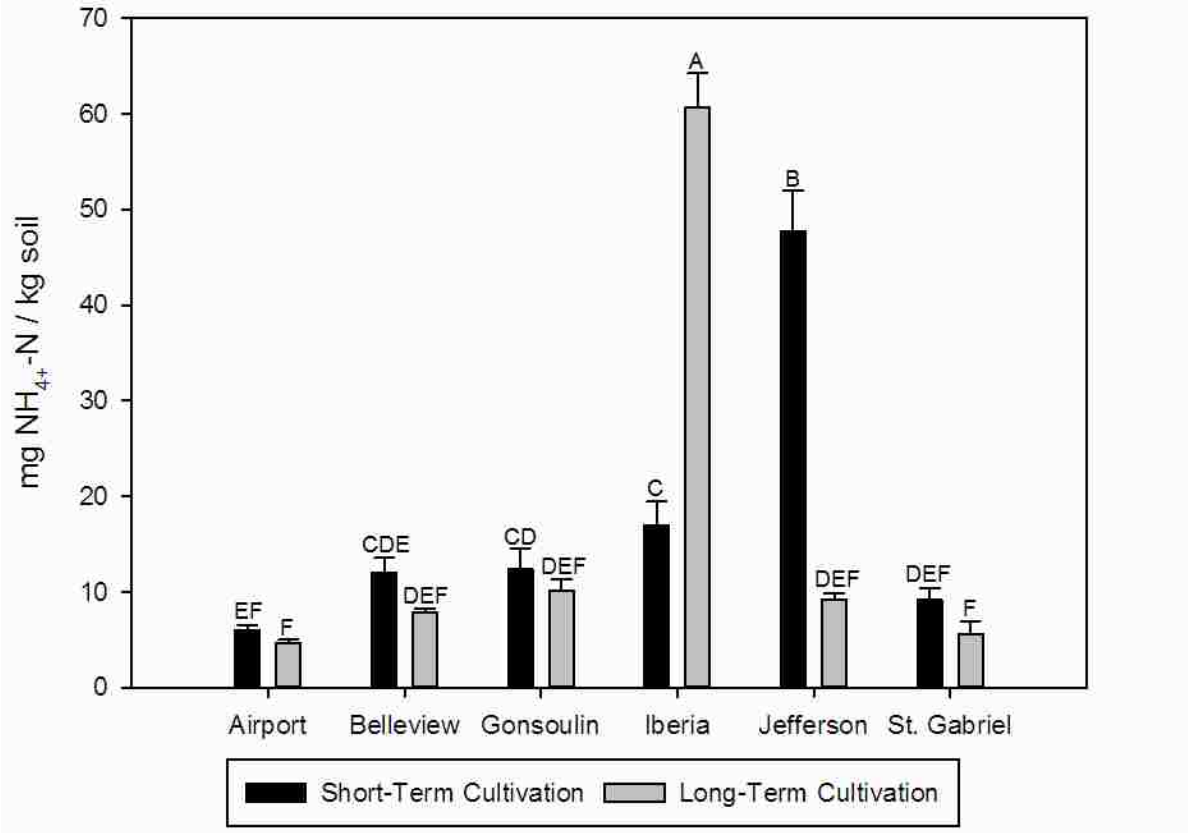

Figure 2.5. Ammonium-N comparison in bulk soils from paired sites with short and long-term sugarcane cropping histories at six locations. Letters represent significance class by least significant difference across all treatments. α = 0.05.

The use of FAME biomarkers as indicators for the relative abundance of broad taxonomic groups allows for broad profiling of soil-associated microbial communities (Buyer and Drinkwater, 1997; Cavigelli et al., 1995; Schutter and Dick, 2002). Distance-based redundancy analysis of the relative abundance of FAMEs produced variable

results for bulk and rhizosphere soils. Bulk soils exhibited differences largely based on location with no discernable clustering patterns to indicate similarities among short or long-term cultivation soils from different locations. The bulk soils from the Gonsoulin, Belleview, and Airport locations exhibited similar FAME profiles that were not strongly affected by cropping history. Co-plotting of biological and environmental variables revealed possible causes for cluster patterns of different soils based on location. In particular, overall similarities in the structure of microbial communities in bulk soils at Gonsoulin, Belleview, and Airport were all strongly correlated with having higher soil pH, while the FAME profile for the short-term cultivation soil at Jefferson was strongly influenced by the most acidic soil pH.

Table 2.12. ANOVA p-values for soil extracellular enzyme activities as affected by sugarcane cropping history and location of paired sites. α = 0.05.

Enzyme	Cropping history	Location	Location x Cropping history
Glucosidase	0.0001	0.0001	0.0001
Glucosaminidase	0.0001	0.0001	0.0001

α = 0.05. NS = not significant.

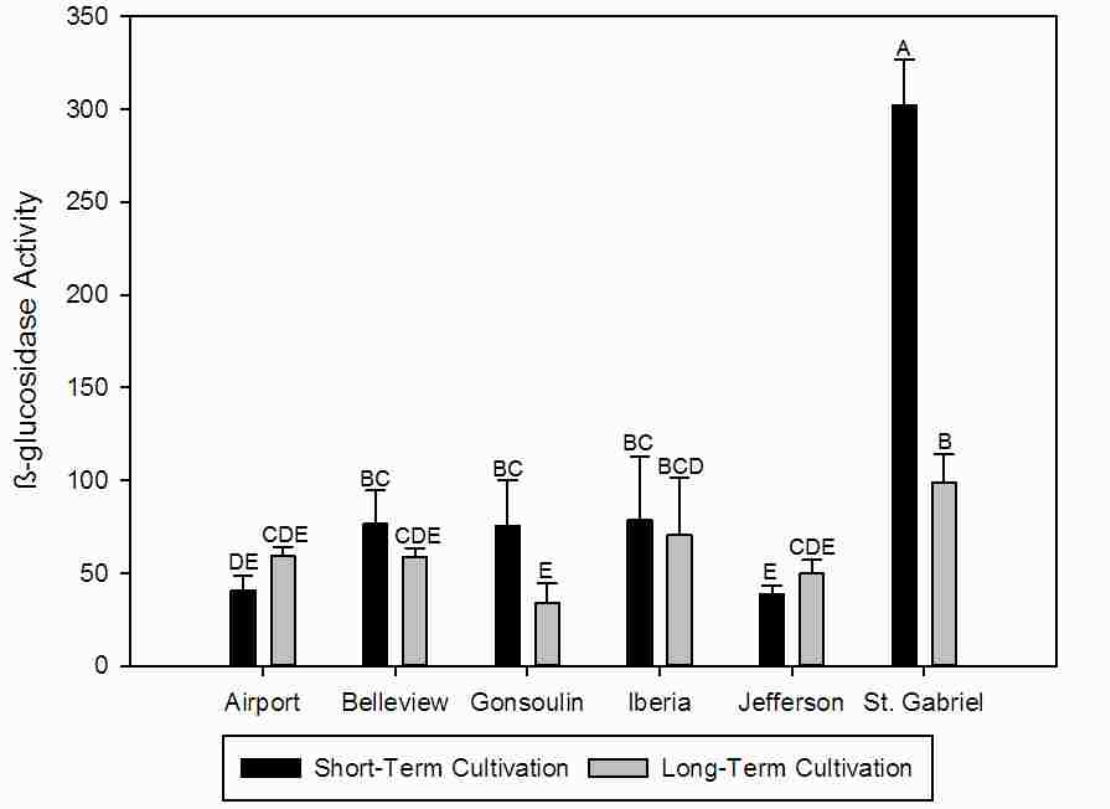

Figure 2.6. β-glucosidase extracellular enzyme activity comparison in bulk soils from paired sites with short and long-term sugarcane cropping histories at six locations. Letters represent significance class by least significant difference across all treatments. α = 0.05.

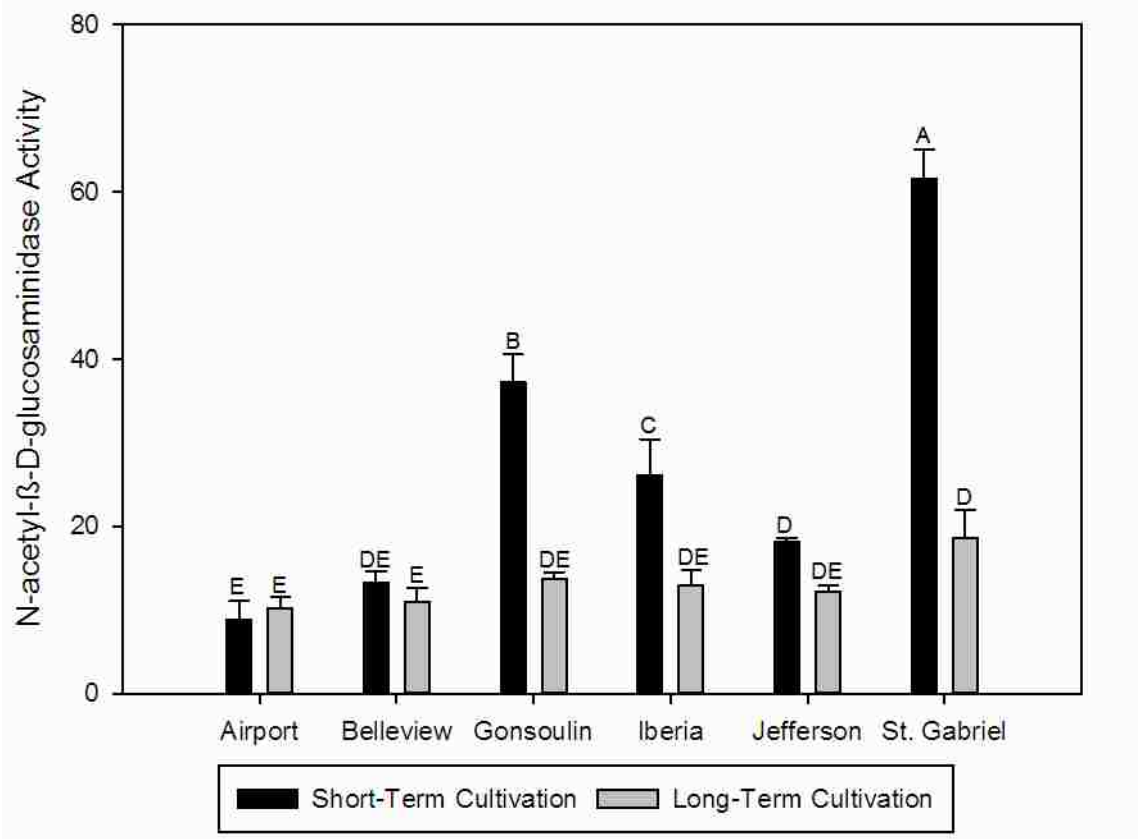

Figure 2.7. N-acetyl-β-glucosaminidase extracellular enzyme activity comparison in bulk soils from paired sites with short and long-term sugarcane cropping histories at six locations. Letters represent significance class by least significant difference across all treatments. α = 0.05.

Table 2.13. ANOVA p-values from root staining to determine fungal endophyte colonization for roots collected from short and long-term sugarcane cultivation paired sites at three locations. α = 0.05. NS = not significant

	Cropping history	Location	Location x Cropping history
Root endophyte colonization	0.0001	NS	0.0208

α = 0.05. NS = not significant.

Distance-based redundancy analysis of rhizosphere FAME profiles revealed greater cropping history differences than for bulk soils, likely due to the direct, long-term influence of sugarcane root exudates (Broeckling et al., 2008, Grayston et al., 1998). Clustering of paired sites for different locations still occurred but was much less evident than for bulk soils. Clustering was more suggestive of similarity for sites with the same cropping history from different locations. Correlation of biomarker variables with FAME profile ordination revealed possible microbial community differences that distinguished short and long-term cultivation in rhizosphere soils and revealed five of six locations

under long-term cultivation clustered into two groups. Long-term cultivation was correlated with a predominance of fungi in the rhizosphere at the Belleview and Jefferson locations, whereas soils under continuous sugarcane cultivation at the Gonsoulin, Iberia, and St. Gabriel locations exhibited higher relative abundances of bacterial taxa with proportionally more Gram-negative bacteria at Gonsoulin and more Gram-positive bacteria and actinobacteria at St. Gabriel. The FAME biomarkers did not provide evidence for a higher relative abundance of any taxa for short-term cultivation soils collected from four of six locations suggesting similarity in the rhizosphere microbial community for soils with no recent history of sugarcane cultivation.

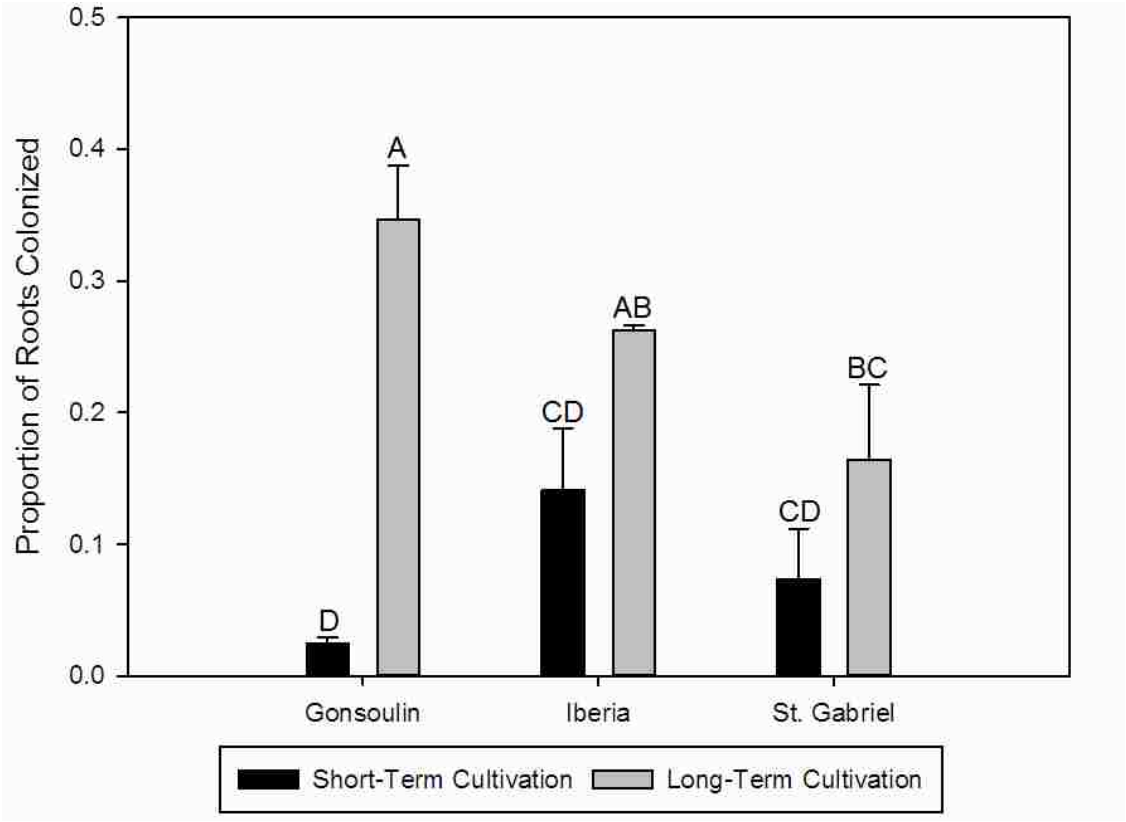

Figure 2.8. Estimated proportion of sugarcane roots colonized by fungal endophytes in paired sites with short and long-term sugarcane cropping histories at three locations. Letters represent significance class by least significant difference across all treatments. α = 0.05.

Table 2.14. ANOVA p-values for factors affecting total fatty acid methyl esters (FAMEs) extracted from bulk and rhizosphere soil of paired sites with short and long-term sugarcane cropping histories at six locations. α = 0.05. NS = not significant.

	Cropping history	Location	Cropping history x Location
Bulk Soil	0.0002	0.0001	0.0001
Rhizosphere	NS	0.0001	0.0001

α = 0.05. NS = not significant.

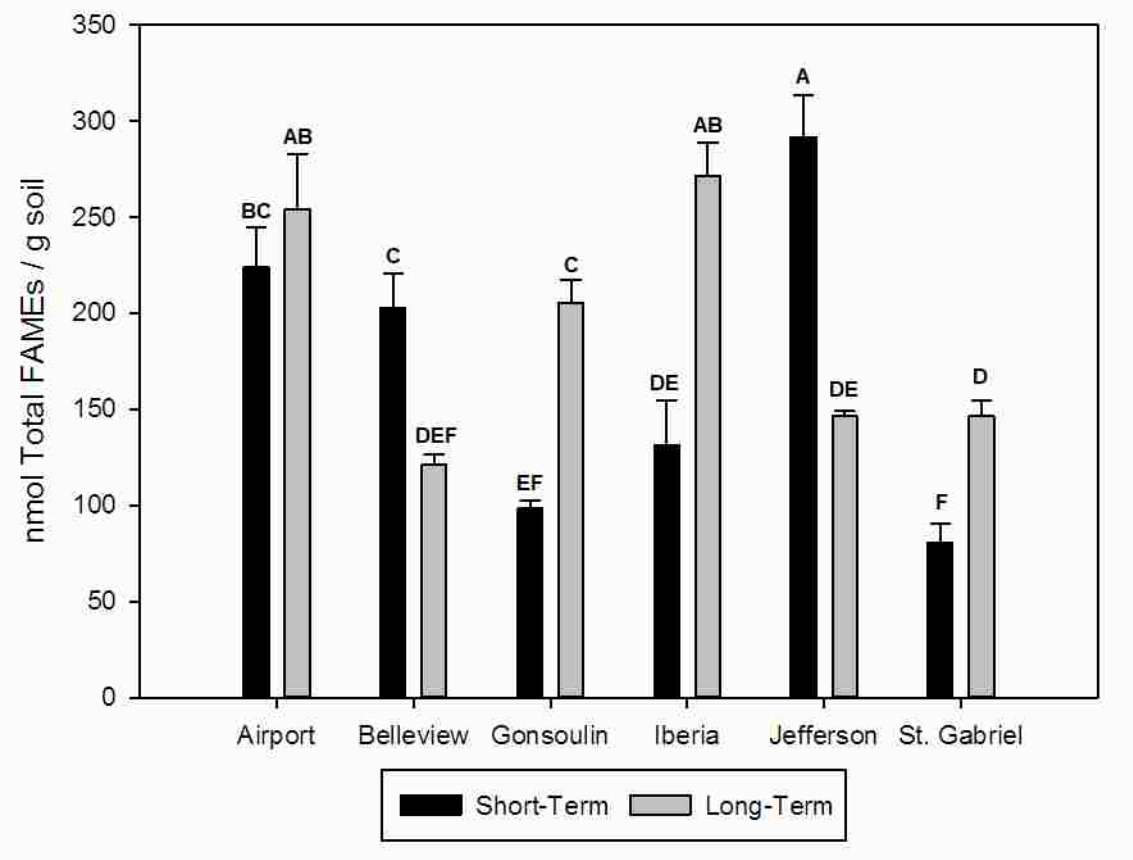

Figure 2.9. Total FAMEs in bulk soil from paired sites with short and long-term sugarcane cropping histories at six locations. Letters represent significance class by least significant difference across all treatments. α = 0.05.

Table 2.15. ANOVA p-values for factors affecting Bray-Curtis distance matrices for relative abundance of fatty acid methyl ester (FAME) profiles from bulk and rhizosphere soils of paired sites with short and long-term sugarcane cropping histories at six locations. α = 0.05.

	Cropping history	Location	Location x Cropping history
Bulk soil relative FAME abundance	0.0001	0.0001	0.0001
Rhizosphere soil relative FAME abundance	0.006	0.0001	0.0001

α = 0.05. NS = not significant.

While long-term cultivation has been shown to increase the diversity of culturable rhizosphere bacteria (Pankhurst et al. 2000), the influence of cropping history on rhizosphere communities and the higher relative abundance of a broad taxonomic group, though not consistently the same group among locations, suggests long-term sugarcane cultivation may alter the balance and diversity of broader taxa to favor one group of microorganisms. While FAME analysis cannot account for changes in specific

taxa, the broad level community changes detected may result in a higher prevalence of deleterious soil microorganisms (Barazani and Friedman, 2001; Nehl et al., 1996; Schippers et al., 1987; Suslow and Schroth, 1982) causing the community level effect associated with yield decline.

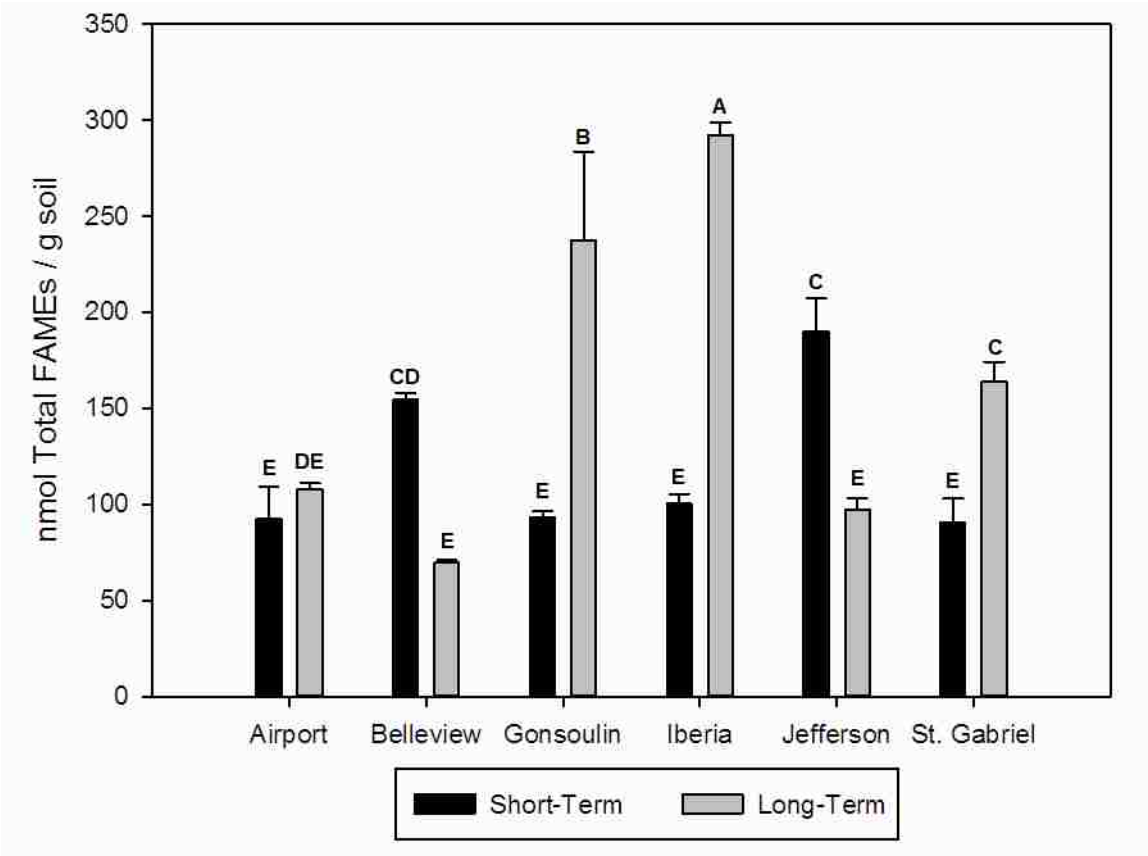

Figure 2.10. Total FAMEs in rhizosphere soil from paired sites with short and long-term sugarcane cropping histories at six locations. Letters represent significance class by least significant difference across all treatments. α = 0.05.

Previous efforts to describe soil chemical property changes associated with continuous sugarcane cultivation and yield decline found acidification, decreased cation exchange capacity and less soil organic matter to be the most consistent trends (Bramley et al., 1996; Garside et al., 1997; Wood, 1985). Organic matter was greater in soils under short-term sugarcane cultivation at four of six locations in this study and four of four locations in the previous Louisiana study (Savario and Hoy, 2010), supporting the conclusion that higher organic matter is a consistent, important factor affecting sugarcane rhizosphere microbial communities. Soil organic matter also increased during in first ratoon at both locations. This short-term gain of soil organic matter from plant cane to first ratoon contradicts the long-term losses associated with monoculture and may have been influenced by crop residue management at the two resampled locations.

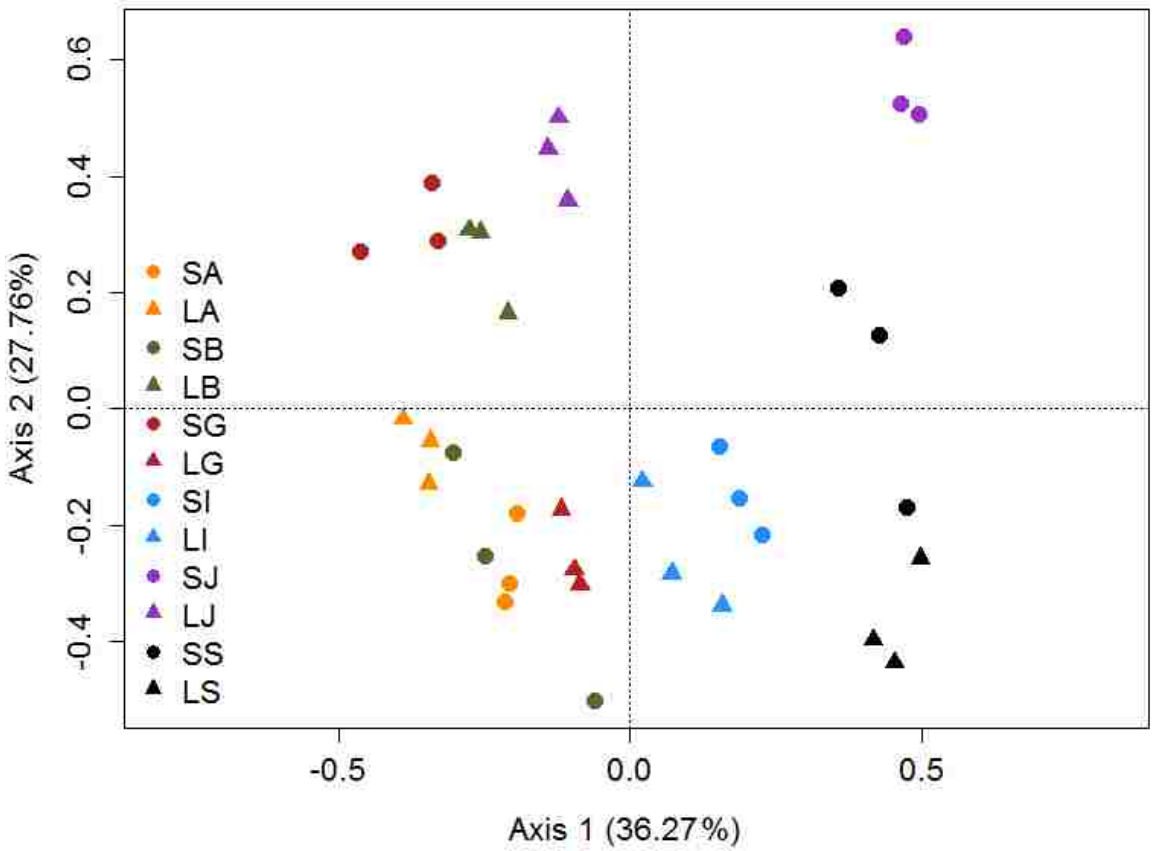

Figure 2.11. Distance-based redundancy analysis of Bray-Curtis distance matrix from relative abundance of bulk soil FAMEs extracted from soils of paired sites with short and long-term sugarcane cropping histories at six locations. Cropping History: S = short-term cultivation, L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel.

Other soil chemical properties, including total carbon, soil acidification, soil nitrogen form and total nitrogen, did not show any consistent association with sugarcane cropping history or any other variable. Similar to the results from the previous study in Louisiana (Savario and Hoy, 2010), the trend of general soil acidification associated with continuous sugarcane cultivation was less supported in this study. Soil samples ranged from slightly alkaline at the Airport location to strongly acidic at the Jefferson short-term cultivation site where pasture was recently converted to sugarcane. Nitrate-N and ammonium-N were not affected by cropping history, exhibiting interactions between locations and cropping history. Similar mixed results were obtained for total nitrogen.

Paired-site comparisons of soil nutrients under short and long-term sugarcane cultivation detected large location-specific differences in the availability of individual Melich-3 and DTPA extractable nutrients based on cropping history and some consistent trends. Overall findings were in accordance with previous reports of a depletion of micronutrients, such as iron, copper, and zinc, associated with the long-term cultivation of sugarcane (Bramley et al., 1996, Wood, 1985). Soil nutrient

differences would be expected to affect the soil microbial community structure and function. In response to scarcity of essential elements, such as iron, soil microbes can produce siderophores, low molecular weight chelating compounds, to sequester soil micronutrients (Ahmed and Holmstrom, 2014). Soils under long-term sugarcane cultivation in Louisiana have shown site-specific differences with a greater abundance of culturable siderophore producing bacteria (Savario and Hoy, 2010).

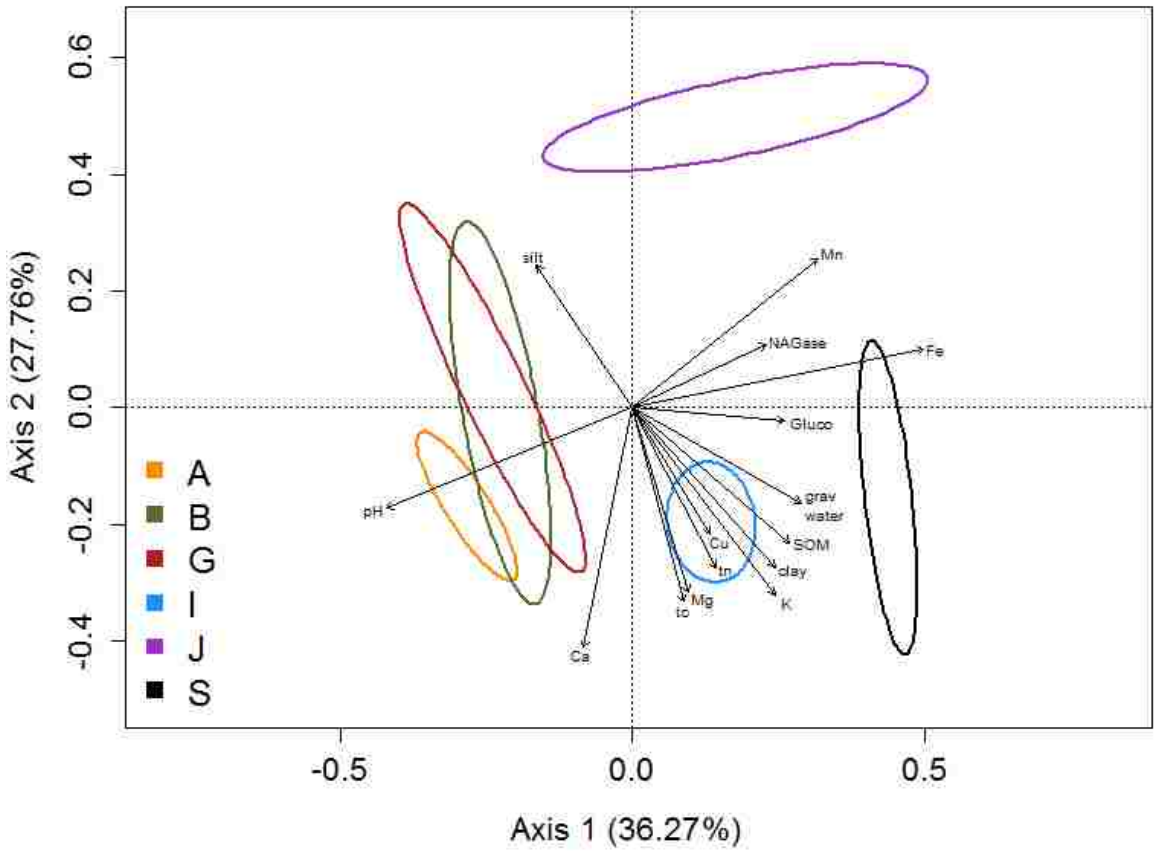

Figure 2.12. Distance-based redundancy analysis of Bray-Curtis distance matrix from relative abundance of bulk soil FAMEs extracted from soils of paired sites with short and long-term sugarcane cropping histories at six locations with maximum correlation of soil environmental variables plotted as vectors (p = 0.05). Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel. Environmental variables: SOM = soil organic matter, tn = total nitrogen, tc = total carbon, Gluco = β-glucosidase, NAGase = *N*-acetyl-β-glucosaminidase, grav water = gravimetric water.

