FINAL TECHNICAL REPORT

Developing a high throughput protocol for using soil molecular biology as trace evidence

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Abstract

Soil has a long and successful history as trace evidence. Originally, the use of soil as trace evidence was accomplished via light microscopy. But the value of soil as trace evidence has expanded significantly with the development of new techniques. Most recently these include using the biochemical molecules from soil microbial communities to make a fingerprint of the specific soil. The current research examines the changes to the microbial community profile that take place during storage of a soil sample. *The purpose of this research was to determine the optimal conditions for storing soil when using biochemical molecules as trace evidence*. The examination of the soil microbial biochemical molecules was divided into two sections: DNA profiles and fatty acid profiles.

The DNA profiles were made with capillary electrophoresis-single stranded conformation polymorphism (CE-SSCP). After statistical analysis using Bray-Curtis distances and ANOSIM (analysis of similarity) *it was observed that storage treatment did not have a significant impact on the genetic profile of soil bacteria*. However, significant differences were observed between soils collected from different sites. This shows that different soils can have different genetic profiles and treatment of soil storage has little effect in their characterization.

The fatty acid profiles were analyzed as fatty acid methyl esters (FAMEs) using gas chromatography-mass spectrometry. Data were analyzed using canonical correlation analysis, squared Mahalanobis distance, and repeated measures. *The FAME data show*

that -80˚C is the best storage treatment to preserve the integrity of the microbial community fatty acid profile, although storage at -20˚C was an acceptable alternative.

We conclude that analysis of fatty acid profiles is more sensitive to changes in the microbial habitat than that of DNA profiles. This is probably because soil microbes' fatty acid composition will change rapidly in response to changes variables such as temperature and nutrient availability. *In contrast, genetic changes in the microbial community require longer periods of time.* With the data from these two methods, using soil microbial community profiling is closer to becoming a viable option for forensic science.

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Executive Summary

The growing interest in using the soil microbial community as trace evidence has opened the door to further investigate the details of this potential trace evidence. Theoretically, the soil microbial community can be used to corroborate testimonial statements by comparing the soil microbial profile found on a person, or on an object, to a location of interest. To ensure that the soil sample being tested in the crime laboratory has the same microbial profile as the soil collected from the crime scene, it is necessary to know if the storage conditions play a role in alterations of the microbial profile. After all, it is common for physical evidence to be stored for some period of time prior to analysis. To understand the effects of storage and handling on the soil microbial community we collected soil samples from four different locations. These samples were then subsampled and either processed immediately or subjected to a range of storage conditions (-80˚C, -20˚C, 4˚C, air dry, oven dry, and freeze dry) for five weeks. We also collected a soil sample from the same locations two weeks later to determine if the microbial community fluctuates sufficiently to cause a significant change in the genetic and fatty acid profile; this was our attempt to simulate the time that lapses between the start of an investigation and the location and processing of a location of interest. We also investigated the effect of seasonal changes by sampling soil during three different time points over a period of one year.

For the amplification of the microbial DNA the target region needed to be common enough to be present in all bacteria, but also have variability among different species. We

selected the V3 region of the 16S rDNA. This region was amplified and fluorescently tagged to characterize the genetic profile of the soil bacterial community. Capillary electrophoresis-single strand conformation polymorphism (CE-SSCP) was used to accomplish this. Fatty acid methyl ester (FAME) analysis was also used as a comparison method of fingerprinting microorganisms within soil samples. These two methods are not currently used in forensic science, but have been used in published literature. However they both could be incorporated into a forensic laboratory; the majority of necessary instruments and techniques are already used in forensic laboratories. The FAME method is currently used to study microbial ecology for a variety of applications.

The two methods used in the current study gave contrasting results, which leads to different implications for the use of soil microbial communities as trace evidence. Both CE-SSCP and FAMEs can be used to identify soils. However the FAME method was more sensitive to storage and handling, in that the FAME data showed distinct differences among the storage treatments. Because of this FAMES should only be used to compare soils that have been stored at similar storage parameters as the soil of interest. Also, we acknowledge that the lack of change in the genetic profile in our soils does not mean that all soils around the world will respond similarly. It is likely that some soil genetic profiles will show significant response to storage and handling, but the four that we tested did not.

One particularly interesting observation was that freeze-dried soils and air-dried soils contained similar microbial profiles. Thus, it might not be necessary to use relatively

elaborate instruments such as a lyophilizer instead of air-drying on a laboratory bench. This could save significant time and money for the analyst and agency.

We suggest that the best way to store soils when using microbes as trace, as to study the microbial community, is by freezing them. Storing soils at -80˚C will ensure the most preservation, while storing at -20˚C is an acceptable alternative. Although air-drying is a common method of storing soils for long term it significantly altered the microbial community profile when examining the fatty acid profile. For both the FAME and CE-SSCP methods there was variation in the results of what was significantly different and what was not significantly different for each soil type and for each season. This illustrates that at each soil collection site and time it is imperative to record and understand the environmental variables as wells as obtaining the appropriate reference and control samples to ensure accurate and reliable results during analysis.

Introduction

The forensic value of soil is due to the facts that soils are widespread and they can vary chemically, physically, and biologically over small units of space. Soil formation is the result of six key factors: climate, biology, parent material, topography, drainage, and time (Jenny 1941). These factors lead to the formation of horizons within the soil. Horizons are layers of soil that are distinct from the soil above and below them. Typically from top to bottom there is A, B, C, and R horizons. The A is the surface horizon that typically contains the most organic material. The B horizon contains less organic matter along with clay, lime, and salts that have been leached from above horizons. The C horizon is the loose parent material, where the R horizon is solid rock parent material. While these horizons are important for many soil processes, soil microorganisms are largely contained in the A horizon (Voroney, 2007).

Soil is a complex matrix composed of three particles: sand, silt, and clay. The relative distribution of these particle sizes is what defines a soil's texture (Figure 1). When investigating soil from a biological perspective, soil texture is an important factor in microbial population and activity. The ideal habitat for a soil microbe is within a soil aggregate. A soil aggregate is a collection of soil particles that cohere to each other more strongly than to other surrounding particles. Plant roots are most important in the formation of soil aggregates. Organic matter, fungi, and Actinomycetes (Actinomycetales: Actinomycetaceae) are also important for aggregate formation and the stabilization of aggregates. Clay particles hold soils together to form an aggregate, acting like glue. This causes soils with a higher clay content to have a higher aggregate content

(Wuddivira et al., 2009). Thus, abundance of microorganisms tends to be positively correlated to soil clay content. Bacteria are generally located within aggregates, which contain air spaces (pores) that allow for a flow of water and nutrients to promote metabolism and growth. Aggregates also serve to provide protection from predators such as nematodes and amoebae (Voroney, 2007).

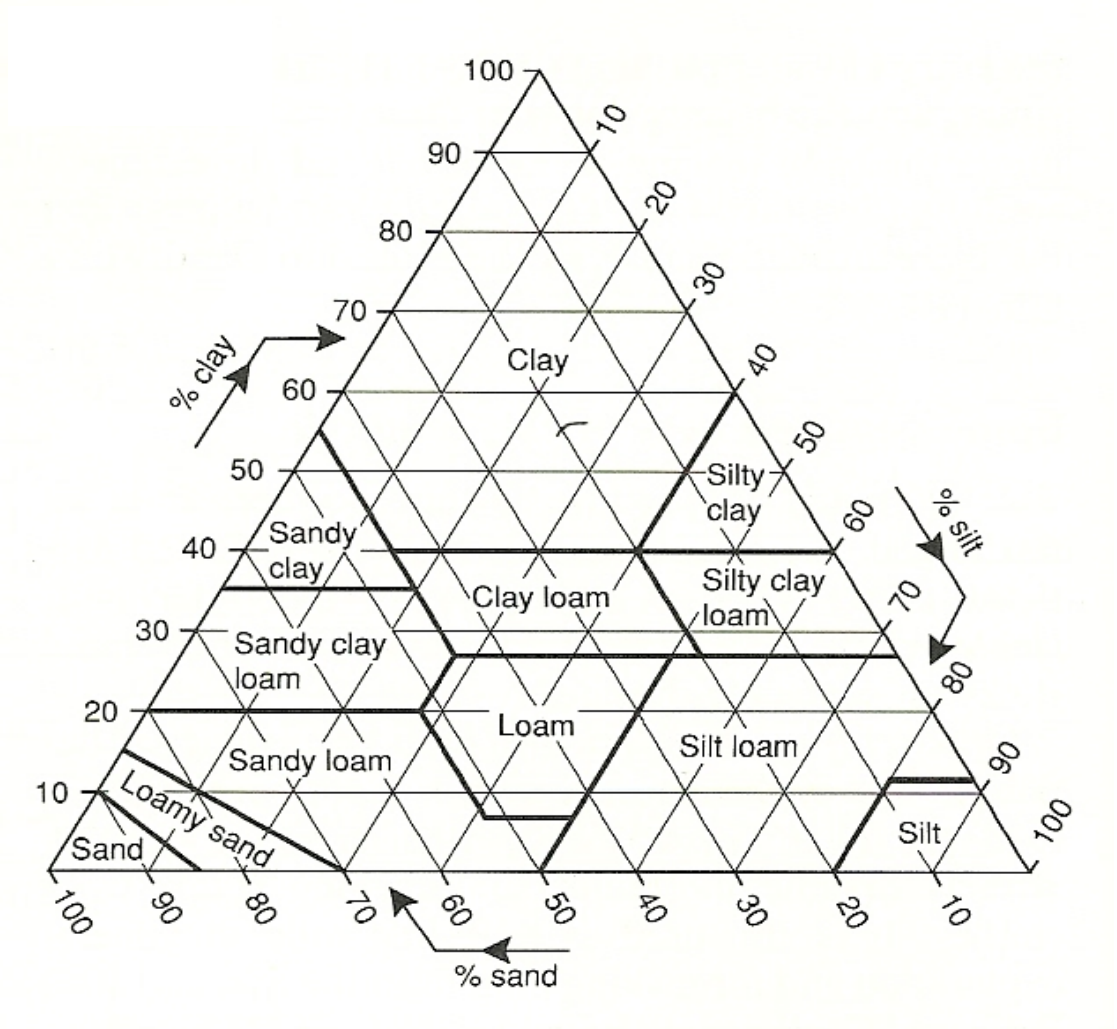

Figure 1. United States Department of Agriculture soil texture chart. This chart allows for the identification of soil classification by utilizing the percent of each soil particle (sand, silt and clay).

Soil for Forensic Science

Trace evidence, although often found in small quantities, can be vital in a forensic investigation. The primary contribution of this form of physical evidence is to trace the movement of an object, including a person. In doing so, trace evidence allows an investigator to connect suspects and victims to the crime scene or to confirm an alibi. Traditionally, there are five main forms of trace evidence (hair, fiber, paint, glass, and soil). However trace evidence is not simply limited to these five categories. Soil has been recognized as trace evidence since the early days of forensic science. In the late 19th century the concept of using soils as trace evidence in forensic science was acceptable to the general public and was incorporated into popular literature. In 1887, the fictional character Sherlock Holmes used soils to trace the movements of Dr. John Watson (Doyle 1887).

