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Effect of Controlled Burns on the Bacterial Communities Composition over Time at Four Sites in the Yawkey Forest on Cat Island in Georgetown, S.C.

Richard Thomas Pepple
Clemson University, tetron4@yahoo.com

EFFECT OF CONTROLLED BURNS ON THE BACTERIAL COMMUNITIES COMPOSITION OVER TIME AT FOUR SITES IN THE YAWKEY FOREST ON CAT ISLAND IN GEORGETOWN, S.C.

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Richard Thomas Pepple
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Tzuen-Rong Tzeng, Committee Chair
Barbara Campbell
Alex Chow

ABSTRACT

Fire is known to be capable of disturbing the composition of soil microbial communities due to changes in soil chemistry post-fire. These fire based disturbances leave behind higher levels of inorganic nutrients and toxic byproducts from incomplete combustion. Ecosystems are seen to recover from these disturbances given enough time. To determine if the controlled burns utilized on Cat Island in Georgetown South Carolina caused a disturbance, and if so to track the recovery from that disturbance, samples were collected from Cat Island over the course of 12 months from 03/07/2016 – 03/06/2017 from four separate locations. The pre-fire, post-fire and first four weeks of sampling were collected with a half-inch corer. The remaining monthly samples were collected with a 2 cm slidehammer. Each of the monthly timepoints were collected in quadruplicate. Samples of the soil immediately prior to the controlled burn were collected and compared to those acquired immediately after those locations had cooled enough for sample collection purposes. The purpose of the controlled burns on Cat Island is for the promotion of longleaf pine (*Pinus palustris*) growth in order to provide greater habitat for the Red Cockaded Woodpecker (*Leuconotopicus borealis*). To determine if there was an effect on the microbial communities from these controlled burns, DNA was extracted from each soil sample and used to measure the communities composition via 16S rRNA gene sequencing. The soil chemistry was also measured to compare to changes in the

soil microbial communities. The specific measurements of soil chemistry included pH, soil moisture content, nitrate, ammonium, phosphorus, carbon and nitrogen.

Examination of the changes in alpha diversity, beta diversity, and phylum level taxonomy of the microbial communities indicate that the severity of the fires that are utilized on Cat Island did not cause a large enough disturbance to the forest soil to create significant shifts in the microbial communities structure. The measurements of soil chemistry proved inconclusive as they did not appear to vary based on location of sampling, time of sampling, burn frequency of sampling location or time past burn.

While the lack of change in relative abundance limits the inferences that can be made into the natural process of post-fire remediation that has evolved over time in forest ecosystems for recovering from fire-based disturbances, it does show that the management practices employed on Cat Island are not causing disturbances in the soil biology or chemistry that might prove detrimental to the ecosystems health. This positive result on the microbiological level means that this management practice can continue to be used without creating undesired changes in the forest habitat.

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CHAPTER ONE

INTRODUCTION

Due to a constantly expanding human population, the need to develop new [1-5] and more effective methods [6] of environmental management [7] and repair [8] is constantly growing. Many of the solutions [9] required for effective management and restoration of natural ecosystems come from observation of those ecosystems in their natural state and under stress [10, 11]. This thesis examines the effect of forest fires on the bacterial communities in a long leaf pine forest. The reaction of the bacterial communities to this stimulus could provide insight into the process of remediation that naturally occurs [12].

Forest fires have been occurring for hundreds of millions of years [13, 14]. The causes of modern fires could be natural [15] or anthropogenic [16, 17]. Fire, while disruptive to a forest ecosystem [18], is a disturbance that forests ecosystems have evolved over millions of years to survive and recover from [19, 20]. Polycyclic aromatic hydrocarbons (PAHs) [21] are generated during this process [22]. PAHs are considered contaminants that require remediation in other locations [23, 24]. Lessons learned from observing this natural process of remediation could be applied to other sites contaminated through anthropogenic processes [25, 26].

The magnitude of the disruption that a fire has is directly related to its severity [27-30]. High severity fires can cause intense disruption and can require decades to recover from [31, 32]; low severity fires, on the other hand, can be

recovered from in only a few years [33]. The severity of a fire is defined by two components. The first component is the duration of the fire [34], the longer the burn, the higher the severity. The second component is the temperature [35]; the higher the temperature, the higher the severity [36, 37].

Low intensity fires with an intentional anthropogenic origin are referred to as controlled burns. These fires are typically initiated under conditions that will cause low severity fires. They are done for various reasons, primarily to prevent the buildup of materials that would cause a high severity fire. Those fires that start naturally are called wildfires, though this term has taken on a more specific connotation to describe fires of high severity.

Regardless of origin or severity, fires cause a disruption in the equilibrium of the environment in which they occur [38]. This disruption in equilibrium causes a shift in traits that will provide the highest competitive advantage [39, 40]. As a result, the communities structure of a forest, post-fire, undergoes both macrobiological and microbiological changes in structure and composition [41, 42].

In this thesis work, the composition of the soil at the Yawkey experimental forest on Cat Island near Georgetown SC was studied on the microbiological scale via 16S rRNA gene profiling of bacterial communities [43-47]. The soil chemistry was also studied by measurements of moisture content, carbon/nitrogen ratio, nitrate, ammonium, phosphorus and pH. Due to the Yawkey site being an experimental forest, with regular controlled burning, the

structure of the pre-fire microbial communities is likely to be different from that of a forest without regular controlled burns [48-51]. The effect of these disruptions was analyzed to determine communities resistance and resilience [52] in the face of fire disruption. The response pattern [53] of the bacterial communities could serve as indicators of whether the controlled burn caused a significant change in the soil chemistry.

CHAPTER TWO

LITERATURE REVIEW

2.1 Controlled Burn

Controlled burns, also known as prescribed fires, are important tools in the management [54] of natural ecosystems [55]. A prescribed burn is defined as the skillful application of fire in a definite place for a specific purpose. These purposes can include maintenance of ecosystem integrity, and the reduction of the chance for wildfires [56]. Prescribed burns have the advantage of low cost and rapid application. Without management, forest ecosystems can suffer from uncontrolled wildfires. Previous management strategies aiming to suppress all fires from occurring leading to accumulation of biomass can actually exacerbate this problem [57]. The use of controlled burns prevents catastrophic fires by reducing the fuel load present in the ecosystem [58]. In addition to a use in controlling wildfires, controlled burns can also be employed to help engineer specific ecosystems for the benefit of plants, animals or humans [59]. For example, in the Yawkey Forest the alteration of the ecosystem for a specific species is conducted to provide more habitat for the benefit of the Red Cockaded Woodpecker.

2.2 Yawkey Forest

The Yawkey Wildlife Center is located approximately 10 miles southeast of Georgetown South Carolina. The Yawkey Wildlife Center is composed of three

islands, North Island, South Island, and Cat Island. Cat Island, the sample site of this thesis work, is not a natural island; it was isolated from the mainland by canals between Winah and Santee bays. See Appendix A for site map and images.

The Yawkey forest originally belonged to William Yawkey, who willed it to his son Tom Yawkey upon his death. Tom Yawkey, upon his death, willed the forest to the South Carolina Department of Natural Resources in 1976. The stipulations of the will required that the property be used, for all time, for wildlife management, education, and research [60]. The habitat of the Yawkey forest is primarily coniferous woods.

The coniferous forest was historically composed of long leaf pines. Due to logging, the old growth pines became rare on the islands. This caused a problem specifically for the Red Cockaded Woodpecker (*Leuconotopicus borealis*). This particular Woodpecker requires live pine trees in which to make its nests. The trees need to at a minimum be 60-70 years of age to contain sufficient heartwood for cavity construction. Suitable habitat is currently found only on Cat Island. This island has 4000 acres that are potentially suitable [60].

In order to promote increased habitat for the Cockaded Woodpecker regular controlled burns are conducted on Cat Island. These burns are primarily aimed at removing oak trees and keeping new growth from decreasing the available nutrients available for the older pines. This burn schedule can be slightly modified by request of research teams. This can include not burning

specific plots or waiting specified time periods between burns. This provides a location where the effect of low intensity fires can be studied in a natural environment. Scientists have used the site to study the effect of fire on water quality that the runoff from fires creates in local waterways [54, 61].

2.3 Longleaf Pine Forests

Longleaf pine (*Pinus palustris*) ecosystems [62] are considered some of the most endangered ones on earth. This loss is considered to be primarily caused by heavy logging that was performed at the start of the 20th century [63]. Additional forest area was lost due to pine resin harvesting and the grazing of free range hogs. In the 1900s, fire suppression plans were implemented that resulted in a suppression of regrowth of pine forests.

Longleaf pines were once prominent across the southeastern United States [64]. They are believed to have totaled 90 million acres at the time of European Colonization. Longleaf Pine forests have declined to less than 3 million acres.

Fire was estimated to occur every 1-3 years during pre-colonial times. Fires were started both by natural actions, such as lightning, and Native American actions. Active fire suppression began between 1910 and 1930. These actions damaged the ecosystems of forests that required fire as part of their growth and renewal [65].

2.4 Pre-Fire Soil Characteristics

The characteristics of the soil present in the Yawkey forest does not currently exist in any database. The soil from a similar site, the Hobcaw Barony [66], is presented instead. This information comes from a database maintained by the USDA under the organization of the Nationals Cooperative Soil Survey. According to the database, the Hobcaw soils consist of deep and very poorly drained, moderately permeable, loamy soils that formed in marine or fluvial sediments on the Lower Coastal Plain. The taxonomic class of the soil is fine-loamy, siliceous, active, thermic Typic Umbraquults. The A horizon on average extends from 0-25.4 cm, which approximates the total depth of sampling which is 20 cm. Within this horizon, the loam is of moderate fine consistency. The granular structure is friable, with many fine and medium roots, few fine holes, very strongly acid, and has clear smooth boundaries [67, 68].