Qualitative microbial community level differences in substrate utilization profiles between short and long-term sugarcane cultivation soils have been documented in Louisiana (Savario and Hoy 2010). The activities of β-glucosidase, that breaks down cellulose, and N-acetyl-β-D-glucosaminidase, that breaks down chitin and is involved in both carbon and nitrogen cycling, can provide indicators of functional diversity in soils (Bandick and Dick 1999). β-glucosidase and N-acetyl-β-D-glucosaminidase potential activity were greater in short-term cultivation soils. β-glucosidase activity was greater in

short-term cultivation at locations where organic matter was also greater. The potential activity of N-Acetyl-β-D-glucosaminidase was lower under long-term cultivation at three locations where nitrate-N, ammonium-N, or total nitrogen content was greater than in recently cultivated soils. The results suggest that soil extracellular activity is affected by soil nutrients and sugarcane land management practices. This activity might be expected to influence the structure of the fungal community in the rhizosphere and subsequently influence plant growth.

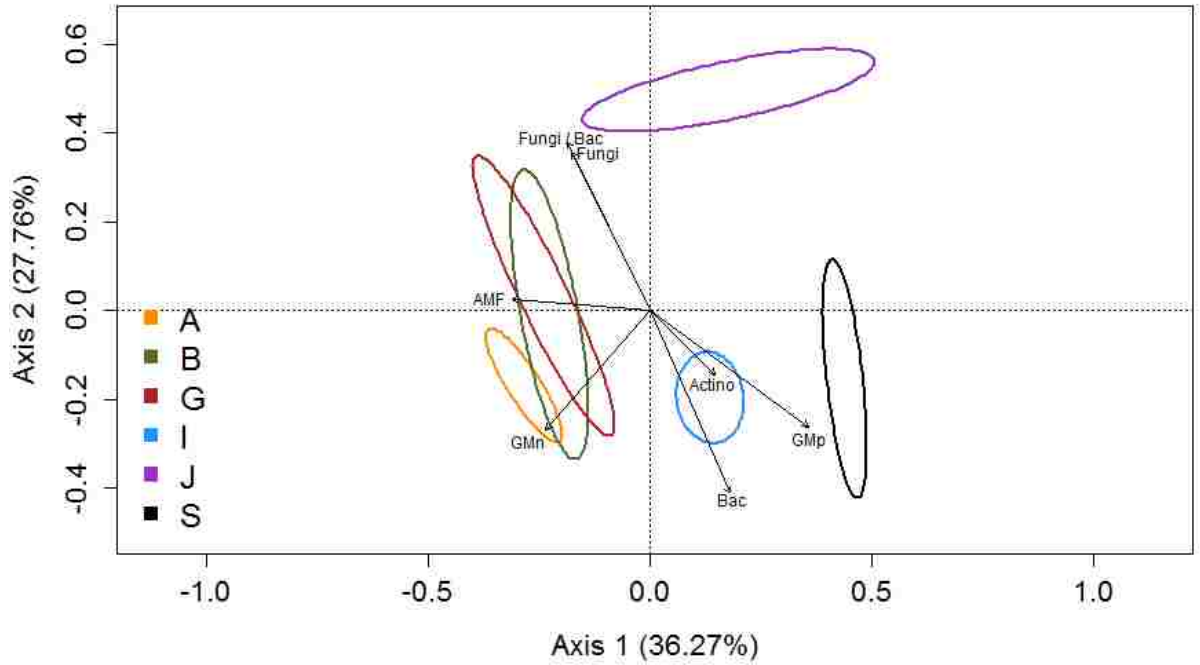

Figure 2.13. Distance-based redundancy analysis of Bray-Curtis distance matrix from relative abundance of bulk soil FAMEs extracted from soils of paired sites with short and long-term sugarcane cropping histories at six locations with maximum correlation of taxonomic environmental variables plotted as vectors (p = 0.05). Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel. Environmental variables: Fungi/Bac = fungal to bacterial ratio, GMn = gram negative bacteria, GMp = gram positive bacteria, Bac = total bacteria, Actino = actinobacteria, AMF = arbuscular mycorrhizal fungi.

2.5 Conclusions

The objective of this study was to analyze differences in soil abiotic and biotic factors in soils under short and long-term sugarcane cultivation then identify any related to soil microbial ecology that might be associated with the yield decline resulting from monoculture. This was done by testing soil chemical properties, determining endophytic fungal colonization of roots, and evaluating differences in soil microbial community structure indicated by FAME biomarkers for different biological taxa. Differences were detected for multiple soil chemical and biological properties related to location. However, differences also were detected for abiotic and biotic factors related to

cropping history. Differences in rhizosphere and bulk soil FAME profiles suggest broad qualitative differences in microbial community structure related to cropping history similar to those found in previous studies. Higher levels of soil organic matter and micro- and macro nutrients were confirmed in non-agricultural soils that can affect microbial ecology. These results improve our understanding of differences between recently and continuously cultivated soils associated with detrimental effects on soil health resulting from sugarcane monoculture. Further research is needed to elucidate in greater detail any consistent differences in rhizosphere microbial communities related to yield decline. The ultimate goal will be to identify management practices that might alter microbial communities in ways more favorable for plant growth in soils with a long-term cropping history.

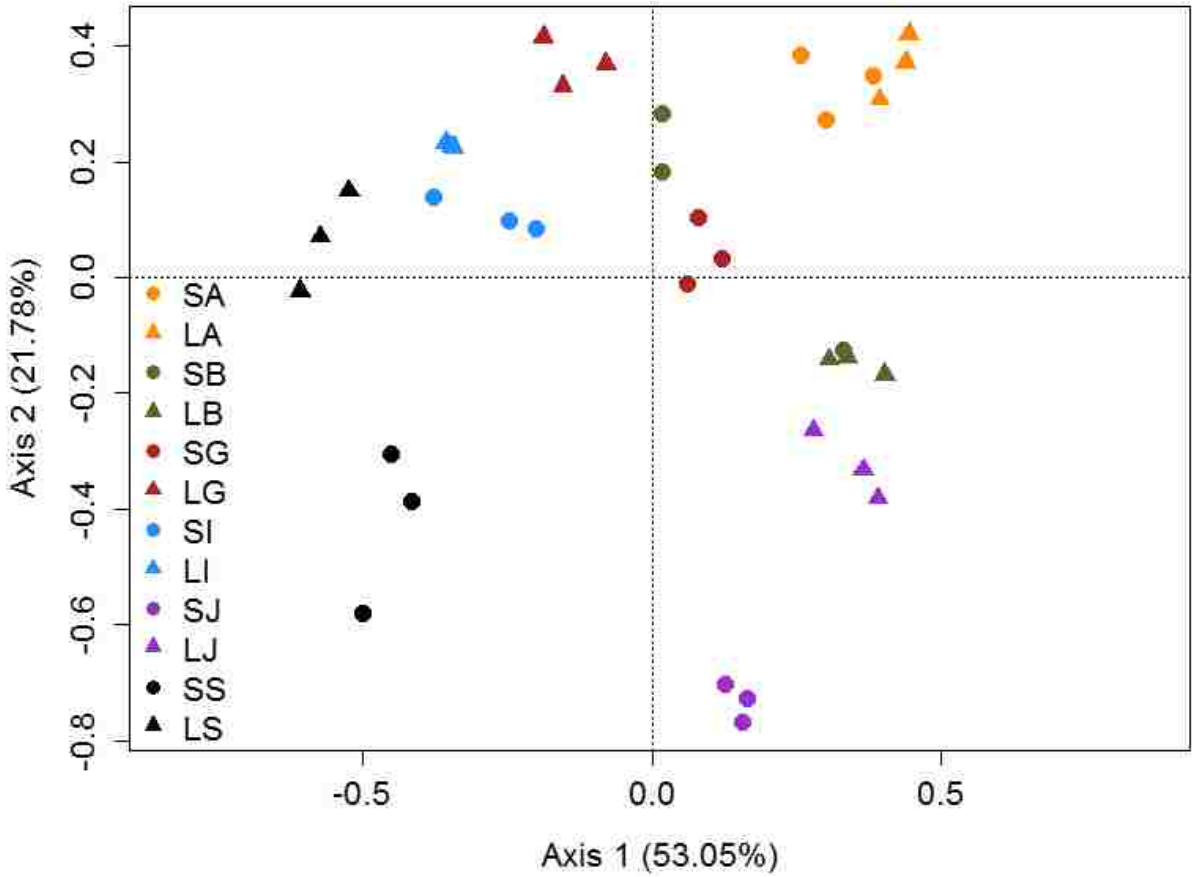

Figure 2.14. Distance-based redundancy analysis of Bray-Curtis distance matrix from relative abundance of rhizosphere soil FAMEs extracted from soils of paired sites with short and long-term sugarcane cropping histories at six locations. Cropping History: S = short-term cultivation, L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel.

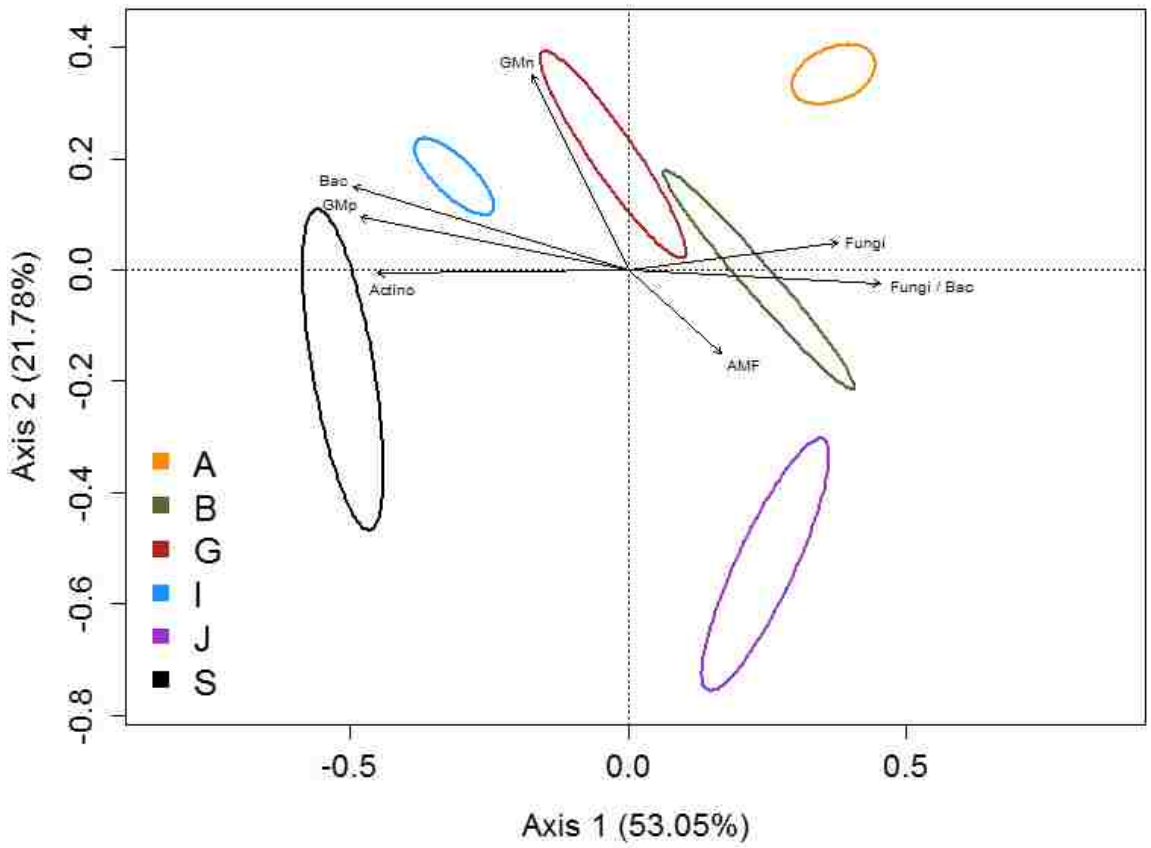

Figure 2.15. Distance-based redundancy analysis of Bray-Curtis distance matrix from relative abundance of rhizosphere soil FAMEs extracted from soils of paired sites with short and long-term sugarcane cropping histories at six locations with maximum correlation of environmental variables plotted as vectors (p = 0.05). Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel. Environmental variables: Fungi/Bac = fungal to bacterial ratio, GMn = gram negative bacteria, GMp = gram positive bacteria, Bac = total bacteria, Actino = actinobacteria, AMF = arbuscular mycorrhizal fungi.

Chapter 3: Metagenomic Analysis of Soils with Short and Long-term Sugarcane Cropping Histories

3.1 Introduction

Sugarcane, *Saccharum* spp. inter-specific hybrids, is a tropical to sub-tropical crop commercially grown for its ability to store sucrose in its stalks and as a source of bioenergy. Sugarcane is often grown as a monoculture. In Louisiana, production is restricted to the south-central portion of the state and has largely been cultivated in the same soils for over 200 years (Hilliard 1979). Previous research in Australia described a "loss of productive capacity of sugarcane soils under long-term monoculture" and termed it "yield decline" (Garside et al. 1997). The phenomenon of reduced yields associated with long-term sugarcane cultivation has been documented in sugarcane growing countries across the globe (Magarey 1996; Martin,et al. 1959; Innes and Manser 1958) and additional evidence that this soil health issue occurs in Louisiana was described in this study (Chapter 2).

The state of diminished yield potential in soils where sugarcane has been continuously cultivated has largely been attributed to a detrimental soil microbiota based on increases in yields following soil sterilization or solarization, and additional support for a biological causal role was obtained from the application of selective biocides (Egan et al. 1984; Garside et al. 1997; Hoy and Schneider 1988a; Magarey and Croft 1995, Pankhurst et al. 2005b, Reghenzani 1988). Positive sugarcane growth responses in long-term cultivation soils treated with the fungicide mancozeb suggested fungi are a major component of yield decline (Magarey et al. 1997a). A study focused on isolating fungi from roots of plants under long-term sugarcane cultivation and characterizing their pathogenicity isolated *Penicillium, Curvularia, Humicola, Phoma, Gongronella, Mortierella, Chaetomium, Aspergillus, Fusarium, Trichoderma, Cladosporium, Thielaviopsis, Rhizoctonia*, and sterile, dematiaceous fungi, but only sterile isolates significantly reduced root growth (Magarey and Croft 1995). An additional study of fungal root colonization from Taiwan consisting of 1,010 isolates found 81% belonged to ten genera: *Fusarium, Trichoderma, Rhizoctonia, Marasmius, Pythium, Monilia, Collectotrichum, Phialophora, Humicola*, and *Thielaviopsis* (Watanabe 1974). While not all isolates were tested, isolates of *Fusarium, Rhizoctonia, Marasmius, Pythium*, and *Thielaviopsis* were found to have some pathogenicity to sugarcane in field or growth chamber experiments.

Further research investigated the etiology of yield decline by evaluating and determining differences in culturable microorganisms associated with soils under short and long-term sugarcane cultivation in Australia and Louisiana (Magarey et al. 1997a, Pankhurst et al. 2000; Savario and Hoy 2010). Fusarial fungi were more abundant in long-term cultivation soils in Louisiana, and a sterile, dematiaceous fungus was associated with sugarcane roots under long-term cultivation and reduced plant growth in Australia. Comparisons of culturable bacteria revealed variation by location and cropping history. Actinobacteria and bacteria in the genera *Bacillus* and *Pseudomonas* were more

abundant in some soils with no recent history of sugarcane cultivation, while siderophore producing bacteria were more abundant in a soil with a long-term sugarcane cultivation history. Research reliant on the culturing of bacteria and fungi may overestimate the importance of those organisms that are readily culturable. *In situ* enumeration of bacterial cells through epiflourescent micrography suggests a single gram of soil may contain as many as 10^{10} bacterial cells distributed amongst 10^4 species, whereas only 10^6 colony forming units were detected by plating (Roesch et al. 2007; Torsvik and Øvreås 2002). There are roughly 3,150 described and readily culturable species of free-living soil fungi, but estimates as to the total number of species range as high as 5.1 million (Blackwell 2011; Gams 2007).

Handelsman et al. (1998) proposed the use of cloning and DNA sequencing in order to study the putative gene functions and chemistry of culturable and non-culturable microbes as a way to bridge the gap between genomics and microbial ecology and coined the term “metagenomics” to refer to the study of collective genomes within an ecosystem. Now, a common focus of amplicon-based metagenomics is to use a single gene to identify microbial community composition. The 16s rRNA gene can be used for phylogenetic analysis and identification of bacteria and archaea (Woese and Fox 1977), while the internal transcribed spacer (ITS) has been proposed as the most reliable single locus for identification across the entire fungal kingdom (Schoch et al. 2012). Qiime (quantitative insights into microbial ecology) is then an open-source pipeline that allows for processing of large amplicon-based metagnomic datasets (Caporaso et al. 2010). Qiime can cluster 16S and ITS amplicons into operational taxonomic units (OTUs), empirically classified groups of sequences at a set level of sequence similarity. Qiime can then provide taxonomic assignment of clustered OTUs, and community diversity can be described by α-diversity metrics that measure the diversity within samples and β-diversity metrics that produce statistics describing the dissimilarity between two samples. The application of this broad approach to analyze the soil microbial community could provide insight and increased understanding about variability in community structure and possible roles of the community in reduced yields associated with continuous sugarcane cultivation.

The most commonly used forms of β-diversity analysis are distance-based redundancy analyses utilizing dissimilarity matrices that transform sets of sequences into a matrix of pairwise comparisons based on the abundance of OTUs, as is the case for Bray-Curtis (Bray and Curtis 1957), or the total phylogenetic distance of communities, as is the case for Unifrac (Lozupone and Knight 2005). Distance-based redundancy analysis can then transform these matrices into an ordination that visualizes the two largest proportions of the total variation to reveal any broad-scale ecological differences in soil microbial communities associated with short and long-term sugarcane cultivation or other land management metadata. *Vegan*, a community analysis package for the statistical computing environment R, allows for post-analysis environmental interpretation of distance-based redundancy analysis by correlating additional metadata, such as soil

nutrient data, with variation in soil microbial community structure to account for both biotic and abiotic differences in samples (Oksanen et al. 2017).

The decline in yields associated with long-term sugarcane cultivation remains an incompletely understood complex soil health issue involving biotic and abiotic factors. The objective of this study was to analyze differences in the soil microbial ecology between paired sites under short and long-term sugarcane cultivation by using culture-independent techniques to identify members and compare structure of microbial communities with an evaluation of the involvement of soil nutrient availability and soil extracellular enzyme activities to better understand how the continuous cultivation of sugarcane affects soil health and crop productivity.

3.2 Methods

3.2.1 Paired Site Selection and Soil Sampling

Paired sites for soil sampling were identified at six locations based on the following criteria: 1) soil with long-term history of sugarcane production was available with neighboring land just converted to sugarcane cultivation, 2) both were planted in a first year crop described as “plant cane”, 3) sugarcane of the same cultivar was planted at both sites, and 4) paired sites had the same or similar soil series (Table 3.1). The soils with no recent history of sugarcane cultivation previously supported mixed plant communities, either pasture or forest.

Three pseudoreplicates were established per site with four subsamples collected per pseudoreplicate for a total of 12 subsamples (250 grams each) of rhizosphere and 12 corresponding subsamples (100 grams each) of bulk soil. Sugarcane plants were dug-out from row beds to expose root systems with intact rhizosphere soil. Rhizosphere soil was considered to be soil closely adhering to roots that could be removed without damaging the root cortex. Roots and rhizosphere soil were cut from plants and placed in a cooler until they could be separated in the lab. Roots from sampling in 2015 were preserved in formalin for later use. Large aggregates wholly adhering to the root system were avoided. Bulk soils were collected at a minimum depth of 150 mm. Samples were bagged and placed in coolers with ice packs during transportation and subsequently stored at -20ºC.

3.2.2 DNA Extraction and Sequencing

Bulk and rhizosphere soils were stored at -80ºC prior to DNA extraction. DNA was extracted from 0.25 grams of soil using the MoBio PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) following the manufacturer’s instructions. DNA vortexing was done using a FastPrep homogenizer (MP Biomedical, Santa Ana, CA) set to 5.5 m/s for 30 seconds for two cycles 30 seconds apart. For 2014 soil samples, a Mobio DNeasy Powerclean (Mo Bio Laboratories, Carlsbad, CA) was used to remove any additional PCR inhibitors remaining following extraction. DNA was quantified (ng/µl) and quality checked by measuring the absorbance ratio of light at 280 nm and 260 nm with a

spectrophotometer. Samples that were submitted for DNA sequencing had 280/260 nm absorbance ratios ranging from 1.3 to 2 and extraction concentrations ranging from 2.0 to 30 ng/µl. A total of 30 ng of DNA extracted from soil samples collected in 2014 and 2015 were sent off for sequencing in 2015. Soil samples collected from 2016 and DNA samples that were not successfully sequenced in 2015 were extracted in triplicate, combined and of 50 ng of DNA was sent off for sequencing.

Table 3.1. Descriptions of paired sites with short and long-term histories of sugarcane cultivation at six locations. First year sampling dates were in plant cane and second year sampling dates were in first ratoon cane at two locations.

Location	Coordinates	Sampling dates	Cultivar	Soil series	Cropping history	Texture
Airport	30.0192ºN 91.8758ºW	6/9/2014	HoCP 04-838	Jeanerette Silt Loam	Sugarcane	Silt Loam
					Forest	Silt Loam
Belleview	30.2256ºN 91.2561ºW	6/24/2014	HoCP 96-540	Commerce Silt Loam	Sugarcane	Silt Loam
					Pasture	Loam
Jefferson	29.9937ºN 91.9180ºW	7/3/2014	HoCP 96-540	Jeanerette Silt Loam	Sugarcane	Silt Loam
					Pasture	Silt Loam
Gonsoulin	30.0150ºN 91.9164ºW	6/5/2015 6/27/2016	HoCP 96-540	Jeanerette Silt Loam	Sugarcane	Silt Loam
					Forest	Silt Loam
Iberia	29.9430ºN 91.7125º W	6/3/2015	HoCP 96-540	Schriever Clay	Sugarcane	Silty Clay
					Pasture	Clay
St. Gabriel	30.2698ºN 91.1035ºW	6/8/2015 6/23/2016 6/30/2016	L 01-299	Commerce Silty Clay Loam	Sugarcane	Silty Clay Loam
					Pasture	Clay Loam

3.2.3 DNA Sequencing

DNA preparation and sequencing were performed at Argonne National Laboratory's Environmental Sample Preparation and Sequencing Facility in Lemont, IL. 16S rDNA was amplified by polymerase chain reaction with modified 515F and 806R primers (Appril et al. 2015) and unique 12 base pair barcodes were added to amplicons from each sample. Amplicons were sequenced on the Illumina MiSeq platform with paired end read lengths of 151 base pairs. The ITS1 gene was amplified with modified ITS1F and ITS2 primers (Smith and Peay 2014) and unique 12 base pair barcodes were

added to amplicons from each sample. Amplicons were sequenced on the Illumina MiSeq platform with paired-end read lengths of 250 base pairs.

3.2.4 Processing of Amplicon Sequencing

Qiime 1.9.1 (Casporaso 2010) was used to process prokaryotic 16S and fungal ITS datasets. Qiime was used for joining paired-end reads, demultiplexing sequencing data, OTU picking, chimera removal, taxonomic assignment, and downstream community analysis.

3.2.4.1 Demultiplexing

Joining of paired-end forward and reverse reads generated from 16S amplicon sequencing was done with Qiime's "join_paired_ends.py" using Fastq-join (Aronesty 2011) using Qiime's default parameter settings. In the case of ITS amplicons, only a small number of reads could be successfully joined and so forward and reverse paired end reads were analyzed individually.

Joined 16S amplicons and ITS forward and reverse amplicons were all demultiplexed using Qiime's "split_libraries_fastq.py script". This step was used to combine data from two sequencing runs as well as to filter low quality data. Reads were also quality filtered at this step based on the phred scores associated with confidence of each individual base call. Reads were trimmed after three consecutive base calls with phred scores below 20. Reads were only retained if the maximum number of consecutive base calls of 20 or greater was 75% of the original read length and contained no more than one barcode error.

3.2.4.2 OTU Picking

All OTU picking was performed using UCLUST (Edgar 2010) and a sequence similarity threshold of 0.97. The OTU picking process followed Qiime's "pick_open_reference_otus.py" workflow, but steps in the workflow were run as several batch jobs to avoid wasting resources by unnecessarily running serial jobs that require few computation resources in a parallel environment. OTUs were first clustered against seeds selected from a reference database. For 16S OTU picking, Silva 128's 97% similarity OTU database (Quast et al. 2012) was used as a reference. ITS reference based OTU picking was performed using the developer's version of the UNITE (Abarenkov et al. 2010) database's dynamic OTUs. Sequences that failed to match the database in the first round of OTU selection were subsampled at 0.01%, clustered using *de novo* OTU picking, and used as a new reference for a second round of closed reference OTU picking. Sequences that failed to cluster against the reference database or the subsampled *de novo* reference were clustered in a final round of *de novo* OTU picking. OTUs from the two rounds of closed reference OTU picking and *de novo* OTUs were combined, and OTUs consisting of a single sequence were removed.

Following OTU picking, the representative set of sequences that served as centroids for OTU clustering were checked for chimeric sequences in Qiime using the "identify_chimeric_seqs.py" script with Usearch 6.1's reference-based detection method (Edgar 2010, Edgar et al. 2011) against the same reference database used for OTU picking and the default parameters set by Qiime. OTU's with centroids identified as chimeras as well as OTUs composed of a single sequence were removed using Qiime's "filter_otus_from_otu_table.py" script.

Following chimera removal, taxonomy was assigned to each OTU using representative sequences. 16S taxonomy was assigned using the UCLUST algorithm (Edgar 2010) and default parameter values: a minimum required similarity of 90% to be considered a match, a maximum of three database hits to be considered, and required majority consensus for taxonomic assignment at a given depth. Taxonomic assignment for ITS OTUs resulted in the majority of sequences not being assigned any taxonomy, so taxonomic assignment was implemented using blast with a default maximum e value of 0.001 (Altschul et al. 1990). OTUs with no taxonomic identity, or, in the case of ITS OTUs, taxonomic identity outside the fungal kingdom, were removed from further analysis.

Due to variability within the fungal kingdom, short read ITS sequences generated on the Illumina MiSeq platform cannot be aligned or used for phylogenetic analysis (Lindahl et al. 2013). 16S representative sequences were aligned using PyNast (Caporsao et al. 2010). The aligned rep set was used to construct a phylogeny using FastTree (Price et al. 2009) for downstream analysis.

3.2.5 Statistical Analysis

In order to compare microbial communities of samples at an even depth, all samples were rarefied to an even sequencing depth determined by the sample containing the fewest of sequences. In order to determine if rarefaction of all samples at an even sequencing depth captured a sufficient proportion of diversity contained within unrarefied samples, multiple rarefactions were performed at increasing sampling depths up to the depth of the sample containing the fewest reads using Qiime's "multiple_rarefactions.py" The slopes of rarefaction curves represent the rate of additional OTUs were sampled at increasing sampling depths to determine if all diversity captured in sequencing was represented.

Differences in taxonomic assignment for samples from separate DNA extractions, resequencing of DNA extractions, and forward and reverse ITS amplicons were examined using Qiime's "comepare_taxa_summaries.py" for Pearson's correlation coefficient and permutation test p-values constructed using 999 iterations. In order to identify taxa that differed in abundance based on cropping history, Kruskal–Wallis one-way ANOVA was used to test ITS and 16S taxa at all levels of assignment (Kruskal and Wallis 1952).

In order to compare pairwise sample dissimilarity in microbial community structure, OTU tables were converted into distance matrices using Qiime's "beta_diversity.py" function. ITS data was transformed into a Bray-Curtis distance (Bray and Curtis 1957). 16S data was transformed into Bray-Curtis and both weighted and unweighted forms of UniFrac (Lozupone and Knight 2005) using the phylogenetic tree constructed with FastTree.

Analysis of varianace was performed on distance matrices in R (R Core Team 2016) to test for effects of cropping history, location, soil niche (bulk or rhizosphere), and crop year on microbial community structure. Distance-based redundancy analysis was "capscale" in R using the *vegan* package (Oksanen et al. 2017). Ordination was plotted using distance-based redundancy analysis to show differences in soil microbial community structure. Soil environmental variables determined previously for the paired sites included in this study (Chapter 2) were co-plotted as vectors based on maximum correlation using the *envfit* function (Oksanen et al. 2017) to show association between metagenomics microbial community structure and other environmental factors.

3.3 Results

3.3.1 16S Prokaryotic Metagenomics

3.3.1.1 Rarefaction of 16S Sequencing Data

Processing of 16S amplicons generated on the Illumina MiSeq platform in Qiime produced 153,314 operational taxonomic units from 19,121,373 sequences from 131 samples. Individual samples ranged from 52,627 to 332,612 sequences. To compare the structure of 16S prokaryotic communities, all samples were rarefied to an equal depth of 52,500, a value below that of the sample containing the fewest sequences. The rarefied dataset contained a total of 121,535 OTUs. Multiple rarefactions at increasing sequencing sampling depths were performed on bulk and rhizosphere samples from plant cane (Figure 3.1) and locations sampled in plant cane and first ratoon (Figure 3.2).

3.3.1.2 Prokaryotic Community Composition

The composition of prokaryotic communities varied at the phylum level in bulk soils (Figure 3.3) and rhizosphere soils (Figure 3.4). Proteobacteria, Acidobacteria, and Actinobacteria made up the majority of the community in both bulk and rhizosphere soils in plant cane. Actinobacteria were more abundant in bulk and rhizosphere soils with a short-term sugarcane cropping history and Firmicutes were more abundant in bulk soils with a short-term sugarcane cropping history ($p < 0.05$).

Differences in prokaryotic phyla based on cropping history at the two locations sampled in plant cane and first ratoon were not observed in rhizosphere or bulk soils. However, changes in soil prokaryotic phyla were observed between plant cane and first ratoon in bulk soil (Figure 3.5) and rhizosphere soil (Figure 3.6). Proteobacteria and Gemmatimonadetes were more abundant in plant cane in both bulk and rhizosphere soils, whereas Bacteriodetes, Chloroflexi, and Firmicutes were more abundant in first

ratoon rhizosphere and bulk soil. Verrucomicrobia were also greater in first ratoon bulk soils ($p < 0.05$).