Soil is useful as trace evidence because it has complex physical, mineralogical, chemical, and biological properties that can be specific to its location (Jamieson and Moenssens 2009). Current uses of forensic geosciences still involve the use of soil properties, but with recent technological advances and improved techniques (Ruffell 2010). Physical, mineralogical, chemical, and biological properties of soils can be assessed to provide a systematic method of identification.

Physical Analysis

Many physical properties can be used to compare soils, such as particle size and shape, color, density, texture, porosity, and consistency (Murray and Tedrow 1992). These factors can help to determine the relative geographic location of the soils origination (Saferstein 2009). Many of these physical characteristics can be identified by a forensic soil scientist using the naked eye or a low power microscope (Fitzpatrick et al. 2009). This makes the method cost effective, while still yielding significant detail(s) to contribute reliable evidence to the case at hand. A tool that can help with the analysis of physical soil properties is the World Reference Base for Soil Resources (WRB). This resource can help to identify soils on a local, national or even international level.

Soil can contain anthropogenic material visible to the naked eye, which can be helpful in identifying a unique characteristic within a given area. Murray (1991) illustrates the usefulness of unique objects found in soil within a case study: “In a rape case in Upper Michigan three flower pots were knocked over and spilled during the struggle. The suspect had soil on his shoe and within the soil was a unique blue thread that was also present within the soil of one of the flower pots.” Without the blue thread present in the soil it would have been harder to convict the suspect. Again because the method uses only the naked eye or a low power microscope (a nondestructive approach) it does not damage the sample, which allows for the sample to also be processed further by another method of choice. The major drawback to this method is most often seen when the soil

composition is similar for a large distance around the crime scene. If there is no unique or distinguishable feature in the soil, then a more detailed method may be necessary.

Mineralogy

Soil generally contains at least 3-5 mineral varieties, and with numerous optical properties and morphologies that allow for distinct identification, minerals are a vital part of soil analysis (Weinger et al. 2009). Mineralogy is an accurate way to distinguish between soil samples; however there are few scientists that can identify minerals accurately so this method is usually reserved for high profile cases. Identification of minerals is becoming more accessible due to new techniques within the field. The use of a light microscope with infrared spectroscopy creates infrared microprobe analysis, which is a powerful method that incorporates microstructure with chemistry. With the use of the diamond attenuated total reflection (D-ATR) microscope objective individual minerals can be isolated and examined with little to no sample preparation (Weinger et al. 2009). This method, however, is not efficient if only basic minerals are contained within the soil as other methods are less expensive and yield more productive results for common minerals.

Minerals can also be identified in other ways, such as X-ray Diffraction (XRD) or Diffuse Reflectance Infrared Fourier Transform (DRIFT). XRD can provide diffraction patterns of crystalline or even poorly crystalline soil minerals, as well as mixed crystals (Tilstone et al. 2006). DRIFT spectrum is particularly sensitive to clay minerals and quartz, due to its absorption spectrum of infrared light (Jamieson and Moenssens 2009).

These methods do require expensive laboratory equipment and involve detailed data analysis, making them more expensive, but they give details that cannot be detected by the naked eye. When both methods are used together their overlap in data analysis strengthens the results, making them more definitive.

Chemical and Elemental Analysis

Naturally occurring elements in soil can be quantified by spectroscopy. To identify metals found in the soil, to give a unique fingerprint, inductively coupled plasma optical emission spectrometry (ICP-OES) can be used. This method can detect more than 13 elements in minute concentrations (Moreno et al. 2006). With such precision soil samples can be compared and analyzed to narrow down location, but it is highly important that collection of the reference or control sample is complete and representative. Meaning multiple samples should be taken to encompass all possible points of interest. This is also the case with all detailed analysis of soil samples.

More current research has suggested using chromatography for analysis of organic and water soluble molecules in soil. Reverse-phase high-performance liquid chromatography (HPLC) is capable of separating fractions of soil and differentiating soil samples both qualitatively and quantitatively. Ion chromatography (IC) has also been utilized for forensic purposes and gave similar results as HPLC (Bommarito et al. 2007). Using HPLC and IC, soils can be identified by quantitative analysis of the anion concentrations showing a significant difference in soil samples within a 1 m^2 grid (Bommarito et al.). Analysis for forensic purposes has been done using this technique by focusing on

acetonitrile extracts of soil, and analyzing the number, location, and relative intensities of peaks (Bommarito et al. 2007).

Biological Analysis

Plant material such as pollen and seeds can help to distinguish between soils that have similar mineral and chemical properties. Scanning Electron Microscopes (SEM) and Transmission Electron Microscopes (TEM) can be used to identify unique morphology of pollen grains, plant seeds, and fungal spores. This is a precise method of identifying biological matter. This expensive method is destructive to the sample, thus rendering the sample unusable for other analysis methods. A specialist is generally required for the identification of pollen grains. Other parts of the plant can be useful in an investigation. Plant waxes can provide unique profiles from soil samples and plant fragment DNA analysis also helps to obtain a unique characteristic in which the soil samples can be distinguished (Jamieson and Moenssens 2009).

As of 2001 the INTERPOL forensic science team had only acknowledged one case where soil microorganisms were helpful in solving a case; multisubstrate testing method (MT) was used for forensic soil comparisons. Recent research has shown the potential for soil microorganisms to become a practical and reliable form of trace evidence (Heath and Saunders 2006, Bommarito et al. 2007, Hirsch et al. 2010). Within a soil sample there is a wide variety of microorganisms including bacteria, archaea, fungi, microscopic animals, microscopic plants, and viruses (Pye 2007). According to Curtis and Sloan (2005) a sample of soil can contain up to 10^{10} to 10^{17} bacteria and are possibly composed of more

than 10^7 taxa. Because the soil microbial community is diverse the identification of a rare or unique taxon is not necessary to make a unique fingerprint for a given soil. The overall community structure is all that is needed.

Although the microbial community is dynamic, soils from the same samples might not to change significantly over fall, winter, and spring; although in summer there can be a significant difference within the samples (Griffiths et al. 2003). Perhaps more to the point, in a more recent publication by Moreno et al. (2006), it was shown that there is an apparent difference in the wet and dry seasons in the soil microbial community using Bray-Curtis similarity coefficients with multidimensional scaling analysis. The work done by Moreno et al. (2006) would be more applicable to a wider geographic range as compared to the work by Griffiths et al. (2003), because temperature and seasonal variation differs greatly across many geographic regions.

Soil Microbial Identification via Lipids

The membranes of soil microbes are made of phospholipid fatty acids and can be unique to species. By isolating these fatty acids it is possible to examine the soil microbial community (Carpenter-Boggs et al. 1998). Two of the more common methods of examining the fatty acid profile use Fatty Acid Methyl Esters (FAMEs) and Phospholipid Fatty Acids (PLFAs). FAME profiles are based on all ester-linked fatty acids extracted from the soil, an example of this can be found in Cavigelli et al. (1995). Some recently dead microbes may also be included in this method, but it is of note that fatty acids are labile and are most likely be degraded rapidly by other microorganism for energy (Bossio

and Scow, 1998). The fatty acid extraction may include plant waxes; however, these peaks can be removed during analysis. The FAME method examines shorter-chain fatty acids (C < 20) because microorganisms generally have fatty acids from C10 to C20.

The PLFA method is similar to the FAME method in that it examines C10 to C20 fatty acids. However, PLFA separates the polar and non-polar fatty acids by an exchange column, whereas FAME examines both polar and non-polar fatty acids together (Marschner, 2007). According to White (1993) phospholipids in soil can be degraded within minutes. This means that PLFA profiles are used to represent viable microorganisms. However, the PLFA method is more tedious and time consuming than the FAME method (Marschner, 2007). And when examining the fatty acids only, it is generally not possible to identify the species of microorganisms. However, there are several signature fatty acids that correspond to specific groups of microorganisms. As an example, iso16:0 is a known marker of Gram-positive bacteria (Kandeler, 2007). These signature fatty acids are then used to characterize the soil microbial community.

Methyl ester fatty acids can be examined in other ways than just FAME. A new method that has the potential for routine use in a laboratory for studying microbial fuel cells was recently developed in the Kiely Lab (Nelson et al. 2010). They use rapid agitation of the sample within a biological activity test known as SLYM-BART followed by a FAME extraction. With this modified method Nelson et al. (2010) obtained consistent and reliable results.

To strengthen the robustness of results the use of an additional method is often necessary. When examining a river floodplain for redox related soil microbial communities Song et al. (2008) used two different methods, which helped to increase the robustness of his results. Song et al. (2008) used both FAME and terminal-restriction fragment length polymorphism (T-RFLP) to illustrate the differences in types of microorganisms from oxic to anoxic conditions on the river floodplain. An advantage of FAME over T-RFLP is that all microorganism ester-linked fatty acids are extracted at once and can be distinguished by analysis. With T-RFLP there are more tedious steps: extraction of DNA, amplification of target region, cutting of target region with restriction enzymes, then analysis on a genetic analyzer. With the increased number of steps, expenses and error rates also increase.

Soil Microbial Identification via DNA

Some currently used methods for researching the microbial community in soil using DNA are identified using the acronyms TRFLP, DGGE, ARISA or SSCP. The most commonly used method in the literature for fingerprinting soils with microbial community DNA is terminal restriction fragment length polymorphism (TRFLP) (Heath and Saunders, 2006; Meyers and Foran, 2008; Quaak and Kuiper, 2011). This method generally uses the whole 16S ribosomal DNA gene (rDNA) for amplification that is then cut by one or more restriction enzymes (Liu et al., 1997). This cutting by a restriction enzyme should be at a slightly different location on the DNA fragment for each bacterial genus and possibly species. The fragments are processed on a genetic analyzer, giving a peak for each fragment, which represents a ribotype, while the height of that peak

represents the abundance of that ribotype (Singh et al. 2006). This set of peaks becomes the fingerprint for that soil sample.

Denaturing Gradient Gel Electrophoresis (DGGE) has been used for a multitude of microbial ecology studies; more specifically it is used to identify microbial community structure (Lagomarsino et al.,2007; Muyzer and Smalla, 1998; Nakatsu et al., 2000). This is done by using PCR to amplify the region of interest from microbial DNA extracted from soil. The amplicons are then run on a denaturing gradient acrylamide gel, which partially denatures the double stranded DNA. When the electrophoresis is applied, the semi-denatured amplicon begins to migrate based on its size and sequence (Hirsh et al., 2010). The reason it is also based on sequence is that Guanine and Cytosine form a tighter bond with three hydrogen bonds, while Adenine and Thymine are bound by only two hydrogen bonds. This means a high GC content in the sequence of an amplicon would not denature as readily as a high AT content. Thus the GC rich sequence would migrate at a faster rate than the AT rich sequence of two amplicons of the same size. Individual bands from the gel can be isolated and sequenced for identification of the microbial species. This method has recently been used to examine, not only community structure, but also functional groups of microorganisms, by using specific functional genes as a DNA target (Tabatabai et al. 2009).