2.5 Soil Structure

A direct effect of moderate fires is the creation of a water repelling front on the surface of the soil [69, 70]. This front which rarely exceeds 6-8 cm in depth decreases the soil permeability to hydrophilic compounds [71]. The stability of the soil can be increased post-fire by the formation of hydrophobic layers on soil aggregates [72], decreasing their ability to be dissolved. Fires tend to temporarily

increase the amount of soil erosion and runoff [73] due to the increased hydrophobicity of the soil surface [74]. Huffman et al. [75] found that there was a weakening of this hydrophobic layer after 3 months, but it was still detectable a total of 22 months post-fire. The bulk density of the soil also tends to increase as demonstrated by Giovannini et al. [76] as a result of the collapse of the organo-mineral aggregates, and the clogging of soil pores with the ash from the fire, as discussed by Durgin and Vogeslang [77]. According to Boyer and Miller [62], such changes would cause a decrease in the water holding capacity of the soil, which will lead to increased runoff during heavy rain events. These changes are transient with their duration being dependent on the intensity of the fire and the resilience of the original biosystem [78].

2.6 Soil pH

Soil pH is increased by soil heating, but only significantly at high temperatures [79] 450-500 ^{o}C. The immediate effect of fire on soil pH can be quite extreme [80]. Ulery et al. [81] found that the pH of topsoil immediately after fire could increase as much as three units due to the production of K and Na oxides, hydroxides, and carbonates. These compounds though do not persist long if the site receives precipitation. The importance of soil pH on the composition of the microbial communities was discussed by Fierer and Jackson [82] who concluded that among the samples taken from locations all over North America, the strongest predictor of communities composition was the pH of the

soil. In addition, research by Wang et al. [83] showed that there was a strong correlation between pH and beta bacterial diversity in late successional forests, though it was less predictive in early successional forests.

2.7 Carbon/Nitrogen Ratio

The Carbon/Nitrogen (C:N) [84] ratio is a measurement of the ratio of the mass of carbon compared to the mass of nitrogen in a substance [85]. This ratio can be used for a variety of purposes such as identifying the source of sediments between terrestrial and marine, and even identifying the type of organisms which created that sediment [86]. In a soil setting this ratio gives an indicator of the productivity of the soil in question. Soil microorganisms have a carbon nitrogen(C:N) ratio of approximately 8:1 [87]. They are, on average, maximally productive when the soil they inhabit has a C:N ratio of 24:1. Deviations from this ratio caused by the controlled burn therefore could alter the dynamics of the soil communities [88]. Nitrogen is typically the limiting nutrient in terrestrial ecosystems [89, 90] so the deposit of nitrogen from a fire will move the ratio closer to, or sometimes beyond the ideal C:N ratio promoting greater growth rates [91].

There is a slight loss of soil organic nitrogen from fires due to volatilization. A substantial portion survives low intensity fires and is released in a bioavailable form into the soil. Studies such as that done by Ginzburg and Steinberger [92] indicate that post-fire total soluble nitrogen (TSN) can increase to a point where it

inhibits microbial activity. Generally though, as discussed by Knicker [36], the release of nitrogen into the soil causes a fertilization of that soil and, at least temporarily, an increase in the soils metabolic activity [93]. This increase in soil nitrogen is generally coupled to a decrease in soil organic carbon [94]. Examination of the change in the Carbon/Nitrogen ratio should initially see a decrease in the Carbon/Nitrogen ratio followed by an increase back to pre-fire levels. Changes in the Carbon/Nitrogen ratio will be one way of tracking the level of integration of the ash from the fire into the soil.

2.8 Phosphorus

During fire, combustion of organic matter releases bound phosphorus (P) [95]. However, according to Hume et al. [84], there is a lack of consensus about the availability of P post-fire [96]. Phosphorus levels are also known to be highly variable as they are affected by the state of the chemical, biological and physical [97, 98] properties of the soil; all of which can be altered during a controlled burn. What is known is that the intensity of the fire affects the amount, and source, of the P liberated from the ecosystem [99]. According to Ketterings et al. [96], low intensity fires generally liberate P from surface organisms and deposit this P in the ash layer. High intensity fires, while still liberating P from the combusted materials, also cause increased soil sorption of P due to changes in the mineralogical composition of the soil itself. This reduces its availability while this new mineralogical profile persists.

Combustion of organic matter converts organic P to orthophosphate according to Cade-Menum et al. [99] The peak availability of P to soil organisms is around pH 6.5, thus any changes the fire induces in pH towards neutrality increase the availability of P to the soil communities according to Sharpley [100]. This creates, according to Serrasolas and Kahanna [101], a temporary enrichment of available P. The exact effect of phosphorus availability is difficult to quantify as most terrestrial systems are not phosphorus limited [102].

2.9 Soil Moisture

The impact of fire on the biological properties of soil is related to soil moisture content according to Choromanska and DeLuca [103]. Choromanska and DeLuca showed that soils at three different levels of moisture content experienced different levels of microbial biomass loss. The highest loss was observed in the soil with the highest moisture content. Heat in moist soils is transported faster, due to the high thermal conductivity of water, but so long as there is water present in the soils the temperature cannot rise above the latent heat of vaporization of 95 ^{o}C [104].

Soil moisture is also important in the regulation of microbial growth rates [105]. According to Skoop et al. [106], aerobic microbial activity depends on achieving a moisture level where the limiting effects of substrate diffusion and oxygen supply are equal. Drenovsky et al. [107] concluded that along with

organic carbon availability, soil water content was a primary driver of microbial communities composition [108-111].

2.10 PAH Degrading Organisms

As discussed by Chikere et al. [112], the use of microorganisms to bioremediate PAH contaminated locations is advantageous because of the low costs [113] involved in their use [114-116]. These same organisms can cause damage and loss of product, such as crude oil, [117] when they exist in sites of oil extraction as discussed by Vasconcellos et al. The number of genera that contain PAH [118] degrading organisms are relatively limited [119], but their members are present in most environments [120, 121]. The genera with widely distributed PAH degrading members are *Pseudomonas*, *Mycobacterium* [122], *Sphingomonas* [123], and *Bacillus*, just to name a few [124-128].

Experiments with PAH degrading organisms by Chadhain et al. [129] show that individual PAHs would favor the growth of different bacterial genera, and species. This indicates that not only do these PAH degrading organisms gain a competitive advantage [130, 131] from the addition of PAHs to the soil [132, 133], individual microorganisms within this group specialize even further in the degradation of specific PAHs. The study of these PAH degraders therefore must take into account the need to examine the communities's contribution to the remediation, and not just that of the individual organisms. Kampfer et al. [134] examined the response of PAH degrading bacteria to an influx of nutrients in a

PAH contaminated site and saw changes in the whole communities. These changes were beyond those associated directly with PAH degrading bacteria. The enzymatic pathways required to breakdown PAHs are complex but the majority start with a dioxygenase enzyme [135, 136]. These dioxygenase enzymes utilize molecular oxygen to break the aromatic ring structures of PAHs [137]. This creates two adjacent hydroxyl groups [138]. From there further enzymes are recruited depending upon the new chemistry of the broken ring structure [139]. The majority of PAHs require their own pathways, or at least branched pathways to deal with their end products [140]. In particular, a well-studied PAH, that is a significant component of the black carbon left after a fire, is naphthalene [129, 141, 142]. It is the simplest and most soluble of PAHs making it one of the easiest to metabolize. The complexity for the remediation of PAHs also comes about due to the fact that the composition of PAH contamination varies based on its source [143-146].

2.11 Soil Microbial Communities Structure

The most immediate effect of fire on microbial communities structure is a reduction of biomass [147]. The peak temperatures of a fire considerably exceed those required for killing of most living organisms [148]. Additional loss of soil organisms can occur due to the release of toxic combustion byproducts such as

PAHs [149, 150]. These products, as mentioned by Mrozik et al. [151] can be altered by organisms present in the soil.

These changes in the nutrient makeup of the soil can alter the assembly process that the microbial communities [83] will undergo as it attempts to return to an equilibrium state. Ferrenberg et al. [152] studied the effect of wildfire on soil bacterial communities and chemical composition at 4 weeks, and 16 weeks post-fire. Their research showed a statistically significant change in microbial communities composition between the 4-week and 16-week samples post-fire, between the 4-week burn, and unburned, and between the 16-week burn and unburned samples. In the burned samples at 4 weeks post-fire, the two largest phyla were Actinobacteria, and Firmicutes. For the unburned samples the two largest are Actinobacteria and Alphaproteobacteria. The burned samples displayed a less even distribution of the 7 phyla identified than the unburned samples indicating a level of selective enrichment due to fire. The 16-week data showed that burned site as having Firmicutes and Betaproteobacteria as its largest phyla, while the unburned sites had Actinobacteria and Alphaproteobacteria. This indicated a significant change between the 4- and 16-week samples for the burned sites, and very little, if any, change between the 4- and 16-week samples for the unburned sites. Their examination of unburned controls also indicated that magnitude of the shifts due to fire far outweighed those due to seasonal change.

Hypothesis

The controlled burns on Cat Island near Georgetown SC will cause a change in the composition of the microbial communities due to changes in soil chemistry.

Aims

Track the changes in microbial community composition via alpha diversity measurements, beta diversity measurements, and taxonomy measurements for a one year period after a controlled burn using 16S rDNA gene sequence analyses.