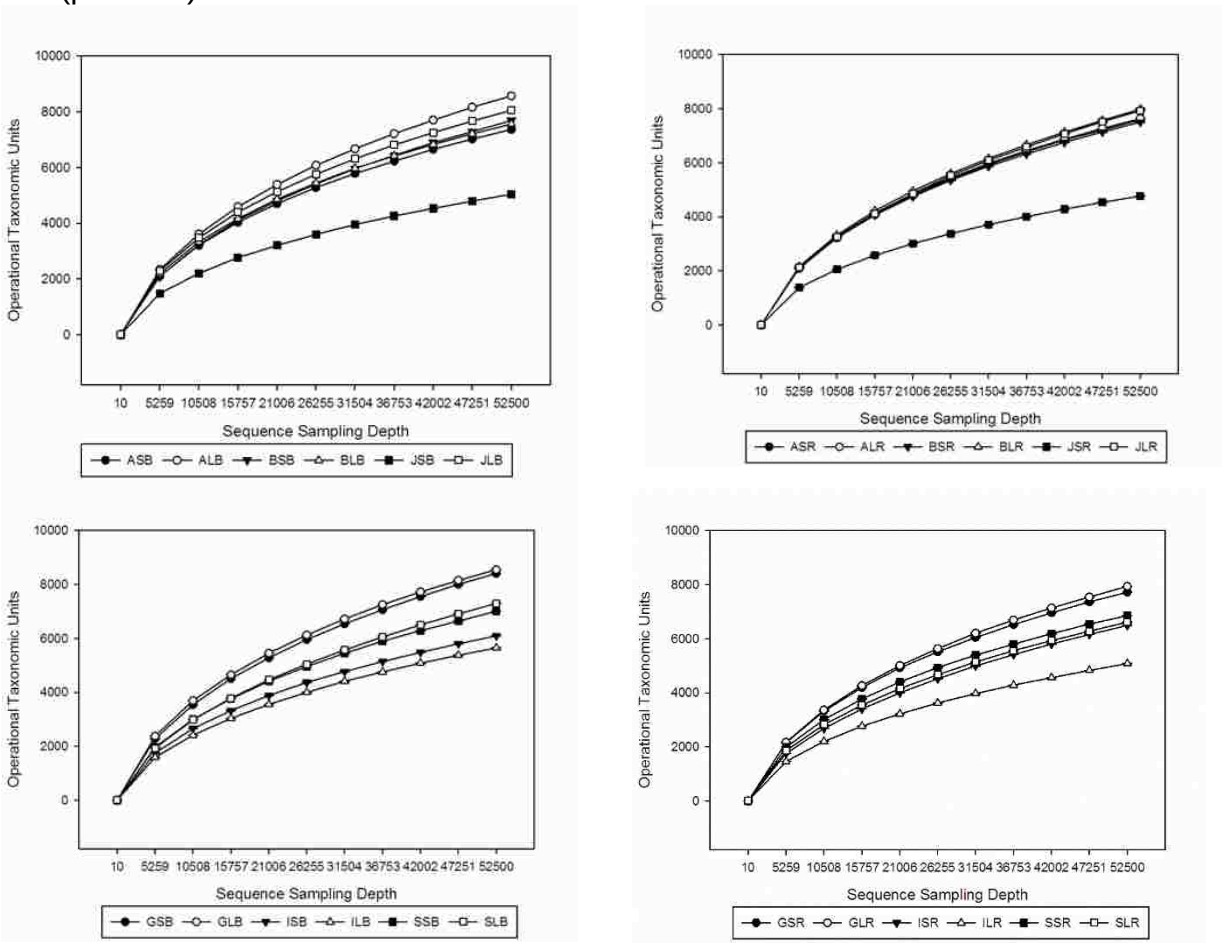

Figure 3.1. Rarefaction curves for the number of 16S OTUs from soils from paired sites in plant cane at six locations with short and long-term sugarcane cropping histories at increasing sequencing depths in bulk and rhizosphere soils. Top left. Three 2014 locations bulk soil samples; Top right. Three 2014 locations rhizosphere soil samples; Bottom left. Three 2015 locations bulk soil samples; Bottom right. Three 2015 locations rhizosphere soil samples. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel. Cropping history: S = short-term cultivation, L = long-term cultivation. Soil niche: B = bulk soil, R = rhizosphere soil.

A total of 16 soil samples were sequenced in both 16S Illumina MiSeq runs. Five samples were re-extracted and re-sequenced and eleven samples were re-sequenced using the same extractions. Permutation tests resulted in no significant difference in taxonomic assignment at any levels for both re-extracted and re-sequenced samples ($p < 0.001$). Pearson's correlation coefficient was greater for samples where the same sample was re-extracted rather than re-extracted and re-sequenced (Table 3.2).

Taxonomic assignment of 68,093 16S OTUs present in bulk soils from six locations of sugarcane with short and long-term cropping histories resulted in 844 unique taxonomic assignments at the family level, of which 74 were significantly greater in long-term

cultivation soils and 30 were greater in short-term cultivated soils ($p < 0.05$). The most abundant families associated with short-term cultivation (Table 3.3) were of interest as candidate beneficial soil microbes, whereas the most abundant families more associated with long-term cultivation (Table 3.4) were of interest as candidates for being detrimental to sugarcane yields. At the genus level, 1,978 unique taxons were assigned to OTUs from plant cane bulk soils. Of which, 107 genera were greater in short-term cultivation soils ($p < 0.05$), with the 25 most abundant listed in Table 3.5, and 117 were greater in long-term cultivation soils ($p < 0.05$), with the 25 most abundant listed in Table 3.6. The most abundant taxa without an association with either cropping history were listed in Table A.1 and Table A.2.

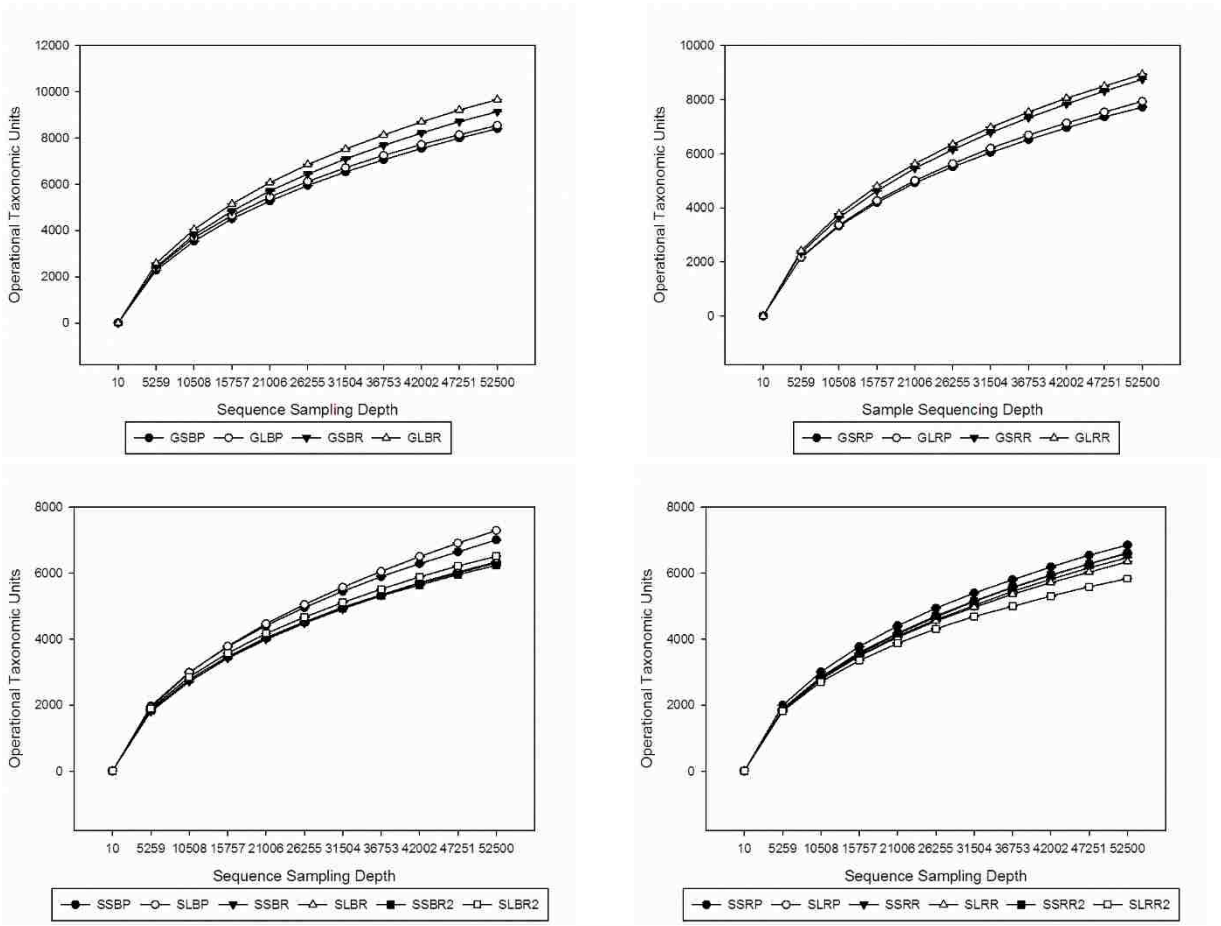

Figure 3.2. Rarefaction curves of the number of 16S OTUs from soils from paired sites at two locations with short and long-term sugarcane cropping histories in plant cane and first ratoon at increasing sequence sampling depths. Top left. Gonsoulin location bulk soil samples; Top right.Gonsoulin location rhizosphere soil samples; Bottom left. St. Gabriel location bulk soil samples; Bottom right. St. Gabriel rhizosphere soil samples. Locations: G = Gonsoulin and S = St. Gabriel. Cropping history: S = short-term cultivation, L = long-term cultivation. Soil niche: B = bulk soil, R = rhizosphere soil. Crop year: P = plant cane, R = first ratoon, R2 = first ratoon second sampling.

Taxonomic assignment of 65,509 16S OTUs present in rhizosphere soils from six locations of sugarcane with short and long-term cropping histories resulted in 942

unique taxonomic assignments at the family level, of which 54 were significantly greater long-term cultivation soils and 42 were greater in short-term cultivated soils ($p < 0.05$). The most abundant families associated with short-term cultivation (Table 3.7) were of interest as candidate beneficial soil microbes, whereas the most abundant families more associated with long-term cultivation (Table 3.8) were of interest as candidates for being detrimental to sugarcane yields. At the genus level, 1,955 unique taxons were assigned to OTUs from plant cane bulk soils. Of which, 97 genera were greater in short-term cultivation soils ($p <0.05$), with the 25 most abundant listed in Table 3.9, and 94 were greater in long-term cultivation soils ($p < 0.05$), with the 25 most abundant listed in Table 3.5. The most abundant taxa without an association with either cropping history were listed in Table A.3 and Table A.4.

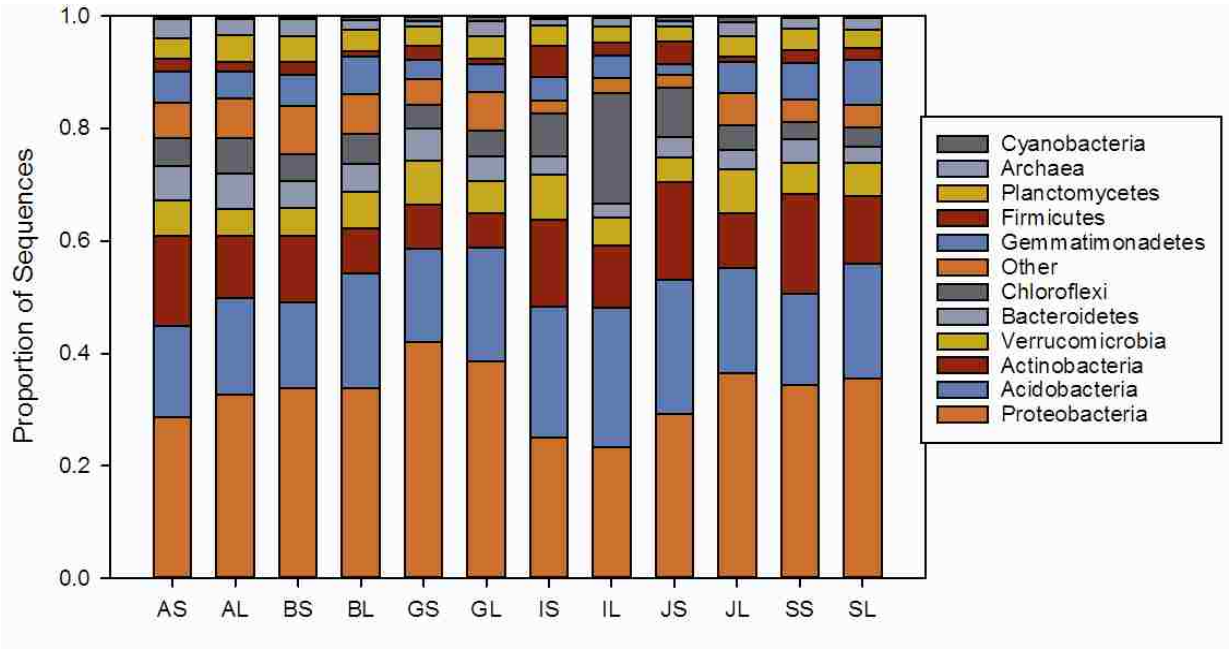

Figure 3.3. Relative abundance of bacterial phyla from bulk soils from paired sites with short and long-term sugarcane cropping histories at six locations in plant cane. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel. Cropping history: S = short-term cultivation, L = long-term cultivation.

3.3.1.3 Prokaryotic Community Structural Analysis

3.3.1.3.1 Prokaryotic Community Structural Analysis for Bulk Soils in Plant Cane

Analysis of variance of weighted UniFrac, unweighted UniFrac, and Bray-Curtis OTU β-diversity revealed significant effects on the structure of sugarcane prokaryotic communities in bulk soil based on cropping history, location, and their interaction (Table 3.11).

Weighted UniFrac distance-based redundancy analysis revealed differences in bacterial communities driven by differences in cropping history only at the Jefferson and Iberia locations (Figure 3.7). Axis 1 captured 57.64% of variance and axis 2 contained

11.08%. Maximum correlation of soil pH was a factor in a larger-scale cluster of three locations, while manganese affected the clustering of short and long-term cultivation sites at the Iberia and Jefferson locations, respectively (Figure 3.8). Soluble salts, silt, calcium, sulfur, magnesium, potassium, clay, soil organic matter, nitrate, iron and manganese were all significantly correlated with variance in prokaryotic community structure by location (Figure 3.8).

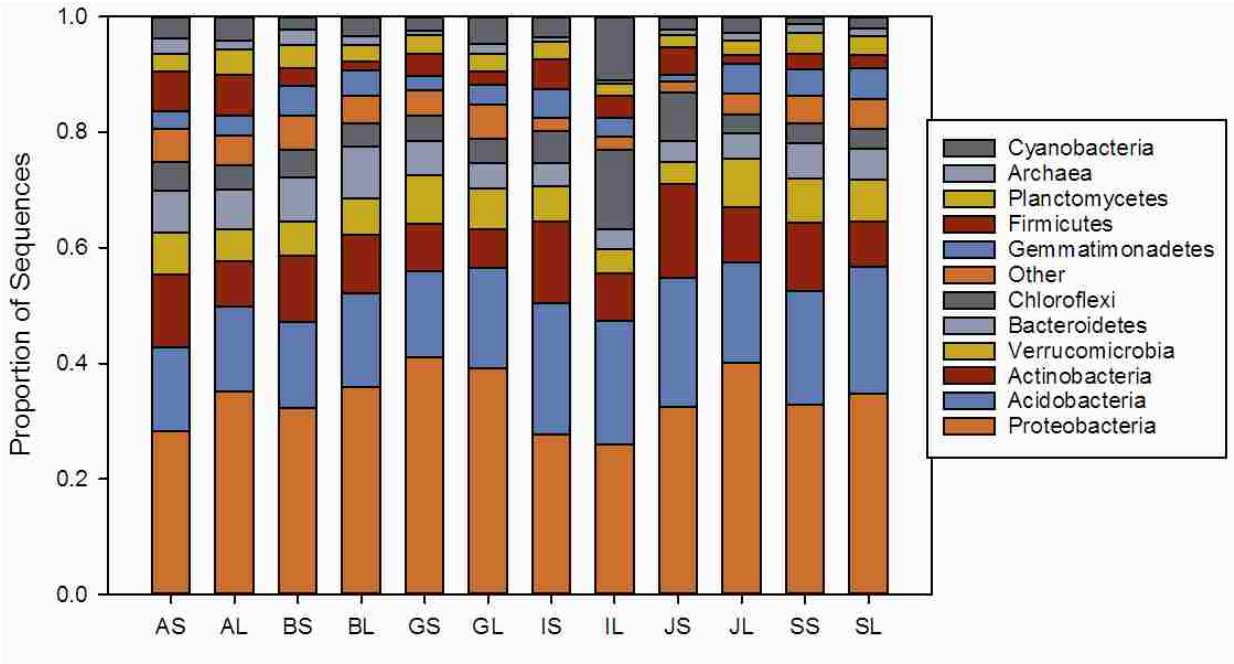

Figure 3.4. Relative abundance of bacterial phyla from rhizosphere soils from paired sites with short and long-term sugarcane cropping histories at six locations in plant cane. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel. Cropping history: S = short-term cultivation, L = long-term cultivation.

Distance-based redundancy analysis of Bray-Curtis captured 41.73% of variance on axis 1 and 16.59% on axis 2 (Figure 3.9). Bray-Curtis ordination of bulk soils primarily distinguished community composition based on location, but separated two locations, Belleview and Jefferson, based on cropping history. Three locations formed tight clusters containing short and long-term cropping histories. Correlation of environmental variables was primarily location specific (Figure 3.10). Maximum correlation of nitrate, gravimetric water, manganese, iron, sulfur, soil organic matter, clay, potassium, pH, silt, N-Acetyl-β-D-glucosaminidase, and β-glucosidase were all associated with no more than two locations or specific cropping histories for given locations.

Unweighted UniFrac distance-based redundancy analysis of prokaryotic OTUs from bulk soil produced a near identical ordination as unweighted UniFrac (Figure A.1). Axis 1 captured 34.46% of variance and axis 2 14.41%. Similar to unweighted UniFrac, Maximum correlation of environmental variables was also similar to Bray-Curtis: ammonium, gravimetric water, manganese, iron, sulfur, soil organic matter, clay, potassium, pH, silt, N-Acetyl-β-D-glucosaminidase, and β-glucosidase were all significantly correlated with Bray-Curtis ordination (Figure A.2).

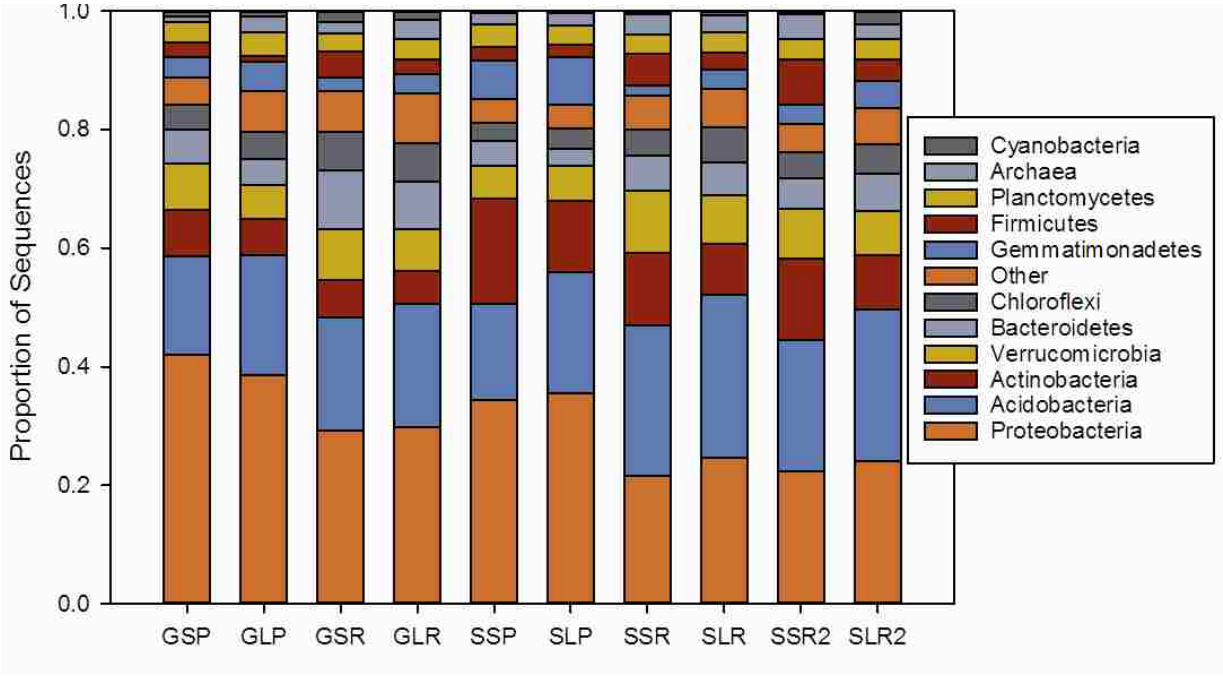

Figure 3.5. Relative abundance of bacterial phyla from bulk soils from paired sites with short and long-term sugarcane cropping histories at two locations sampled in plant cane and first ratoon. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R = first ratoon, Fa= first ratoon, R2= first ratoon second sampling.

3.3.1.3.2 Prokaryotic Community Structural Analysis for Rhizosphere Soils in Plant Cane

Analysis of variance of weighted UniFrac, unweighted UniFrac, and Bray-Curtis OTU β-diversity for 16S metagenomic community composition in rhizosphere soils all revealed significant effects on prokaryotic community structure due to cropping history, location, and their interaction (Table 3.12).

Distance-based redundancy analysis of weighted UniFrac of rhizosphere soils resulted in loose clusters based on location along axis 1 with varying extents of separation based on cropping history, with the exception of the Jefferson location separated by cropping history along axis 1 (Figure 3.11). The analysis captured 54.66% of variance on axis 1 and 15.63% on axis 2.

Distance-based redundancy analysis of Bray-Curtis distance for rhizosphere soils captured 42.11% of variance on axis 1 and 16.23% on axis 2 (Figure 3.12). Clustering generally occurred based on location with Jefferson, Iberia, and Belleview locations separated by cropping history.

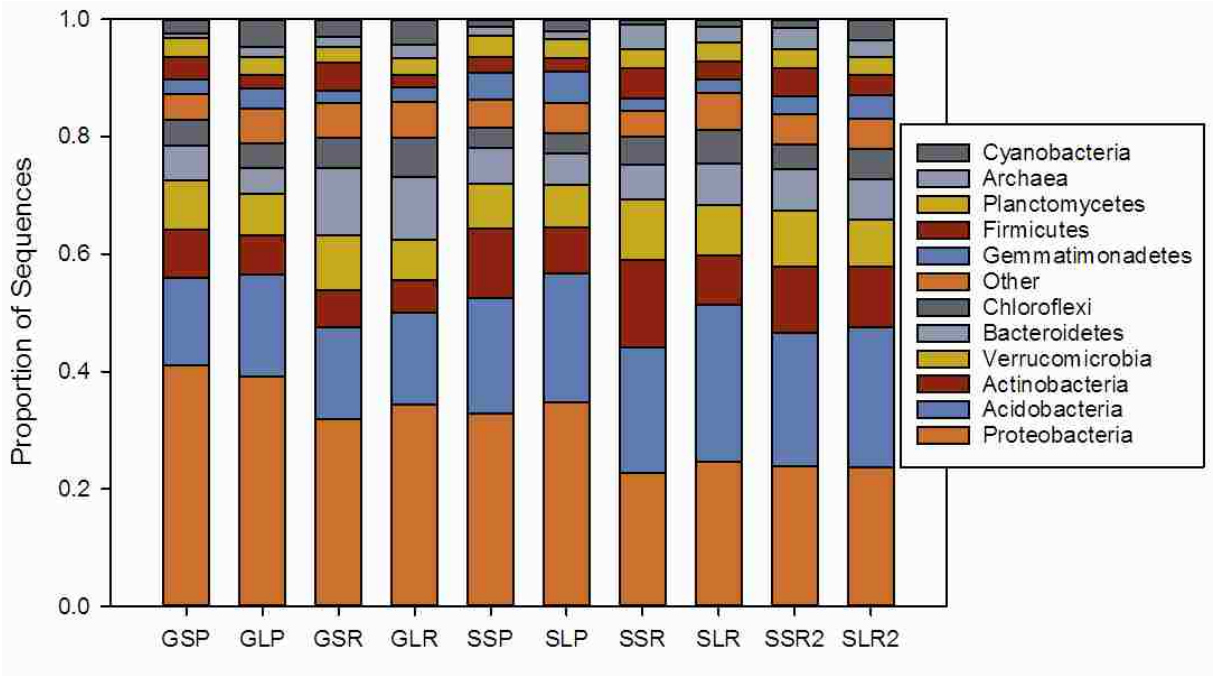

Figure 3.6. Relative abundance of bacterial phyla from rhizosphere soils from paired sites with short and long-term sugarcane cropping histories sampled at two locations in plant cane and first ratoon. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R = first ratoon, R= first ratoon, R2= first ratoon second sampling.

Table 3.2. Pearson's correlation 16S metagenomic community composition for bulk soil from paired sites with short and long-term sugarcane cropping histories in plant cane at six locations.

	Phylum	Class	Order	Family	Genus
Re-extracted Samples	0.9599	0.9184	0.8904	0.8757	0.8741
Re-sequenced Samples	0.9818	0.963	0.9485	0.9422	0.9417

Distance-based redundancy analysis of unweighted Unifrac β-diveristy for rhizosphere soils captured 35.15% of variance on axis 1 and 14.22% on axis 2 (Figure A.3). Ordination of unweighted UniFrac, representing the relatedness of community members, was similar to Bray-Curtis β-diversity in rhizosphere prokaryotic communities, representing the abundance of OTUs, but not weighted UniFrac, which incorporates abundance counts into unweighted UniFrac.

3.3.1.3.2 Prokaryotic Community Structural Analysis in Plant Cane and Ratoon Soils

Analysis of variance revealed significant differences in bulk soil prokaryotic community structure associated with sugarcane cropping history, location, and crop year differences between plant cane and first ratoon (Table 3.13). Interactions between

cropping history x location and crop year x location were also significant for all three β-diversity metrics.

Table 3.3. Prokaryotic families more associated with bulk soils under short-term sugarcane cultivation than long-term cultivation ($p < 0.05$) and their abundance and relative rank among families for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of prokaryotic community	Rank among families	Proportion of prokaryotic community	Rank among families
Xanthobacteraceae	0.026569	5	0.020719	11
Bacillaceae	0.018365	15	0.007101	32
Hyphomicrobiaceae	0.008089	33	0.006103	39
Soil Crenarchaeotic Group(SCG) Other	0.007760	34	0.003874	52
Solirubrobacterales Elev-16S-1332	0.007304	35	0.003977	50
Streptomycetaceae	0.004022	53	0.002752	74
Alicyclobacillaceae	0.001452	114	0.000525	204
Planococcaceae	0.001366	118	0.000534	201
Ktedonobacterales 1959-1	0.000854	156	0.000523	205
Methylocystaceae	0.000766	168	0.000402	226
Acidimicrobiaceae	0.000676	179	0.000474	213
Ambiguous Chloroflexi KD4-96	0.000705	175	0.000444	218
Methylococcaceae	0.000617	185	0.000083	387
Ruminococcaceae	0.000428	224	0.000241	272
Ambiguous Gemmatimonadetes S0134 terrestrial group	0.000370	236	0.000152	321
Nitrospirales FW13	0.000283	260	0.000084	385
Thermoactinomycetaceae	0.000235	279	0.000055	434
Nocardiaceae	0.000131	338	0.000077	392
Ambiguous Planctomycetes vadinHA49	0.000138	328	0.000056	432
Peptostreptococcaceae	0.000138	329	0.000044	459
Methanobacteriaceae	0.000128	343	0.000015	565
Cyanobacteria ML635J-21 Other	0.000081	391	0.000035	472
Ignavibacteriaceae	0.000091	383	0.000001	775
Clostridiales Other	0.000054	430	0.000001	776
Cyclobacteriaceae	0.000046	447	0.000005	655

Table 3.4 Prokaryotic families more associated with bulk soils under long-term sugarcane cultivation than short-term cultivation ($p < 0.05$) and their abundance and relative rank among families for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of prokaryotic community	Rank among families	Proportion of prokaryotic community	Rank among families
Gemmatimonadaceae	0.037745	2	0.051656	2
Nitrosomonadaceae	0.017378	16	0.033256	4
Rhizobiales Incertae Sedis	0.011954	23	0.016905	15
Verrucomicrobia OPB35 soil group Other	0.004506	47	0.006098	40
Coxiellaceae	0.003607	56	0.005531	42
Uncultured Ktedonobacteria JG30-KF-AS9	0.002802	77	0.006168	38
Soil Crenarchaeotic Group(SCG) Unknown Family	0.002425	87	0.005000	44
Fimbriimonadaceae	0.002386	88	0.003495	58
Rhizobiales KF-JG30-B3	0.002343	90	0.003402	60
Uncultured Thaumarchaeota	0.000833	157	0.003460	59
Burkholderiaceae	0.001223	129	0.002172	90
Uncultured Chloroflexi SBR2076	0.000932	149	0.001434	116
Thermoplasmatale Marine Group II	0.000559	200	0.001748	100
Simkaniaceae	0.000574	195	0.001617	105
Phycisphaeraceae	0.000810	160	0.001378	123
Chlamydiales cvE6	0.000515	212	0.001668	103
Solimonadaceae	0.000528	206	0.001618	104
Uncultured Acidobacteria Subgroup 15	0.000672	180	0.001456	114
Unculutred Acidobacteria Subgroup 13	0.000719	171	0.001335	127
Uncultured Omnitrophica	0.000560	199	0.001385	121
Rhodospirillales JG37-AG-20	0.000164	315	0.000994	155
Uncultured Elusimicrobia Lineage IV	0.000326	247	0.000817	168
Elusimicrobia Lineage IIa Other	0.000292	256	0.000769	175
Ambiguous Acidobacteria Subgroup 13	0.000062	418	0.000730	179
Ignavibacteriale BSV26	0.000135	332	0.000590	192

Table 3.5. Prokaryotic genera more associated with bulk soils under short-term sugarcane cultivation than long-term cultivation ($p < 0.05$) and their abundance and relative rank among genera for each cropping history.

	Short-term cropping history		Long-term cropping history	
Genus	Proportion of prokaryotic community	Rank among genera	Proportion of prokaryotic community	Rank among genera
Bacillus	0.017618	10	0.006953	30
Soil Crenarchaeotic Group(SCG) Other	0.007760	28	0.003874	52
Uncultured Solirubrobacterales	0.006075	37	0.003114	72
Streptomyces	0.003928	56	0.002689	82
Defluviicoccus	0.004998	45	0.001542	125
Xanthobacteraceae uncultured	0.003593	58	0.001691	107
Nocardioides	0.003156	66	0.002011	101
Myxococcales P3OB-42	0.002869	76	0.001600	114
Tepidisphaeraceae Other	0.001979	111	0.001144	169
Solirubrobacterales Elev-16S-1332	0.001228	152	0.000860	199
Rhodomicrobium	0.001412	138	0.000492	272
Planococcaceae Other	0.000941	181	0.000381	310
Tumebacillus	0.000923	184	0.000386	305
Uncultured Ktedonobacterales 1959-1	0.000796	205	0.000503	269
Isosphaera	0.000877	188	0.000333	335
Actinomadura	0.000875	189	0.000323	346
Ambiguous Chloroflexi KD4-96	0.000705	220	0.000444	283
Methylosinus	0.000655	235	0.000349	327
Fibrobacteraceae possible genus 04	0.000674	229	0.000192	424
Uncultured Solirubrobacterales TM146	0.000531	273	0.000286	361
Bacillaceae Other	0.000499	285	0.000089	576
Ambiguous Gemmatimonadetes S0134 terrestrial group	0.000370	319	0.000152	463
Ambiguous Planctomycetaceae	0.000341	334	0.000095	556
Luteimonas	0.000302	352	0.000072	617
Lysinibacillus	0.000283	370	0.000028	839

Table 3.6. Prokaryotic genera more associated with bulk soils under long-term sugarcane cultivation than short-term cultivation ($p < 0.05$) and their abundance and relative rank among genera for each cropping history.