ARISA (Automated Ribosomal Intergenic Spacer Analysis) is a technique that uses the intergenic space (ITS) in the ribosome to examine community structure (Kent and Triplett, 2002; Ranjard et al., 2001). This technique uses PCR to amplify the ITS region

and then processes those amplicons on a genetic analyzer. The unique length of the ITS regions corresponds to microbial species. With modifications it can be used to identify species of bacteria and shifts or changes in small microbial communities within a micro environment (Kennedy et al., 2005). It is unable to identify these same community shifts in a large and dynamic community because the primers for the PCR will tend to favor a selective group of microorganisms (Rochelle et al., 1994). This will result in a biased community profile. In addition, if the species is unknown its specific peaks may not be identified, to identify the peaks the whole 16S rDNA will have to be sequenced. This in turn requires more time and makes the method less cost effective. Popa et al. (2009) suggest pairing this method with another microbial community fingerprinting method to obtain optimal results. ARISA is also an ideal method for following a specific species of microorganism both evolutionarily or spatially.

A new and potentially more accurate technique is CE-SSCP (capillary electrophoresis-single stranded conformation polymorphism). CE-SSCP uses the 16S rDNA, but only a small region of the gene to provide slightly more variable fragments for the genetic analyzer. However, in CE-SSCP the conformation (secondary structure) of the fragments are formed allowing for a more detailed analysis. Thus, each DNA fragment is separated by size and secondary conformation. This gives a detailed profile of the microbial community structure. This profile then makes a fingerprint of that soil sample by the number of peaks (the abundance of bacteria, selection of ribotype) and the relative peak height (the relative number of a given bacterial ribotype).

The details of the CE-SSCP method are as follows. DNA extraction, polymerase chain reaction (PCR), and capillary electrophoresis are the three steps required for data in CE-SSCP. The DNA extraction may be done numerous ways, but it is important to obtain good quality of DNA for a non-bias profile (Thakuria et al., 2008). The PCR requires a target sequence that would ideally be in a conserved region with variable segments of sequence. A study from Kourkine et al. (2002) showed that CE-SSCP works best using a target sequence of 175 to 400 base pairs in length. The target sequence is fluorescently labeled by 5' tags on the primers to be detected by the laser during CE. The amplicons are added to a mix of formamide and an internal size standard. This mixture is then heated to separate the double stranded DNA into single stranded DNA. After heating the amplicon mixture it is placed directly on ice to ensure that the single stranded DNA forms into its unique secondary conformation based on its sequence. A current is applied to the amplicon mixture that causes the DNA to travel through the capillary and past the laser, which detects the fluorescence. The smallest amplicons travel the fastest; however the conformation of the amplicon can alter its speed. This allows for a different organism with the same length of target sequence to travel at different speeds making a unique peak for each ribotype. Each time the laser detects fluorescence it makes a peak; the more fluorescence detected the larger the peak.

Although CE-SSCP is high throughput and relatively inexpensive, it does have a few shortcomings. It is known that temperature can cause alterations to the migration speed of DNA. Given that SSCP is in nondenaturing conditions a lower temperature is best for accuracy of size, while a higher temperature can give more precise peaks (Zinger et al.

2008). Most studies use a temperature of 32˚C as a compromise to obtain accurate size and precise peaks. The base line of the peaks has also been reported to rise, which may make analysis more difficult (Loisel et al. 2006, Zinger et al. 2008).

This method has been tested and validated through multiple publications. Recently a standard protocol was published to help establish uniform methodology in the scientific community (Larsen et al. 2007). The SSCP method has been shown to be reliable and reproducible (King et al. 2005) in analyzing microbial communities within natural settings (Zinger et al. 2009) and industrial settings (Duthoit et al. 2003). In one of the early studies using CE-SSCP scientists were able to identify several bacterial from lung cultures from cystic fibrosis patients. This study was crucial because the ability to identify the bacteria allows for a more appropriate antibiotic to be prescribed to the patient (Ghozzi et al. 1999). Being a newer method CE-SSCP has been compared to several other methods for microbial community profiling. When CE-SSCP was compared to denaturing gradient gel electrophoresis (DGGE) it was found that CE-SSCP gave better resolution of peaks, took less time to prepare samples and analyze data, and showed less artifacts than DGGE (Hong et al. 2007). This comparison shows that CE-SSCP is a good high-throughput method for analyzing microbial community profiles. Hiibel et al. (2010) recently developed a newer method called active community profiling (ACP) which utilizes CE-SSCP. This method looks at both the DNA and RNA of the 16S through PCR and CE-SSCP to determine what microbes are active within the community. This method provides advantages to DNA profiling alone, by illustrating which community members are active (the RNA profile) from the community members that are

dormant or dead (potential peaks from the DNA profile). It is “active” community profiling because the RNA profile is only present in a cell that is alive and active (using its metabolic functions). While, the DNA profile can contain cells that are dormant or recently deceased, thus not having a major impact on the ecosystem.

Clearly, there are multiple techniques used to examine the microbial community structure. The key is to use a method that is statistically valid, works well with your lab equipment, and is most cost effective. When trying to extract all the valuable information from one source of trace evidence it would be ideal to have multiple methods that examine different components of that source of trace evidence to provide the most compelling argument for the criminal case. In other words, it is of most use and significant to a criminal case to have a multitude of analyses with different methodologies to provide the most robust conclusion. With CE-SSCP as an additional method to examine soil evidence, the current project aims to provide a more robust understanding of the storage and handling requirements of soils as trace evidence. CE-SSCP is a positive complementary method to current soil analytical methods.

Project Aims

To assess the investigative value of soil bacterial DNA and fatty acids we conducted a study to determine the potential changes to the microbial community following a range of storage and handling parameters. *We tested the hypothesis that the storage of soil samples will not significantly alter the microbial community profiles.* To examine this we collected soil from four different grassland sites during three seasons over the period of

one year. Soils were stored at one of six treatments (4˚C, -20˚C, -80˚C, air dried, freeze dried, and oven dried) for five weeks. Field fresh samples were compared to the stored samples along with samples collected two weeks following initial collection to analyze the changes in the soil at the collection.

Methods

Soils

Four contrasting soils from southeastern Nebraska were used in this study. It is important to test a variety of soils because soil type can have a strong influence on the structure of the soil microbial community (Singh e al., 2007). The four soil names are Soil 1 : Morrill soil; Soil 2: Aksarben soil; Soil 3: Muir soil; Soil 4: Malcolm soil. Three of the four soil types (Morrill, Aksarben, and Muir) were collected at Twin Lakes on a Nebraska Game and Parks Reserve, while the fourth soil type (Malcolm) was collected in a pasture near the town of Raymond, Nebraska. Soil samples were sent to Ward Laboratories, Inc., (Kearney, Nebraska) for physicochemical testing. Soil particle size distribution was determined using the hydrometer method. Soil physicochemical characteristics are presented in Table 1.

Experimental Procedure

Sample Collection

Three 1 m x 1 m plots were constructed approximately 3 meters apart at each soil site. This study was replicated during three different seasons, so the same three plots were used for all three replications at an individual site. From within each of the three plots 20 cores were taken from a depth of 0 cm to 5 cm. Soil cores were placed into a plastic zip-lock bag in a cooler with ice until they reached the lab (approximately 60 min). After reaching the lab the soils were placed at 4 °C overnight. Within 24 hours of collection the 20 soil cores from each plot were sieved (4 mm). The three different plots of sieved soil core samples were mixed and placed into storage or used for immediate extraction of DNA. Soils were collected during three seasons: harvest season (September 2010), dormant season (November 2010), and growing season (July / August 2011).

Soil Storage and Handling

Soil samples were exposed to one of six storage treatments: 4 °C, -20 °C, -80 °C, air drying, freeze drying, oven drying. Soils stored at 4 ˚C, -20 ˚C, and -80 ˚C were stored in sealed plastic bags. Air-dried samples were dried on a countertop for 7 days then placed in a sealed plastic bag. Oven dried samples were placed in metal tins in an oven at 160 °C for 2 days then placed in a sealed plastic bag. Freeze dried samples were sealed in a bag, placed at -20 °C for 2 days, then lyophilized on a Freezone6 (Labconco, Kansas City, MO) for approximately 3 days then placed into a sealed bag. All samples were in storage

for 5 weeks. At the end of the five weeks DNA extraction was done. In addition, DNA was extracted from the mixed and sieved soils the day of samples being placed into their storage treatments. This allowed for a reference sample for the effect of storage and handling on soil microbial communities.

Sites Revisited

The investigation of a criminal act always occurs after the criminal act has been committed. As a result, crime scene investigators always arrive at a crime scene some time after the crime has been committed. To address this, all sites were visited 14 days after initial soil collection and soil was collected again in the same manner as before. Collected soils were processed fresh, i.e. DNA was extracted from these soils upon return to the laboratory. This allowed insight into the effect of time on the structure of the soil microbial community. This permitted us to ask the question: is it possible for soil collected two weeks later to still represent the soil at the time of the crime?

DNA Analysis

Approximately 5 g soil was ground in liquid nitrogen by mortar and pestle. From the ground soil 0.2 g of soil was used to extract DNA using Powersoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer instructions, with two modifications. The first modification was: 0.2 grams of 0.1 mm glass beads (BioSpec Products, Inc., Bartlesville, OK) were added to the tube with the soil and first solution, and then placed in a mini-beadbeater (BIOSPEC Products, Inc., Bartlesville,

OK) at 4600 rpm for 3 min. This was to ensure all soil aggregates were broken and cells were lysed. The second modification to the manufactures instructions was that the DNA was eluted into water and, not into the provided solution. DNA was stored at -80 °C.

Polymerase Chain Reaction (PCR)

The PCR contained 0.26 µM of forward primer, W49 (Duthoit et al. 2003) labeled with FAM (Intergrated DNA Technologies, Coralville, Iowa) and 0.26 µM of reverse primer, W34 (Duthoit et al. 2003) labeled with VIC (Applied Biosystems, Carlsbad, CA). The forward and reverse primers were 5'-ACGGTCCAGACTCCTACGGG-3' and 5'-TTACCGCGGCTGGCAC-3', respectively. To the same tube 0.10 mM dNTP (Promega, Madison, WI) were added along with 2.5 U/µl Pfu Turbo DNA Polymerase AD (Agilent Technologies, Inc., Santa Clara, CA). The Pfu Turbo reaction buffer was diluted to 1x and 1 µl of template DNA that was a concentration of 10 ng/µl was added. Sterile water was added to a final volume of 20 µl.

The PCR cycles were as follows: activation of enzyme at 94 °C for 2 m; 25 cycles of denaturation at 94 °C for 15 s; hybridization at 61 °C for 15 s, extension at 72 °C for 15 s; and final extension cycle at 72 °C for 10 m. The PCR cycle times were suggested from Zinger et al. (2007) and the temperatures and cycle numbers were suggested from Hong et al. (2007). The PCR was run on a GeneAmp PCR system 9700 (Applied Biosystems, Carlsbad, CA).

Capillary electrophoresis single-strand conformation polymorphism (CE-SSCP)

The PCR products were diluted 1:70 before being used on the genetic analyzer, if peaks were too intense PCR products were further diluted and rerun on the genetic analyzer. Each sample was run with 10 µl of Hi-Di Formamide (Applied Biosystems), 0.3 µl internal DNA size standard Genescan-LIZ600 (Applied Biosystem), and 1 µl of 1:70 diluted PCR product. The samples were denatured at 95 °C for 3 m then placed directly on ice to cool for 15 min before being placed on the genetic analyzer.