Track the changes in moisture, pH, elemental composition, nitrogen levels and phosphorus concentrations over the same period.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Site Sampling

Soil cores were obtained from Cat Island in Georgetown, South Carolina. Site maps are available in Appendix A. Previous studies had already subdivided locations on the island into 10 square meter experimental sections. Temperature of the controlled burn was measured by thermocouple with vertical thermocouple measurements showing a mean of 148.2ºC and horizontal thermocouples showing measurements of a mean temperature of 214.3ºC [153]. The sections sampled for this experiment are sites 401, 428, 201 and 217. Sites 401 and 428 were loamy soils while sites 201 and 217 were sandy soils. Additionally sites 401 and 201 were burned approximately every three years, while sites 428 and 217 were burned approximately every three years. Samples were obtained from 3/7/2016 until 3/6/2017. The sampling period started with samples taken immediately pre-fire, and immediately post-fire. These samples were designated pre-fire and post-fire along with their site designation. The next four samples were taken at weekly intervals. The designation of these samples was week 1, week 2, week 3, and week 4. Samples were then taken at monthly intervals starting with month 2 and proceeding until month 12. Month 10 was not acquired due to complications with personnel schedules on site. These issues did not appear in time for the researcher to reach the site within the sampling window.

These samples were designated month 2, month 3, etc. up to month 12 (Table 1: Sampling dates, soil types, and burn schedules of four sites on the Cat Island).

Table 1: Sampling dates, soil types, and burn schedules of four sites on Cat Island.

	Loamy Soil		**Sandy Soil**	
	Site 401 (1-Y Burn)	**Site 428 (3-Y Burn)**	**Site 201 (1-Y Burn)**	**Site 217 (3-Y Burn)**
Pre-Fire	03/07/2016	03/07/2016	03/15/2016	03/15/2016
Post-Fire	03/07/2016	03/07/2016	03/15/2016	03/15/2016
Week 1	03/15/2016	03/15/2016	03/22/2016	03/22/2016
Week 2	03/22/2016	03/22/2016	03/29/2016	03/29/2016
Week 3	3/29/2016	3/29/2016	04/05/2016	04/05/2016
Week 4	04/05/2016	04/05/2016	04/12/2016	04/12/2016
Month 2	05/06/2016			
Month 3	06/06/2016			
Month 4	07/08/2016			
Month 5	08/01/2016			
Month 6	09/06/2016			
Month 7	10/14/2016			
Month 8	11/07/2016			
Month 9	12/16/2016			
Month 10	NA			
Month 11	02/03/2017			
Month 12	03/06/2017			

The pre-fire, post-fire, and weekly samples were obtained through the use of a half centimeter hand core. Prior to harvesting the core, the location was brushed to remove loose surface debris. The coring device was then used and the 0-10 cm section was separated from the 11-20 cm section [154]. These sections were stored separately with 5-7 g stored at -80 $^{\circ}$C [101] for 16S rRNA

gene sequencing and the majority being subjected to desiccation in a drying oven at 70 °C for a minimum of 3 days [77].

The monthly samples were obtained using a 2-inch slide hammer coring device. This coring device contained a 5 cm cup to collect the core sample and the 0-5 cm and 6-10 cm samples were combined to form a 0-10 cm sample, with the 11-15 cm and 16-20 cm [155] samples also combined. The monthly samples were also obtained in quadruplicate from each site. These replicates were designated S1, S2, S3, and S4 respectively.

The resulting designation for each sample therefore contained its site, time point, replicate, and depth measurement, for example 401 M2S1 0-10 cm.

3.2 DNA extraction

DNA was extracted from samples stored at -80 °C utilizing a MO BIO Power Soil kit (MO BIO Carlsbard, CA) following the manufacturer's instructions. This kit was chosen because of its ease of use, low time requirement and high fidelity. Other methods [156-158] such as those presented by Zhou et al. [159] and Yeates et al. [160, 161] were also examined but deemed less effective for this experiment.

The harvested DNA was then measured by NanoDrop™ (Waltham MA) spectrography to determine the DNA concentration and the level of RNA contamination [162]. This was accomplished through the measurement of the 260:280 ratio. This measurement method was selected due to its ubiquity in the

literature, specifically used to assess the quality of other DNA extraction methods as discussed by Hu et al [163] Upon completion of measurement sample was stored at -80 °C until needed for PCR amplification.

3.3 PCR amplification

PCR amplification utilized primers for the V4 hypervariable [164, 165] region of the 16S rRNA gene [166] component of the bacterial ribosomal RNA [167]. This primer was chosen due to its versatility and coverage [164, 168-170]. PCR amplification consisted of 30 cycles of denaturing, annealing, and amplification at a starting concentration of 1 ng/ul. After which, samples of the PCR product were run on a 2% w/v agarose gel to confirm amplification. PCR amplicons were then pooled at equimolar concentrations by using the SequalPrep Normalization Plate kit (Invitrogen, Carlsbad, CA), following the manufacturer's instructions.

3.4 Sequencing

Pooled PCR amplicons were sent to Dr. Richards' facility at Clemson University for sequencing utilizing a Illumina MiSeq sequencer (San Diego CA) [171-173]. The resulting data were processed with QIIME [174]. The first step was the joining of paired ends using a custom Flash script. The maximum overlap was set at 250 bp. The multiple split libraries command was then used with a quality threshold of 19. Chimeric sequences were removed using

usearch61 [175]. OTU picking was then performed using an open reference frame method of sortmerna_sumaclust. The nonbacterial taxa were then filtered out and alpha (Chao1 and Shannon) and beta diversity metrics (PCoA plots) created [176]. The core diversity analyses command was then used with a subsampling depth of 10,000. The metrics used for alpha diversity analysis were chao1 and Shannon diversity metrics utilized through the QIIME package [174]. The processed data were then analyzed using STAMP [177] to produce PCoA plots to examine beta diversity. PCoA plots were created at the genus level with ANOVA testing using the Tukey-Kramer post-hoc test with significance set at less than 0.05.

Prior to final analyses, the data from site 217 harvested in month three for the first biological replicate was removed as a significant outlier in the data set. This was done because of a failure to achieve accurate reads from the sample during the Illumina MiSeq run.

3.5 Soil Moisture

Soil moisture was determined by comparing the mass of samples before and after desiccation. Soil samples were bagged on site and weighed at facilities located at the Baruch Institute. The samples were then left to desiccate in a drying oven at 70 $^{\circ}$C for a minimum of 3 days and then weighed again immediately after removal from the drying oven. Post desiccation weight was

then divided by pre-desiccation weight to determine the percent of weight that was composed of water.

3.6 Soil pH

Soil pH was determined by measurement with a glass electrode pH meter with the soil prepared in a 100 mM calcium chloride solution [178]. The procedure involved the mixing of 10 mL of 100 mM calcium chloride with 0.5g of sample [179]. The sample was allowed to desorb for 30 minutes without shaking and then measured with the glass electrode pH meter and recorded. Calibration standards for the pH meter were pH 4 and pH 10.

3.7 Elemental Composition (C,H,N)

C,H, and N were measured by using an elemental CHN analyzer. Standards were used to calibrate the instrument prior to each run. Samples were prepared by weighing each sample inside tinfoil cups, each sample was kept between 8-12 mg and then the tinfoil cups were sealed by folding prior to being placed into a rotary sampler.

3.8 Inorganic Nitrogen Measurement

The measurement of soil ammonium [180, 181] was conducted with the use of an ammonium assay reagent. 200 mL of reagent was prepared by combing 1.08 g of pthalaldehyde, 100 uL of β-mercaptonal, 20 mL of 100%

ethanol, 80 mL of 1 M potassium phosphate buffer and 99.9 mL of dH_2O. Ammonium standards were also created from NH_4Cl at concentrations of 500, 100, 30, 5, and 1 nM ammonium. These were used to create a standard curve for comparison with the samples. Samples were prepared by suspending 0.25 g of soil in 250 uL of dH_2O. This was allowed to desorb for 30 minutes without shaking then centrifuged at 10,000 xg for 2 minutes. Then 30 uL of supernatant was then added to 570 uL of ammonium reagent and allowed to react for 30 minutes. Samples were then measured at 420 nm using a NanoDrop™ spectrophotometer.

The measurement of soil nitrate was determined through the use of 16.5 mM sulfamic acid, salicylic acid-sulfuric acid, and 4 M NaOH. Twenty uL of aliquot obtained from samples as prepared above were placed into microcentrifuge tubes. One uL of sulfamic acid was added to remove nitrate. Next 100 uL of salicylic acid-sulfuric acid was then added and mixed by pipetting. Immediately afterwards, 1 mL of cold NaOH was added and the solution allowed to react for 30 minutes. The solution was then measured at 420 nm using a NanoDrop™ spectrophotometer [182].

3.9 Phosphorus measurement

Phosphate measurements were obtained by using a Malachite Green Phosphate Assay kit (Cayman Scientific Ann Arbor, MI). A plate reader was used and the sample measured at 620 nm. Alternative extraction methods were

examined [183] and the Malachite Green Phosphate Assay kit was determined to be most reliable. The detailed instructions contained in the kit allowed for rapid collection of phosphorus levels in the soil samples. The resulting plates were read using a plate reader with dH_2O as the buffering solution.

CHAPTER FOUR

RESULTS

All 4 sites sampled in this study were subjected to chemical [184] and microbial analysis. The chemical analysis consisted of measurements of pH, soil moisture, ammonium concentration, nitrate concentration, phosphorus concentration and C, H, N (carbon, hydrogen, nitrogen) contents. The microbial measurements involved the sequencing of the 16S rRNA gene [185] to determine identity of the microbial communities [186]. This data set gives only relative abundance but was deemed sufficient for the purpose of this study [187].

Observation of the soil during sampling shows a loam layer that is approximately 15 cm deep for the 401 and 428 sites which will be called loamy soils, and less than 10 cm in depth for the 201 and 217 sites which will be called sandy soils. See Appendix A for site locations. Additionally, the soil shows clear boundaries between layers and has an acidic pH.