	Short-term cropping history		Long-term cropping history	
Genus	Proportion of prokaryotic community	Rank among genera	Proportion of prokaryotic community	Rank among genera
Uncultured Gemmatimonadaceae	0.019399	9	0.032525	2
Uncultured Nitrosomonadaceae	0.016784	11	0.032501	3
Verrucomicrobia OPB35 soil group Other	0.004506	50	0.006098	36
Uncultured Ktedonobacteria JG30-KF-AS9	0.002802	79	0.006168	34
Aquicella	0.003091	69	0.005038	39
Candidatus Nitrososphaera	0.002425	91	0.005000	40
Uncultured Rhizobiales KF-JG30-B3	0.002338	94	0.003399	65
Uncultured Fimbriimonadaceae	0.002286	96	0.003344	67
Mizugakiibacter	0.000929	183	0.003856	53
Uncultured Thaumarchaeota Gp 1(SAGMCG-1)	0.000833	198	0.003460	62
Burkholderia-Paraburkholderia	0.000992	173	0.001825	103
Reyranella	0.001077	166	0.001559	122
Uncultured Chloroflexi SBR2076	0.000932	182	0.001434	136
Uncultured Acidobacteria Subgroup 15	0.000672	231	0.001456	133
Polycyclovorans	0.000493	286	0.001586	117
Ambiguous Acidobacteria Subgroup 13	0.000719	217	0.001335	148
Uncultured Simkaniaceae	0.000524	276	0.001497	129
Rhizobium	0.000677	228	0.001328	149
Uncultured Omnitrophica	0.000560	269	0.001385	141
Uncultured Rhizobiales	0.000485	288	0.001347	146
Chlamydiales cvE6 Other	0.000293	360	0.001141	170
Acidobacterium	0.000361	324	0.000869	198
Uncultured Elusimicrobia 3__Lineage IV	0.000326	341	0.000817	210
Rhodospirillales JG37-AG-20	0.000079	612	0.000838	206
Ambiguous Acidobacteria Subgroup 13	0.000062	677	0.000730	221

Table 3.7. Prokaryotic families more associated with rhizosphere soils under short-term sugarcane cultivation than long-term cultivation ($p < 0.05$) and their abundance and relative rank among families for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of prokaryotic community	Rank among families	Proportion of prokaryotic community	Rank among families
Uncultured Gaiellales	0.024743	6	0.017502	12
Uncultured Acidimicrobiales	0.007383	35	0.005149	42
Soil Crenarchaeotic Group(SCG) Other	0.006487	37	0.002784	67
Solirubrobacterales Elev-16S-1332	0.005104	43	0.002574	77
Myxococcales P3OB-42	0.003789	57	0.002879	65
Pseudonocardiaceae	0.003453	62	0.002444	82
Nocardioidaceae	0.003224	67	0.002315	85
Planococcaceae	0.002697	74	0.002171	88
Gaiellaceae	0.002813	72	0.001838	99
Mycobacteriaceae	0.002066	94	0.001176	128
Alicyclobacillaceae	0.002269	86	0.000862	158
Streptosporangiaceae	0.000812	151	0.000491	201
Acidimicrobiaceae	0.000608	181	0.000436	211
Ktedonobacterales 1959-1	0.000724	163	0.000270	258
Ambiguous Chloroflexi KD4-96	0.000614	179	0.000379	224
Ambiguous Rhizobiales	0.000678	167	0.000230	274
Ruminococcaceae	0.000364	224	0.000222	278
Methylococcaceae	0.000401	220	0.000054	422
Ambiguous Gemmatimonadete S0134 terrestrial group	0.000321	240	0.000116	336
Bacteroidetes VC2.1 Bac22 uncultured bacterium	0.000228	283	0.000109	344
Thermoactinomycetaceae	0.000196	295	0.000068	400
Nitrospirales FW13	0.000198	294	0.000032	464
Gracilibacteraceae	0.000180	302	0.000047	436
Peptostreptococcaceae	0.000139	327	0.000039	447
Deltaproteobacteria 43F-1404R Other	0.000143	322	0.000005	633

Table 3.8. Prokaryotic families more associated with rhizosphere soils under long-term sugarcane cultivation than short-term cultivation ($p < 0.05$) and their abundance and relative rank among families for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of prokaryotic community	Rank among families	Proportion of prokaryotic community	Rank among families
Nitrosomonadaceae	0.017260	17	0.028516	7
Oxalobacteraceae	0.004212	54	0.009716	26
Verrucomicrobia OPB35 soil group Other	0.005180	42	0.007833	32
Coxiellaceae	0.004278	53	0.006143	39
Sphingobacteriaceae	0.002260	87	0.004924	46
Uncultured Ktedonobacteria JG30-KF-AS9	0.002073	93	0.004751	48
Burkholderiaceae	0.002372	85	0.004280	50
Fimbriimonadaceae	0.002459	81	0.003752	55
Soil Crenarchaeotic Group(SCG) Unknown Family	0.001949	97	0.003352	57
Rhizobiaceae	0.001638	110	0.003080	60
Uncultured Obscuribacterales	0.000857	146	0.001184	127
Solimonadaceae	0.000636	178	0.001331	118
Chlamydiales cvE6	0.000748	158	0.001210	124
Neisseriaceae	0.000338	236	0.001598	106
Chthoniobacterales LD29	0.000359	228	0.001543	108
Uncultured Thaumarchaeota	0.000420	214	0.001393	114
Uncultured ElusimicrobiaLineage IIa	0.000733	161	0.000959	143
Uncultured Omnitrophica	0.000566	187	0.000929	147
Uncultured Chthonomonadales	0.000525	198	0.000877	156
Patulibacteraceae	0.000344	233	0.000590	184
SAR324 clade(Marine group B) Other	0.000135	331	0.000502	199
Uncultured Elusimicrobia Lineage IV	0.000171	307	0.000466	207
Ambiguous Acidobacteria Subgroup 13	0.000040	446	0.000393	218
Uncultured Elusimicrobia Lineage IIc	0.000065	399	0.000224	277
Uncultured Bellilinea	0.000057	412	0.000231	273

Table 3.9. Prokaryotic genera more associated with rhizosphere soils under short-term sugarcane cultivation than long-term cultivation ($p < 0.05$) and their abundance and relative rank among genera for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of prokaryotic community	Rank among families	Proportion of prokaryotic community	Rank among families
Uncultured Gaiellales	0.017574	9	0.012109	16
Soil Crenarchaeotic Group(SCG) Other	0.006487	32	0.002784	71
Uncultured Acidimicrobiales	0.004758	43	0.002898	68
Uncultured Solirubrobacterales Elev-16S-1332	0.004096	57	0.001960	96
Defluviicoccus	0.004612	45	0.001071	162
Gaiella	0.002813	74	0.001838	107
Uncultured Xanthobacteraceae	0.002937	72	0.001370	137
Planococcaceae Other	0.002234	94	0.001959	97
Myxococcales P3OB-42 Other	0.002423	86	0.001333	140
Mycobacterium	0.002066	103	0.001176	157
Solirubrobacterales Elev-16S-1332	0.001007	178	0.000607	242
Rhodomicrobium	0.001241	162	0.000307	334
Tumebacillus	0.000855	190	0.000439	288
Isosphaera	0.000782	202	0.000251	385
Ambiguous Chloroflexi KD4-96	0.000614	242	0.000379	307
Uncultured Ktedonobacterale 1959-1	0.000650	236	0.000260	380
Amibuous Rhizobiales	0.000678	222	0.000230	401
Kribbella	0.000560	260	0.000262	378
Uncultured Solirubrobacterales TM146	0.000526	273	0.000193	434
Luteimonas	0.000580	254	0.000128	500
Chthoniobacterales DA101 soil group	0.000400	306	0.000216	414
Bacillaceae Other	0.000384	310	0.000090	584
Ambiguous Gemmatimonadetes	0.000321	345	0.000116	522
Microbispora	0.000290	363	0.000089	589
Ambiguous Planctomycetaceae	0.000251	386	0.000059	669

Table 3.10. Prokaryotic genera more associated with rhizosphere soils under long-term sugarcane cultivation than short-term cultivation ($p < 0.05$) and their abundance and relative rank among genera for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of prokaryotic community	Rank among families	Proportion of prokaryotic community	Rank among families
Uncultured Nitrosomonadaceae	0.016537	11	0.027515	4
Uncultured Gemmatimonadaceae	0.017324	10	0.022727	6
Verrucomicrobia OPB35 soil group Other	0.005180	41	0.007833	25
Mizugakiibacter	0.003158	66	0.007285	31
Massilia	0.003041	70	0.004861	44
Uncultured Ktedonobacteria JG30-KF-AS	0.002073	102	0.004751	46
Mucilaginibacter	0.001723	121	0.004460	47
Burkholderia-Paraburkholderia	0.002043	107	0.003897	54
Uncultured Fimbriimonadaceae	0.002304	93	0.003563	59
Candidatus Nitrososphaera	0.001949	111	0.003352	62
Rhizobium	0.001121	173	0.002307	88
Polycyclovorans	0.000602	248	0.001292	144
Noviherbaspirillum	0.000384	311	0.001434	132
Uncultured Thaumarchaeota	0.000420	299	0.001393	135
Unculutred Omnitrophica	0.000566	258	0.000929	184
Oxalobacteraceae Other	0.000216	418	0.001211	152
Uncultured Chthonomonadales	0.000241	391	0.000061	663
Pseudogulbenkiania	0.000270	372	0.001053	166
Sphingobacteriales env OPS 17 Other	0.000311	349	0.000971	180
Chthoniobacterales LD29 Other	0.000223	411	0.000992	177
Uncultured Oxalobacteraceae	0.000061	675	0.000993	176
Uncultured Chthoniobacterales LD29	0.000135	490	0.000551	258
DeltaproteobacteriaSAR324 clade(Marine group B) Other	0.000135	489	0.000502	269
Paucimonas	0.000119	516	0.000493	271
Ambiguous Acidobacteria Subgroup 13	0.000546	265	0.000363	314

Table 3.11. ANOVA p-values for factors affecting pair-wise dissimilarity of three distance metrics for 16S metagenomic community composition for bulk soil from paired sites with short and long-term sugarcane cropping histories in plant cane at six locations.

	Weighted UniFrac	Unweighted UniFrac	Bray-Curtis
Cropping history	0.001	0.004	0.001
Location	0.001	0.001	0.001
Cropping history x Location	0.001	0.001	0.001

α = 0.05. NS = not significant.

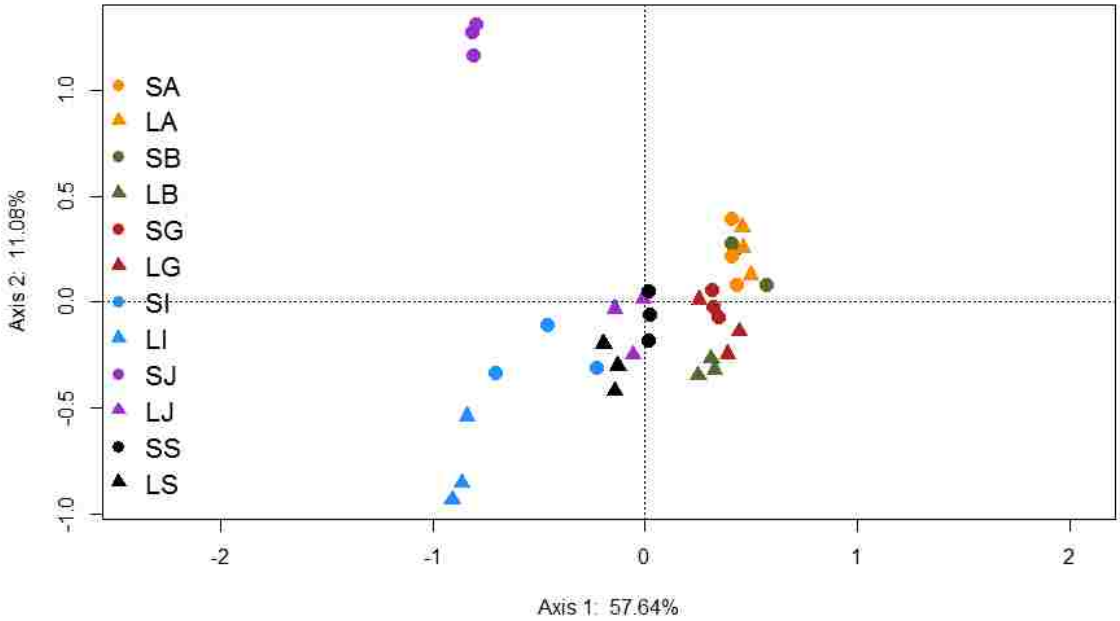

Figure 3.7 Distance-based redundancy analysis of weighted UniFrac distance matrix of 16S prokaryotic OTUs for bulk soils from paired sites with short and long-term sugarcane cropping histories in plant cane at six locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel.

Distance-based redundancy analysis of weighted UniFrac for bulk soils in plant cane and first ratoon separated microbial communities into four distinct clusters by location and crop year (Figure 3.13). Prokaryotic communities separated along axis 1 by location and axis 2 by crop year. Axis one captured 42.64% of variance, and axis two captured 33.47 % of variance. Differences in copper, potassium, iron, magnesium, calcium, salts, and pH were all significantly correlated with community structure differences between the two locations, while soil organic matter, manganese, and sodium were correlated with first ratoon prokaryotic communities at one or both locations (Figure 3.14).

Principal component analysis of microbial community structure in bulk soil as determined by Bray-Curtis was separated along axis 1 by location and axis 2 by cropping history (Figure 3.15). Axis 1 captured 51.25% of variance, and axis 2 18.07%.

Potassium, copper, calcium, magnesium, soil organic matter, and phosphorus all correlated with short-term cultivation for prokaryotic communities at one location (Figure 3.16). Zinc and sulfur were correlated with microbial communities from short-term cultivation. Manganese was correlated with long-term cultivation at one location, and pH and soluble salts were correlated with location specific differences.

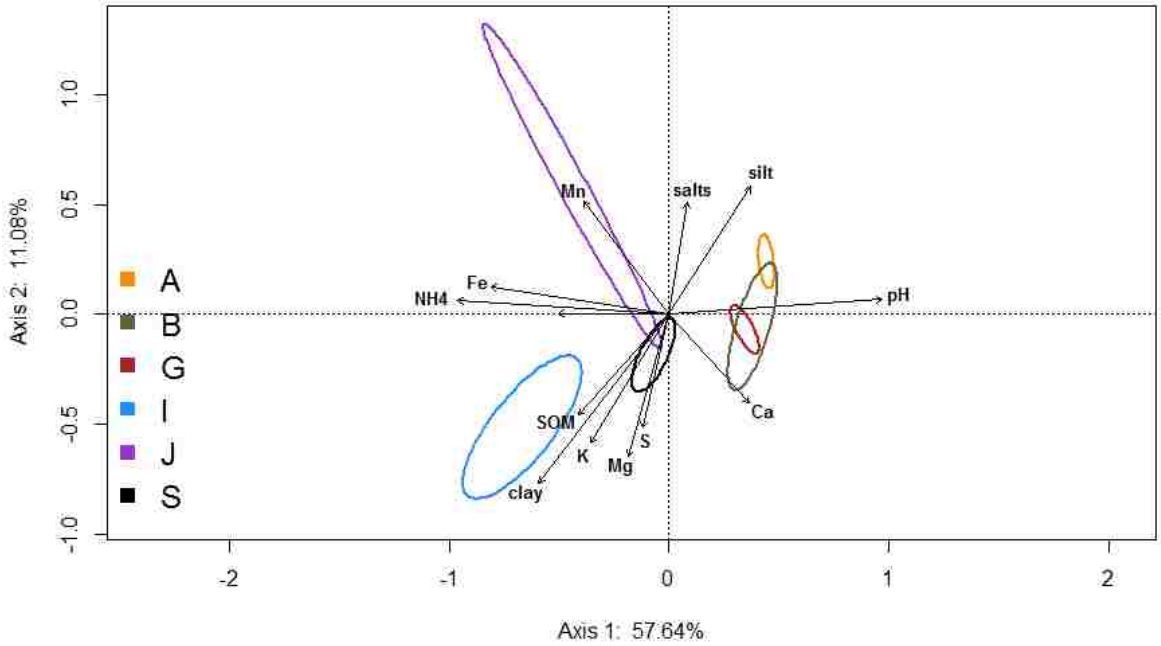

Figure 3.8 Distance-based redundancy analysis of weighted UniFrac distance matrix of 16S prokaryotic OTUs for bulk soils from paired sites with short and long-term sugarcane cropping histories in plant cane at six locations with maximum correlation of soil and nutrient environmental variables plotted as vectors ($p < 0.05$). Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel. Variables: NH4 = ammonium, grav water = gravimetric water, NAGase = N-Acetyl-β-D-glucosaminidase, gluco = β-glucosidase.

Distance-based redundancy analysis of unweighted UniFrac also separated bulk soil prokaryotic community composition along axis 1 by location and axis 2 by cropping history (Figure A.4). Axis 1 captured 41.85% of the variance, and axis 2 captured 15.07%. Maximum correlation of environmental variables detected association with potassium, copper, calcium, magnesium, soil organic matter, and iron with prokaryotic community composition at one location and pH and soluble salts at the other (Figure A.5). Zinc, sulfur, and phosphorus were correlated with microbial communities from short-term cultivation.

UniFrac of rhizosphere microbial communities revealed community structure was unrelated to cropping history, location, crop year, or any interaction thereof, whereas rhizosphere weighted UniFrac and Bray-Curtis diversity was affected by cropping history, location, crop year, cropping history x location, and location x crop year (Table 3.14).

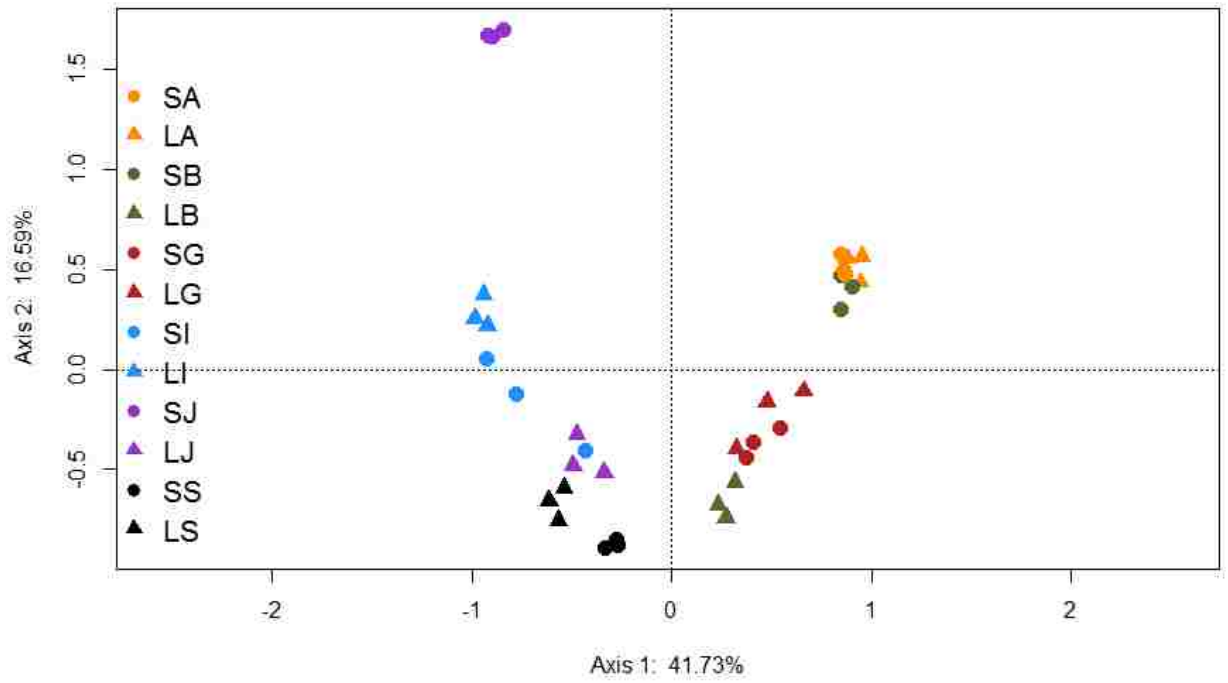

Figure 3.9. Distance-based redundancy analysis of Bray-Curtis distance matrix of 16S prokaryotic OTUs for bulk soils from paired sites with short and long-term sugarcane cropping histories in plant cane at six locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel.

Weighted UniFrac β-diveristy analysis of rhizosphere community structure separated along axis 1 by location and along axis 2 by crop year (Figure 3.17). Axis 1 captured 49.92% of variance, and axis 2 captured 24.26%. Unweighted UniFrac distance analysis of rhizosphere communities captured 26.86% of variance along axis 1 and 16.50% along axis 2 (Figure A.6), but samples did not cluster in any discernable way. Bray-Curtis principal coordinate analysis of rhizosphere communities separated samples by location along axis 1, while axis 2 separated samples by cropping history (Figure 3.18). Axis one captured 52.23% of variance, and axis 2 captured 18%.

3.3.2 ITS Fungal Metagenomics

3.3.2.1 Rarefaction of ITS Sequencing Data

Processing of ITS forward read amplicons generated on the Illumina MiSeq platform in Qiime 57,398 OTUs from 14,461,180 sequences from 125 samples. Individual samples ranged from 24,976 to 285,417 sequences. To compare the structure of ITS fungal communities, all samples were rarefied to an equal depth of 24,800, a value below that of the sample containing the fewest sequences. The rarefied dataset contained a total of 40,309 OTUs. ITS reverse reads could only be rarefied to 8,100 sequences per sample. Multiple rarefactions of ITS forward amplicons at increasing sequence sampling depths were performed on bulk and rhizosphere samples from six locations in plant

cane (Figure 3.19) and two locations sampled in plant cane and first ratoon (Figure 3.20). Rarefied samples typically ranged between 400 and 1,000 OTUs. Communities continued to increase in the number OTUs as sequencing depth increased up to the sampling depth used for pairwise comparisons, suggesting additional coverage may capture additional diversity lost in rarefaction.

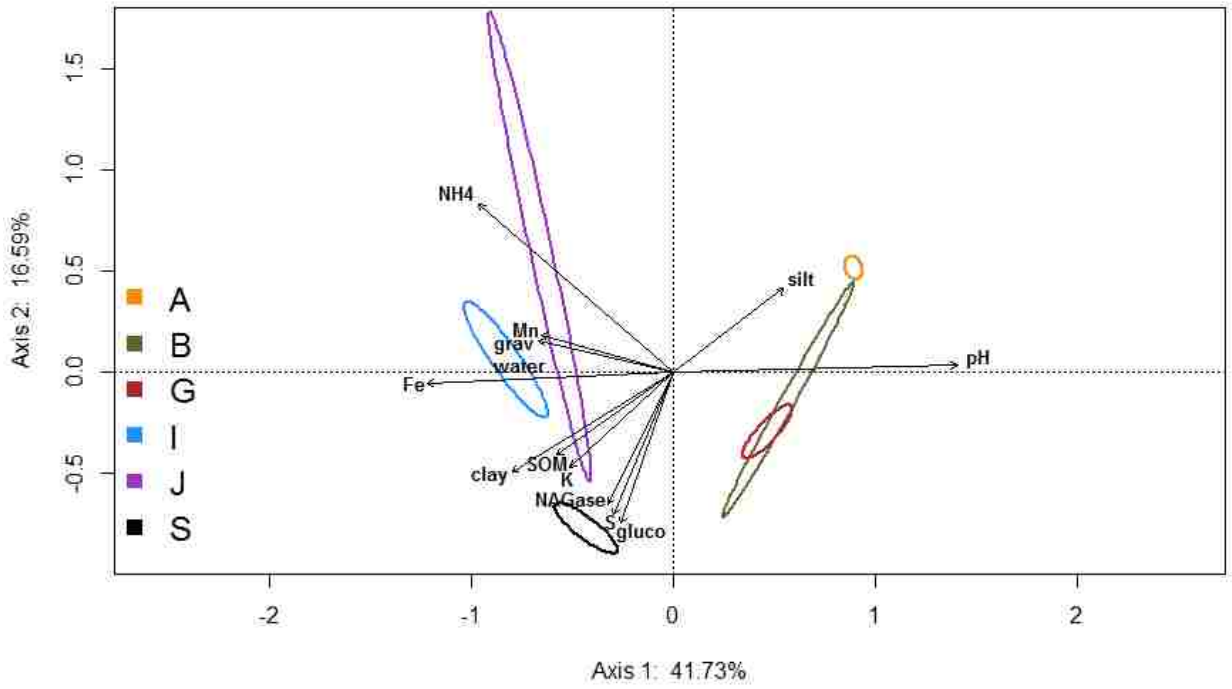

Figure 3.10. Distance-based redundancy analysis of Bray-Curtis distance matrix of 16S prokaryotic OTUs for bulk and rhizosphere soils from paired sites with short and long-term sugarcane cropping histories in plant cane at six locations with maximum correlation of soil and nutrient environmental variables plotted as vectors ($p < 0.05$). L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel. Variables: NH4 = ammonium, grav water = gravimetric water, NAGase = N-Acetyl-β-D-glucosaminidase, gluco = β-glucosidase, SOM = soil organic matter.

Table 3.12. ANOVA p-values for factors affecting pair-wise dissimilarity of three distance metrics for 16S metagenomic community composition for rhizosphere soil from paired sites with short and long-term sugarcane cropping histories in plant cane at six locations.

	Weighted UniFrac	Unweighted UniFrac	Bray-Curtis
Cropping history	0.001	0.007	0.001
Location	0.001	0.001	0.001
Cropping history x Location	0.001	0.001	0.001

α = 0.05. NS = not significant.

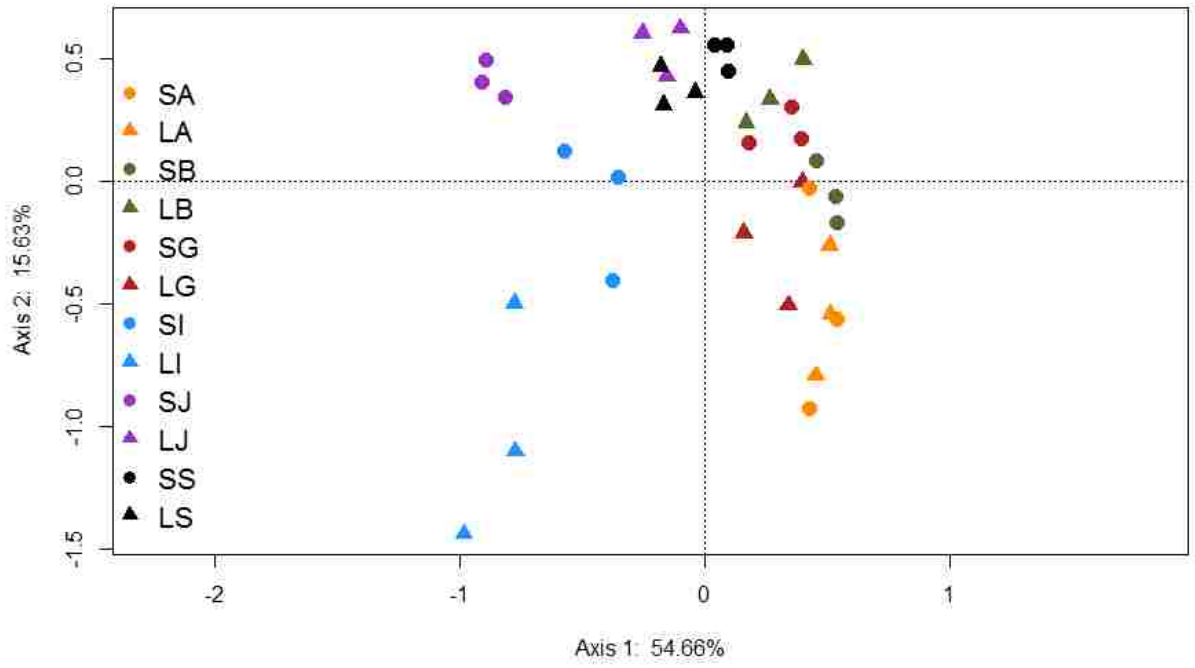

Figure 3.11 Distance-based redundancy analysis of weighted UniFrac distance matrix of 16S prokaryotic OTUs for rhizosphere soils from paired sites with short and long-term sugarcane cropping histories in plant cane at six locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel.

3.3.2.2 Fungal Community Composition

Forward and reverse ITS amplicons were both processed through the Qiime pipline. Given the greater coverage of fungal community provided by a larger number of forward reads than reverse and low probability of taxonomic correlation ($p < 0.001$), only forward reads were used in the analysis. Eleven DNA extractions were sequenced in both ITS Illumina MiSeq runs. Eleven samples were re-sequenced using the same extractions. Permutation tests resulted in no significant difference in taxonomic assignment at any levels for both re-extracted and re-sequenced samples ($p < 0.001$). Pearson's correlation coefficient was lowest at the class level and increased with data sorted by subsequent taxonomic divisions (Table 3.15).

Blast taxonomic assignment of ITS amplicons was determined at the phylum level for sugarcane fungal communities from plant cane bulk soils (Figure 3.21), plant cane rhizosphere soils (Figure 3.22), bulk soils from plant cane and first ratoon (Figure 3.23), and rhizosphere soils from plant cane and first ratoon (Figure 3.24). The composition of fungal communities in all soils was dominated by Ascomycota, Basidiomycota, and unidentified fungi.

With regards to cropping history, variation of broad fungal taxa in plant cane did not exhibit any pattern within soil types, except a minor component phylum, Glomeromycota, was more abundant in rhizosphere soils with a short-term sugarcane

cropping history ($p < 0.05$). In comparisons of plant cane and first ratoon, variation of phyla was extensive. In both bulk and rhizosphere soils, ascomycetes were greater in short-term cultivated soils than long-term. In rhizosphere samples, unidentified fungi were more abundant in short-term cultivation soils in first ratoon, whereas Blastocladiomycota and Zygomycota were more abundant in long-term cultivation soils ($p < 0.05$).

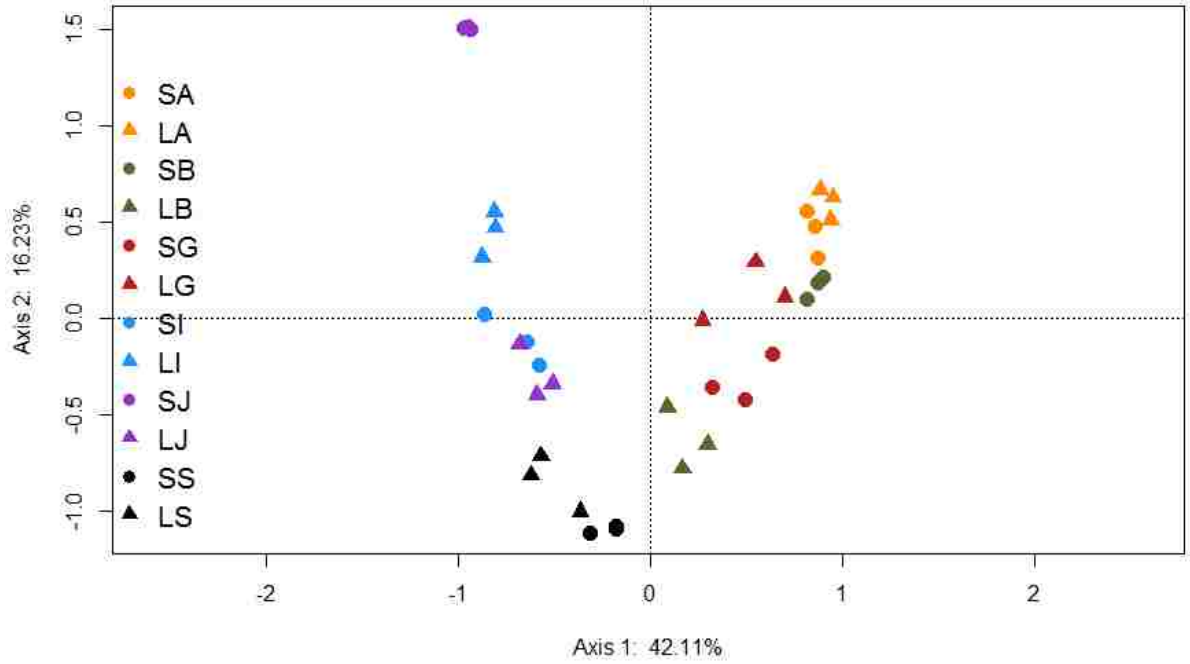

Figure 3.12 Distance-based redundancy analysis of Bray-Curtis distance matrix of 16S prokaryotic OTUs for rhizosphere soils from paired sites with short and long-term sugarcane cropping histories in plant cane at six locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel.

Taxonomic assignment of 15,751 ITS fungal OTUs present in bulk soil from six locations with the Unite database documented 359 unique taxa at the family level, of which 35 were significantly greater under long-term cultivation and 18 were greater under short-term cultivation (Table 3.16) ($p < 0.05$). The 25 most abundant families associated with long-term cultivation shown in Table 3.17 were of interest as candidates involved in the community effect that is detrimental to sugarcane yields. At the genus level, 876 unique taxons were assigned to OTUs present in bulk soils. Of these, 37 genera were greater under short-term cultivation and 58 were greater under long-term cultivation ($p <0.05$). The most abundant families associated with short-term cultivation (Table 3.18) were of interest as candidate beneficial soil microbes negatively affected in long-term sugarcane cultivation, whereas the most abundant families more associated with long-term cultivation (Table 3.19) were of interest as candidates possibly detrimental to sugarcane yield. The most abundant taxa without an association with either cropping history were listed in Table A.5 and Table A.6

Table 3.13. ANOVA p-values for factors affecting pair-wise dissimilarity of three β-diveristy metrics for 16S metagenomic community composition for bulk soil from paired sites with short and long-term sugarcane cropping histories in plant cane and first ratoon at two locations.