Capillary electrophoresis was done on a 3130 Genetic Analyzer (Applied Biosystems) using a capillary array of 36 cm in length. Samples were run using Conformation Analysis Polymer (Applied Biosystems, Foster City, CA) made according to Applied Biosystems instructions. Samples were run with an injection time of 22 seconds and injection voltage of 1 kV. Electrophoresis was set to 32 °C for 30 m.

Fatty Acid Analysis

Mild alkaline hydrolysis was used to examine the soil microbial community structure based on the total FAMEs. In using this procedure only ester-linked fatty acids are freed for further examination. Briefly, 10 g soil was placed in a 50 ml Teflon centrifuge tube. Freshly made 0.2 M KOH in methanol was added to the soil. Then samples were placed into a 37 °C water bath for 1 hr with the samples mixed every 15 min. After the water bath soils were neutralized, hexane was used to partition the freed fatty acids. The tubes were then centrifuged at 6000 rpm for 10 minutes. The hexane layer was then filtered through a PTFE 0.2 µm syringe filter into a clean Pyrex tube. The hexane solvent was

evaporated under N_2 to a small volume. At this point 3-4 drops of benzene was added, mixed, and evaporated to dryness. The residue was dissolved in hexane and transferred to a vial to be placed on the gas chromatograph (GC). Samples were prepared for the GC by evaporating solvent under N_2 and redissolving in 250 µl to 500 µl (depending on biomass concentration) containing nonadecanoic acid (C19:0, 0.05 mg/ml) as an internal standard. Fifty µl was transferred to the conical GC vial and capped.

Gas chromatography was used to separate FAMEs, with helium as the carrier gas and an Ultra 2 HP (50 m, 0.2 mm I.D., 0.33 µm film thickness) capillary column. Split mode (44:1) was used, with a 45 s purge time. Injector and flame ionization detectors were maintained at 280 ˚C and 300 ˚C, respectively. The oven temperature was ramped from 50 ˚C to 160 ˚C at 40 ˚C m^{-1} and held for 2 m, followed by a ramp at 3 ˚C m^{-1} to 300 ˚C and held for 30 m. Fatty acids were identified by retention time and the concentrations of FAMEs was calculated from the peak areas relative to the internal standard. Concentrations are reported as nmol g^{-1} soil. The fatty acids are described using the IUPAC-UIB (1987), thus the total number of C atoms followed by a colon, and if unsaturated the number of double bonds is listed followed by the position of the double bond from the carboxyl end of the fatty acid.

Statistical Analyses

The DNA profiles from the 3130 genetic analyzer were aligned with T-Align (Smith et al., 2005). T-Align is a freeware that calculates the peak numbers and peak areas into a matrix. To do this, two analysis of the same sample are used to provide an accurate

reading of the peak numbers and areas. With the matrix from T-Align Bray-Curtis untransformed distances were obtained and analysis of similarity (ANOSIM) was used to discriminate significant differences between soil storage treatments with R software. To visualize the data spatially nonmetric multidimensional scaling was done with the data from the Bray-Curtis index using R software. The fatty acids were transformed to nmol% and analyzed by a stepwise discriminant procedure, which statistically isolates the fatty acids for uniqueness and discrimination among samples based on the treatment selected: storage, location, season, or a combination of these. The model allows for fatty acids to enter or be removed for the highest discrimination power. The fatty acids that were identified, were then used in a canonical correlation analysis and squared Mahalonobis distances were used to identify significant changes when comparing the storage and handling treatments. All analysis was done using the SAS software, version 9.1. Plots were made in Origin, version 7.5.

Results

Soil Physicochemistry

The four soil collection sites all had the same soil classification of a loam texture but contained different distributions of sand, silt and clay (Table 1). There are differences in some chemical and physical properties between the soils, such as Bray P, geographic location, and land management. Table 1 highlights the differences and similarities between the soils at each collection site.

Table 1. Soil characteristics from the four soil collection sites. All four soils are classified as loam soil, but contain a unique set of chemical and physical characteristics.

	Soil1	Soil2	Soil3	Soil4
% Sand	48	30	32	36
% Silt	36	50	50	42
% Clay	16	20	18	22
pH	6.3	6.3	6.3	6.1
Organic Matter Content	2.9	6	4.9	2.8
Cation Exchange Capacity	13.2	14.5	13.7	14.7
Bray P	2	8	20	6
Current Vegetation Management	Grassland Pasture	Grassland Pasture	Grassland Prairie	Grassland Brome Grass
GPS Coordinates	40˚ 49.788N, 96˚ 56.800W	40˚ 50.014N, 96˚ 56.672W	40˚ 50.577N, 96˚ 57.140W	40˚ 57.714N, 96˚ 44.644W

DNA Analysis

When processing oven-dried samples on the 3130 genetic analyzer, no profile was obtained due to the quality of DNA available after the sample was oven dried. With no profile oven dried samples were not analyzed statistically. Processing samples on the genetic analyzer allowed for the visualization of soil microbial community profiles with the Genemapper software (Applied Biosystems, Foster City, CA). The electropherograms are standardized using an internal size standard, in this case LIZ600, with modifications noted in the discussion. The forward and reverse primers are labeled with different fluorescence tags so the profiles can be distinguished.

In order to analyze the electropherograms peaks and peak area data it needs to be converted to a tangible number. To do this we used freeware called T-Align. This output data can be further processed using the Bray-Curtis similarity index. Figures 2a – 2f are a nonmetric multidimensional scaling (MDS) graph using the data from the Bray-Curtis index to examine the fresh samples from the four different soil collection sites over the three collection seasons. The four soils are represented by color and number (the circular shapes are placed by hand to help illustrate the overlap of samples). When the samples are separated and group with their own soil location it indicates that, that particular soil is distinguishable. When soils overlap it illustrates that there are some similarities to them. The MDS graphs also examine both the forward and reverse primers. Due to the nature of the CE-SSCP method (it uses secondary conformation to separate single stranded DNA fragments) the forward and reverse primers are unable to be put together for combined analysis. Thus there are separate sets of data for the forward and reverse primers. Soil4 grouped more consistently than the other soils. Soil2 grouped somewhat consistently, but is spread across coordinate 2 on most of the plots. Soils 1 and 3 grouped together or close to each other. Both the forward and reverse primers give profiles that group similarly. September samples grouped with the most overlapping samples, while November and July / August samples separated in to more distinguishable groups.

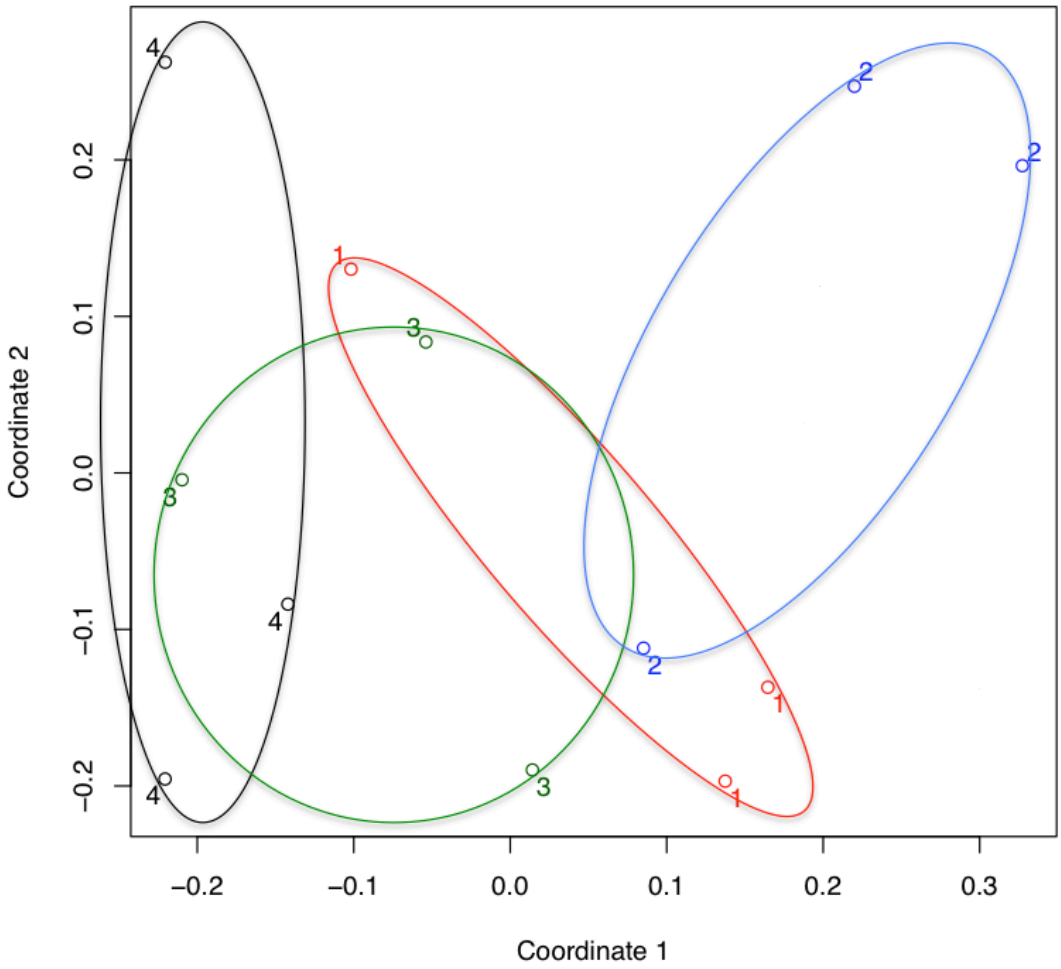

Figure 2a. Bray-Curtis untransformed forward primer September fresh samples. (1 = Soil1, 2 = Soil2, 3 = Soil3, 4 = Soil4).