4.1 Microbial Alpha Diversity

The Shannon and Chao1 [174] indexes were used as nonparametric estimates of alpha diversity. This measurement allows for the comparison of diversity and richness of microbial communities between soil parameters. The Shannon and Chao1 indexes showed a nearly identical level of diversity and richness when comparing sandy soils to loam soils. The Shannon average for the

sites are 8.555±0.350, 8.521±0.718, 8.681±0.484, and 8.491±0.629 for sites 201, 217, 401, and 428 respectively. The Shannon and Chao1 indexes showed some variation of richness in the comparison between the different sites form which samples were taken but none outside the margin for error. The comparison between sites burned every three years vs. sites burned every year also showed a Shannon and Chao1 index that indicates nearly identical richness between the sites (Figure 1: Shannon and Chao1 alpha diversity measurements).

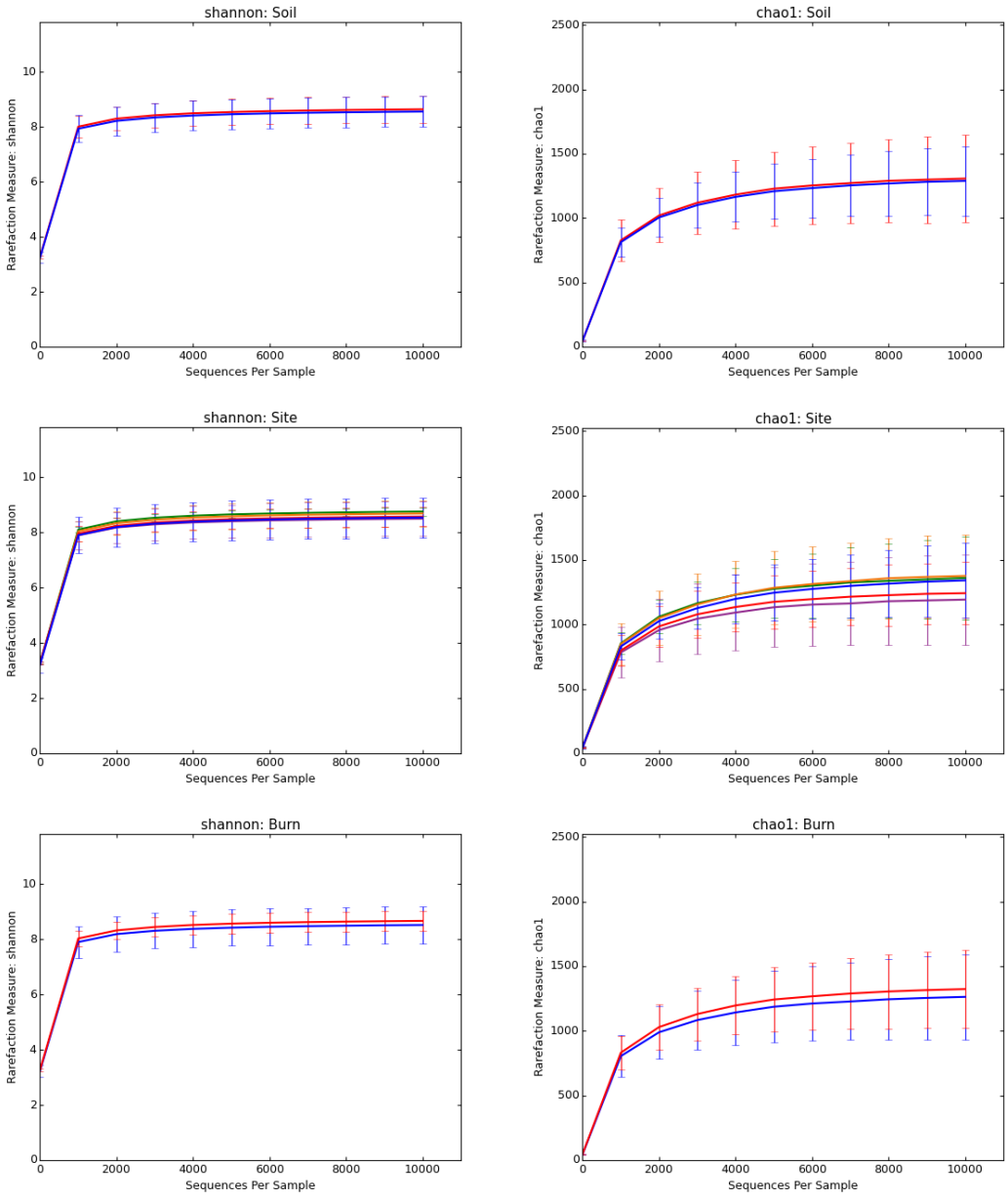


Figure 1: Shannon and Chao1 alpha diversity measurements. Soil measurements have blue for sandy soil and red for loamy soils. Site measurements have red for site 201, blue for site 217, orange for site 401, purple for site 428 and green for site 401 depth 10-20 cm. All other samples are from the depth of 0-10 cm. Burn frequency has blue for sites burned every three years and red for sites burned every year.

This examination of alpha diversity indicated that the species diversity and richness of locations was not significantly influenced by the type of soil the sample was obtained from, the frequency of controlled burns performed at the site, or the specific location the sample was obtained from.

4.2 Microbial Beta Diversity

Examination of the PCoA plots of the 16S rRNA gene sequencing results showed little grouping of the sampling locations based on the site it was harvested from. Samples appeared to form a single pool with little effect generated from the site location parameter (Figure 2A:PCoA plots of site, burn frequency, soil type and time post burn utilizing all sample data points). The comparison between burning every year versus every three years with all four sites included did not appear to show a difference based on burn frequency (Figure 2B). The effect of soil type on the beta diversity between the two different soil types also did not appear to demonstrate a difference in the two populations when comparing all 4 locations being sampled (Figure 2C). The final parameter the date of sampling was then examined to determine if there were any populations that clustered among themselves. With the exception of week 1 for the sites 401 and 428 which appeared in the upper right there did not appear to be any grouping of the sites based on the time of harvest (Figure 2D).

With four possible parameters the effect of different parameters on the beta diversity of the locations could be concealed. This required the examination of the sample sets in smaller combinations in an attempt to reduce the number of confounding variables applicable to each comparison. The first parameter eliminated from examination was soil type. This involved the comparison between sites 201 and 217 which contain sandy soils and sites 401 and 428 which contain loamy soils. Sites 201 and 217 showed a slight divergence from each other (Figure 3A: PCoA plots of location and burn frequency) as did sites 401 and 428 (Figure 3B). As sites 201 and 401 were burned every year and sites 217 and 428 are burned every three years the separation between sites could be a result of either location or burn type (Figure 3C/3D).

To determine if there were significant differences in diversity between soil types that might be occluded by the confound variable of burn frequency the samples were grouped based on burn frequency for a comparison. The comparison between sites 201 and sites 401 involve the parameters of soil type and location (Figure 4A: PCoA plots of soil type). There was a separation between populations which could be the result of either soil type or location. The comparison between sites 217 and 428 showed no apparent separation (Figure 4B). The separation between soil type comparison for 401 and 201 is larger than that of the separation seen between locations of the same soil type such as 401

and 428, or 201 and 217. Given this further examination of location would be most accurate when using the same soil types.

The effect of time on the combined data from sites 201 and 217 did not appear to show a difference based on the time period of sampling (Figure 5A: PCoA plots of time post burn). This was indicated by the fact that the different time points did not cluster together and were instead distributed randomly. The comparison between sites 401 and 428 showed a similar lack of clustering with the exception of the week 1 samples for sites 401 and 428 (Figure 5B).