	Weighted UniFrac	Unweighted UniFrac	Bray-Curtis
Cropping history	0.001	0.001	0.001
Location	0.001	0.001	0.001
Crop year	0.001	0.004	0.001
Cropping history x Location	0.001	0.004	0.001
Cropping history x Crop year	NS	NS	NS
Location x Crop year	0.035	0.039	0.021
Cropping history x Location x Crop year	NS	NS	NS

α = 0.05. NS = not significant.

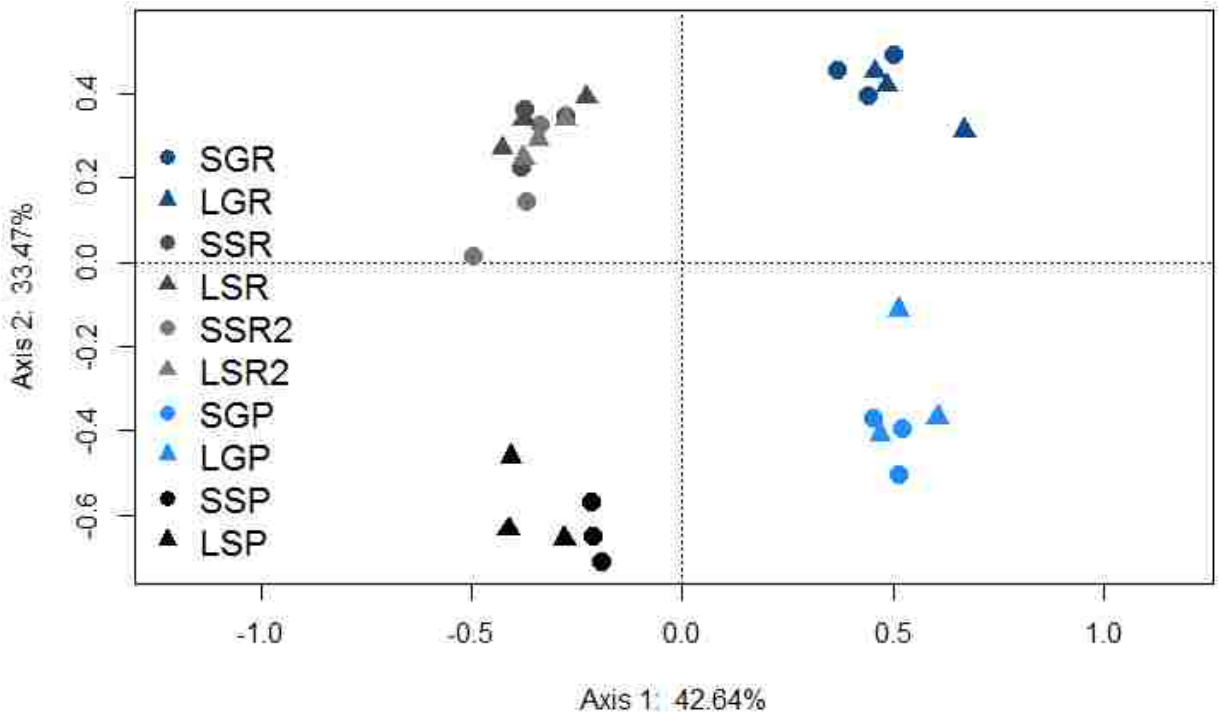

Figure 3.13 Distance-based redundancy analysis of weighted UniFrac distance matrix of 16S prokaryotic OTUs for bulk soils from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon at two locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R = first ratoon, R2 = first ratoon second sampling.

Rhizosphere soils from six locations of sugarcane under short and long-term cropping histories contained 346 unique assignments at the family level, of which 22 were more abundant ($p < 0.05$) under short-term cultivation (Table 3.20) and 26 were more abundant under long-term cultivation (Table 3.21). Additionally, of 824 genera of

rhizosphere fungi, 46 were more frequently associated with short-term sugarcane cultivation and 40 with long-term cultivation. Similarly, the most abundant genera associated with short-term cultivation (Table 3.22) were of interest as candidate beneficial soil microbes negatively affected in long-term sugarcane cultivation, whereas the most abundant genera more associated with long-term cultivation (Table 3.23) were of interest as candidates for being detrimental to sugarcane yields. The most abundant taxa without an association with either cropping history were listed in Table A.7 and Table A.8.

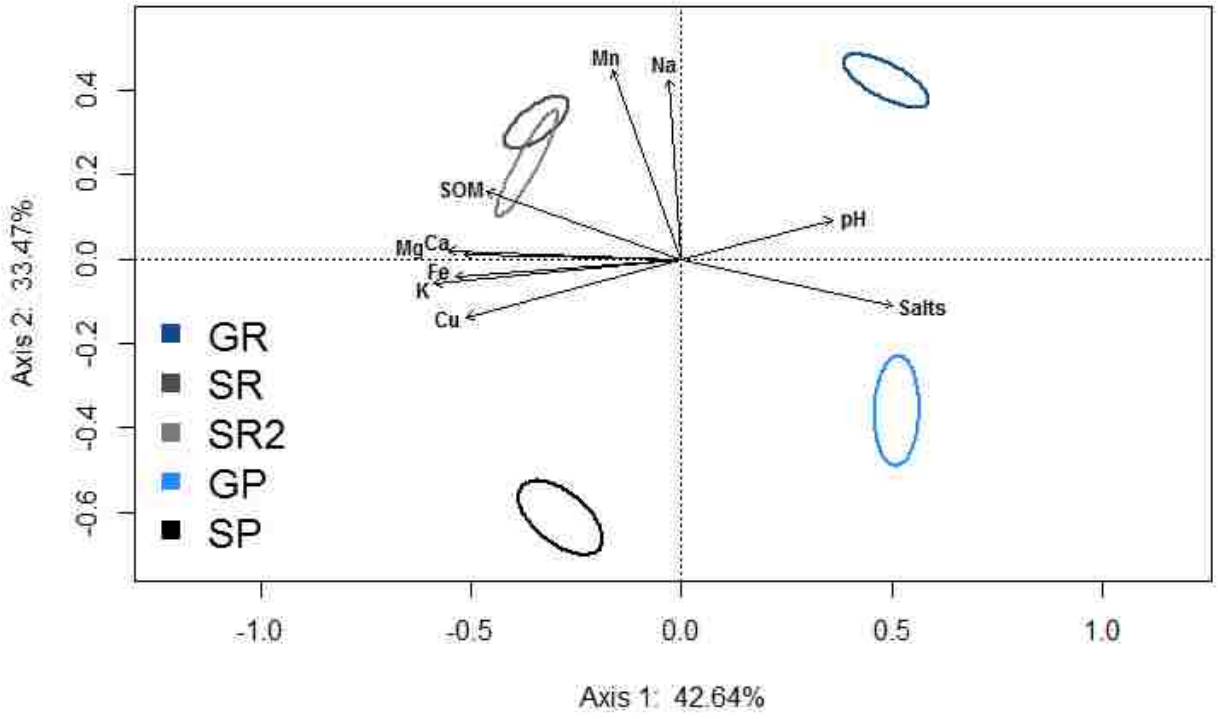

Figure 3.14 Distance-based redundancy analysis of weighted UniFrac distance matrix of 16S prokaryotic OTUs for bulk soils from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon at two locations with maximum correlation of soil and nutrient environmental variables plotted as vectors ($p < 0.05$). Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R = first ratoon, R2 = first ratoon second sampling. Variables: SOM = soil organic matter, salts = soluble salts.

3.3.2.3 Fungal Community Structural Analysis

3.3.2.3.1 Fungal Community Structural Analysis for Bulk Soils in Plant Cane

Analysis of variance of ITS fungal community structure in bulk soil using Bray-Curtis revealed significant effects associated with sugarcane cropping history, location, and their interaction (Table 3.24).

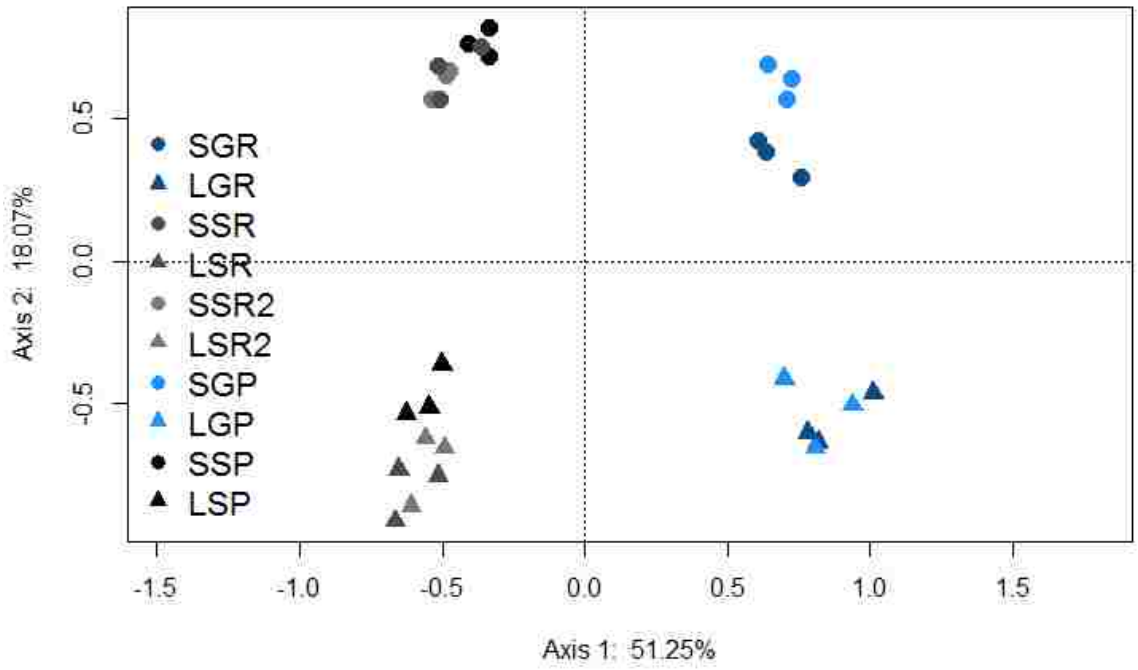

Figure 3.15. Distance-based redundancy analysis of Bray-Curtis distance matrix of 16S prokaryotic OTUs for bulk soils from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon at two locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R = first ratoon, R2 = first ratoon second sampling.

Distance-based redundancy analysis of ITS fungal community structure in bulk soils separated samples by cropping history for four of six locations (Figure 3.25). Axis 1 captured 13.99% of variance and axis 2 captured 12.90%. Environmental variables correlated with several loose clusters of short-term cultivated bulk soil fungal communities or location associated communities. Short-term sugarcane cultivation community structure at St. Gabriel and Iberia correlated with soil organic matter, iron, sulfur, N-Acetyl-β-D-glucosaminidase, and β-glucosidase (Figure 3.26). Short-term cultivation fungal community structure at Gonsoulin and Jefferson correlated with zinc, soluble salts, nitrate, and silt. Clay, magnesium, potassium, sand, and pH were correlated with differences in community structure based on location.

3.3.2.3.2 Fungal Community Structural Analysis for Rhizosphere Soils in Plant Cane

Analysis of variance of ITS fungal community structure in rhizosphere soils using Bray-Curtis revealed significant effects due to sugarcane cropping history, location, and their interaction (Table 3.25). Fungal community structure in rhizosphere soils separated individual locations, with the exception of the Airport location, but did not cluster rhizosphere fungal communities across all sites based on cropping history (Figure 3.27). Fungal community structure under long-term sugarcane cultivation at St. Gabriel was

more similar to short-term cultivation at other locations. Axis 1 captured 14.69% of the variance and axis 2 captured 12.58%.

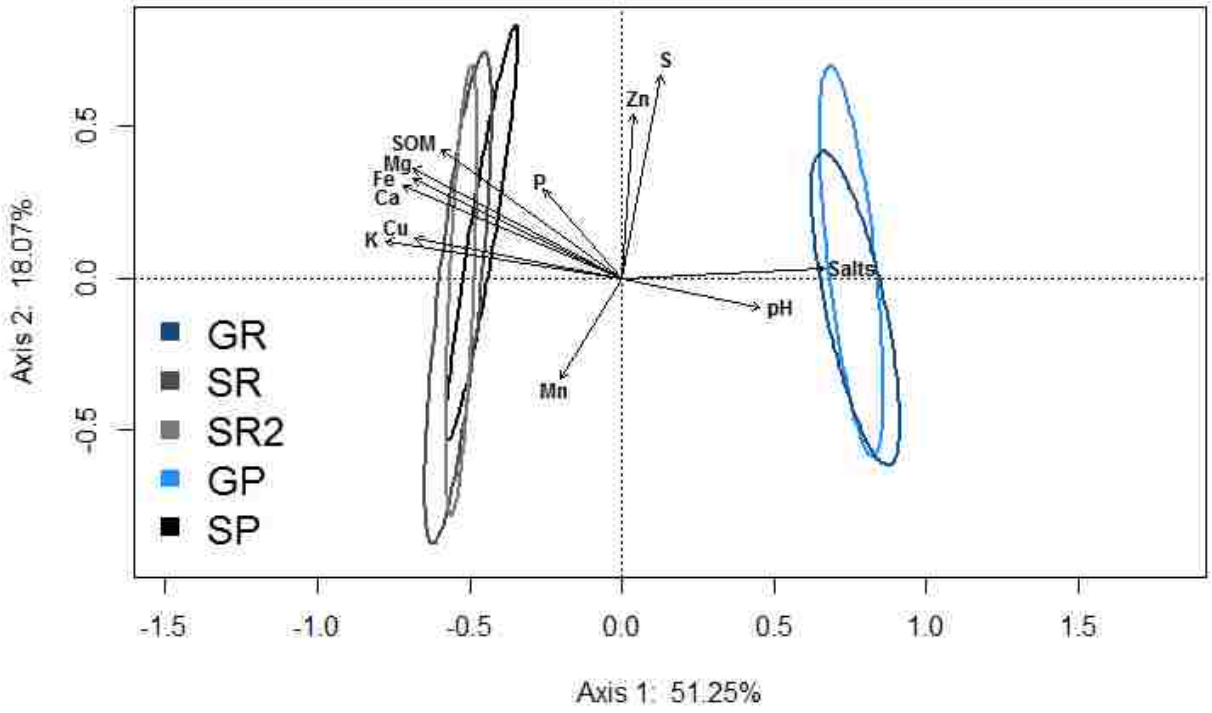

Figure 3.16. Distance-based redundancy analysis of Bray-Curtis distance matrix of 16S prokaryotic OTUs for bulk soils from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon at two locations with maximum correlation of soil and nutrient environmental variables plotted as vectors ($p < 0.05$). Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R = first ratoon, R2 = first ratoon second sampling. Variables: SOM = soil organic matter, salts = soluble salts.

Table 3.14. ANOVA p-values for factors affecting pair-wise dissimilarity of three β-diversity metrics for 16S metagenomic community composition for rhizosphere soils from paired sites with short and long-term sugarcane cropping histories in plant cane and first ratoon at two locations.

	Weighted UniFrac	Unweighted UniFrac	Bray-Curtis
Cropping history	0.001	NS	0.001
Location	0.001	NS	0.001
Crop year	0.001	NS	0.003
Cropping history x Location	0.004	NS	0.001
Cropping history x Crop year	NS	NS	NS
Location x Crop year	0.005	NS	0.009
Cropping history x Location x Crop year	NS	NS	NS

α = 0.05. NS = not significant.

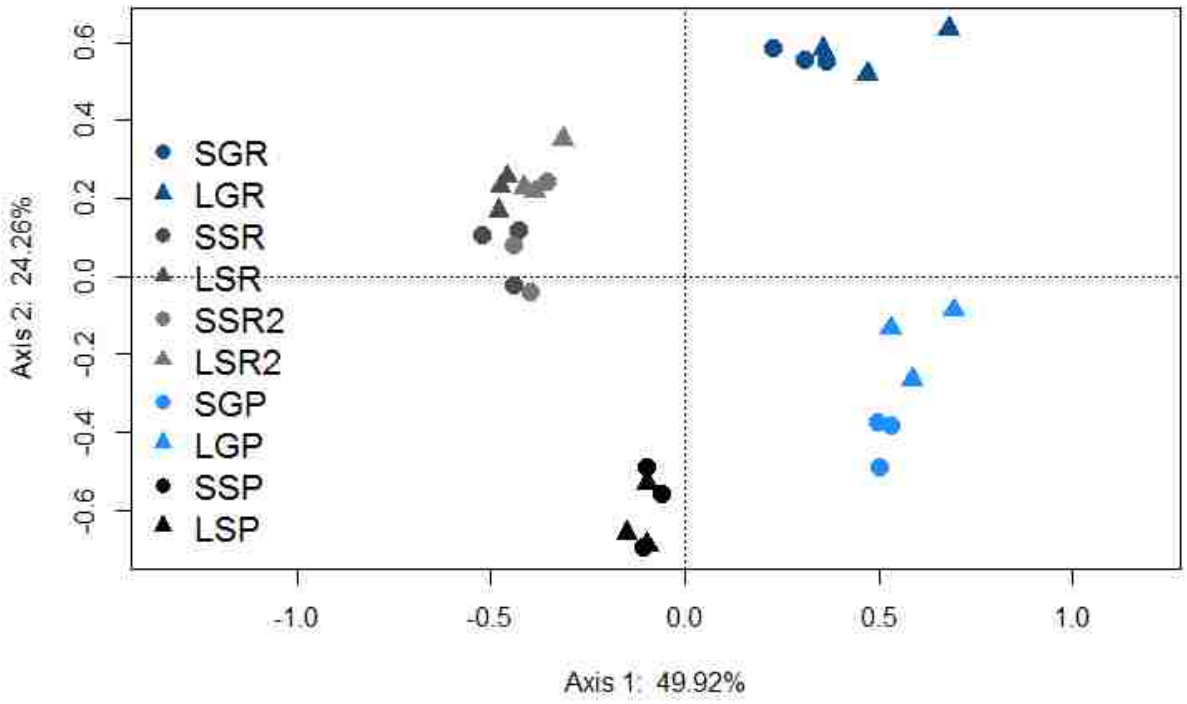

Figure 3.17 Distance-based redundancy analysis of weighted UniFrac distance matrix of 16S prokaryotic OTUs of rhizosphere soils combined from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon at two locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R = first ratoon, R2 = first ratoon second sampling.

3.3.2.3.2 Fungal Community Structural Analysis in Plant Cane and Ratoon Soils

Analysis of variance of Bray-Curtis β-diversity for fungal communities in bulk soils from plant and ratoon cane under short and long-term sugarcane cultivation revealed significant effects on community structure based on cropping history, location, crop year and all possible interactions (Table 3.26). Distance-based redundancy analysis clustered communities along axis 1 first by location then by crop year, and axis 2 by cropping history. Axis 1 captured 21.56% of constrained variance, and axis 2 captured 19.64% (Figure 3.28). Zinc and sulfur were correlated with short-term cropping history at both locations, while soil organic matter, magnesium, iron, and calcium were correlated with short-term cultivation at St. Gabriel ($p < 0.05$) (Figure 3.29). Higher soil pH was associated with community structure at the Gonsoulin site in ratoon cane under long-term cultivation, whereas manganese was correlated with long-term cultivation in first ratoon at St. Gabriel.

Analysis of variance of Bray-Curtis β-diversity for rhizosphere communities revealed significant effects due to cropping history, location, crop year, cropping history x site, cropping history x crop year, and location x crop year interactions (Table 3.27).

Distance-based redundancy analysis of rhizosphere fungal communities revealed differences across axis 1 was based on location and crop cycle, and axis two was based on cropping history (Figure 3.30). Axis 1 captured 25.46% of variance and axis 2 17.90%.

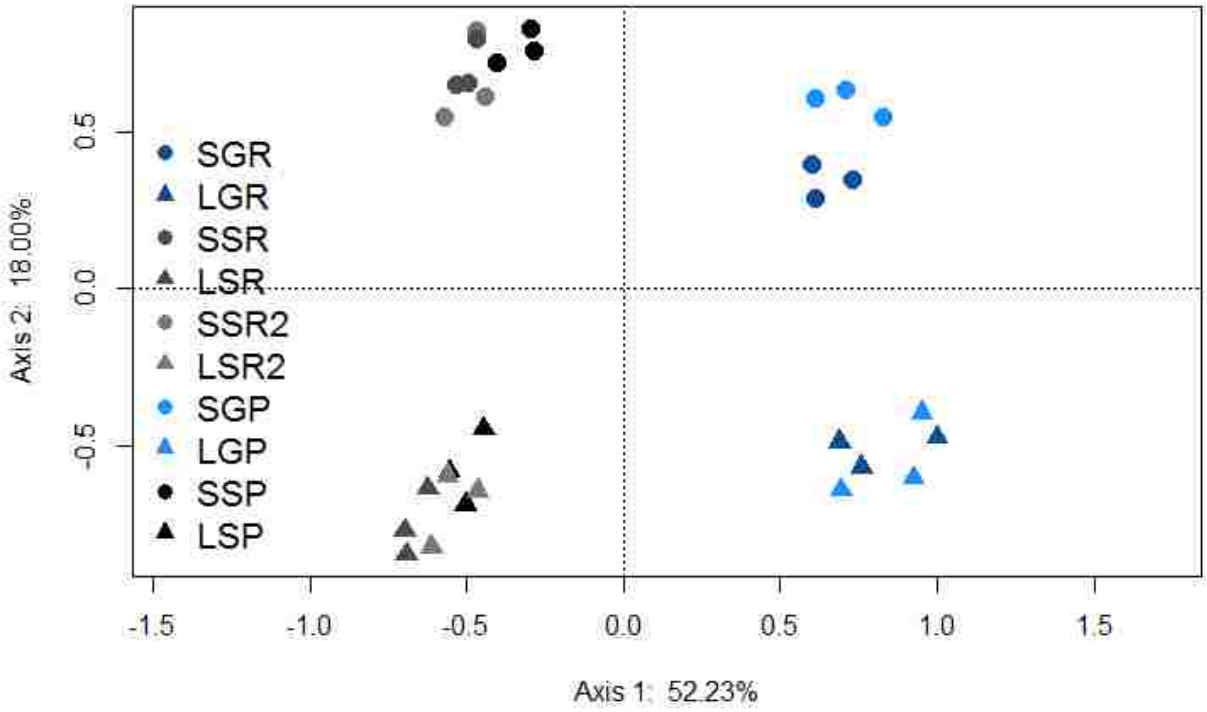

Figure 3.18 Distance-based redundancy analysis of Bray-Curtis distance matrix of 16S prokaryotic OTUs of rhizosphere soils combined from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon at two locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R = first ratoon, R2 = first ratoon second sampling.

3.4 Discussion

In order to better understand changes in the soil prokaryotic and fungal communities associated with the yield decline resulting from continuous sugarcane cultivation, this study examined soil 16S and ITS metagenomes from paired sites with short and long-term sugarcane cropping histories. This method allowed for the high-throughput profiling of soil microbial community members that both can and cannot be readily cultured. Differences in soil microbial communities based on cropping history, location, and additional factors were detected for both bulk and rhizosphere communities at six locations in the first season of sugarcane cultivation and in two of those communities in first ratoon or the second season of cultivation. Changes in microbial communities associated with sugarcane monoculture were quantified with different diversity metrics: α-diversity metrics, which examine the diversity within communities of individual

samples, and β-diversity metrics, which calculate the pairwise dissimilarity of communities between any two samples.

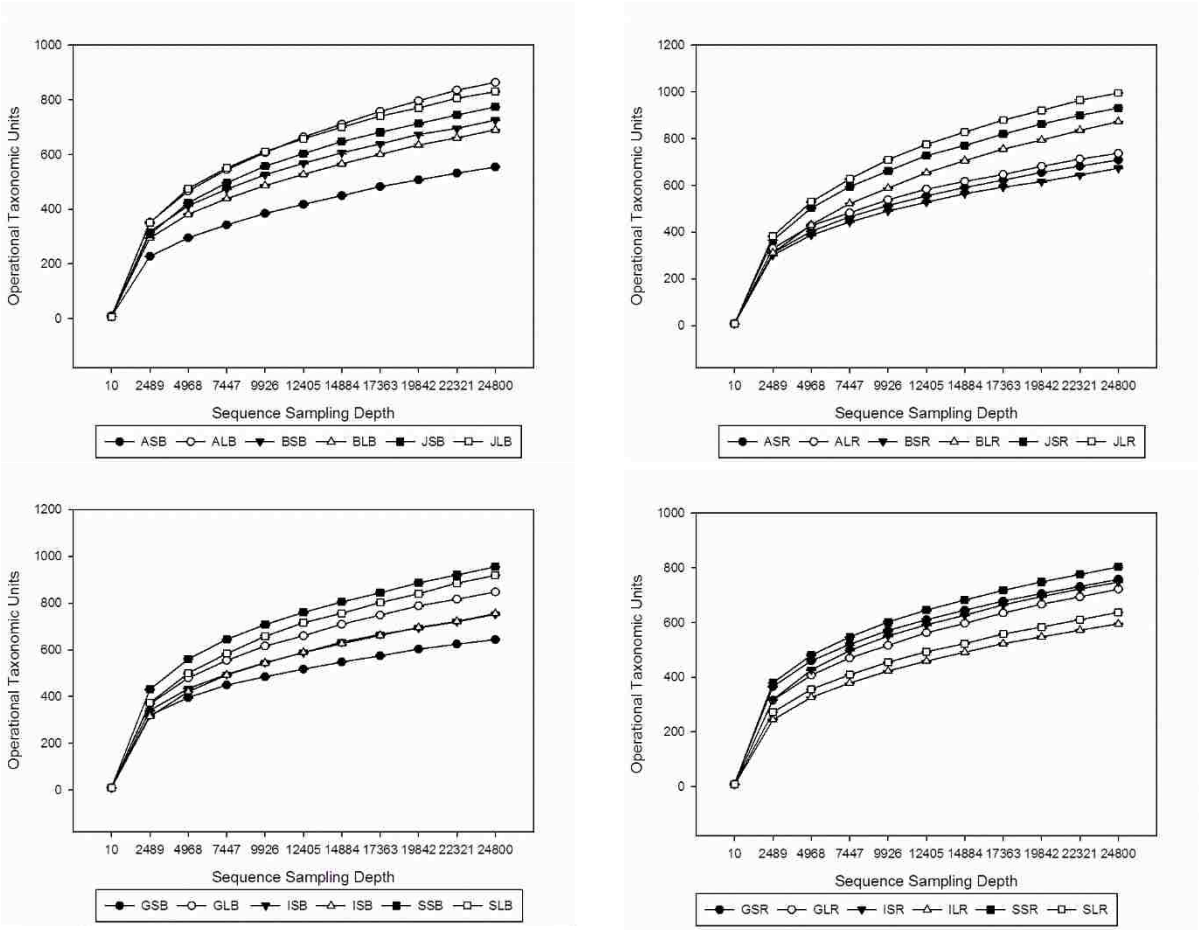

Figure 3.19 Rarefaction curves of the number of ITS OTUs from bulk and rhizosphere soils from paired sites at six locations in plant cane with short and long-term sugarcane cropping histories at increasing sequence sampling depths. Top left. Three 2014 locations bulk soil samples; Top right. Three 2014 locations rhizosphere soil samples; Bottom left. Three 2015 locations bulk soil samples; Bottom right. Three 2015 locations rhizosphere soil samples. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel. Cropping history: S = short-term cultivation, L = long-term cultivation. Soil niche: B = bulk soil, R = rhizosphere soil.

The α-diversity of individual soil samples contained a small portion of the gamma diversity, which represents the total diversity of sugarcane associated microbial communities observed in this study. Metagenomes from individual soil samples typically consisted of approximately 1/12 of all bacterial and 1/40 of all fungal OTUs contained within this study. This metagenomics survey found a greater total number of OTUs than a previous study examining bacterial and fungal communities associated with endophytic and exophytic compartments of the sugarcane microbiome (De Souza et al 2016). Here, approximately six times as many bacterial and twice as many fungal OTUs were observed. In addition to differences in study design and niches examined, the difference in the number of OTUs may also in part reflect the “greedy” nature of the

UCLUST algorithm used for OTU picking (Edgar 2010). Given the large OTU to genera ratios of prokaryotes and fungi observed in both bulk and rhizosphere soils, many of these OTUs belong to the same genera or even the same taxon. Additionally, rarefaction curves of ITS and 16S data showed a continued rate of increase in the number of OTUs at increasing sample depths. Therefore, the sugarcane associated soil metagenomes used in this study apparently contain a large number of low abundance OTUs, some of which were excluded following rarefaction.

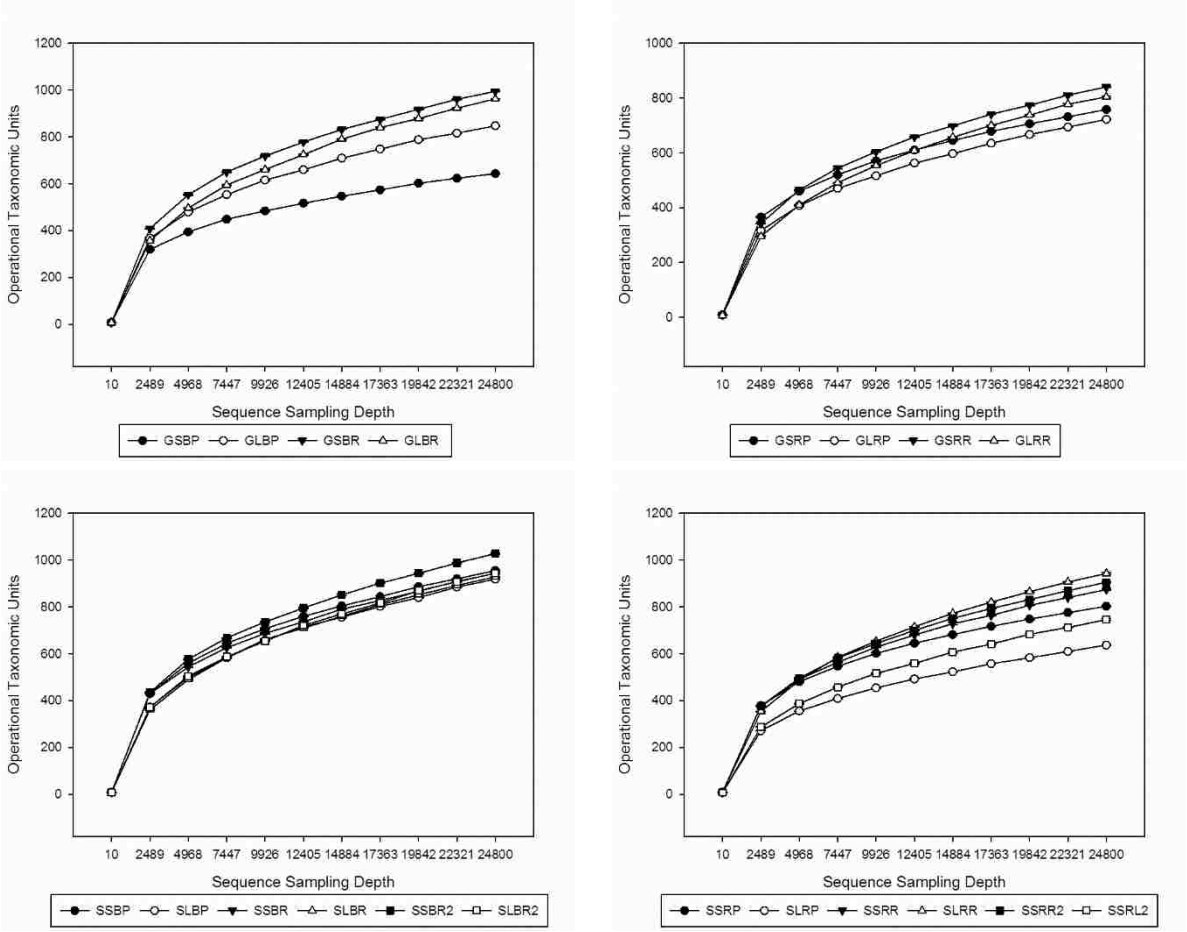

Figure 3.20. Rarefaction curves of the number of ITS OTUs from bulk and rhizosphere soils from paired sites at two locations with short and long-term sugarcane cropping histories in plant cane and first ratoon at increasing sequence sampling depths. Top left. Gonsoulin location bulk soil samples; Top right. Gonsoulin location rhizosphere soil samples; Bottom left. St. Gabriel location bulk soil samples; Bottom right. St. Gabriel rhizosphere soil samples. Locations: G = Gonsoulin, S = St. Gabriel. Cropping history: S = short-term cultivation, L = long-term cultivation. Soil niche: B = bulk soil, R = rhizosphere soil. Crop year: P = plant cane, R = first ratoon, R2 = first ratoon second sampling.