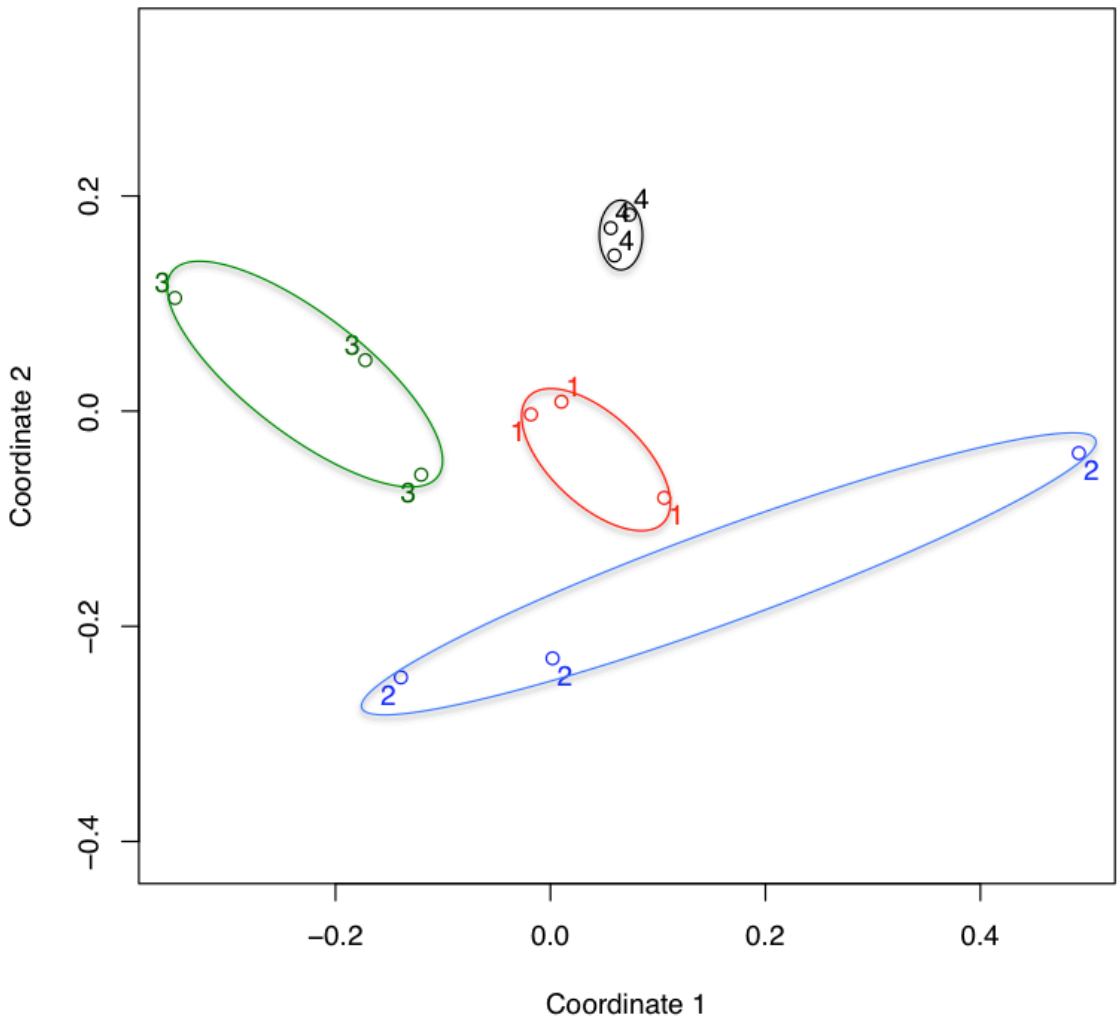

Figure 2b. Bray-Curtis untransformed forward primer November Fresh samples. (1 = Soil1, 2 = Soil2, 3 = Soil3, 4 = Soil4).

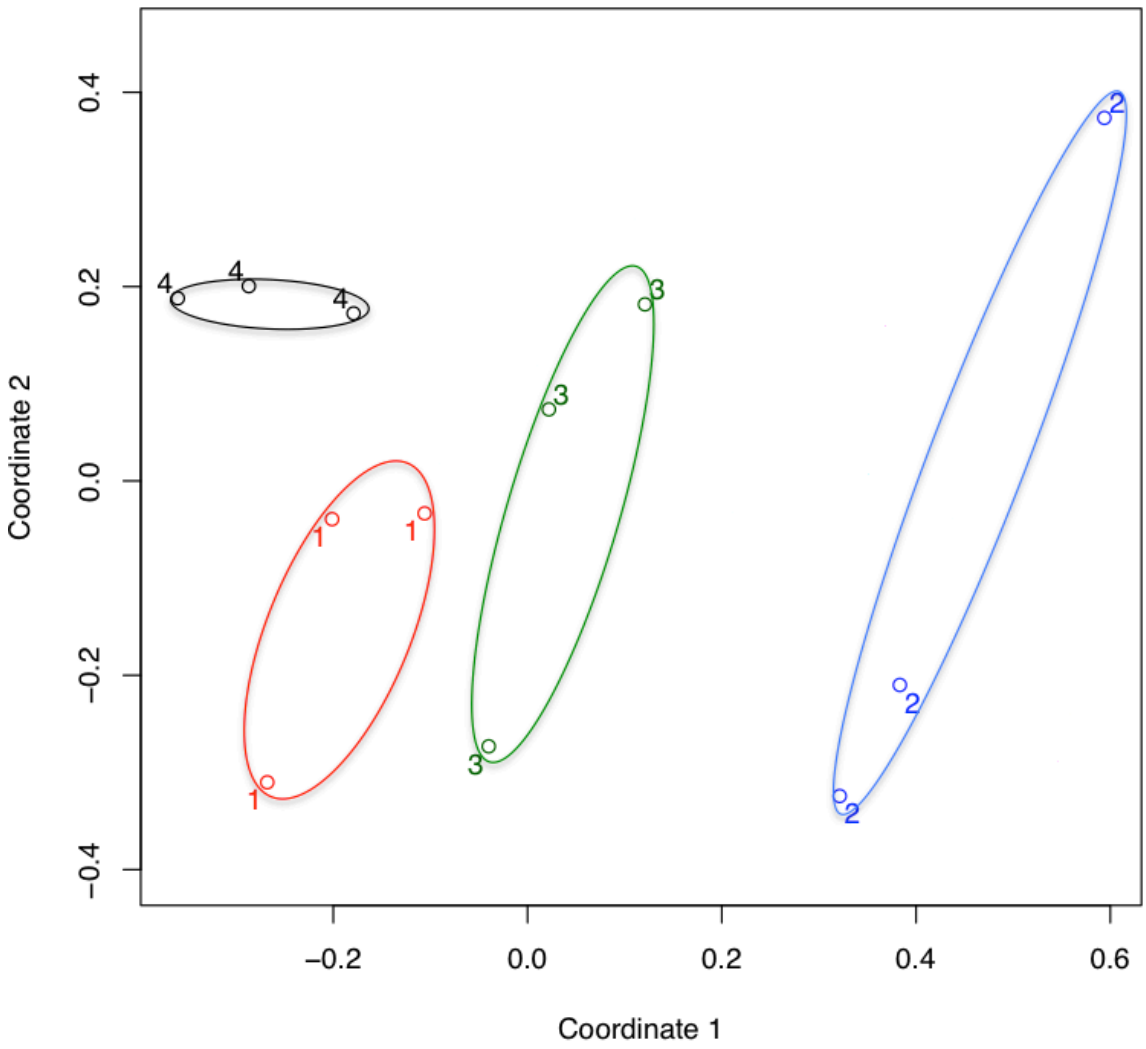

Figure 2c. Bray-Curtis untransformed forward primer July August Fresh samples. (1 = Soil1, 2 = Soil2, 3 = Soil3, 4 = Soil4)

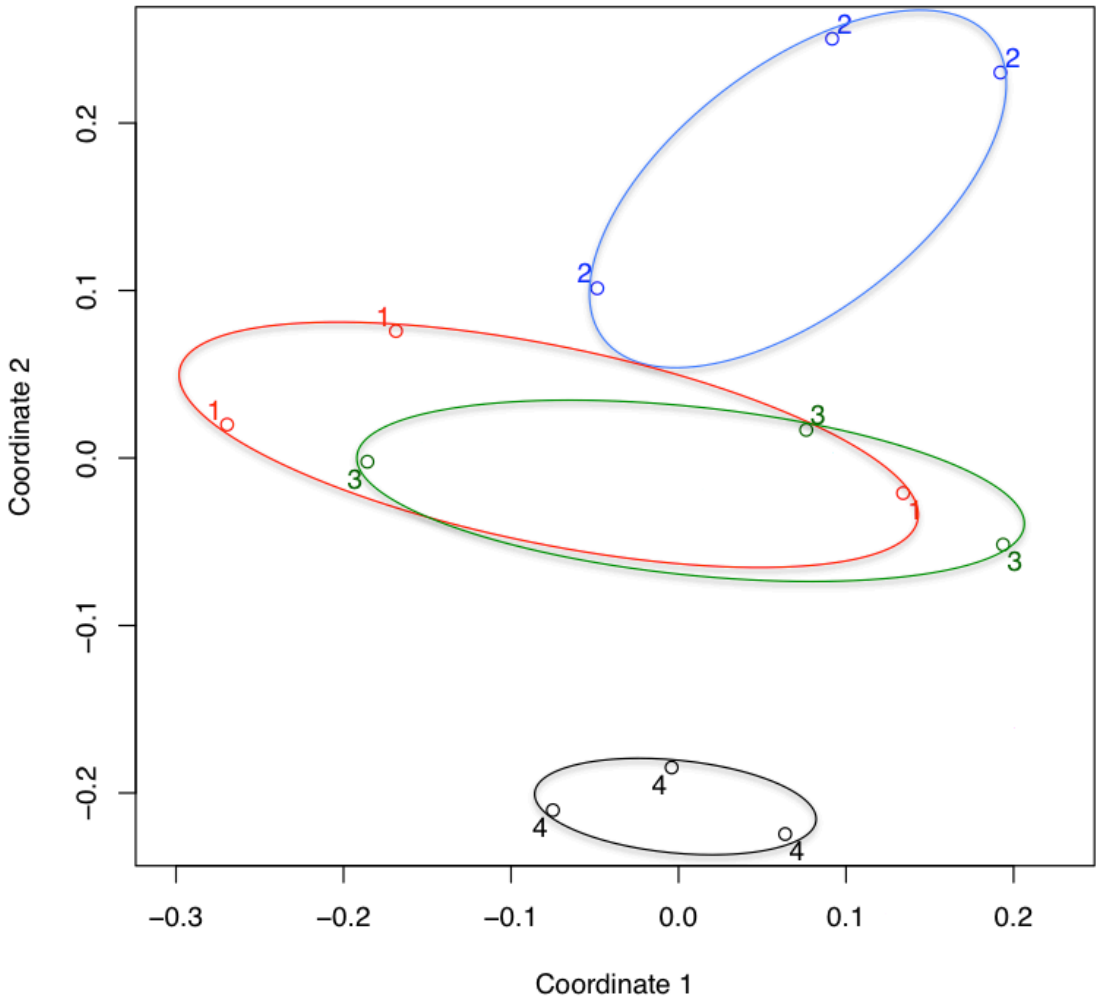

Figure 2d. Bray Curtis untransformed reverse primers September Fresh samples. (1 = Soil1, 2 = Soil2, 3 = Soil3, 4 = Soil4)