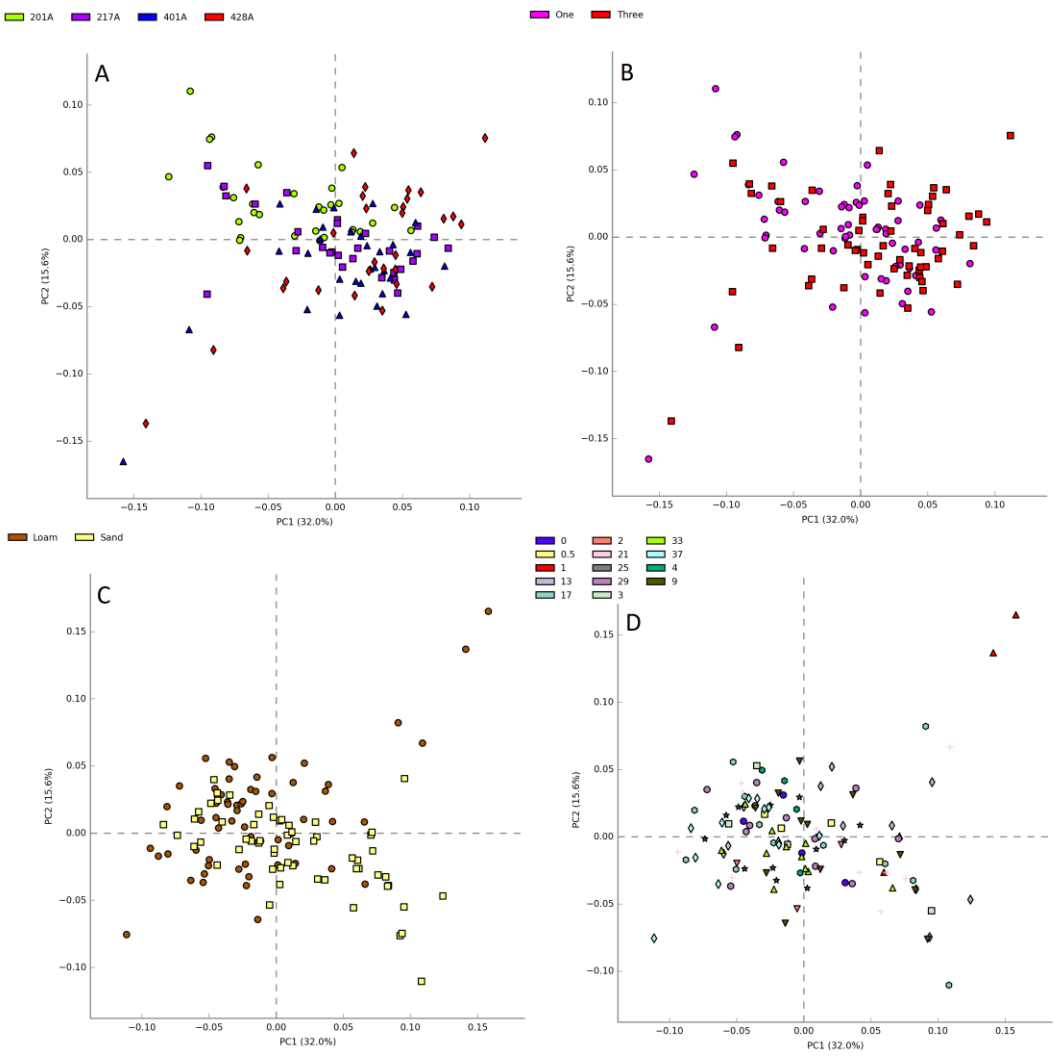


Figure 2: PCoA plots of site, burn frequency, soil type and time post burn utilizing all sample data points. Genus level PCoA plot using 16S rRNA gene sequence data to identify and quantify the microbial communities, **A.** site 201(Lime), site 217(Purple), site 401(Blue), and site 428(Red), **B.** yearly burning in magenta, with burning every three years in red, **C.** loam soils in brown, sandy soils in yellow, **D.** Pre-fire in dark blue, Post-fire in yellow, week 1 in red, week 2 in brick, week 3 soft green, week 4 forest green, week 9 dark green, week 13 grey, week 17 teal, week 21 pink, week 25 dark grey, week 29 purple, week 33 light green, week 37 light blue. Site 201 is a yearly burned sandy soil, Site 217 is burned every three years and is a sandy soil, site 401 is burned yearly and is a loam soil, and site 428 is burned every three years and is a loam soil.

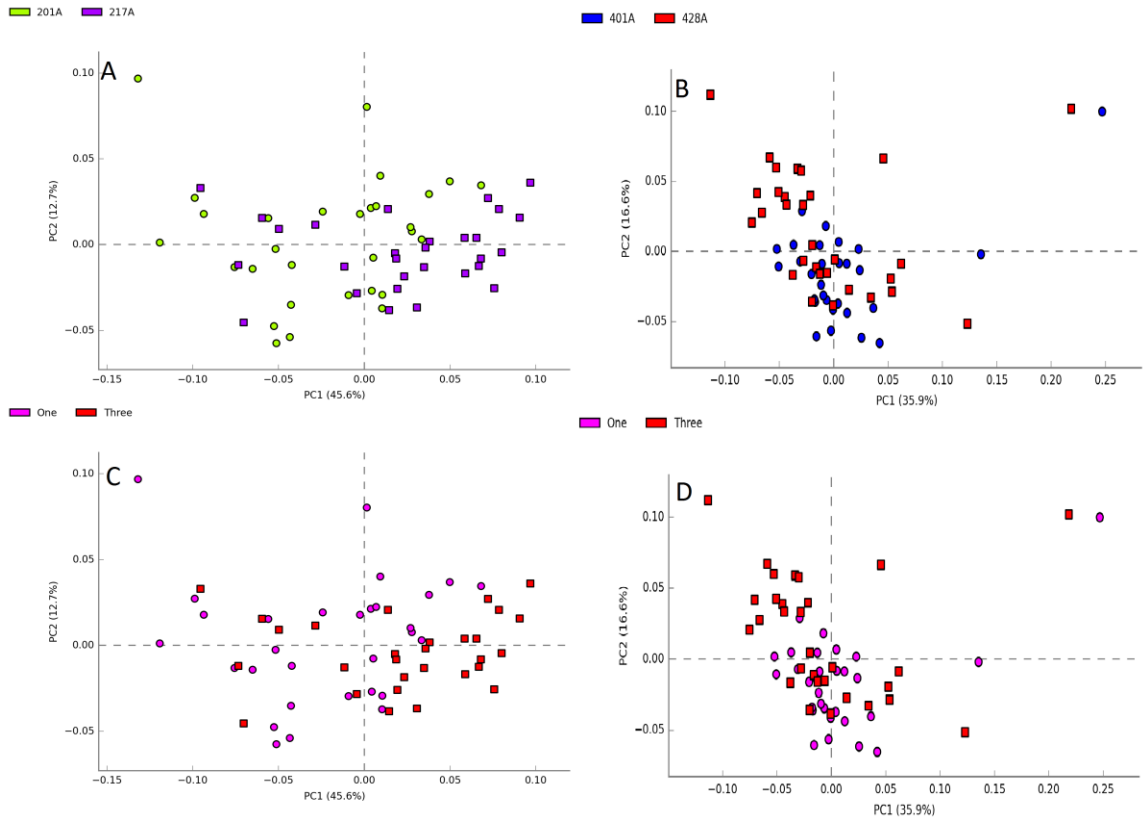


Figure 3: PCoA plots of location and burn frequency. Genus level PCoA plot using 16S rRNA gene sequence data to identify and quantify the microbial communities, **A.** site 201(Lime), site 217(Purple), **B.** site 401(Blue), and site 428(Red), **C.** and **D.** yearly in magenta, every three years in red.

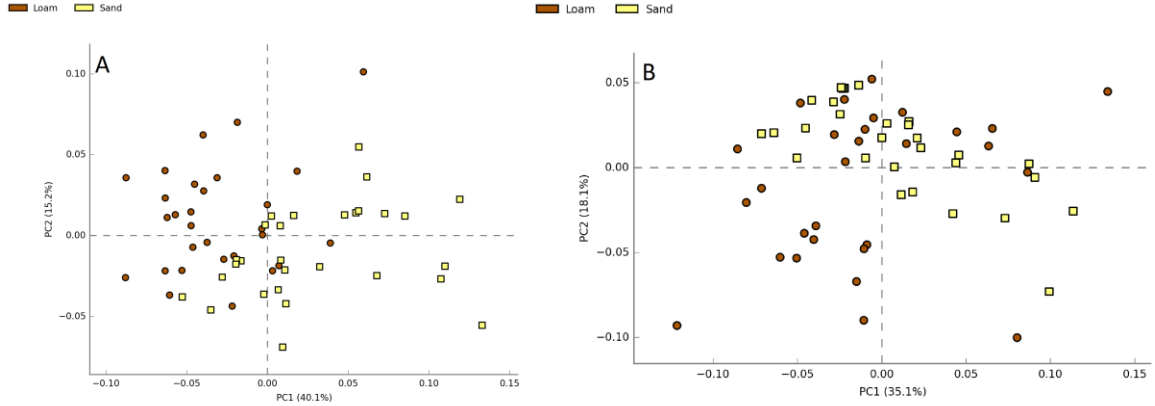


Figure 4: PCoA plots of soil type. Genus level PCoA plot using 16S rRNA gene sequence data to identify and quantify the microbial communities, **A.** site 201(Yellow) site 401 (Brown), **B.** site 217 (Yellow) site 428 (Brown). Week 1 samples were removed for clarity of view.

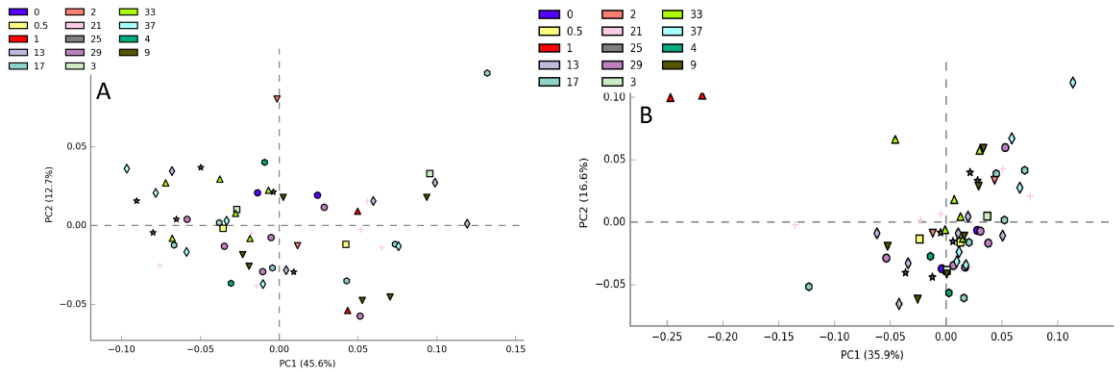


Figure 5: PCoA plots of time after burn. Genus level PCoA plot using 16S rRNA gene sequence data to identify and quantify the microbial communities, **A.** compares sites 201 and 217; **B.** compares sites 401 and 428. Pre-fire in dark blue, Post-fire in yellow, week 1 in red, week 2 in brick, week 3 soft green, week 4 forest green, week 9 dark green, week 13 grey, week 17 teal, week 21 pink, week 25 dark grey, week 29 purple, week 33 light green, week 37 light blue. Site 201 is a yearly burned sandy soil, Site 217 is burned every three years and is a sandy soil, site 401 is burned yearly and is a loam soil, and site 428 is burned every three years and is a loam soil.