Numerous low abundance OTUs are unlikely to affect β-diversity metrics that consider abundance, such as Bray-Curtis (Bray and Curtis 1957) and weighted UniFrac but could affect unweighted UniFrac that is calculated by summation of the total phylogenetic distance of OTUs present in only one of a pair of samples (Lozupone and Knight 2005).

This was evident in the unweighted UniFrac analysis comparing rhizosphere soils in plant cane and first ratoon where none of the factors cropping history, location, or crop year significantly affected variation in the OTUs present. In contrast, differences in the structure of microbial communities were influenced by all three factors when analyzed using Bray-Curtis and weighted UniFrac. This difference in results between the β-diversity metrics suggests an analysis that equally weights rare and abundant taxa across multiple locations, cropping histories and years can result in misleading conclusions when there were many rare taxa present in the community. However, cropping history and location were related to differences in the structure of microbial communities in all plant cane comparisons analyzed with all three β-diversity metrics.

Table 3.15. Pearson's correlation of ITS taxonomic assignment of from forward and reverse reads and eleven samples sequenced in both Illumina Miseq runs.

	Phylum	Class	Order	Family	Genus
Forward and reverse reads	0.8949	0.8926	0.8526	0.8056	0.7391
Re-sequenced samples	0.8111	0.6683	0.6909	0.7038	0.7107

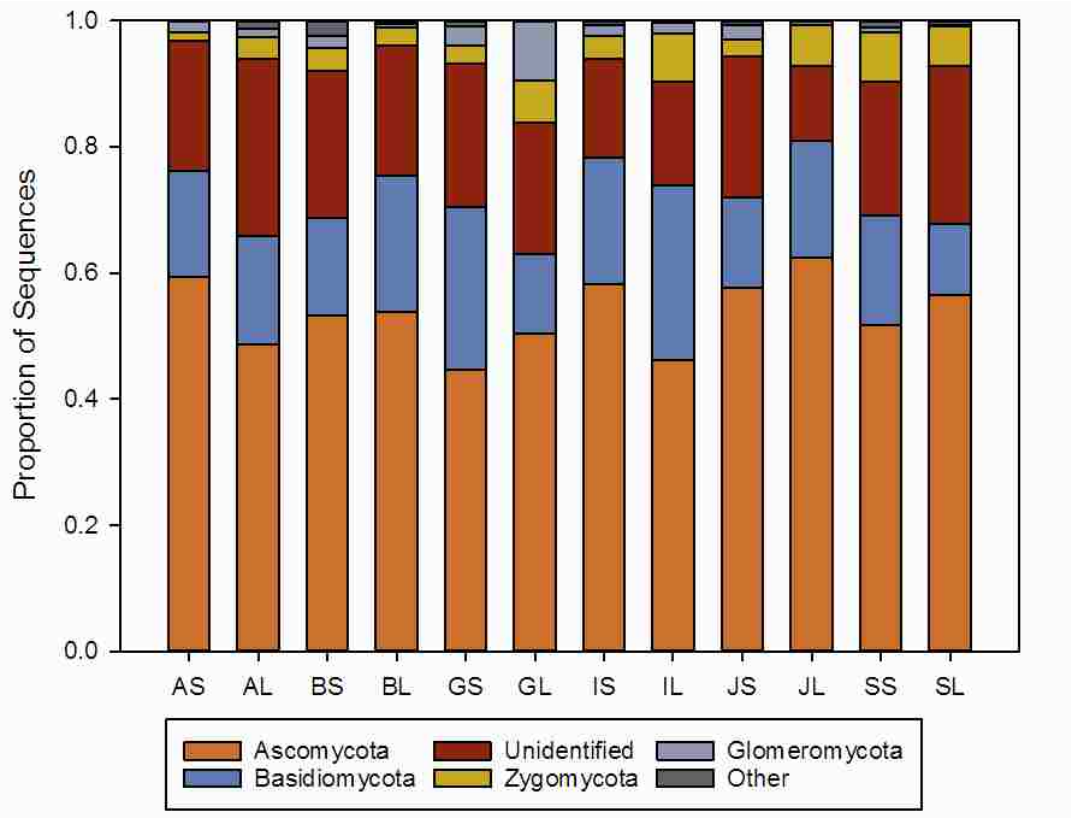

Figure 3.21 Relative abundance of fungal phyla for bulk soils from paired sites in plant cane with short and long-term sugarcane cropping histories at six locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel.

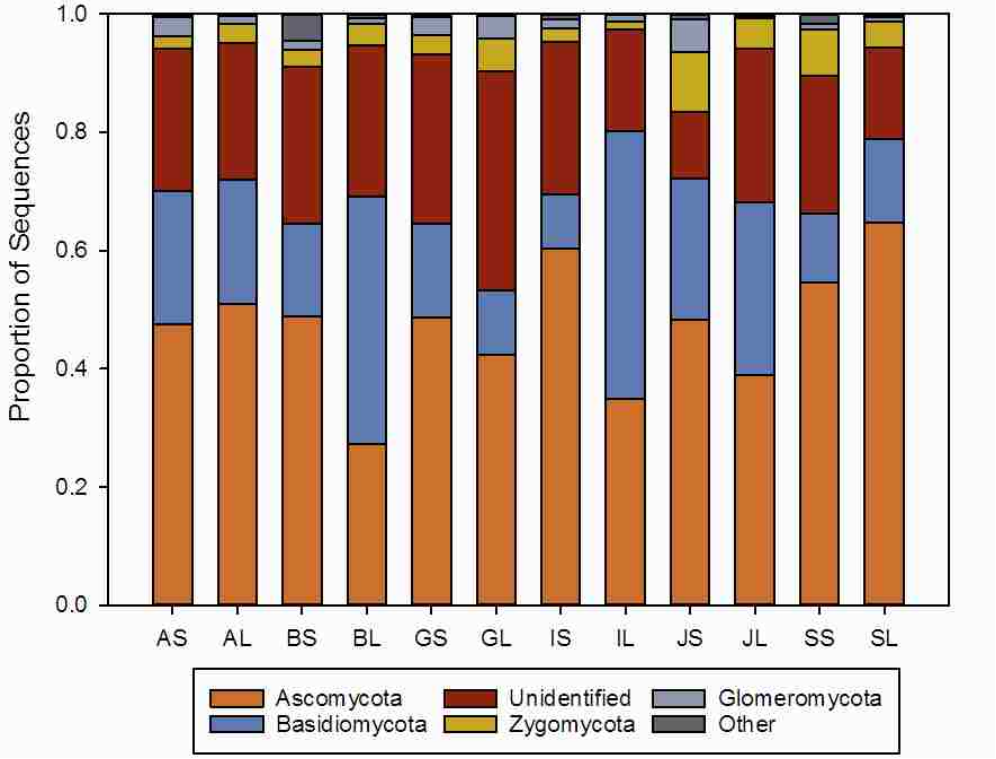

Figure 3.22 Relative abundance of fungal phyla for rhizosphere soils from paired sites in plant cane with short and long-term sugarcane cropping histories at six locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel.

Despite large numbers of low abundance OTUs, 16S amplicon sequencing, and to a lesser extent ITS sequencing, exhibited a high level of technical reproducibility. Taxonomic assignment of 16S sequences re-sequenced had Pearson's correlation coefficients higher than 0.9 at all levels and re-extracted samples had Pearson's correlation coefficients higher than 0.87. Taxonomic assignments of ITS amplicons from re-sequenced samples were lower than 16S data. Differences in Pearson's correlation coefficients between ITS forward and reverse reads and forward reads of samples sequenced in two separate MiSeq runs might in part be explained by the stochasticity of PCR which can distort the amplicon pool and produce unique low abundance OTUs that are not biologically representative of soil microbial communities and are therefore difficult to assign taxonomically (Kebschull and Zador 2015). Regardless, differences in 16S and ITS taxonomic assignments were not significantly different for re-sequenced samples at any level.

Visualization of prokaryotic communities through distance-based redundancy analysis revealed different clustering patterns for different β-diversity metrics. Both Bray-Curtis and unweighted UniFrac produced horseshoe shaped ordination patterns for rhizosphere and bulk soil prokaryotic communities from plant cane across all six locations. This pattern can occur in ordination of metagenomics data when too few similarities exist between samples (Morton et al. 2017). In contrast, the horseshoe pattern did not occur in weighted UniFrac. The high levels of dissimilarity in prokaryotic

community β-diversity calculated from Bray-Curtis that led to the formation of a horseshoe clustering pattern were likely reduced in weighted UniFrac through the incorporation of phylogenetic distance. This suggests the difference in results between these two β-diversity metrics was driven by differences in abundant OTUs that were closely related to each other. Closely related OTUs may represent redundant OTUs in 16S dataset, something that would be expected when considering the high ratio of OTUs to genera. The high level of dissimilarity in unweighted UniFrac β-diversity was probably due to variation in the presence and absence of the large number of low abundance OTUs. This high level of dissimilarity was minimized in weighted UniFrac by factoring in the abundance of OTUs. Further, the horseshoe pattern was not observed in ordinations that only included the two locations sampled in plant cane and first ratoon. This suggests datasets that include a large number of low abundance OTUs associated with samples collected at multiple, specific locations may be poorly suited to unweighted UniFrac analysis.

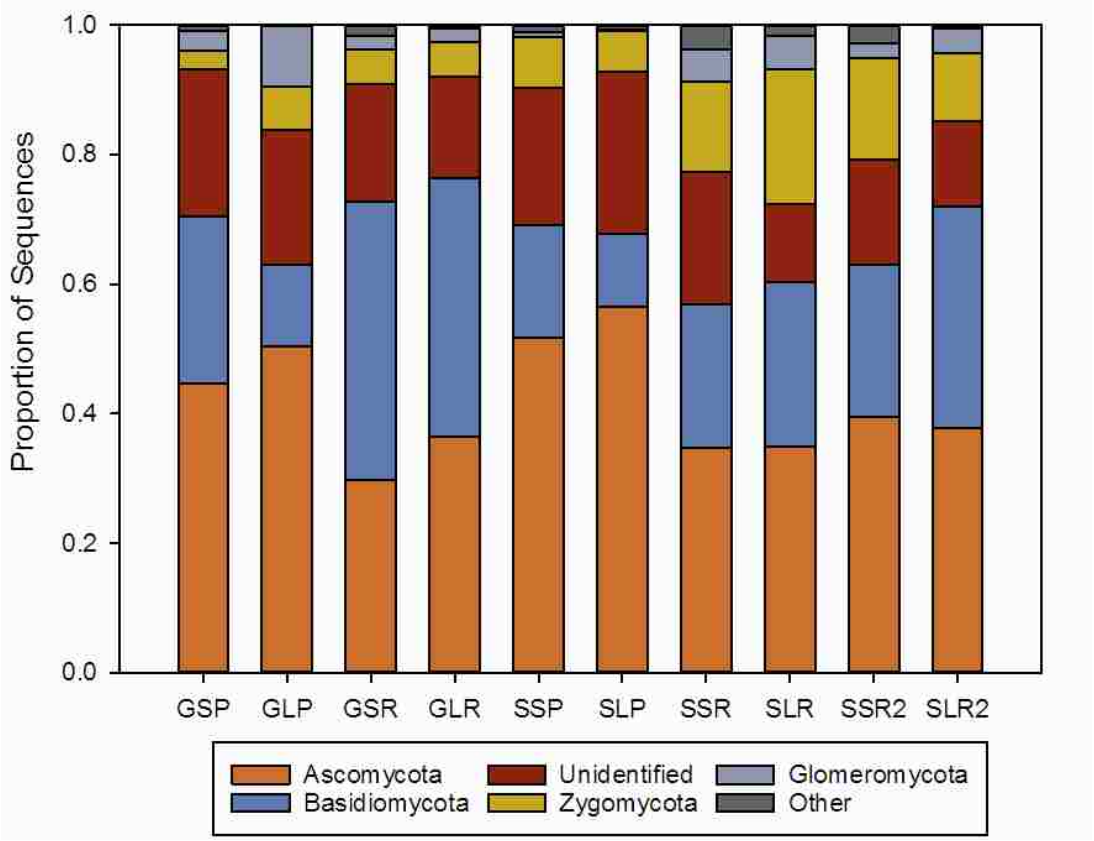

Figure 3.23 Relative abundance of fungal phyla for bulk soils from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R = first ratoon, R2= first ratoon second sampling.

Distance-based redundancy analysis of prokaryotic communities in plant cane and first ratoon produced very different ordinations using different β-diversity metrics, but produced similar ordinations for bulk and rhizosphere communities when using the

same β-diversity metric. All ordinations separated communities by location along axis 1. Weighted UniFrac ordination of both bulk and rhizosphere prokaryotic communities visualized differences between plant cane and first ratoon along axis 2 regardless of cropping history, whereas Bray-Curtis ordinations for both soil niches and bulk soil unweighted UniFrac ordination separated communities based on cropping history. These opposing results in β-diversity ordinations may be due to fluctuation in proportions of broad level taxa between plant cane and first ratoon crops that influenced the outcome for unweighted UniFrac compared to long-term changes in prokaryotic community structure under continuous sugarcane cultivation occurring as changes in large numbers of more closely related OTUs that influenced β-diversity determined by Bray-Curtis and bulk soil unweighted UniFrac.

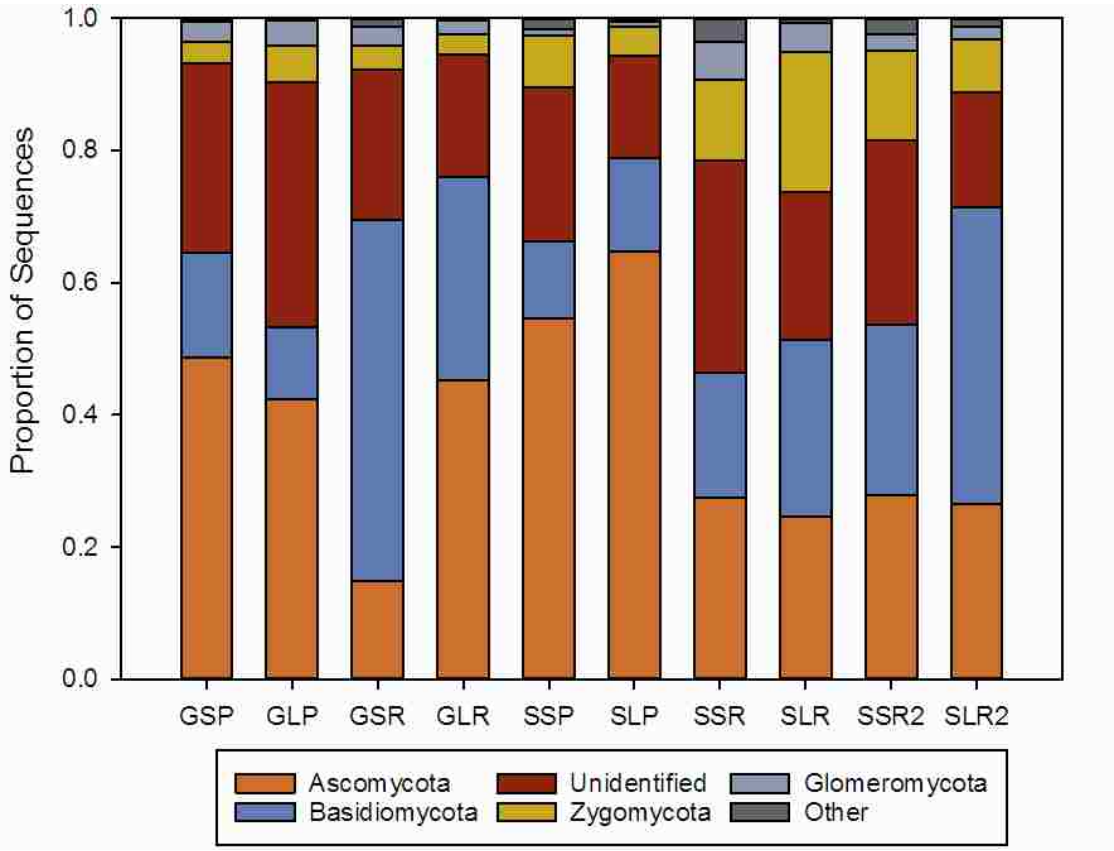

Figure 3.24 Relative abundance of fungal phyla for rhizosphere soils from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R = first ratoon, Fa= first ratoon, R2= first ratoon second sampling.

Because distance-based redundancy analysis of prokaryotic weighted UniFrac primarily clustered communities by location, co-plotting of environmental variables (Chapter 2) suggested community structure is influenced by soil factors, such as pH, nutrient availability, and texture in a location-dependent manner. In plant cane bulk soils,

similarities in prokaryotic community structure at the Airport, Belleview, and Gonsoulin locations were consistently associated with higher soil pH. At the Airport and Gonsoulin locations, communities associated with short and long-term cropping histories were indistinguishable. These same three locations also grouped together in fatty acid methyl ester profile ordinations and were similarly correlated with high soil pH (Chapter 2). These results suggest that soil pH strongly influenced prokaryotic community structure in bulk soil across locations. Other environmental variables correlated with differences in ordination of community structure based on location or on cropping history within a single location. The prokaryotic community at the Jefferson location under short-term cultivation was separated from all other communities. This location had the lowest pH (Chapter 2), but the unique prokaryotic community associated short-term cultivation was correlated with manganese availability in this analysis. The Iberia location was correlated with higher soil organic matter and potassium in short-term and greater clay content in the long-term cultivation soil. Other soil properties, such as soluble salts, iron, ammonium, and silt, had ambiguously placed vectors with respect to prokaryotic community ordinations, and their relation to prokaryotic community structure was less clear.

Table 3.16. Fungal families more associated with bulk soils under short-term sugarcane cultivation than long-term cultivation ($p < 0.05$) and their abundance and relative rank among families for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of fungal community	Rank among families	Proportion of fungal community	Rank among families
Nectriaceae	0.034418	4	0.020255	12
Sordariales	0.022256	8	0.009644	26
Pleosporaceae	0.020065	10	0.004534	37
Microascaceae	0.015269	15	0.000114	182
Lentitheciaceae	0.012608	20	0.000542	116
Magnaporthaceae	0.010354	24	0.002133	62
Archaeorhizomycetaceae	0.011776	22	0.000130	173
Entorrhizaceae	0.001779	75	0.000076	204
Schizosaccharomycetaceae	0.001039	97	0.000002	285
Claroideoglomeraceae	0.000820	108	0.000132	171
Sebacinales Group B	0.000793	109	0.000085	199
Sordariomycetes Incertae sedis	0.000441	135	0.000029	241
Boliniaceae	0.000450	133	0.000000	na
Onygenaceae	0.000195	170	0.000025	245
Venturiales unidentified	0.000143	184	0.000000	na
Trichomonascaceae	0.000094	209	0.000000	na
Rhizophlyctidaceae	0.000081	214	0.000000	na
Albatrellaceae	0.000060	227	0.000000	na

Ordinations of soils from plant cane and first ratoon revealed largely location specific differences in the relationship between community structure and soil properties. Co-plotted environmental variables for bulk soil Bray-Curtis and unweighted UniFrac ordinations indicated shared features of prokaryotic communities in short-term cultivation soils were correlated with greater sulfur and zinc content. Co-plotting of the environmental variables with weighted UniFrac ordination showed greater availability of sodium and manganese correlated with first ratoon communities.

Table 3.17. Fungal families more associated with bulk soils under long-term sugarcane cultivation than short-term cultivation ($p < 0.05$) and their abundance and relative rank among families for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of fungal community	Rank among families	Proportion of fungal community	Rank among families
Chaetomiaceae	0.019503	11	0.098098	2
Mortierellaceae	0.027912	7	0.046263	4
Sporormiaceae	0.015468	14	0.047489	3
Tubeufiaceae	0.007204	35	0.027442	6
Tremellomycetes unidentified	0.003761	51	0.025426	7
Piskurozymaceae	0.000168	176	0.017413	13
Cystofilobasidiaceae	0.000345	146	0.015766	16
Coniochaetaceae	0.000148	182	0.007209	28
Orbiliaceae	0.002713	61	0.004581	36
Phallaceae	0.000515	125	0.004131	39
Chaetosphaeriaceae	0.000276	160	0.003743	44
Capnodiaceae	0.000119	195	0.003595	46
Clavulinaceae	0.000914	101	0.002263	61
Lipomycetaceae	0.000296	158	0.001682	71
Sporidiobolales unidentified	0.000311	153	0.001577	73
Atheliaceae	0.000367	143	0.001452	77
Togniniaceae	0.000166	177	0.001476	75
Xenasmataceae	0.000374	142	0.001066	87
Ophiostomataceae	0.000204	169	0.001015	88
Debaryomycetaceae	0.000190	171	0.000921	93
Geoglossaceae	0.000038	242	0.000836	98
Cryphonectriaceae	0.000000	na	0.000867	96
Legeriomycetaceae	0.000034	250	0.000773	101
Cephalothecaceae	0.000112	201	0.000428	133
Orbiliales unidentified	0.000025	258	0.000251	150

While ordination of fungal β-diversity in plant cane at six locations explained less of the constrained variation than prokaryotic β-diversity ordinations, communities of bulk and rhizosphere soils from all locations loosely clustered based on cropping history. Lower total variance in distance-based redundancy analysis may be due to differences in

community structure between locations not being as well represented in the ordination. In bulk soils, fungal communities associated with long-term sugarcane cultivation clustered more closely than communities under short-term cultivation. In plant cane rhizosphere soils, short-term cultivation communities were more closely clustered with the exception of the St. Gabriel location, where the fungal community under long-term cultivation appeared to share characteristics with communities from recently cultivated soils at other locations. Distance-based redundancy analysis of rhizosphere and bulk soils from locations resampled during first ratoon distinguished fungal communities by location and crop year along axis 1 and cropping history along axis 2, though this pattern was less clear in rhizosphere soils.

Table 3.18. Fungal genera more associated with bulk soils under short-term sugarcane cultivation than long-term cultivation ($p < 0.05$) and their abundance and relative rank among genera for each cropping history.

	Short-term cropping history		Long-term cropping history	
Genus	Proportion of fungal community	Rank among genera	Proportion of fungal community	Rank among genera
Sordariales unidentified	0.022256	6	0.009644	24
Fusarium	0.014821	10	0.003853	42
Archaeorhizomyces	0.011776	13	0.000130	294
Scedosporium	0.011561	14	0.000056	390
Lentitheciaceae unidentified	0.005007	42	0.000007	546
Clitopilus	0.004503	50	0.000260	222
Alternaria	0.003132	61	0.000739	137
Pseudallescheria	0.002717	67	0.000029	455
Preussia	0.002592	70	0.000141	285
Robillarda	0.002552	71	0.000000	na
Phialophora	0.002231	75	0.000184	259
Chlorophyllum	0.002052	81	0.000018	493
Clavicipitacea unidentified	0.001893	87	0.000040	421
Ganoderma	0.001651	97	0.000269	219
Tomentella	0.001523	103	0.000087	350
Talbotiomyces	0.001543	101	0.000054	395
Pseudophialophora	0.001006	125	0.000105	321
Schizosaccharomyces	0.001039	123	0.000002	599
Arachnion	0.000925	131	0.000000	na
Pleosporales unidentified	0.000865	136	0.000000	na
Clonostachys	0.000789	144	0.000036	431
Peziza	0.000609	171	0.000141	286
Halosphaeriaceae unidentified	0.000739	151	0.000000	na
Microdochium	0.000735	152	0.000000	na
Thanatephorus	0.000390	208	0.000000	na

Table 3.19. Fungal genera more associated with bulk soils under long-term sugarcane cultivation than short-term cultivation ($p < 0.05$) and their abundance and relative rank among genera for each cropping history.

	Short-term cropping history		Long-term cropping history	
Genus	Proportion of fungal community	Rank among genera	Proportion of fungal community	Rank among genera
Sporormiaceae unidentified	0.005685	35	0.039220	3
Helicoma	0.006548	33	0.026310	6
Chaetomium	0.005632	36	0.024379	8
Tremellomycetes unidentified	0.003761	57	0.025426	7
Solicoccozyma	0.000168	301	0.017287	12
Guehomyces	0.000159	307	0.015473	13
Lasiosphaeris	0.000002	647	0.007903	27
Lecythophora	0.000148	312	0.007032	28
Mariannaea	0.000195	285	0.003909	40
Leptoxyphium	0.000119	344	0.003595	45
Orbiliaceae unidentified	0.000603	173	0.002825	53
Metarhizium	0.000497	188	0.002352	62
Clathrus	0.000078	402	0.002657	57
Clavulinaceae unidentified	0.000721	155	0.001664	78
Chloridium	0.000146	314	0.002198	64
Lipomyces	0.000296	248	0.001682	76
Sporidiobolales unidentified	0.000311	238	0.001577	82
Spirosphaera	0.000000	na	0.001682	77
Rhexoacrodictys	0.000054	446	0.001602	81
Phaeoacremonium	0.000166	304	0.001476	87
Unidentifed Atheliaceae	0.000007	605	0.001127	100
Priceomyces	0.000190	292	0.000921	119
Ijuhya	0.000049	462	0.000939	116
Trichoglossum	0.000038	496	0.000831	122
Cylindrotrichum	0.000002	646	0.000750	133

Similarities in community structure based on cropping history across all locations and crop years sampled suggest that fungi are likely involved in the whole soil microbial community effect associated with yield decline in soils under continuous sugarcane cultivation. The detrimental effects of fungi associated with long-term sugarcane cultivation previously demonstrated by the positive plant growth response to mancozeb (Magarey et al. 1997a), the association and pathogenicity of sterile, dematiaceous fungi (Magarey and Croft 1995), and higher endophytic root colonization by fungi (Chapter 2) considered together with the current results support the concept that fungi play a major role in yield decline.

Because ordinations of fungal communities clustered based on cropping history, co-plotting of environmental variables (Chapter 2) was able to reveal a number of soil nutrients for which availability correlates with changes in fungal communities due to long-term sugarcane cultivation. Fungal community structure in short-term sugarcane production was associated with higher soil organic matter, sulfur, zinc, copper, and soil extracellular enzyme activities in one set of locations and zinc, soluble salts, silt, and nitrate in another. Similar to Bray-Curtis and unweighted UniFrac ordinations in prokaryotic communities, fungal communities in recently cultivated soils from both plant cane and first ratoon were correlated with greater sulfur and zinc content.

Table 3.20. Fungal families more associated with rhizosphere soils under short-term sugarcane cultivation than long-term cultivation ($p < 0.05$) and their abundance and relative rank among families for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of fungal community	Rank among families	Proportion of fungal community	Rank among families
Nectriaceae	0.029783	7	0.010390	21
Pleosporaceae	0.014599	13	0.008078	25
Agaricomycetes unidentified	0.011971	19	0.008418	24
Bolbitiaceae	0.012688	15	0.005594	35
Archaeorhizomycetaceae	0.016476	12	0.000179	159
Ascobolaceae	0.008999	28	0.003094	46
Saccharomycetales Incertae sedis	0.009953	25	0.000670	108
Microascaceae	0.007986	29	0.000397	129
Blastocladiaceae	0.007294	32	0.000376	131
Agaricaceae	0.004711	43	0.000235	148
Magnaporthaceae	0.003649	51	0.000871	98
Lentitheciaceae	0.002984	59	0.000560	115
Ambisporaceae	0.003125	56	0.000260	138
Basidiomycota unidentified	0.001286	88	0.000948	92
Sordariomycetes Incertae sedis	0.001761	71	0.000101	181
Sebacinales Group B	0.000952	99	0.000867	99
Hymenochaetaceae	0.000889	101	0.000289	137
Olpidiales unidentified	0.000482	125	0.000000	na
Archaeosporaceae	0.000417	133	0.000000	na
Buckleyzymaceae	0.000370	138	0.000000	na
Vibrisseaceae	0.000358	141	0.000000	na
Archaeosporales unidentified	0.000341	144	0.000016	239
Claroideoglomeraceae	0.000208	165	0.000108	175
Glomerellaceae	0.000202	167	0.000000	na
Metschnikowiaceae	0.000168	178	0.000020	233

Table 3.21. Fungal families more associated with rhizosphere soils under long-term sugarcane cultivation than long-term cultivation ($p < 0.05$) and their abundance and relative rank among families for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of fungal community	Rank among families	Proportion of fungal community	Rank among families
Hypocreaceae	0.009319	27	0.043266	3
Sporormiaceae	0.012668	16	0.025672	7
Tubeufiaceae	0.011281	21	0.024892	10
Tremellomycetes unidentified	0.004449	45	0.025152	9
Sordariaceae	0.001602	75	0.012950	18
Piskurozymaceae	0.000363	140	0.009554	23
Cystofilobasidiaceae	0.000159	182	0.007899	26
Capnodiaceae	0.000087	212	0.005853	33
Coniochaetaceae	0.000105	202	0.004597	40
Clavulinaceae	0.001205	91	0.002319	51
Lophiostomataceae	0.001584	76	0.001707	68
Sporidiobolales unidentified	0.000112	198	0.002867	47
Chaetosphaeriaceae	0.000390	136	0.001906	59
Sebacinales_Group_B	0.000952	99	0.000867	99
Ophiostomataceae	0.000166	179	0.001254	80
Xenasmataceae	0.000175	176	0.001098	85
Mytilinidiaceae	0.000349	143	0.000918	93
Lipomycetaceae	0.000027	260	0.000874	97
Geoglossaceae	0.000004	286	0.000509	121
Lyophyllaceae	0.000000	na	0.000511	119
Cryphonectriaceae	0.000000	na	0.000155	165
Cunninghamellaceae	0.000034	250	0.000004	266
Lindgomycetaceae	0.000002	296	0.000018	238
Verrucariaceae	0.000000	na	0.000011	247

Maximum correlation of fungal β-diversity ordinations, in conjunction with trends in soil nutrient data (Chapter 2), suggest changes in fungal community structure due to cropping history coincide with depletions in specific soil nutrients based on location. Similar reports of location specific depletion of soil nutrients associated with long-term sugarcane cultivation have been reported elsewhere (Bramley et al. 1996, Wood 1985). Whether these changes in soil chemical properties directly affect plant health or indirectly affect it by leading to changes in fungal community structure that are detrimental to sugarcane yields in unclear, but long-term sugarcane monoculture appears to affect both as productive capacity decreases.

Table 3.22. Fungal genera more associated with rhizosphere soils under short-term sugarcane cultivation than long-term cultivation ($p < 0.05$) and their abundance and relative rank among genera for each cropping history.