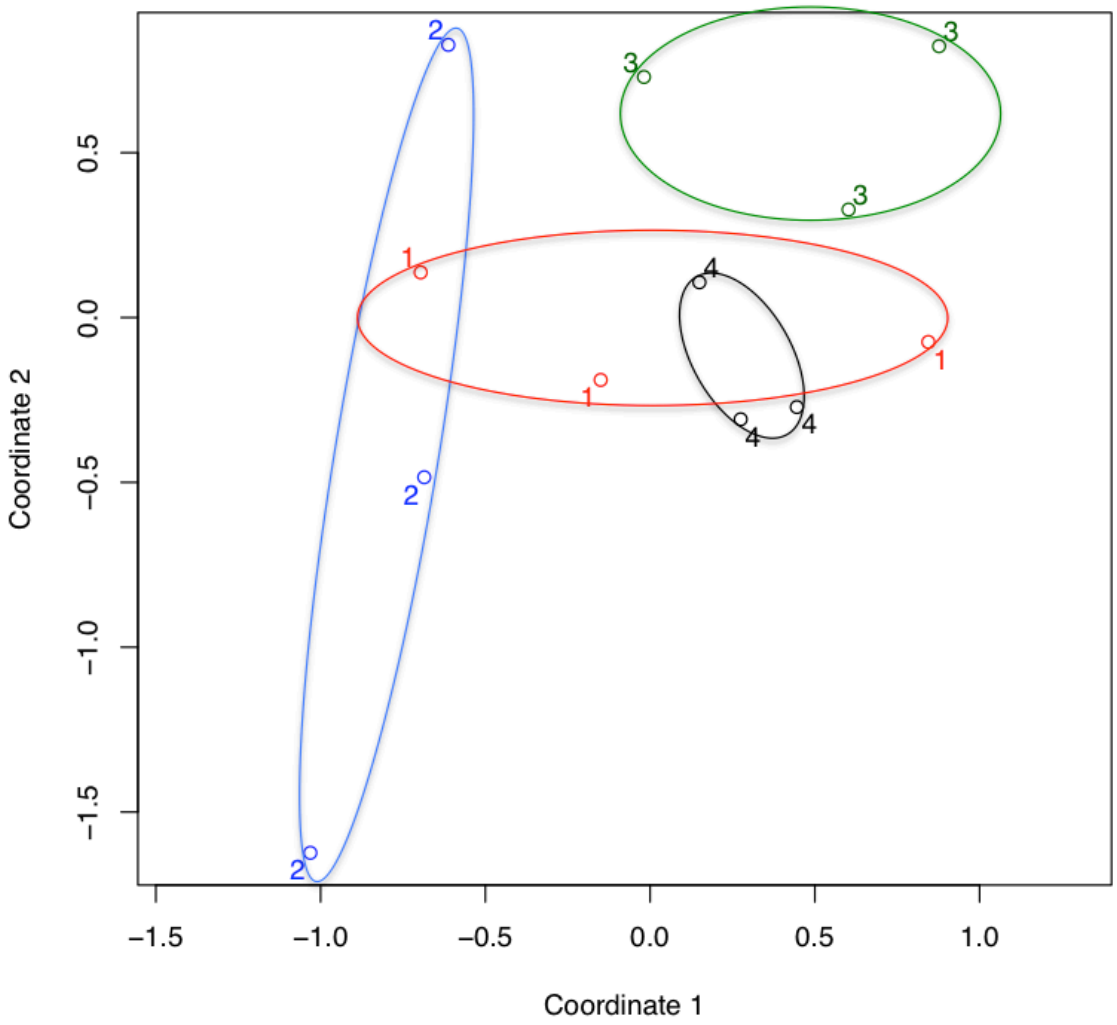

Figure 2e. Bray-Curtis untransformed reverse primers November Fresh samples. (1 = Soil1, 2 = Soil2, 3 = Soil3, 4 = Soil4).

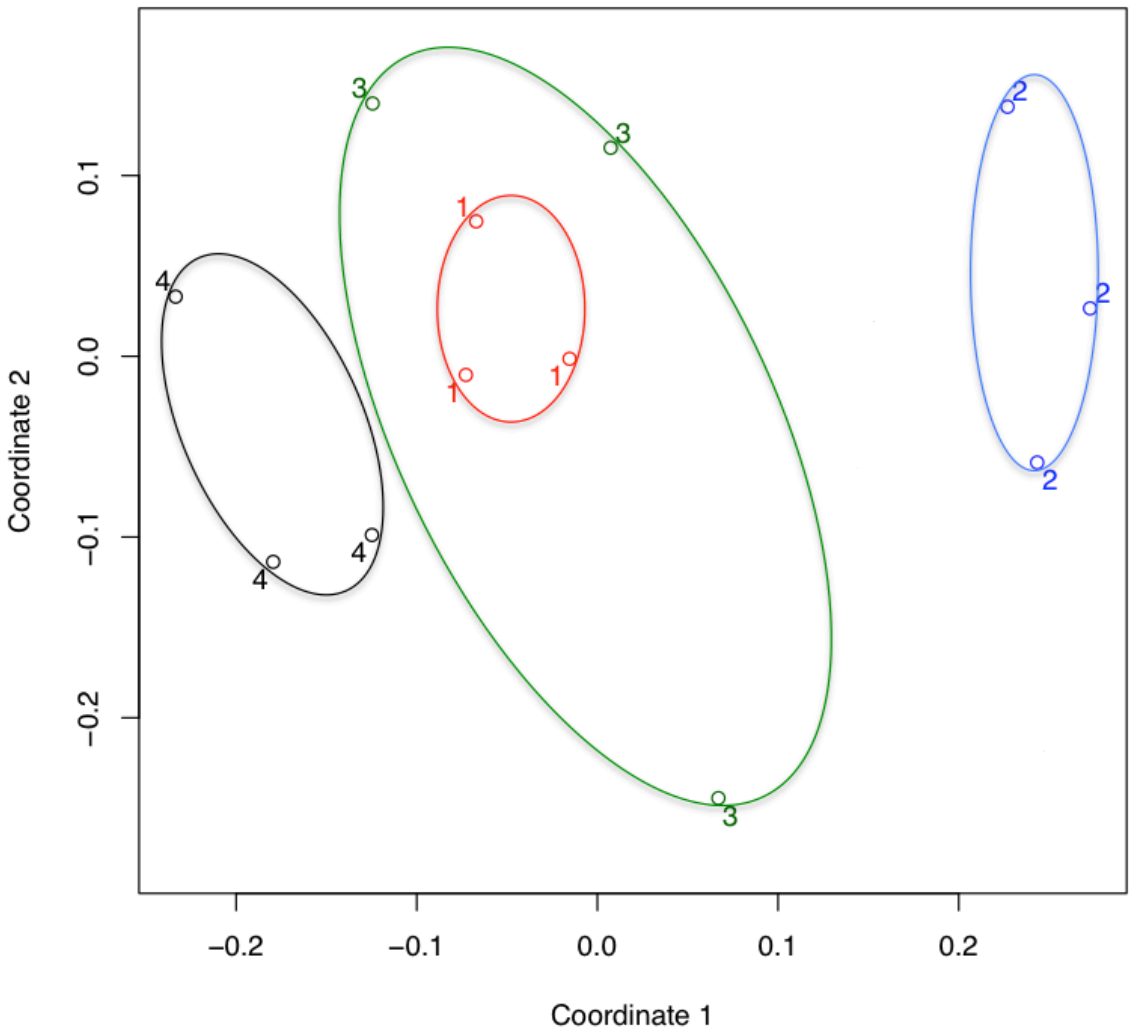

Figure 2f. Bray-Curtis untransformed reverse primers July August Fresh samples. (1 = Soil1, 2 = Soil2, 3 = Soil3, 4 = Soil4).

The storage samples were compared to the fresh sample to identify significant changes to the microbial community. The statistics were done using the data from the electropherograms converted to a matrix by the freeware T-Align. Then using the software R the matrix was transformed to Bray-Curtis index and then analyzed via Analysis of Similarity (ANOSIM). The combined section puts all four soils together to

more fully examine the storage treatment compared to fresh soils over one year's time (Table 2). When all the soil collection sites are combined for the forward primer the overall sample is significantly different for all sample seasons, as well was fresh vs. fresh revisited. Also in the combined fresh vs 4C and fresh vs -80C are significantly different for November and fresh vs. freeze-dried is significantly different during July / August. In the combined soils for the reverse primer the overall sample is significantly different in all three seasons as well as the fresh vs. fresh revisited. Also in the combined for the reverse primer during November the fresh vs 4C and the fresh vs -80C are significantly different.

Table 2. Analysis of Similarity significance values for combined soil sites of storage samples compared to fresh sample over three seasons for both forward and reverse primers. Values in bold are significant, indicating that the corresponding storage method yields changes to the microbial community that make it significantly different from the fresh sample. ($P < 0.05$ = *, $P < 0.001$ = ** where n = 3)

Forward Primer	**September**	**November**	**July/August**
Combined			
Overall	**0.002***	**0.001****	**0.001****
fresh vs. -20C	0.266	0.366	0.683
fresh vs. 4C	0.077	**0.010***	0.261
fresh vs. -80C	0.111	**0.012***	0.566
fresh vs. air dried	0.429	0.050	0.098
fresh vs. freeze dried	0.539	0.804	**0.040***
fresh vs. fresh revisited	**0.001****	**0.001****	**0.002***
Reverse Primer			
Combined			
Overall	**0.005***	**0.001****	**0.008***
fresh vs. -20	0.701	0.221	0.464
fresh vs. 4C	0.479	**0.014***	0.351
fresh vs. -80	0.160	**0.002***	0.825
fresh vs. air dried	0.805	0.195	0.361
fresh vs. freeze dried	0.734	0.100	0.176
fresh vs. fresh revisited	**0.001***	**0.002***	**0.001****

Table 3, using the same statistical analysis as before, shows the forward primer soil microbial community profiles for all four soil collection sites over all three seasons. The overall sample examines all storage and handing samples to identify if they are different from each other. For soil1 all samples were not significant. Soil2 the overall sample during July / August was significantly different. Soil3 the overall samples during November was significantly different. For soil4 the overall sample was significantly different during September and November.

Table 4, using the same statistical analysis as above, shows the reverse primer soil microbial community profiles for all four soil collection sites over all three seasons. The overall sample is significantly different in July / Augusts for soil1 and soil2. In soil2 the overall samples is also significantly different in November. In soil3 only the overall sample is significantly different in September. In soil4 the overall sample is significantly different in both September and November.

Table 3. Analysis of Similarity significance values for forward primers of storage method compared to the fresh sample for four soil sites over three seasons. Values in bold are significant, indicating that the corresponding storage method yields changes to the microbial community that make it significantly different from the fresh sample. ($P < 0.05$ = *, $P < 0.01$ = ** where n = 3).

	September	**November**	**July/August**
Soil1			
Overall	0.126	0.103	0.092
fresh vs. -20C	0.179	0.914	0.897
fresh vs. 4C	0.200	0.812	0.295
fresh vs. -80C	0.313	0.418	0.908
fresh vs. air dried	0.185	0.294	0.505
fresh vs. freeze dried	0.418	0.399	0.223
fresh vs. fresh revisited	0.095	0.078	0.099
Soil2			
Overall	0.190	0.130	**0.001****
fresh vs. -20C	0.797	0.095	1.000
fresh vs. 4C	0.589	0.104	0.712
fresh vs. -80C	0.916	0.387	0.602
fresh vs. air dried	0.201	0.804	0.579
fresh vs. freeze dried	0.893	0.095	0.407
fresh vs. fresh revisited	0.366	0.108	0.309
Soil3			
Overall	0.089	**0.037***	0.643
fresh vs. -20C	0.715	0.796	0.890
fresh vs. 4C	1.000	0.399	0.889
fresh vs. -80C	0.415	0.275	0.806
fresh vs. air dried	0.599	0.311	0.208
fresh vs. freeze dried	0.500	0.800	0.409
fresh vs. fresh revisited	0.101	0.211	0.672
Soil4			
Overall	**0.024***	**0.001****	0.187
fresh vs. -20C	1.000	0.580	0.890
fresh vs. 4C	0.097	0.105	0.189
fresh vs. -80C	0.380	0.092	0.405
fresh vs. air dried	0.081	0.100	0.483
fresh vs. freeze dried	0.401	0.111	0.311
fresh vs. fresh revisited	0.298	0.099	0.481

Table 4. Analysis of Similarity significance values for reverse primers of storage method compared to the fresh sample for four soil sites over three seasons. Values in bold are significant, indicating that the corresponding storage method yields changes to the microbial community that make it significantly different from the fresh sample ($P < 0.05$ = *, $P < 0.01$ = ** where n = 3).