4.3 Microbial Taxonomy

The phylum level taxonomic makeup of the microbial communities did not appear to change based on any of the parameters studied (Figure 6). Comparisons between site 201 which was burned yearly and site 217 which was burned every three years showed approximately the same phylum level composition over the time period studied (Table 2). The examination of each site individually also showed very little variation over the time period studied. The major phylum's found in the soil were Acidobacteria (average 30.75%±6.65%), Actinobacteria (average 7.75%±2.70%), Planctomycetes (average

10.69%±2.95%), Porteobacteria (average 27.41%±4.03), and Verrucomicrobia (13.34%±3.00%). The exception to this variance was shown in the samples for week 1 for the sites 401 and 428 where Bacteroidetes (11.1%) and Cyanobacteria (13.5%) increase their percentage of the communities makeup with an additional though smaller increase in Proteobacteria (42.4%). This alteration in communities composition is transient as it existed only in week 1 and then the communities composition returned to that seen in the pre and post-fire samples.

Figure 6: Taxonomic comparison between samples. Red boxes indicate samples from week 1. Black arrow indicates 217 month 3 sample 1 which was determined to be an outlier.

Table 2: Average and standard deviation of each phylum within the taxonomic analysis of sites 201, 217, 401, and 428 for the depth 0-10 cm.

Phylum	201 Avg±STD	217 Avg±STD	401 Avg±STD	428 Avg±STD
Unassigned;Other	0.48%±0.21%	0.59%±0.36%	0.77%±0.55%	0.76%±0.50%
AD3	0.09%±0.11%	0.08%±0.10%	0.15%±0.18%	0.06%±0.07%
Acidobacteria	30.80%±4.52%	31.13%±7.14%	29.84%±6.56%	31.24%±8.36%
Actinobacteria	10.30%±2.84%	7.28%±3.39%	6.93%±2.32%	6.50%±2.23%
Armatimonadetes	0.66%±0.36%	0.48%±0.17%	0.44%±0.21%	0.37%±0.22%
BHI80-139	0.01%±0.02%	0.02%±0.03%	0.01%±0.02%	0.01%±0.02%
Bacteroidetes	1.83%±1.12%	1.81%±1.32%	2.06%±2.10%	2.68%±2.96%
Chlamydiae	0.18%±0.10%	0.40%±0.29%	0.19%±0.10%	0.30%±0.17%
Chlorobi	0.06%±0.07%	0.05%±0.03%	0.06%±0.15%	0.08%±0.11%
Chloroflexi	0.51%±0.42%	0.75%±1.23%	1.69%±1.66%	1.37%±1.37%
Cyanobacteria	1.15%±0.44%	1.83%±1.44%	1.78%±3.45%	1.23%±1.31%
Elusimicrobia	0.40%±0.18%	0.51%±0.22%	0.42%±0.58%	0.31%±0.18%
FBP	0.01%±0.02%	0.01%±0.02%	0.01%±0.01%	0.01%±0.05%
FCPU426	0.52%±0.54%	0.64%±0.36%	0.34%±0.38%	0.38%±0.27%
Fibrobacteres	0.01%±0.02%	0.00%±0.00%	0.00%±0.00%	0.00%±0.01%
Firmicutes	0.08%±0.07%	0.11%±0.20%	0.06%±0.06%	0.21%±0.74%
GAL15	0.04%±0.03%	0.03%±0.03%	0.02%±0.03%	0.03%±0.04%
Gemmatimonadetes	0.13%±0.09%	0.13%±0.07%	0.16%±0.07%	0.23%±0.29%
Lentisphaerae	0.00%±0.00%	0.00%±0.00%	0.00%±0.01%	0.00%±0.00%
Nitrospirae	0.00%±0.01%	0.02%±0.04%	0.02%±0.03%	0.02%±0.03%
OD1	0.03%±0.06%	0.02%±0.03%	0.01%±0.02%	0.02%±0.03%
OP3	0.03%±0.03%	0.05%±0.04%	0.01%±0.02%	0.01%±0.02%
PAUC34f	0.00%±0.00%	0.00%±0.01%	0.00%±0.01%	0.00%±0.00%
Planctomycetes	12.69%±3.17%	11.48%±2.73%	9.92%±3.12%	8.68%±2.79%
Proteobacteria	24.67%±3.50%	26.49%±4.35%	30.57%±2.99%	27.89%±5.29%
SAR406	0.00%±0.00%	0.00%±0.00%	0.00%±0.01%	0.01%±0.06%
SBR1093	0.00%±0.00%	0.00%±0.00%	0.00%±0.00%	0.00%±0.00%
Spirochaetes	0.00%±0.01%	0.02%±0.07%	0.39%±2.09%	0.00%±0.01%
TM6	0.24%±0.34%	0.29%±0.15%	0.22%±0.15%	0.30%±0.20%
TM7	0.04%±0.05%	0.02%±0.03%	0.01%±0.02%	0.03%±0.04%
Tenericutes	0.11%±0.10%	0.04%±0.06%	0.11%±0.08%	0.03%±0.04%
Verrucomicrobia	11.66%±2.62%	13.66%±3.05%	12.19%±2.85%	15.87%±3.46%
WPS-2	3.28%±1.21%	2.06%±0.99%	1.60%±0.76%	1.37%±0.95%
ZB3	0.00%±0.01%	0.00%±0.00%	0.00%±0.02%	0.00%±0.00%

4.4 Chemical Analysis

The soil samples were subjected to six physical and chemical measurement techniques; measurements of pH, moisture, phosphorus, nitrate, ammonium and carbon/nitrogen ratio. Each of these measurements was plotted over time to examine the changes in these components of soil composition and compare them to the changes in bacterial communities composition. While techniques were developed for the extraction, purification and measurement of PAHs, lack of GCMS sensitivity available for the project prevented those measurements from being obtained.

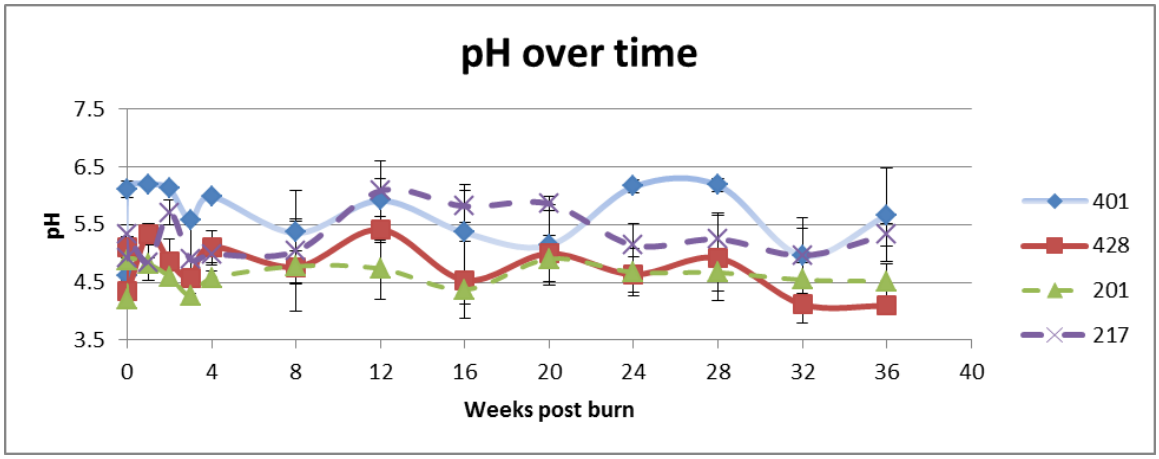
The measurements of pH over time indicate that site 401 has a pH one higher than that of site 428. Site 201 has a pH measurement one lower than that of site 217 (Figure 7A: Graphs of chemical analysis). This difference in pH appears to simply be a site specific variable as the only other difference between the sites in question is burn frequency. The sites 401 and 201 are burned yearly while sites 217 and 428 are burned every three years. As the pH difference does not match up to the burn frequency differences, that parameter is unlikely to be the cause of the observed differences. This, in conjunction with previous results in alpha diversity, beta diversity, and taxonomy would also indicate that this difference in pH is not significant enough to effect communities composition between those samples that share a soil type.The moisture measurements show a highly variable moisture level for the loam soils, with a more stable moisture

measurement for the sandy soils (Figure 7B). The phosphorus measurements change unpredictably over time with only site 201's measurements being stable (Figure 7C). Nitrate and Ammonium over time show relatively stable levels with a ratio of 10:1 Ammonium:Nitrate in all samples (Figure 7D/7E). Finally carbon/nitrogen ratios where measureable show an average measurement within the range of health soils (Figure 7F).