	Short-term cropping history		Long-term cropping history	
Genus	Proportion of fungal community	Rank among genera	Proportion of fungal community	Rank among genera
Fusarium	0.017993	10	0.003810	42
Agaricomycetes unidentified	0.011971	13	0.008418	22
Archaeorhizomyces	0.016476	11	0.000179	241
Ascobolus	0.008911	23	0.003094	49
Conocybe	0.011864	14	0.000038	395
Naganishia	0.009541	19	0.000000	na
Allomyces	0.006154	31	0.000291	198
Scedosporium	0.006181	30	0.000101	300
Ambispora	0.003087	57	0.000260	203
Aspergillus	0.001913	73	0.000618	146
Lentitheciaceae unidentified	0.001938	72	0.000195	234
Pseudophialophora	0.001866	75	0.000002	547
Entoloma	0.001044	117	0.000493	162
Clavicipitaceae unidentified	0.001326	95	0.000170	244
Amaurodon	0.001322	97	0.000000	na
Clonostachys	0.001299	99	0.000000	na
Agaricaceae unidentified	0.001221	109	0.000000	na
Xylariaceae unidentified	0.001073	114	0.000065	349
Pseudallescheria	0.000860	138	0.000186	237
Blastocladiella	0.000894	133	0.000085	317
Cochliobolus	0.000905	132	0.000043	388
Tricharina	0.000887	134	0.000002	549
Hydropus	0.000757	152	0.000000	na
Chlorophyllum	0.000500	179	0.000078	327
Olpidiales unidentified	0.000482	181	0.000000	na

Analyses comparing prokaryotic and fungal α-diversity in soils under short and long-term sugarcane cultivation revealed differences in taxonomic composition in both phyla and genera. In plant cane, only lower abundance phyla varied by cropping history, such as Glomeromycota's greater abundance in recently cultivated rhizosphere soils. Although, these higher populations of arbuscular mycorrhizal fungi or conversely reduced populations in long-term cultivation soils could be related to differences in plant growth. With respect to broad prokaryotic taxa that varied by cropping history, actinobacteria were more abundant in soils with short-term cropping histories. Previous research has documented actinobacteria as more abundant in recently cultivated or nearby non-agricultural soils than in soils under long-term sugarcane production (Savario and Hoy 2010; Magarey et al. 1997b). Actinobacteria commonly produce

antibiotic compounds and are candidate antagonists towards plant pathogens capable of suppressing disease (Garbeva et al. 2004). Further, actinobacteria found in sugarcane soils in Louisiana have been reported to commonly have antibiotic activity towards known root pathogens, such as *Pythium arrhenomanes* (Cooper and Chilton 1950, Hoy and Schneider 1988a). While overall prokaryotic community structure may not be consistently affected by long-term sugarcane cultivation, consistent changes in certain taxa competitive or antagonistic to fungi could contribute to the changes in fungal community structure associated with sugarcane monoculture.

Table 3.23. Fungal genera more associated with rhizosphere soils under long-term sugarcane cultivation than long-term cultivation ($p < 0.05$) and their abundance and relative rank among genera for each cropping history.

	Short-term cropping history		Long-term cropping history	
Genus	Proportion of fungal community	Rank among genera	Proportion of fungal community	Rank among genera
Trichoderma	0.009104	22	0.042899	3
Helicoma	0.010786	17	0.023896	8
Tremellomycetes unidentified	0.004449	41	0.025152	5
Sporormiaceae unidentified	0.002538	66	0.023459	9
Chaetomium	0.004447	42	0.013992	15
Sordaria	0.001501	88	0.012836	17
Pseudorobillarda	0.000058	449	0.011248	18
Solicoccozyma	0.000363	210	0.009496	21
Guehomyces	0.000069	424	0.007769	23
Leptoxyphium	0.000087	388	0.005853	30
Lecythophora	0.000034	495	0.004382	40
Lasiosphaeris	0.000020	544	0.003463	47
Sporidiobolales unidentified	0.000112	353	0.002867	50
Clavulinaceae unidentified	0.000325	227	0.001340	79
Rhexoacrodictys	0.000085	396	0.001420	76
Xenasmatella	0.000175	296	0.001098	98
Taeniolella	0.000349	215	0.000918	114
Clitocybula	0.000000	na	0.001254	85
Stropharia	0.000016	554	0.001205	89
Priceomyces	0.000186	285	0.000972	110
Lipomyces	0.000027	516	0.000874	119
Ijuhya	0.000034	496	0.000804	126
Chloridium	0.000020	543	0.000750	135
Parasarcopodium	0.000009	590	0.000641	145
Sporothrix	0.000000	na	0.000569	154

Broad prokaryotic and fungal taxa tended to vary more greatly with the crop year rather than cropping history. Large decreases in proteobacteria and ascomycetes and

increases in basidiomycetes were detected from plant cane to first ratoon in soils under both short and long-term cultivation. These results were further supported by differences in β-diversity metrics, particularly weighted UniFrac ordinations separating communities by crop year as dissimilarity increases with phylogenetic distance of OTUs. In general, microbial communities tended to vary at broad taxonomic levels between plant cane and first ratoon, while the composition of taxa within broad taxonomic groups changed with long-term sugarcane cultivation.

Table 3.24. ANOVA p-values for factors affecting pair-wise dissimiilarity of Bray-Curtis distance matrix for ITS metagenomic community composition for bulk soil from paired sites with short and long-term sugarcane cropping histories at six locations.

	Bray-Curtis
Cropping history	0.001
Location	0.001
Cropping history x Location	0.006

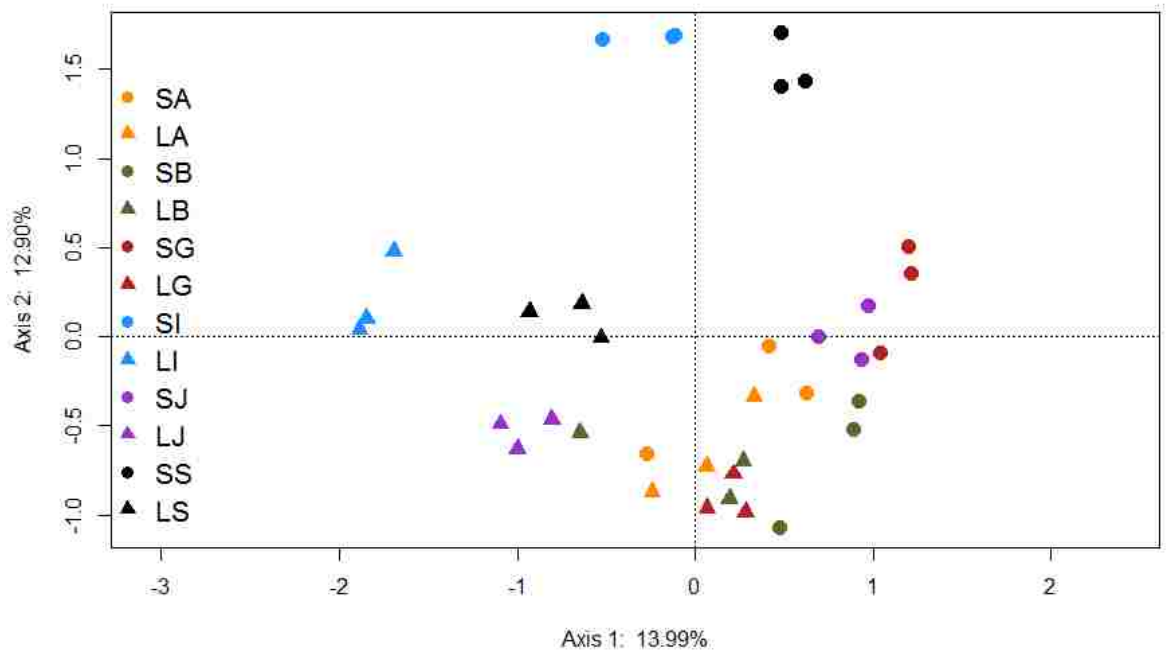

Figure 3.25 Distance-based redundancy analysis of Bray-Curtis distance matrix of ITS fungal OTUs for bulk soils from paired sites in plant cane with short and long-term sugarcane cropping histories at six locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel.

Previous efforts to unravel the etiology of yield decline have largely focused on culturable microorganisms. While amplicon-based metagenomics allows for the incorporation of previously uncultured taxa into studies, database identification is still somewhat limited by culturable taxa. Taxonomic assignment of prokaryotic OTUs

resulted in a large numbers of uncultured accessions from the SILVA 128 database (Quast et al. 2012). Taxonomic assignment of ITS OTUs led to a large proportion of the fungal community being classified as unidentified fungi, and many additional OTUs were classified as unidentified at the genus level. Unidentified fungi may represent unclassified dematiaceous fungi, possibly the pathogenic dark, sterile fungi previously associated with root colonization and reduced yields under long-term sugarcane cultivation (Magarey et al. 2005). These fungi are unlikely to be well represented in the UNITE database (Abarenkov et al. 2010). While challenges in taxonomic identification do not affect β-diversity analysis where taxonomic identity of OTUs is not required, unidentified taxa complicate and limit interpretation of α-diversity comparisons.

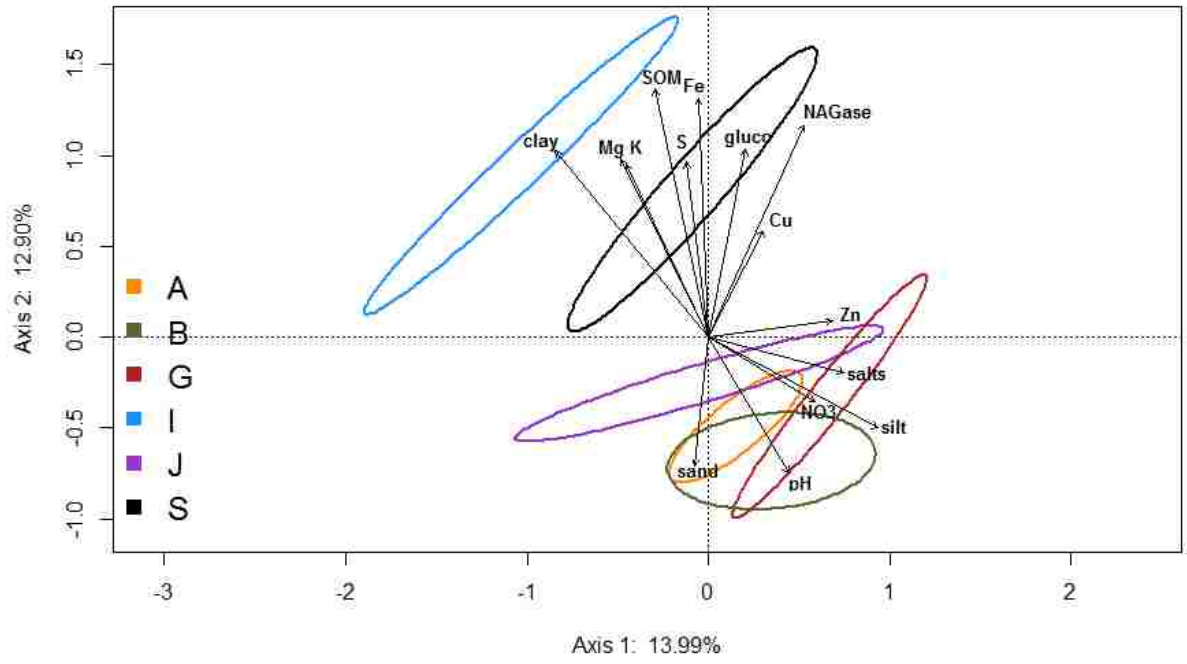

Figure 3.26 Distance-based redundancy analysis of Bray-Curtis distance matrix of ITS fungal OTUs for bulk soils from paired sites in plant cane with short and long-term sugarcane cropping histories at six locations with maximum correlation of soil and nutrient environmental variables plotted as vectors ($p < 0.05$). Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel. Variables: NO3 = nitrate, NAGase = N-Acetyl-β-D-glucosaminidase, gluco = β-glucosidase, SOM = soil organic matter.

In order to identify candidate taxa that are associated with yield decline either as taxa beneficial to sugarcane growth that decrease under long-term cultivation or deleterious taxa that increase during continuous cultivation, taxa significantly more associated with either cropping history were sorted by their abundance. In most cases, taxa more commonly associated with a short or long-term sugarcane cropping history tended to be relatively low in abundance based on their relative rank among taxa. The small subset of abundant taxa that were more commonly associated with short or long-term cropping histories could play large roles in yield differences associated with cropping history.

Additional taxa that were not nominated as candidate pathogens or beneficial microorganisms across all sampled locations may also contribute to declining yields in a site specific manner.

Table 3.25. ANOVA p-values for factors affecting pair-wise dissimiilarity of Bray-Curtis distance matrix for ITS metagenomic community composition for rhizosphere soils from paired sites in plant cane with short and long-term sugarcane cropping histories at six locations.

	Bray-Curtis
Cropping history	0.004
Location	0.001
Cropping history x Location	0.003

α = 0.05. NS = not significant.

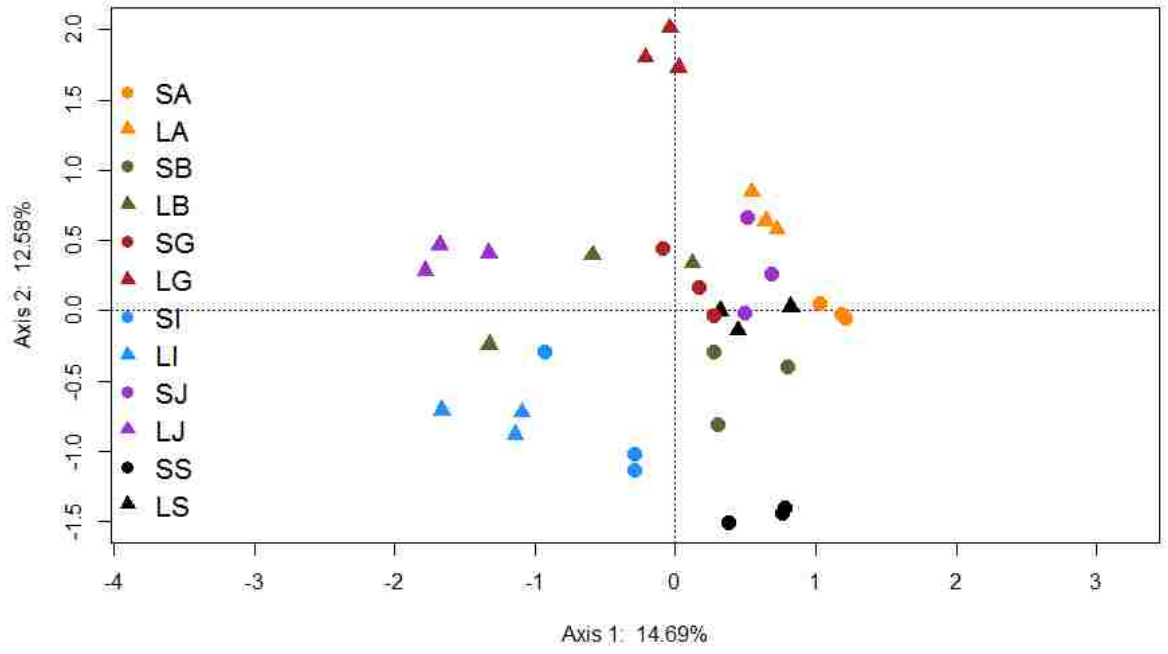

Figure 3.27 Distance-based redundancy analysis of Bray-Curtis distance matrix of ITS fungal OTUs for rhizosphere soils from paired sites in plant cane with short and long-term sugarcane cropping histories at six locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel.

This study identified some fungal genera that were of high abundance in either bulk or rhizosphere soils that previous research had identified as readily culturable from soils under continuous sugarcane cultivation, including *Humicola*, *Marasmius*, *Curvularia*, and *Mortierella*, but these fungi were not more associated with short or long-term cultivation in this study (Watanabe 1974; Pankhurst et al. 2000). Similarly for bacteria, *Sphingomonas* and *Nocardioides* had previously been reported as readily culturable from long-term sugarcane cultivation rhizosphere soil (Pankhurst et al. 2000). These

bacterial genera were abundant in this study but were unassociated and associated with short-term cultivation, respectively. *Pseudomonas*, which has been identified as readily culturable from the sugarcane rhizosphere and more abundant in short-term cultivation soils in previous studies (Savario and Hoy 2010; Pankhurst et al. 2000), was indicated to occur at low relative abundance in this study (data not shown) highlighting differences in quantification between culture-dependent and culture-independent techniques for enumerating members of the microbial community.

Table 3.26. ANOVA p-values for factors affecting pair-wise dissimiilarity of Bray-Curtis distance metrix for ITS metagenomic community composition for bulk soils from two paired sites with short and long-term sugarcane cropping histories in plant cane and first ratoon.

	Bray-Curtis
Cropping history	0.001
Location	0.001
Crop year	0.001
Cropping history x Location	0.001
Cropping history x Crop year	0.015
Location x Crop year	0.005
Cropping history x Location x Crop year	0.049

α = 0.05. NS = not significant.

Notable taxa more associated with short-term sugarcane cultivation soils in this study were *Bacillus* and *Fusarium*. Previous research has shown *Fusarium* to be more readily culturable from soils in Louisiana under short-term sugarcane cultivation than long-term cultivation (Savario and Hoy 2010). Taxa more associated with long-term cultivation in this study were *Burkholderia* and *Trichoderma*. *Burkholderia* and *Fusarium* are capable of both inducing and suppressing plant disease (Alabouvette et al. 1993, Eberl and Vandamme 2016), while *Bacillus* and *Trichoderma* are well known agents for plant disease biocontrol (Harman et al. 2004, Ongena and Jacques 2007). Interestingly, nonpathogenic *Fusarium oxysporum* has been found to increase colonization by arbuscular mycorrhizal fungi (Fracchia et al. 2000). In sugarcane, filtered extracts from *Fusarium* and *Trichoderma* isolated from sugarcane monoculture soils contained phytotoxic compounds that reduced sugarcane germination and root growth (Kao and Hsieh 1986). Although *Fusarium* species can cause multiple diseases in sugarcane, including stalk rot, wilt, and a shoot apex disease named Pokkah boeng (Croft 2000, Rao and Agnihotri 2000, Whittle and Irawan 2000), the higher abundance of *Fusarium* in soils under short-term cultivation suggests a possible positive plant health role. *Trichoderma* has rarely been reported to adversely affect plant growth, so the implication of its greater relative abundance in long-term sugarcane soils is uncertain. Additionally, the positive plant growth response of sugarcane to mancozeb was associated with an increase in root colonization by *Trichoderma* and a decrease in colonization by the sterile, dematiaceous fungus (Magarey et al. 1997a). While amplicon-based metagenomics may identify differences in the abundance of important

prokaryotic and fungal genera in soils under short and long-term sugarcane cultivation, it does not address their niche and function.

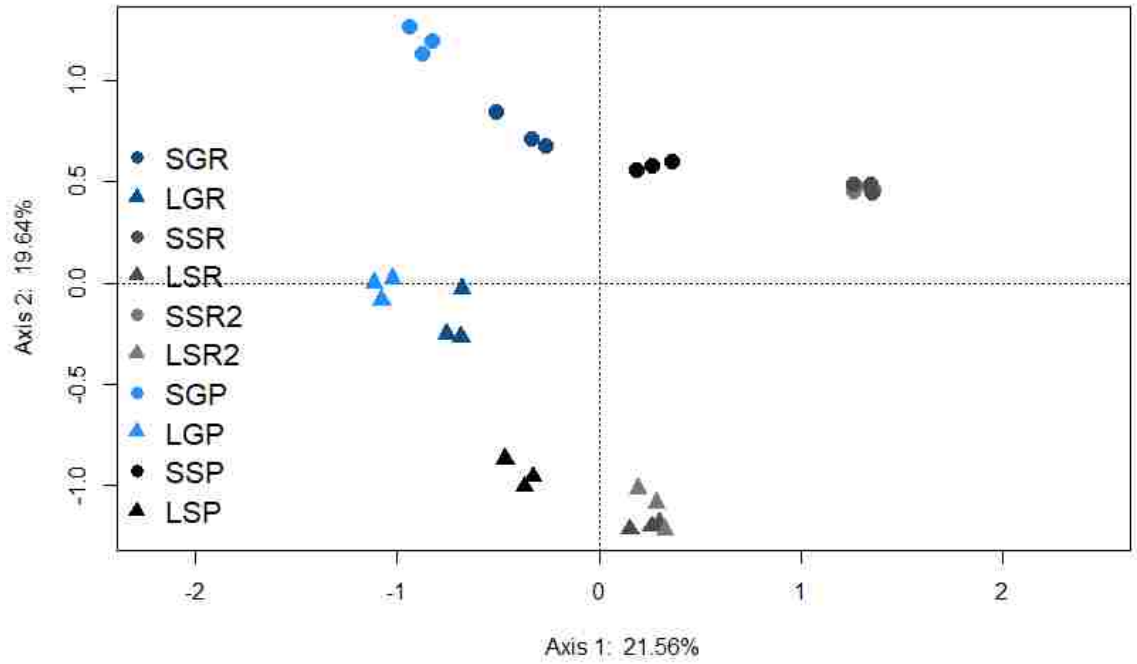

Figure 3.28 Distance-based redundancy analysis of Bray-Curtis distance matrix of ITS fungal OTUs for bulk soils from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R= first ratoon first sampling, R2= first ratoon second sampling.

3.5 Conclusions

The objective of this study was to use 16S and ITS amplicon-based metagenomics to profile the diversity of soil prokaryotic and fungal communities associated with short and long-term sugarcane cultivation in order to better understand the etiology of yield decline related to detrimental soil microbiota. Principal coordinate analyses of soil prokaryotic β-diversity revealed differences based primarily on location, but cropping history also had a significant impact. Ordinations of fungal β-diversity revealed shared differences in microbial community structure based on cropping history across locations. Similar to previous research, these results suggest fungi play a major role in the detrimental effect soil microbial communities associated with long-term sugarcane cultivation have on yield. Correlations of environmental variables with principal coordinate analyses suggest location-specific depletion of soil nutrients coincide with changes in fungal and, to a lesser extent, prokaryotic communities. However, additional research is required to determine if management of soil nutrients can prevent detrimental changes in microbial community structure associated with sugarcane monoculture. Analysis of taxonomic assignments of OTUs identified large numbers of low abundance taxa associated with short and long-term cropping histories across all

six locations that likely contribute to community-level effects on sugarcane yields. Many prokaryotic and fungal taxa previously described as readily culturable from soils under continuous sugarcane cultivation were not associated with either cropping history or even high in abundance, reflecting differences in community estimates from culture dependent and culture independent techniques. However, the greater abundance of soil microbes, such as actinobacteria and fusarial fungi, in recently cultivated soils support previous research on microbial communities associated with sugarcane cultivation in Louisiana.

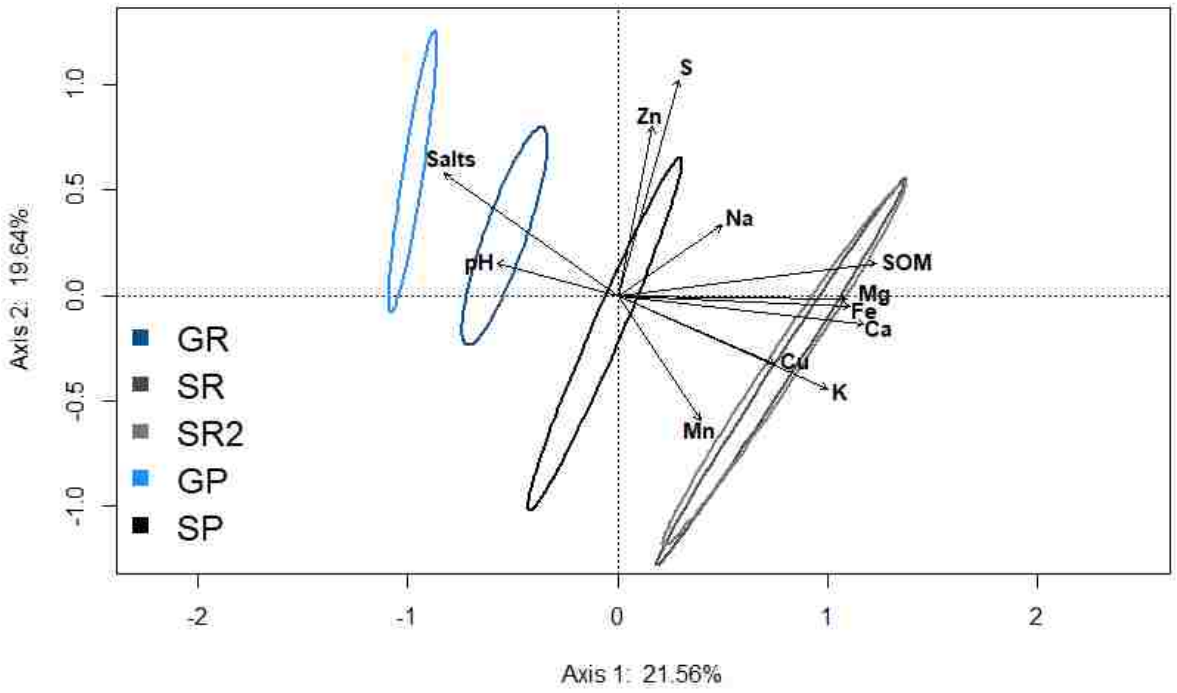

Figure 3.29. Distance-based redundancy analysis of Bray-Curtis distance matrix of ITS fungal OTUs for bulk soils from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon with maximum correlation of soil and nutrient environmental variables plotted as vectors ($p < 0.05$). Locations: G = Gonsoulin, S = St. Gabriel, Crop year: PC = plant cane, FR = first ratoon, FRa= first ratoon first sampling, FRb= first ratoon second sampling. Variables: SOM = soil organic matter, salts = soluble salts.

Table 3.27. ANOVA p-values for factors affecting pair-wise dissimiilarity of Bray-Curtis distance matrix for ITS metagenomic community composition for rhizosphere soils from two paired sites with short and long-term sugarcane cropping histories in plant cane and first ratoon.

	Bray-Curtis
Cropping history	0.001
Location	0.001
Crop year	0.001
Cropping history x Location	0.001
Cropping history x Crop year	0.033
Location x Crop year	NS
Cropping history x Location x Crop year	NS

α = 0.05. NS = not significant.

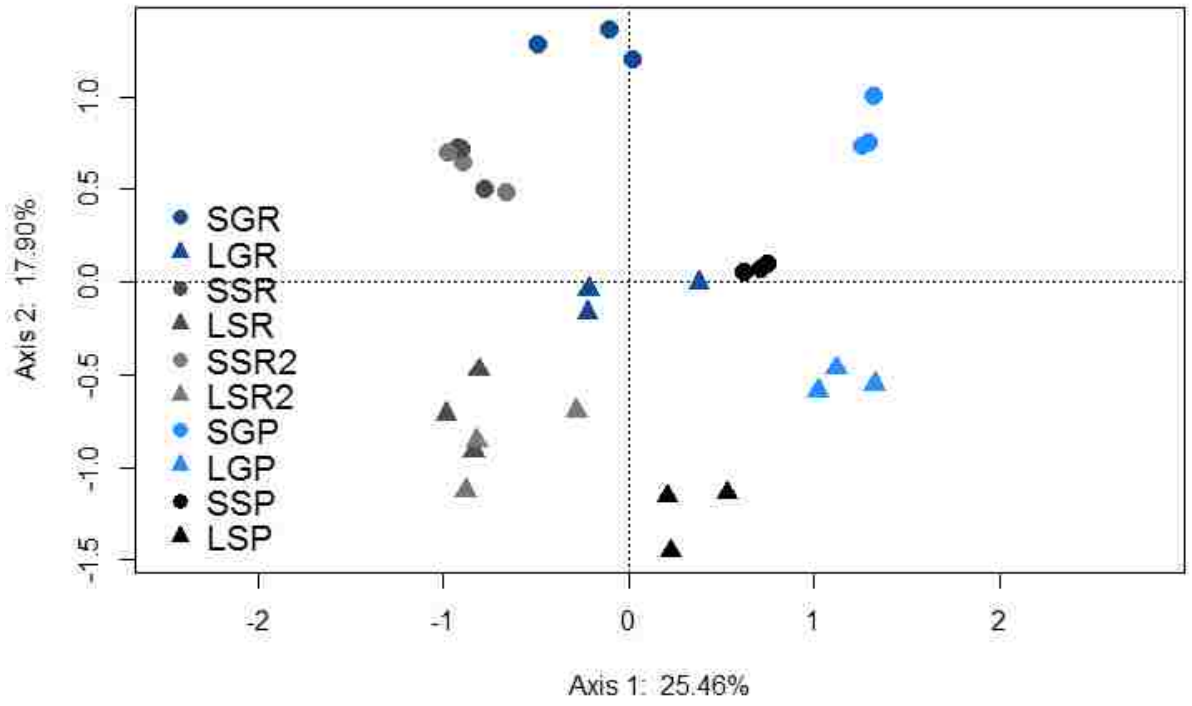

Figure 3.30. Distance-based redundancy analysis of Bray-Curtis distance matrix of ITS fungal OTUs for rhizosphere soils from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R= first ratoon first sampling, R2= first ratoon second sampling.

Chapter 4: Conclusions

- Yield component estimates were generally higher in recently cultivated soils than soil under long-term sugarcane cultivation.

- Soil nutrients were generally greater in soils with a short-term sugarcane cultivation history than their long-term cropping history counterparts, but they varied in a location-dependent manner.
    - Soil organic matter, iron and calcium were greater in short-term cultivation at four of six locations.
    - Copper and sodium were greater in short-term cultivation at three of six locations.

- Soil extracellular enzyme activities were greater in short-term cultivation soils when differences between cropping histories were observed.
    - N-acetyl-β-glucosaminidase was greater in short-term cultivation at three of six locations, and β-glucosidase was greater at two of six locations.

- Quantification of stained roots revealed more extensive colonization by fungal endophytes in soils with a long-term sugarcane cropping history.

- Analysis of fatty acid methyl ester biomarkers revealed differences in soil microbial community structure based primarily on location but also cropping history.

- Maximum correlation of fatty acid methyl ester profile distance-based redundancy analysis revealed high soil pH strongly influenced bulk soil microbial community structure at three of six locations.
    - Similar results were obtained for maximum correlation of pH with β-diversity ordinations of 16S prokaryotic communities in bulk soils.

- An amplicon-based metagenomics analysis of prokaryotic and fungal communities in bulk and rhizosphere soils from paired sites with short and long-term sugarcane cultivation histories identified members and detected differences in the structure of the microbial communities based on cropping history, location and additional factors.

- Distance-based redundancy analysis of prokaryotic β-diversity revealed community structure was primarily influenced by location.
    - Differences in distance-based redundancy analysis of prokaryotic communities using different β-diversity metrics revealed greater fluctuations in the proportions of phyla between plant cane and first ratoon

and greater changes in the composition of phyla between short and long-term cropping histories.

- Distance-based redundancy analysis of fungal β-diversity revealed community structure was primarily influenced by cropping history suggesting fungi play a major role in the yield decline associated with cropping history.
    - Maximum correlation of fungal community structure in recently cultivated bulk soils with environmental variables revealed associations with soil organic matter, sulfur, iron, copper, and soil extracellular activities in one set of locations and zinc, soluble salts, silt, and nitrate in another set.

- α-diversity comparisons of prokaryotic and fungal communities revealed subsets of the community more commonly associated with both short and long-term sugarcane cropping histories.
    - 107 prokaryotic genera and 37 fungal genera in bulk soils and 97 prokaryotic genera and 46 fungal genera in rhizosphere soils were more associated with short-term sugarcane cultivation.
    - 117 prokaryotic genera and 58 fungal genera in bulk soils and 94 prokaryotic genera and 40 fungal genera in rhizosphere soils were more associated with long-term sugarcane cultivation.
    - Notable genera more commonly associated with short-term cultivation included *Bacillus* and *Fusarium*, while genera more associated with long-term cultivation included *Burkholderia* and *Trichoderma*.