	September	**November**	**July/August**
Soil1			
Overall	0.219	0.227	**0.009***
fresh vs. -20	0.406	0.701	0.697
fresh vs. 4C	0.213	0.501	0.304
fresh vs. -80	0.293	0.291	1.000
fresh vs. air dried	0.189	0.402	0.795
fresh vs. freeze dried	0.394	0.805	0.206
fresh vs. fresh revisited	0.208	0.272	0.096
Soil2			
Overall	0.082	**0.002***	**0.001****
fresh vs. -20	1.000	0.087	1.000
fresh vs. 4C	1.000	0.097	0.204
fresh vs. -80	0.694	0.087	0.422
fresh vs. air dried	0.200	0.545	0.399
fresh vs. freeze dried	0.903	0.093	0.213
fresh vs. fresh revisited	0.196	0.193	0.123
Soil3			
Overall	**0.029***	0.170	0.373
fresh vs. -20	0.308	0.578	0.603
fresh vs. 4C	0.793	0.404	0.620
fresh vs. -80	0.460	0.910	0.601
fresh vs. air dried	0.673	0.079	0.577
fresh vs. freeze dried	0.489	1.000	1.000
fresh vs. fresh revisited	0.107	0.104	0.690
Soil4			
Overall	**0.022***	**0.005***	0.268
fresh vs. -20	0.389	0.892	0.491
fresh vs. 4C	0.094	0.326	0.207
fresh vs. -80	0.589	0.304	0.396
fresh vs. air dried	0.099	0.696	0.198
fresh vs. freeze dried	0.128	0.181	0.093
fresh vs. fresh revisited	0.085	0.102	0.220

Fatty Acid Analysis

After the initial data analysis it was observed that the oven dried samples had a lower biomass (data not shown) and a different profile of fatty acids than the other storage methods. It was readily apparent that oven drying samples is not a viable option for storing soils when examining the soil microbial community structure. For this reason we continued the data analysis without the oven dried samples to better examine the significance between the other storage treatments and the fresh sample.

To discuss the data, quadrants of the fatty acid figures will be used. Thus, negative Can1 and positive Can2 is quadrant 1. Positive Can1 and positive Can2 is quadrant 2. Negative Can1 and negative Can2 is quadrant 3. Finally positive Can1 and negative Can2 is quadrant 4. In Figure 3b the quadrants are numbered accordingly as a guide.

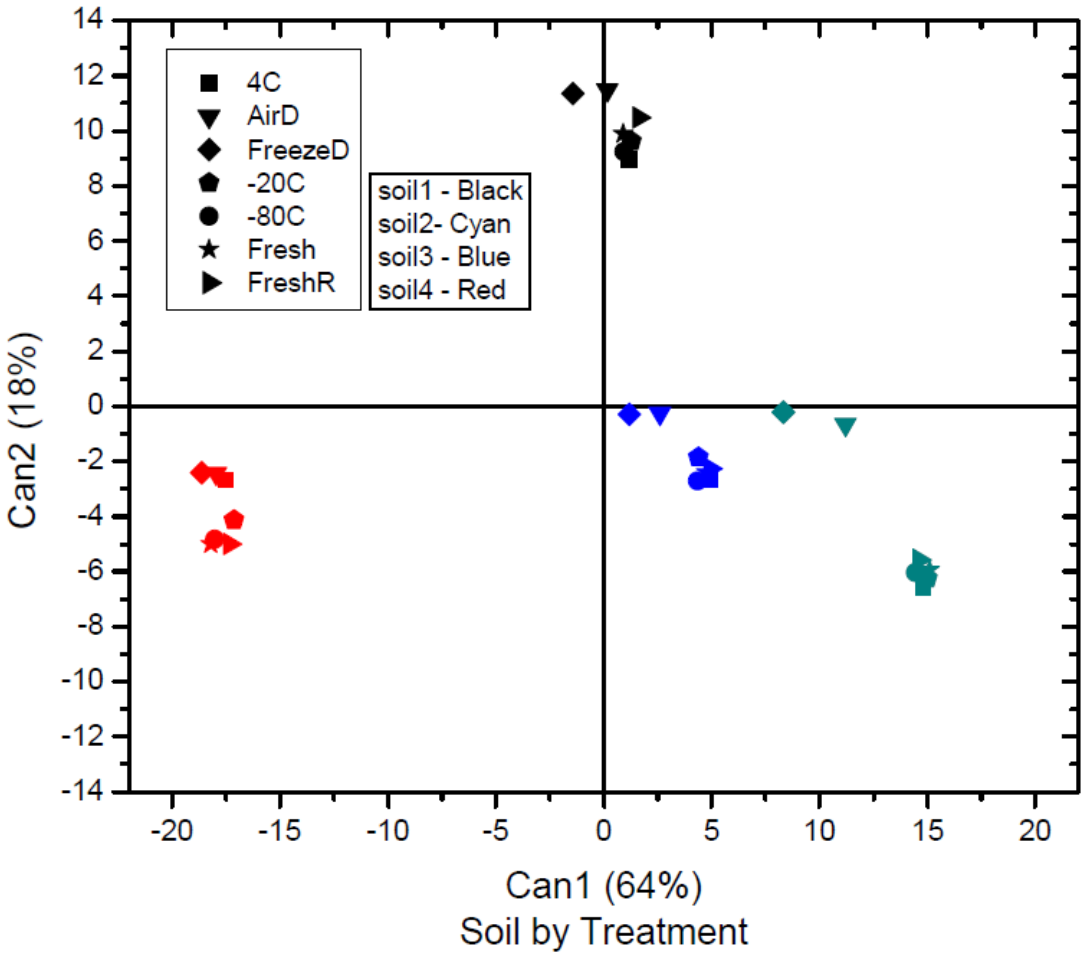

Figure 3a. Canonical correlation analysis class means of soil collection sites by storage treatments.

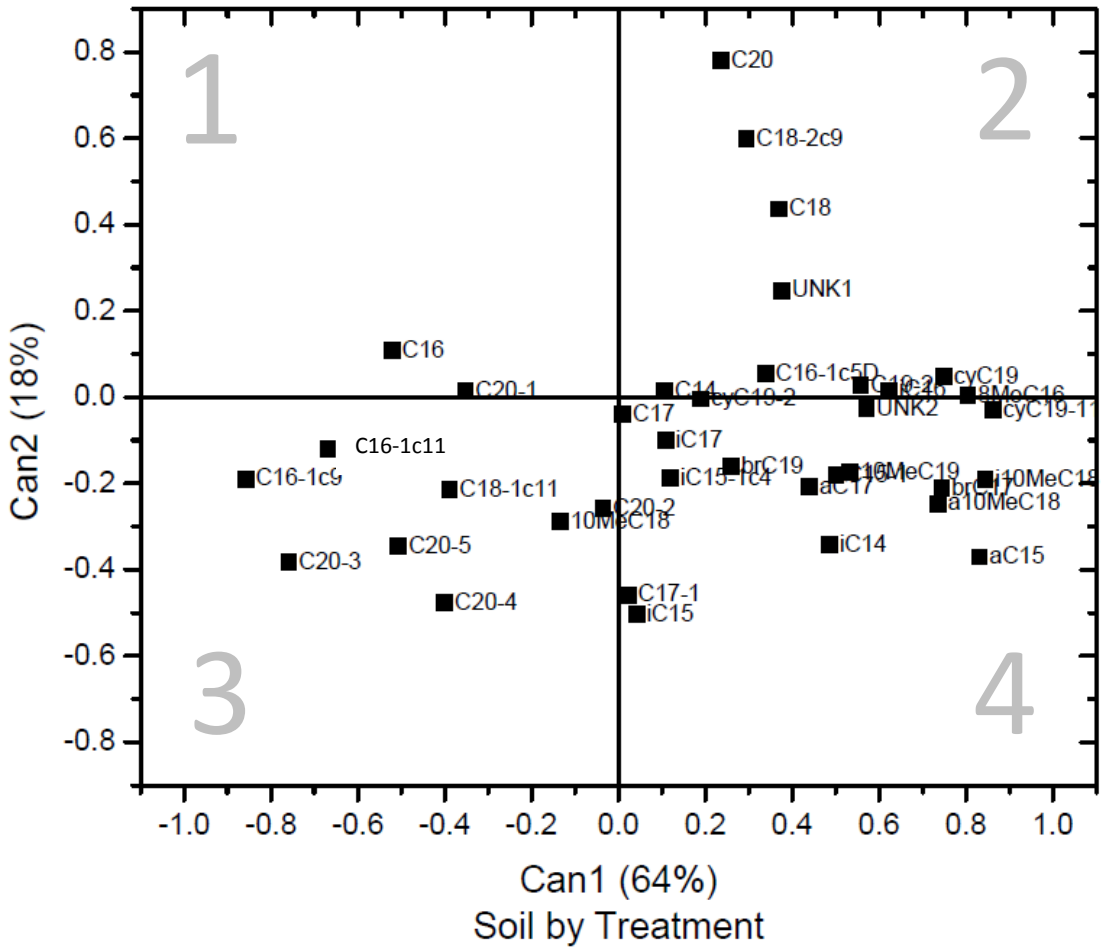

Figure 3b. Canonical correlation analysis of soil collection sites by storage treatments of influential fatty acids.

The soil collection site played a pivotal role in distinguishing between samples. When examining the fatty acids in Figure 3b, along with their corresponding concentrations in each sample, it becomes clear that the soil itself is the main factor in separation of the samples. The total fatty acids extracted for all analysis was 42. After the stepwise discriminant analysis there were 37 fatty acids that were used in producing the canonical correlation graphs. In Figure 3a the eigenvalues for the Can1 and Can2 are 63.80% and 18.21%, respectively. The eigenvalues represent the level of impact of a particular Can; the higher the eigenvalue the higher the impact of that particular Can. When represented as a percent the eigenvalue shows the percent of impact on the overall matrix that that particular Can has. The fatty acids in quadrant 2 were highest in soil 1, which caused the shift of soil 1 to the positive Can2 area. The fatty acids in quadrant 3 were highest in soil 4, this caused soil 4 to shift to the negative Can1. In all four soils the air-dried and freeze dried samples were shifted to a more positive Can2 than their respective fresh sample. The shift is caused by an increase in C:20 in both air dried (Air D) and freeze dried (Freeze D) samples with a decrease in 10MeC18 and UNK2 when compared to the other storage methods. The frozen samples (-20C and -80C) and the fresh revisited (FreshR) samples grouped close to the fresh samples, illustrating the least amount of change to the microbial community within those samples. The 4C sample was near the fresh sample in soil1, soil2, and soil3, but grouped closer to the air-dried and freeze-dried samples in soil4. The squared Mahalanobis distance P values for the four soil collection sites of the storage samples compared to their respective fresh sample are listed in Table 4. The samples highlighted in yellow have no significant difference, which shows the storage methods that produce the most similar profile to the fresh sample.

Table 4. Squared Mahalanobis distance P values for each collection site by storage treatment. (Soil1 = 1; Soil 2 = 2; Soil3 = 3; Soil4 = 4).