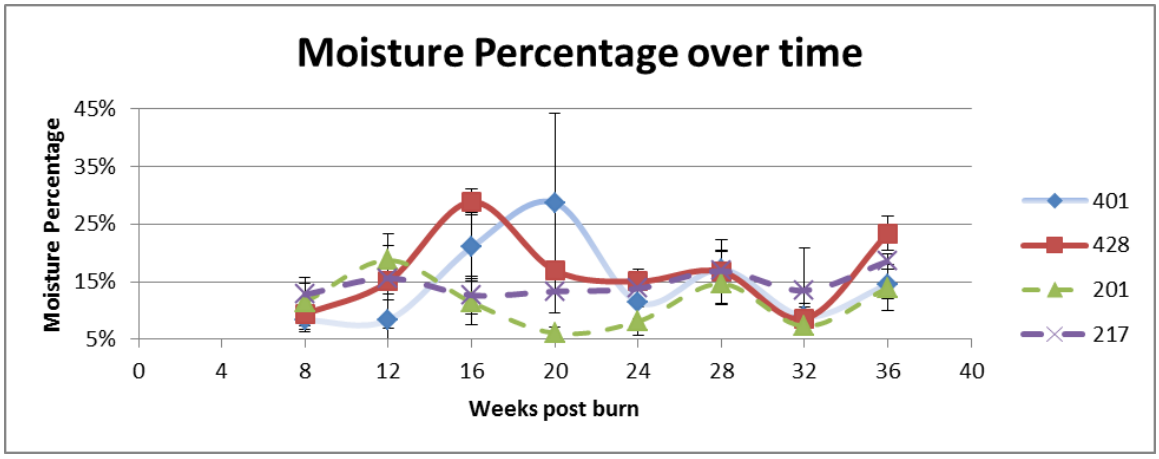
Table 3: Average and standard deviation of measurements of chemical and physical values of the sample sites 401, 428, 201, and 217

Sample	pH Avg±Std	Moisture Avg±Std	Phosphorus µMol/g soil Avg±Std	Nitrate nMol/g soil Avg±Std	Ammonium nMol/g soil Avg±Std	C/N Avg±Std
201	4.63±0.38	11.47%±4.84%	0.0567±0.0044	181.72±79.49	2649.32±539.54	27.53±1.70
217	5.37±0.50	14.64%±3.77%	0.0291±0.0215	177.78±96.45	3050.06±589.13	31.10±9.55
401	5.63±0.66	14.85%±8.67%	0.0110±0.0158	163.01±89.57	2599.23±889.35	25.01±11.58
428	4.73±0.52	16.76%±7.26%	0.0284±0.0197	231.53±149.37	1944.90±1027.02	31.55±6.64

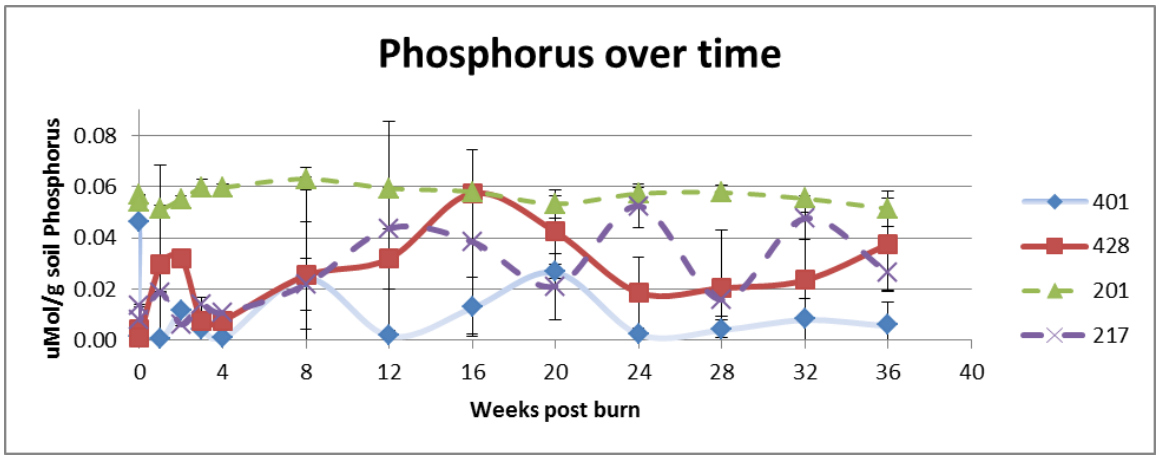
A


pH over time
pH
7.5
6.5
5.5
4.5
3.5
0
4
8
12
16
20
24
28
32
36
40
Weeks post burn
401
428
201
217

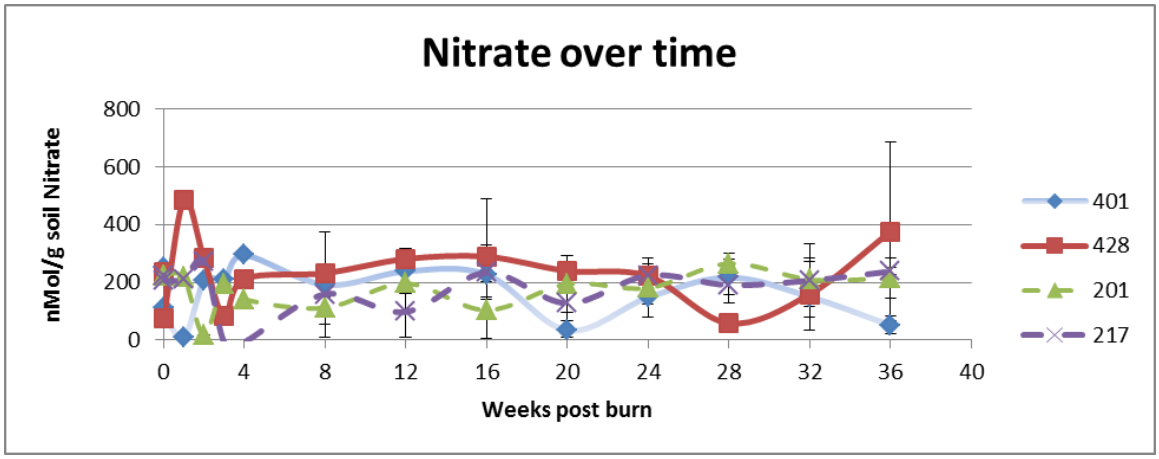
B


Moisture Percentage over time
Moisture Percentage
45%
35%
25%
15%
5%
0
4
8
12
16
20
24
28
32
36
40
Weeks post burn
401
428
201
217

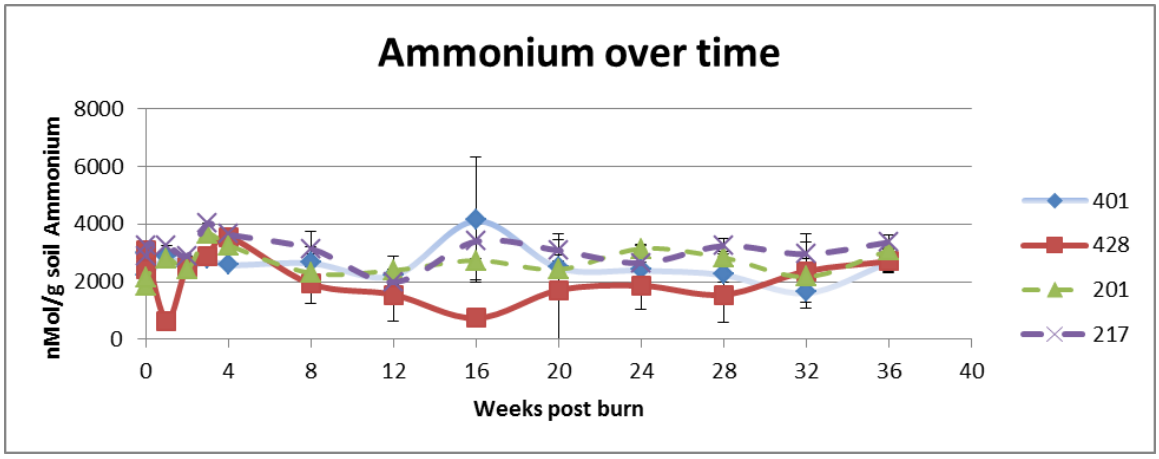
C


Phosphorus over time
uMol/g soil Phosphorus
0.08
0.06
0.04
0.02
0.00
0
4
8
12
16
20
24
28
32
36
40
Weeks post burn
401
428
201
217

D



E



F

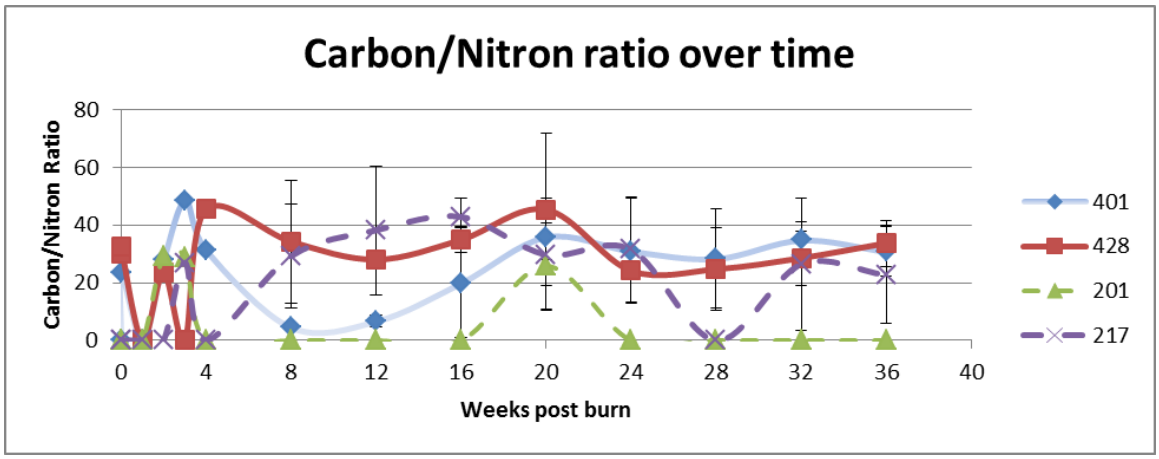


Figure 7: A. pH, **B.** percentage moisture content, **C.** phosphorus concentration, **D.** nitrate concentration, **E.** ammonium concentration**,** and **F.** carbon/nitrogen ratio over time.

CHAPTER FIVE

DISCUSSION

The typical drawback to field studies though is the lack of control of the environment being studied. To cover this gap, field sites can be subjected to specific stressors and the response of the communities to individual stressors can be observed in the field environment.