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Appendix: Figures and Tables

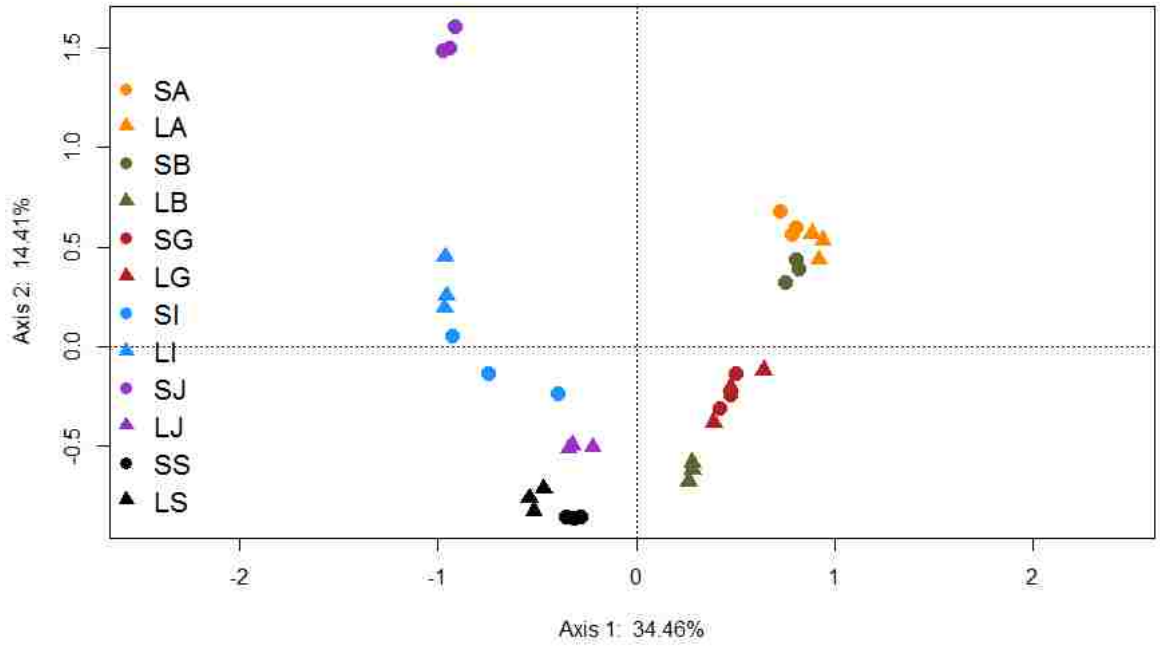

Figure A.1. Distance-based redundancy analysis of unweighted UniFrac distance matrix of 16S prokaryotic OTUs for bulk soils from paired sites with short and long-term sugarcane cropping histories in plant cane at six locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel.

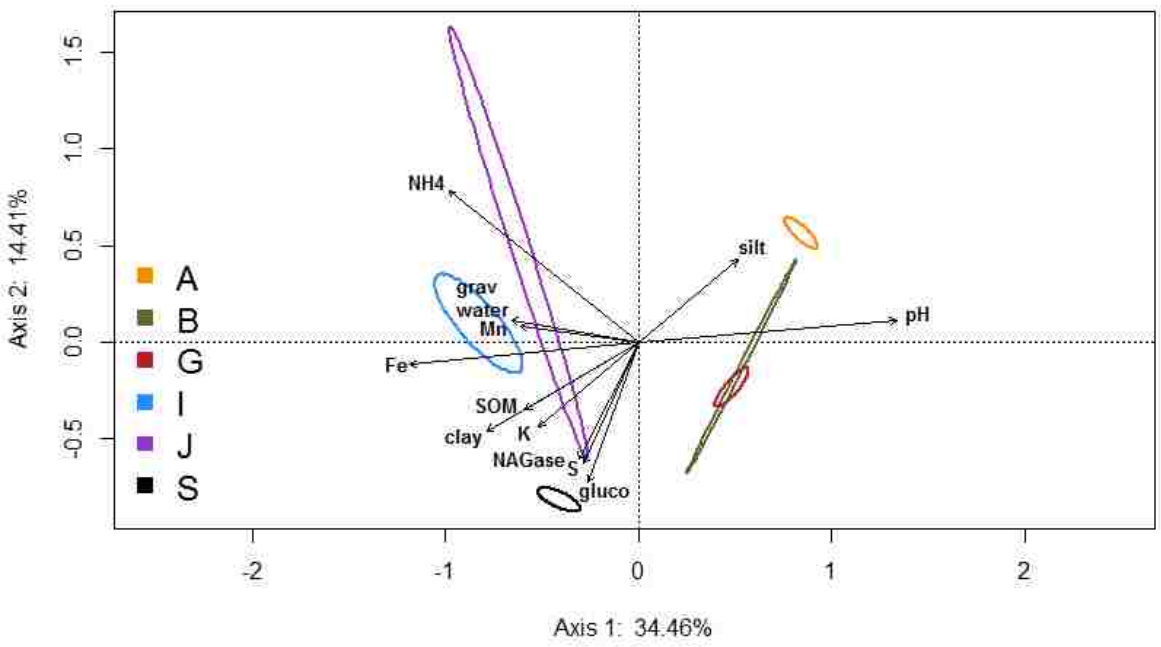

Figure A.2. Distance-based redundancy analysis of unweighted UniFrac distance matrix of 16S prokaryotic OTUs from bulk soils from paired sites with short and long-term sugarcane cropping histories at six locations with maximum correlation of soil nutrient environmental variables plotted as vectors ($p < 0.05$). L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel. Variables: NH4 = Ammonium, grav water = gravimetric water, NAGase = N-Acetyl-β-D-glucosaminidase, gluco = β-glucosidase, SOM = soil organic matter.

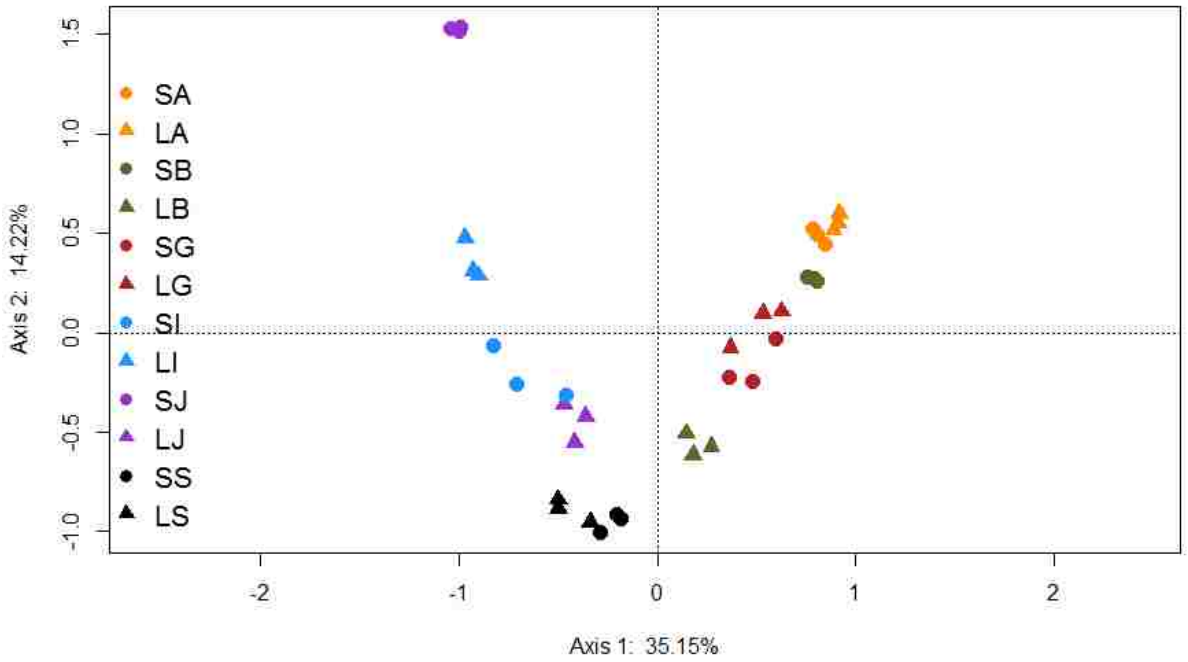

Figure A.3. Distance-based redundancy analysis of unweighted UniFrac distance matrix of 16S prokaryotic OTUs for rhizosphere soils from paired sites with short and long-term sugarcane cropping histories in plant cane at six locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: A = Airport, B = Belleview, G = Gonsoulin, I = Iberia, J = Jefferson, S = St. Gabriel.

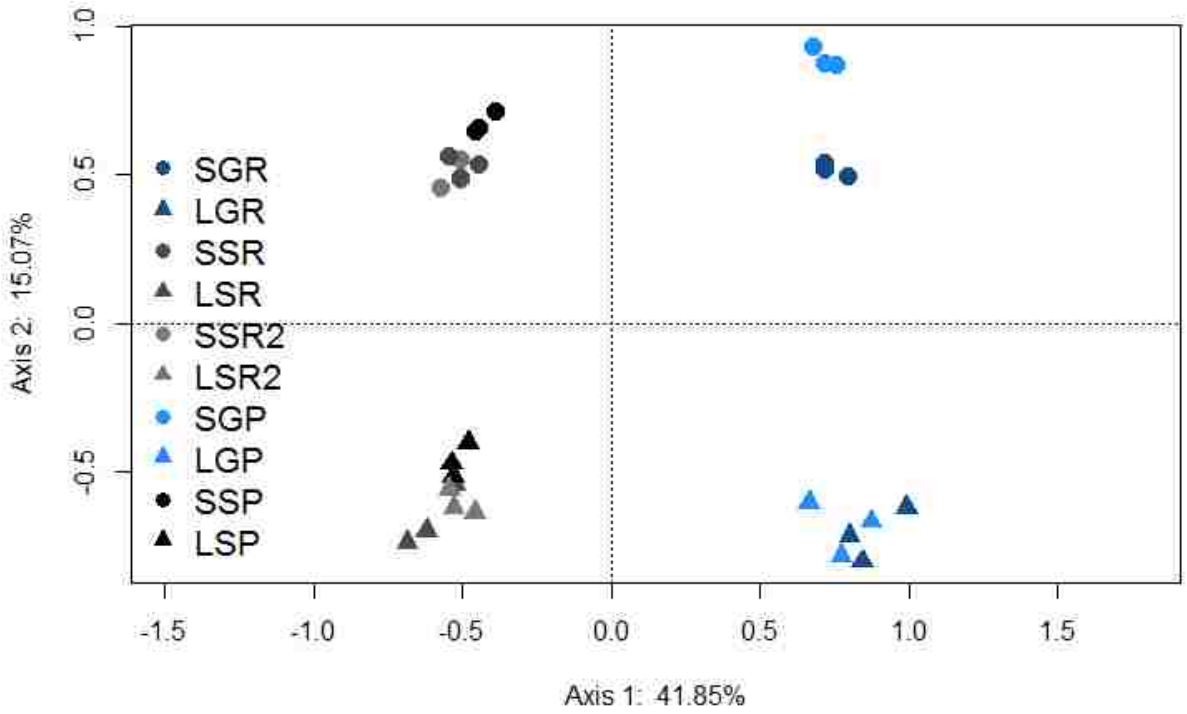

Figure A.4. Distance-based redundancy analysis of unweighted UniFrac distance matrix of 16S prokaryotic OTUs for bulk soils from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon at two locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R = first ratoon, R2 = first ratoon second sampling.

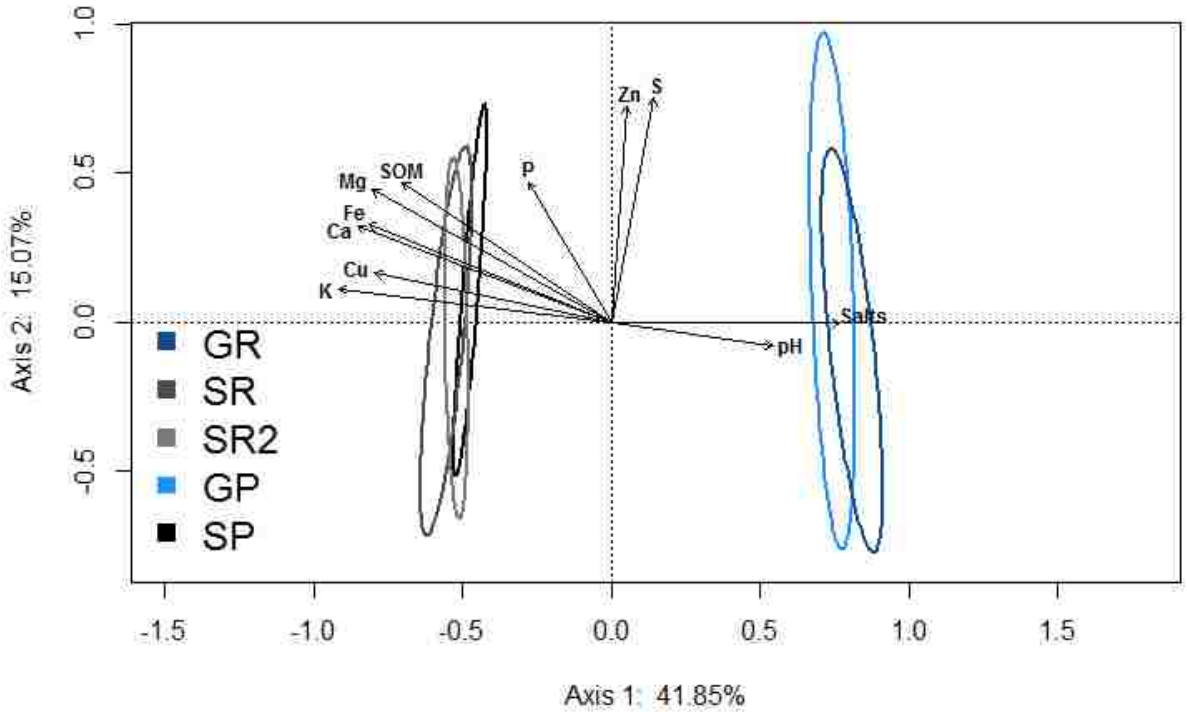

Figure A.5. Distance-based redundancy analysis of unweighted UniFrac distance matrix of 16S prokaryotic OTUs for bulk soils from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon at two locations with maximum correlation of soil and nutrient environmental variables plotted as vectors ($p < 0.05$). Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R = first ratoon, R2 = first ratoon second sampling. Variables: SOM = soil organic matter, salts = soluble salts.

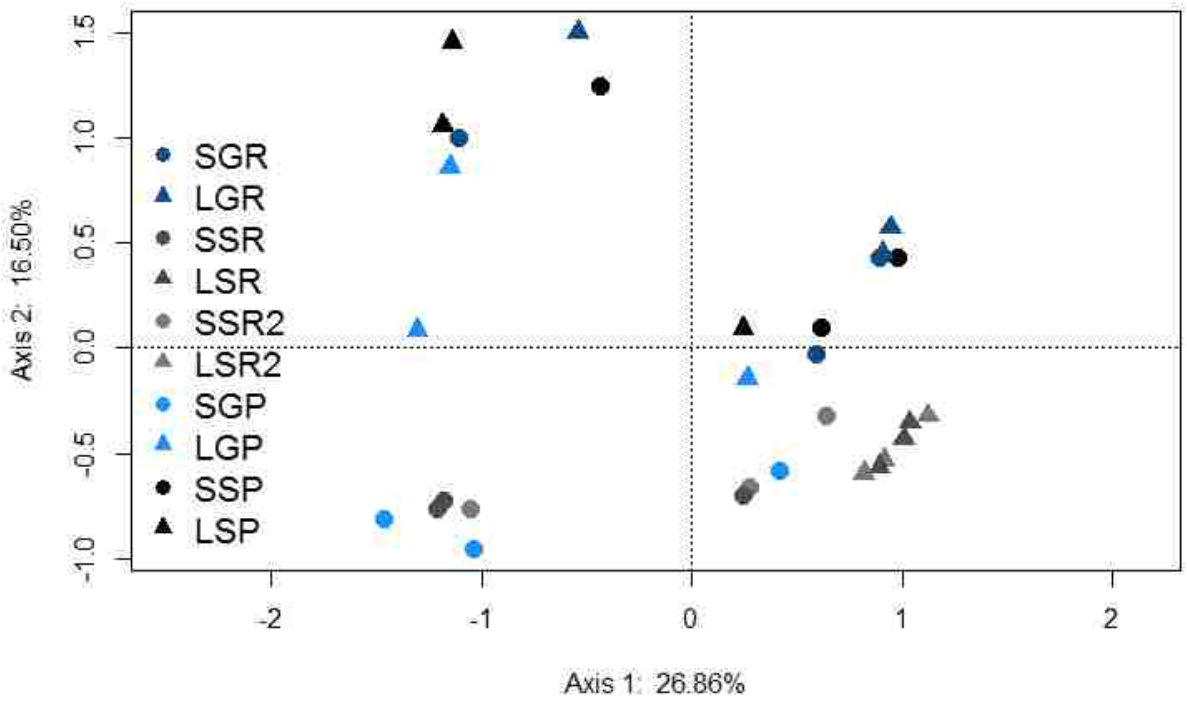

Figure A.6. Distance-based redundancy analysis of unweighted UniFrac distance matrix of 16S prokaryotic OTUs of rhizosphere soils combined from paired sites with short and long-term sugarcane cropping histories sampled in plant cane and first ratoon at two locations. Cropping history: S = short-term cultivation, L = long-term cultivation. Locations: G = Gonsoulin, S = St. Gabriel, Crop year: P = plant cane, R = first ratoon, R2 = first ratoon second sampling.

Table A.1. Abundant prokaryotic families in bulk soil with no significant association in soils under short or long-term sugarcane cultivation ($p > 0.05$) and their relative abundance and rank among families for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of fungal community	Rank among genera	Proportion of fungal community	Rank among genera
Acidobacteriaceae (Subgroup 1)	0.060642	1	0.075063	1
Blastocatellaceae (Subgroup 4)	0.030058	4	0.034578	3
Gaiellale uncultured	0.031011	3	0.023017	8
Chitinophagaceae	0.026005	6	0.023892	7
Verrucomicrobia OPB35 soil group uncultured bacterium	0.023331	8	0.025716	6
Sphingomonadaceae	0.020719	12	0.026180	5
Desulfurellaceae	0.025309	7	0.021426	10
Acidobacteria Subgroup 6 Other	0.021446	10	0.021854	9
Chthoniobacterales DA101 soil group	0.022716	9	0.016101	17
Planctomycetaceae	0.020038	13	0.017967	13
Haliangiaceae	0.016494	17	0.019670	12
Xanthomonadales Incertae Sedis	0.019430	14	0.016480	16
Solibacteraceae (Subgroup 3)	0.015831	18	0.017779	14
Acidothermaceae	0.020843	11	0.009370	24
Anaerolineaceae	0.012805	22	0.014423	18
Rhodospirillaceae	0.014235	19	0.011298	20
Acidobacteria Subgroup 6 uncultured bacterium	0.013863	20	0.011570	19
Micromonosporaceae	0.012961	21	0.010750	21
Tepidisphaeraceae	0.010334	24	0.009872	23
Xanthomonadaceae	0.008340	32	0.010472	22
Myxococcales BIrii41	0.009584	25	0.009125	26
Rhodospirillales DA111	0.009226	26	0.008768	28
Comamonadaceae	0.008367	31	0.009331	25
Nitrospiraceae	0.007256	36	0.009007	27
Xanthomonadales uncultured	0.008988	28	0.006647	35

Table A.2. Abundant prokaryotic genera in bulk soil with no significant association in soils under short or long-term sugarcane cultivation ($p > 0.05$) and their relative abundance and rank among genera for each cropping history.

	Short-term cropping history		Long-term cropping history	
Genus	Proportion of fungal community	Rank among genera	Proportion of fungal community	Rank among genera
Uncultured Acidobacteriaceae Subgroup 1	0.052231	1	0.069238	1
Uncultured OPB35 soil group	0.023331	2	0.025716	5
Sphingomonas	0.020364	7	0.025885	4
Desulfurellaceae H16	0.023249	3	0.020185	7
Acidobacteria Other	0.021446	5	0.021854	6
Uncultured Gaiellales	0.021992	4	0.015828	11
Haliangium	0.016494	12	0.019670	8
Uncultured DA101 soil group	0.020133	8	0.012854	14
Gemmatimonas	0.016356	13	0.016620	10
Blastocatellaceae Subgroup 4 RB41	0.014055	14	0.018186	9
Acidothermus	0.020843	6	0.009370	19
Uncultured Anaerolineaceae	0.012307	17	0.014175	13
Uncultured Chitinophagaceae	0.013816	16	0.012515	15
Uncultured Acidobacteria Subgroup 6	0.013863	15	0.011570	16
Rhizomicrobium	0.010202	22	0.014362	12
Acidibacter	0.011312	18	0.010137	18
Uncultured Planctomycetaceae	0.011030	19	0.010292	17
Xanthobacteraceae;Other	0.010490	21	0.008793	22
Variibacter	0.010495	20	0.008423	24
Uncultured Myxococcales BIrii41	0.009127	23	0.008498	23
Candidatus Solibacter	0.007816	27	0.009062	20
Uncultured Tepidisphaeraceae	0.008066	26	0.008284	25
Nitrospira	0.006892	32	0.008927	21
Bryobacter	0.007559	29	0.008239	27
Blastocatellaceae Subgroup 4 11-24	0.006926	30	0.008246	26

Table A.3. Abundant prokaryotic families in rhizosphere soils with no significant association in soils under short or long-term sugarcane cultivation ($p > 0.05$) and their relative abundance and rank among families for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of fungal community	Rank among genera	Proportion of fungal community	Rank among genera
Acidobacteriaceae (Subgroup 1)	0.068940	1	0.074125	1
Gemmatimonadaceae	0.033733	2	0.038787	2
Sphingomonadaceae	0.026703	5	0.038462	3
Chitinophagaceae	0.031397	3	0.030517	5
Blastocatellaceae (Subgroup 4)	0.028194	4	0.030192	6
Verrucomicrobia OPB35 soil group uncultured bacterium	0.023780	8	0.026006	8
Bacillaceae	0.024537	7	0.018452	11
Xanthobacteraceae	0.023263	9	0.018749	10
Acidobacteria;D_2__Subgroup 6 Other	0.019308	13	0.019472	9
Xanthomonadales Family Incertae Sedis	0.021471	10	0.016233	15
Desulfurellaceae	0.020169	12	0.013903	20
Solibacteraceae (Subgroup 3)	0.016372	18	0.017044	13
Planctomycetaceae	0.017647	16	0.014849	18
Chthoniobacterales;D_4__DA101 soil group	0.018292	14	0.012981	22
Haliangiaceae	0.014924	19	0.016230	16
Xanthomonadaceae	0.013895	20	0.015088	17
Comamonadaceae	0.011956	25	0.016623	14
Rhizobiales Incertae Sedis	0.012095	24	0.014681	19
Acidothermaceae	0.018254	15	0.006980	36
Rhodospirillaceae	0.013492	21	0.011067	23
Acidobacteria; Subgroup 6 uncultured bacterium	0.012825	23	0.010637	24
Anaerolineaceae	0.012931	22	0.010285	25
Micromonosporaceae	0.010303	26	0.008971	27
Myxococcales BIrii41	0.010005	27	0.008604	28
Tepidisphaeraceae	0.008830	30	0.008508	29

Table A.4. Abundant prokaryotic genera in rhizosphere soils with no significant association in soils under short or long-term sugarcane cultivation ($p > 0.05$) and their relative abundance and rank among genera for each cropping history.

	Short-term cropping history		Long-term cropping history	
Genus	Proportion of fungal community	Rank among genera	Proportion of fungal community	Rank among genera
Acidobacteriaceae (Subgroup 1); uncultured	0.057922	1	0.066771	1
Sphingomonas	0.026237	2	0.038015	2
Verrucomicrobia OPB35 soil group uncultured bacterium	0.023780	4	0.026006	5
Bacillus	0.023828	3	0.018211	8
Acidobacteria Subgroup 6 Other	0.019308	6	0.019472	7
Desulfurellaceae H16	0.018490	7	0.012977	14
Chitinophagaceae uncultured	0.015592	13	0.015623	10
Haliangium	0.014924	14	0.016230	9
Gemmatimonas	0.014768	15	0.014144	12
Blastocatellaceae (Subgroup 4) RB41	0.012577	18	0.015208	11
Chthoniobacterales DA101 soil group	0.015925	12	0.010151	19
Acidothermus	0.018254	8	0.006980	33
Acidibacter	0.013704	16	0.010635	18
Acidobacteria;D_2__Subgroup 6 uncultured bacterium	0.012825	17	0.010637	17
Rhizomicrobium	0.010416	20	0.012516	15
Anaerolineaceae	0.012461	19	0.010080	20
Myxococcales BIrii41 uncultured bacterium	0.009651	21	0.008157	22
Planctomycetaceae uncultured	0.009230	23	0.008011	24
Variibacter	0.008906	25	0.007757	26
Xanthobacteraceae Other	0.009289	22	0.007324	30
Candidatus Solibacter	0.007783	29	0.008545	21
Bryobacter	0.008139	27	0.008093	23
Bradyrhizobium	0.008247	26	0.007423	27
Tepidisphaeraceae uncultured bacterium	0.006969	30	0.007095	32
Nitrospira	0.006317	35	0.007416	28

Table A.5. Abundant fungal families in bulk soil with no significant association in soils under short or long-term sugarcane cultivation ($p > 0.05$) and their relative abundance and rank among families for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of fungal community	Rank among genera	Proportion of fungal community	Rank among genera
Unidentified Fungi	0.209250	1	0.204798	1
Unidentified Ascomycota	0.050538	2	0.030043	5
Unidentified Hypocreales	0.030847	5	0.022330	8
Unidentified Pleosporales	0.028333	6	0.021165	11
Unidentified Sordariomycetes	0.037948	3	0.010437	24
Lasiosphaeriaceae	0.017124	13	0.021685	10
Sordariaceae	0.011075	23	0.022112	9
Unidentified Dothideomycetes	0.018586	12	0.011703	21
Herpotrichiellaceae	0.014319	17	0.010690	23
Pezizomycotina Family Incertae sedis	0.008851	28	0.016147	15
Glomeraceae	0.008645	30	0.016165	14
Bolbitiaceae	0.021841	9	0.000569	114
Hypocreaceae	0.007890	34	0.011539	22
Helotiales Family Incertae sedis	0.005768	42	0.012991	20
Ascobolaceae	0.014740	16	0.003770	43
Hypocreales Family Incertae sedis	0.013056	19	0.004931	34
Psathyrellaceae	0.013660	18	0.003692	45
Corticiaceae	0.010090	25	0.007086	29
Amylocorticiaceae	0.002191	69	0.014686	18
Polyporaceae	0.003427	55	0.013042	19
Unidentified Agaricales	0.011989	21	0.003524	47
Unidentified Cantharellales	0.000058	231	0.015334	17
Trichosphaeriales Family Incertae sedis	0.009552	26	0.005222	33
Hydnaceae	0.004209	48	0.009619	27
Trichocomaceae	0.007086	36	0.005793	32
Unidentified Basidiomycota	0.008582	31	0.003887	42

Table A.6. Abundant fungal genera in bulk soil with no significant association in soils under short or long-term sugarcane cultivation ($p > 0.05$) and their relative abundance and rank among genera for each cropping history.

	Short-term cropping history		Long-term cropping history	
Genus	Proportion of fungal community	Rank among genera	Proportion of fungal community	Rank among genera
Unidentifed Fungi	0.209250	1	0.204798	1
Unidentifed Ascomycota	0.050538	2	0.030043	5
Trichocladium	0.006839	31	0.071564	2
Unidentifed Hypocreales	0.030847	4	0.022330	9
Unidentifed Mortierellaceae	0.017482	9	0.032323	4
Unidentifed Pleosporales	0.028333	5	0.021165	11
Unidentifed Sordariomycetes	0.037948	3	0.010437	21
Sordaria	0.011017	15	0.021747	10
Unidentifed Dothideomycetes	0.018586	8	0.011703	19
Mortierella	0.010430	17	0.013940	16
Conocybe	0.021167	7	0.000237	232
Unidentifed Glomeraceae	0.006431	34	0.013873	17
Trichoderma	0.007885	24	0.011084	20
Ascobolus	0.014662	11	0.003766	44
Ceraceomyces	0.002153	78	0.014686	15
Unidentifed Agaricales	0.011989	12	0.003524	46
Unidentifed Cantharellales	0.000058	443	0.015334	14
Westerdykella	0.007191	29	0.008127	26
Polyporaceae	0.002298	74	0.012863	18
Cladophialophora	0.010435	16	0.004680	34
Nigrospora	0.009552	18	0.005222	32
Nectriaceae	0.007798	25	0.005974	30
Unidentifed Hydnaceae	0.004144	53	0.009503	25
Unidentifed Basidiomycota	0.008582	20	0.003887	41
Hyalocladosporiella	0.001178	115	0.009738	22

Table A.7. Abundant fungal families in rhizosphere soils with no significant association in soils under short or long-term sugarcane cultivation ($p > 0.05$) and their relative abundance and rank among families for each cropping history.

	Short-term cropping history		Long-term cropping history	
Family	Proportion of fungal community	Rank among genera	Proportion of fungal community	Rank among genera
Unidentified Fungi	0.232800	1	0.240681	1
Lasiosphaeriaceae	0.052204	2	0.019698	13
Unidentified Cantharellales	0.000072	222	0.070681	2
Unidentified Ascomycota	0.035939	4	0.033571	5
Mortierellaceae	0.036644	3	0.032390	6
Chaetomiaceae	0.024189	10	0.040737	4
Unidentified Hypocreales	0.030426	6	0.020260	12
Unidentified Pleosporales	0.025903	9	0.024279	11
Unidentified Dothideomycetes	0.032366	5	0.012921	19
Unidentified Sordariomycetes	0.028909	8	0.007186	29
Psathyrellaceae	0.017245	11	0.017921	14
Marasmiaceae	0.004850	39	0.025558	8
Ceratobasidiaceae	0.013844	14	0.012307	20
Unidentified Sordariales	0.011147	22	0.014294	17
Corticiaceae	0.007256	33	0.015677	15
Glomeraceae	0.012014	18	0.007289	28
Pezizomycotina Family Incertae sedis	0.003647	52	0.015056	16
Unidentified Agaricales	0.010674	23	0.006519	32
Herpotrichiellaceae	0.010233	24	0.006799	31
Hypocreales Family Incertae sedis	0.011873	20	0.004857	37
Helotiales Family Incertae sedis	0.006382	34	0.009682	22
Tremellales Family Incertae sedis	0.012641	17	0.002083	56
Trichocomaceae	0.009850	26	0.004635	39
Trichosphaeriales Family Incertae sedis	0.007919	31	0.004707	38
Hydnaceae	0.004960	38	0.007592	27

Table A.8. Abundant fungal genera in rhizosphere soils with no significant association in soils under short or long-term sugarcane cultivation ($p > 0.05$) and their relative abundance and rank among genera for each cropping history.

	Short-term cropping history		Long-term cropping history	
Genus	Proportion of fungal community	Rank among genera	Proportion of fungal community	Rank among genera
Unidentified Fungi	0.232800	1	0.240681	1
Unidentified Cantharellales	0.000072	423	0.070681	2
Unidentified Ascomycota	0.035939	2	0.033571	4
Unidentified Hypocreale	0.030426	5	0.020260	11
Unidentified Pleosporales	0.025903	7	0.024279	6
Unidentified Dothideomycetes	0.032366	4	0.012921	16
Arnium	0.035533	3	0.006458	28
Unidentified Mortierellaceae	0.018647	8	0.021750	10
Unidentified Sordariomycetes	0.028909	6	0.007186	25
Mortierella	0.017997	9	0.010641	19
Psathyrella	0.010811	16	0.015459	13
Marasmius	0.001823	78	0.024267	7
Unidentified Sordariales	0.011147	15	0.014294	14
Trichocladium	0.002760	62	0.020081	12
Unidentified Agaricales	0.010674	18	0.006519	27
Humicola	0.016369	12	0.000782	132
Waitea	0.003741	49	0.010114	20
Unidentified Ceratobasidiaceae	0.008340	27	0.004518	38
Unidentified Glomeraceae	0.008600	25	0.004236	41
Nigrospora	0.007919	28	0.004707	37
Westerdykella	0.009301	21	0.002095	61
Acremonium	0.008844	24	0.002482	54
Curvularia	0.008365	26	0.002603	52
Unidentified Hydnaceae	0.002979	59	0.007536	24
Unidentified Nectriaceae	0.005822	34	0.004391	39

Vita

Adam Francis Bigott is from Naperville, Illinois. In 2014, he received his Bachelor of Arts in Biology from Hendrix College in Conway, Arkansas. That same year, he enrolled in the Master of Science program in the department of Plant Pathology and Crop Physiology at Louisiana State University Agricultural and Mechanical College and worked under the direction of Dr. Jeffrey W. Hoy and Dr. Lisa M. Fultz during his time there. Upon completion of his degree, he plans to obtain his Ph.D. in plant pathology at the University of Wisconsin-Madison.