The lack of control in field studies can also be compensated for by performing analysis of those aspects of the field study, in this instance soil chemistry such as nitrate, ammonium, phosphorus, etc., that are possible sources of change in the microbial communities makeup [188, 189]. Observed changes in the soil chemistry can help provide support for observed changes in the microbial communities. Regular systematic sampling is also important and is often lacking in field studies making timelines and responses of the communities difficult to pin down [190].

The Shannon index showed low variation in species richness between locations. This indicates that across locations the richness of the bacterial communities stayed fairly constant. Literature sources such as Kaiser et al. show Shannon indexes of eight to ten approximately [191]. This indicates measurements for the Cat Island samples were at the low end of the average for similar forest soils. Chao1 index's on the other hand were very dissimilar from the literature showing values of approximately 1400 for the samples collected for this thesis but the literature showed values between 4000-8000 [191]. This indicates

the richness of the bacterial communities studied in this thesis is very low compared to similar soil types found in the literature. In addition the Chao1 values did not show any change in value between the treatments or soil types, indicating that the parameters studied for this thesis did not have an effect on the alpha diversity of the bacterial communities. However there was a single exception as the Chao1 values for week 1 of the study, where they were about two thirds of the average value seen previously, indicating a very short loss of richness from which the ecosystem quickly recovered. This was also seen in the work of Ascoli et al. and was attributed to the effect of the heat of the fire on the bacterial communities [192].

The beta diversity measurements were complicated by the fact that each site contained multiple parameters that could be the cause of any observed changes. Each site had the parameter of location, burn frequency, and soil type that could complicate the analysis of the final parameter of change over time. The measurements of beta diversity when all four sites were included in the analysis indicated that no parameter had a significant effect on the communities composition. Week 1 samples for the loamy soils showed a difference from all other time points but as this difference was not continued into future time points it appears to be a transitory, but representative, change in communities structure.

To determine if confounding variables might be hiding changes in beta diversity due to confounding variables sample sets were cut in half in order to reduce the possible confounding variables from three to two. The strongest effect

on beta diversity was found to be the soil type difference of sandy soil vs. loam soil between sites 201 and 401, respectively. This prompted the analysis of the effect on the communities over time post-fire to be separated by soil type in order to reduce the possibility of confounding variables. Even with this confounding variable removed, the results of the analysis remained the same as with all four sites included.

Taxonomic measurements also confirm this lack of significant change in the communities over time at least at the Phylum level, with the only notable divergence seen in week 1 for the loam soils. Those changes though returned to post-fire levels in week 2 indicating a transient change in the communities structure. When compared to other locations the primary phyla present in the soil sampled on Cat Island (Acidobacteria, Actinobacteria, Planctomycetes, Proteobacteria and Verrucomicrobia) were found to be highly similar to those found in other studies. While somewhat similar to the composition of bacterial phyla found from worldwide sampling [193] those samples taken from specifically temperate forests [191, 194] showed a much closer match to the phylum level composition observed on Cat Island. The primary difference was a higher concentrations of Bacteroidetes, and Chloroflexi found in both the world wide, and temperate forest populations compared to the samples obtained from Cat Island.

The lack of general change in the bacterial communities provides an interesting framework for the analysis of the physical and chemical composition

of the sites sampled. The measurements of pH in particular prove interesting as despite a difference in pH of 1 between sites 401 and 428, and between sites 201 and 217, the beta diversity of those sites did not differ significantly. This would seem to indicate that despite the extremely strong effect of pH on bacterial communities composition this change in pH was within the tolerances of the bacterial communities being studied.

While effects of the chemical changes over time on the bacterial communities do not seem to be visible some general conclusions about the soil chemistry can be obtained. The moisture levels of the different soil types for instance appear to behave slightly differently. The loam soils appear to have a higher capacity for moisture compared to the sandy soils and consequently have a higher variation in soil moisture measurements. This is likely due to the higher water retention and slower drainage rates found in loam soils.

The measurements of nitrogen in the form of nitrate and ammonium show in general relatively stable levels of both nitrogen and ammonium with a ratio of approximately 10:1 ammonium to nitrate seen in all samples.

The phosphorous measurements on the other hand show large swings in concentration that do not appear to be linked to any of the parameters being studied. The notable exception being for site 201 which shows extremely stable levels of phosphorus along with the highest phosphorus measurements among the sites.

While standard variables for most measurements were not available, what data was available for carbon/nitrogen ratios seems to indicate that the carbon/nitrogen ratio is within what is considered healthy for temperate soils [36].

5.1 Conclusion

The effect of the controlled burns on Cat Island near Georgetown SC showed no visible effects of the fire on the structure of the bacterial community. The different management practices in the frequency of controlled burning also did not seem to affect the structure of the bacterial community.

While significant changes were not observed in the microbial communities post-fire, the secondary finding of this thesis is that the management practice of controlled burns on Cat Island appears to have no negative effects on soil health. The lack of significant changes in the communities is likely due to the low temperature of the controlled burns performed on Cat Island. To track the process of remediation developed over time for forest soils, higher severity fires would be required.

5.2 Future Directions

Future research into the effects of prescribed burns on the Cat Island soil microbial communities can easily start with those samples which were collected but not utilized in the experiment. Currently, these data sets consist of months 11 and 12 for the 0-10 cm depth and a full set of samples from time 0 to month 12

for the depth 11-20 cm. This second depth could prove useful for determining if the effects of the fire transmit below the 10 cm depth of the already sequenced samples. The fate of PAHs in soils are not currently well known [195, 196]. Additional data such as the level of PAH penetration into soil could help future research on their effects and methods for remediation. Furthermore, while the diversity analysis utilized in this paper did not show a significant change in communities composition it is possible that changes in significant, but low population, communities members could be important. Data analyses examining the specific members of the communities that changed over time could be used as a baseline for changes caused by those seen in future studies that involve higher severity fires. Additionally the examination of the Archaeal populations could be performed by reprocessing of the data and additional primers for the internal transcribed spacer could be used to track the changes in fungal populations.

Controlled burns and wildfires in general are also not well represented in the literature [197-199]. What studies are available also do not tend to contain regular sampling protocols making inferences on the progression of the communities difficult to make [92, 200].

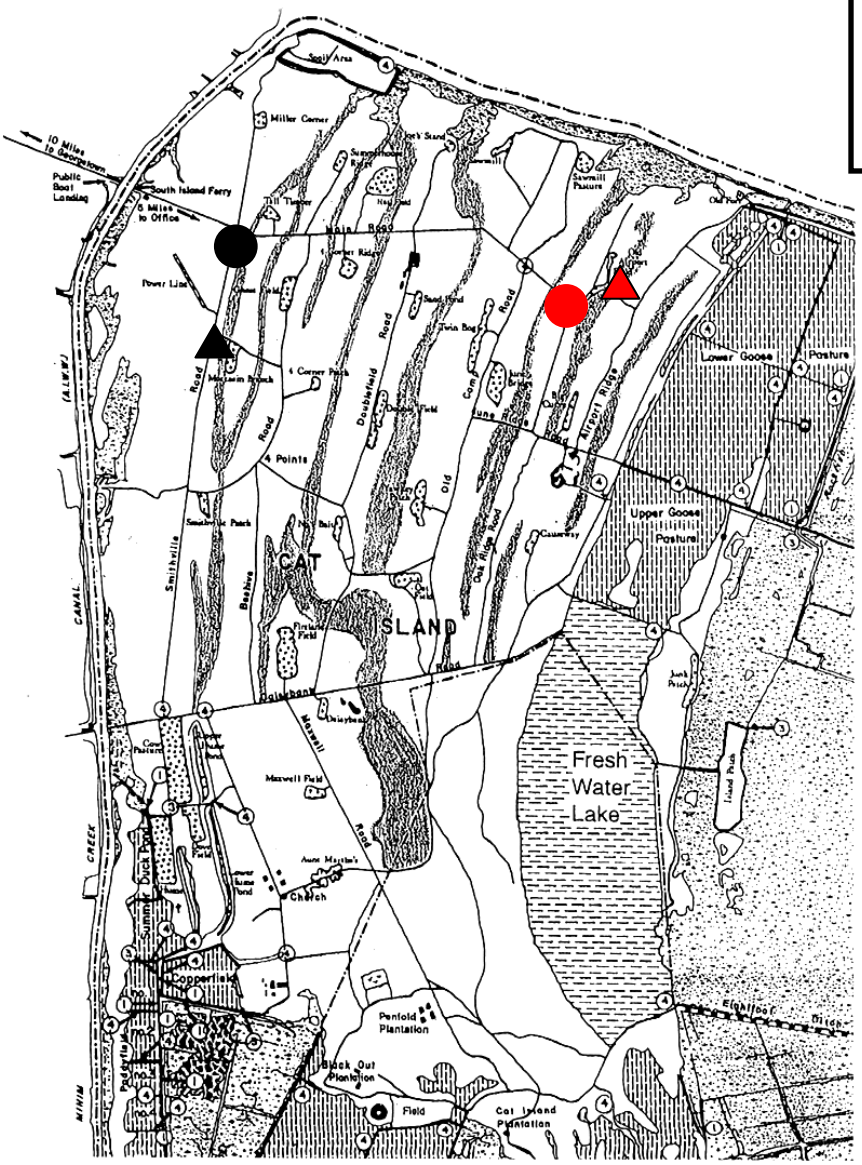
Additionally, multidisciplinary research into the effect of PAHs on the microbial communities would help significantly in understanding the natural processes that already exist for remediation. Including the exaction of PAHs [201] [202] and the measurement of their concentrations [203, 204] to the

demonstrated changes in the microbial communities could provide direct evidence of change and remediation as opposed to the indirect data available from microbiological studies alone.

APPENDIX A

SITE MAP AND IMAGES

401
428
201
217



● Burned Yearly ▲ Burned every three years

Site 401

Site 428

Site 201

Site 217

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