Soil by Treatment	1-Fresh	2-Fresh	3-Fresh	4-Fresh
1- -80	0.1843			
1- -20	0.0014			
1- 4C	<.0001			
1- Air Dried	<.0001			
1- Freeze Dried	<.0001			
1- Fresh Revisit	0.8906			
2- -80		0.0283		
2- -20		0.4396		
2- 4C		<.0001		
2- Air Dried		<.0001		
2- Freeze Dried		<.0001		
2- Fresh Revisit		0.9334		
3- -80			0.0127	
3- -20			0.9777	
3- 4C			<.0001	
3- Air Dried			<.0001	
3- Freeze Dried			<.0001	
3- Fresh Revisit			0.8961	
4- -80				0.7853
4- -20				<.0001
4- 4C				<.0001
4- Air Dried				<.0001
4- Freeze Dried				<.0001
4- Fresh Revisit				0.1740

For the three collection seasons the main discriminating factor is the collection time rather than the soil storage and handling. Samples also sorted by soil collection site as well as by time. The September collection season has an increased concentration of fatty acids C:17 and C:17-1c9 and a decrease in concentration of iC:17 relative to the

November and July collection season, thus the September season is shifted into quadrants 2 and 4 (Figure 4a). The November collection season has shifted into quadrant 3 due to its increase in concentration of the fatty acids located in quadrant 3 of Figure 4b. July collection season (Figure 4a) has shifted up into quadrant 1 due to the increase in concentration of the fatty acids located in quadrant 1 in Figure 4b. The air-dried and freeze dried samples group more positive on Can2 than the fresh samples (Figure 4a), which was also seen in soil collection site by storage treatment discrimination. After the stepwise discriminant analysis 38 fatty acids were selected for the canonical correlation analysis. The eigenvalues for the first to Cans are 55.94% and 23.66%. Table 5 has the squared Mahalanobis distance P values for the seasons by storage treatment when compared to their respective fresh sample. The numbers highlighted in yellow are not significantly different, which shows the storage treatments that preserve the microbial community without change.

Table 5. Squared Mahalanobis distance P values for each collection season by storage treatment.

Season by Treatment	S-Fresh	N-Fresh	J-Fresh
S- -80	<.0001		
S- -20	<.0001		
S- 4	<.0001		
S- Air Dried	<.0001		
S- Freeze Dried	<.0001		
S- Fresh Revisit	<.0001		
N- -80		0.0040	
N- -20		<.0001	
N- 4		<.0001	
N- Air Dried		<.0001	
N- Freeze Dried		<.0001	
N- Fresh Revisit		0.2500	
J- -80			1.000
J- -20			0.3166
J- 4			<.0001
J- Air Dried			<.0001
J- Freeze Dried			<.0001
J- Fresh Revisit			<.0001

The class means for the storage treatments from the canonical correlation analysis were plotted with the first two significant eigenvalues, Can1 and Can2. These two values accounted for 80 to 88 percent of the variability, for the plots in Figure 5a - 5h. The eigenvalues for Figures 5 a – d Can1 and Can2 are 67.82% and 16.68%; 70.87% and 15.26%; 62.26% and 17.71%; and 61.56% and 25.66% respectively. After the stepwise discriminant analysis there was 31, 27, 18, and 27 fatty acids for the canonical correlation analysis for Figures 5 a – d respectively. In Table 4 the yellow highlighted samples are not significantly different from their respective fresh sample for the overall storage treatments and for each storage treatments by each season. The overall treatment (Figure

5a) shows the fresh revisited and -80C are closest to the fresh sample (significance values listed in Table 6). Also of note, the air-dried and freeze dried samples group together. September sampling season canonical correlation analysis plot has fresh revisited and -80C as the two closest storage treatments to the fresh sample, although all samples in the September collection time are significantly different when compared to the fresh sample (Table 6). The November plot again has fresh revisited and -80C as the two closest to the fresh sample. The July collection season plot has -80C, -20C, and fresh revisited samples near the fresh sample, however, only -80C and -20C are not significantly different from the fresh sample (Table 6).

Table 7 contains the biomass from different storage treatments over the three collection seasons for all four soil types. Soil3 has the highest average biomass, followed by soil2, soil1, and then soil4. September collection had the lowest average biomass for all soils out of the three seasons. Soil1 and soil3 have the highest average biomass in July, while soil2 and soil4 have the highest average biomass in November. For the different fixed effects “Soil” has the highest F Value followed by “Season”. Only “Storage + Soil” was not significantly different.

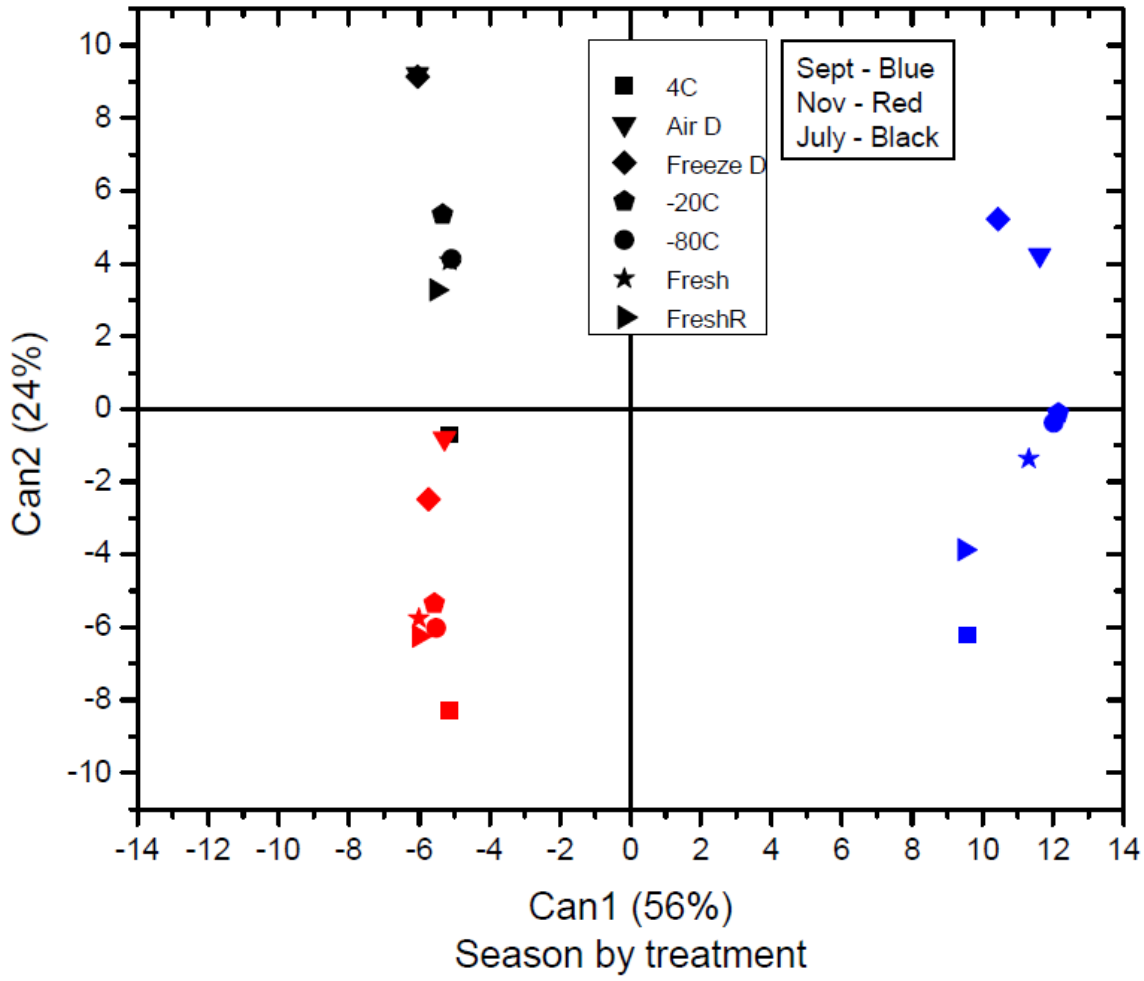

Figure 4a. Canonical correlation analysis class means of seasons of soil collection by storage treatments.

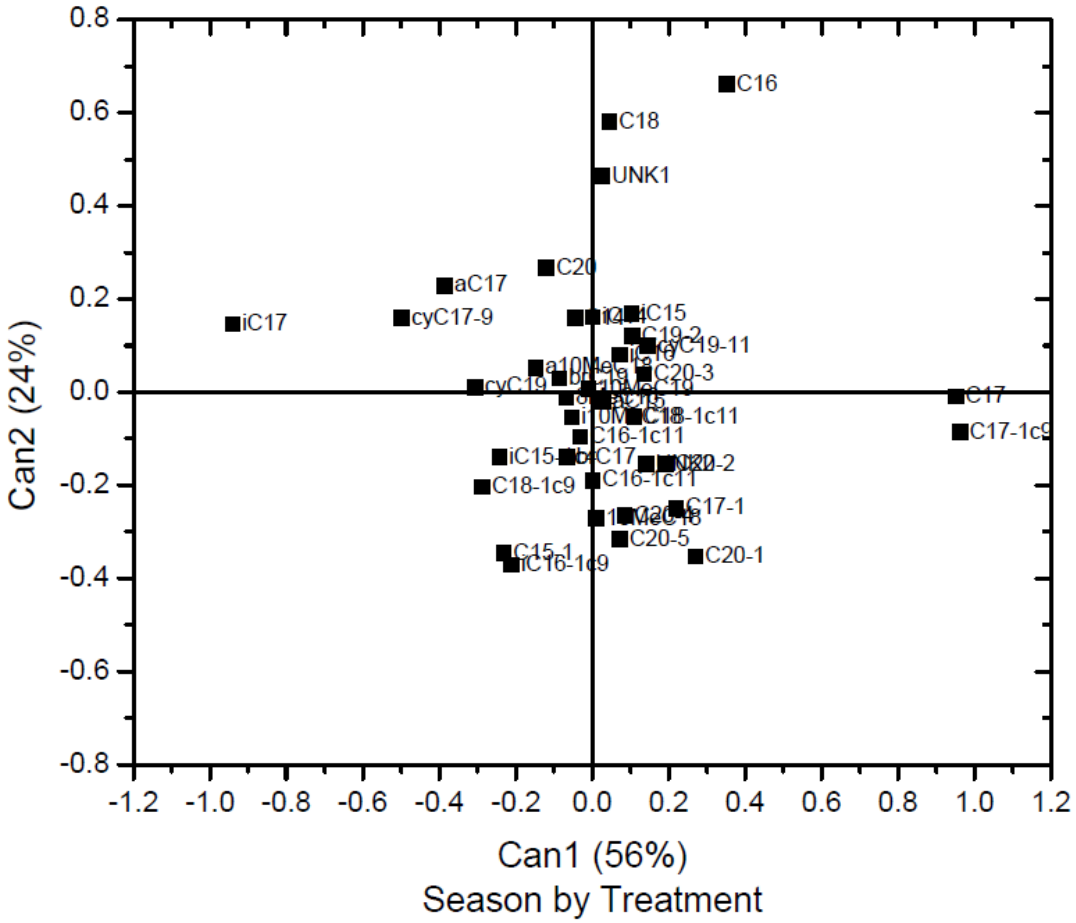

Figure 4b. Canonical correlation analysis of influential fatty acids for season of soil collection by treatment.

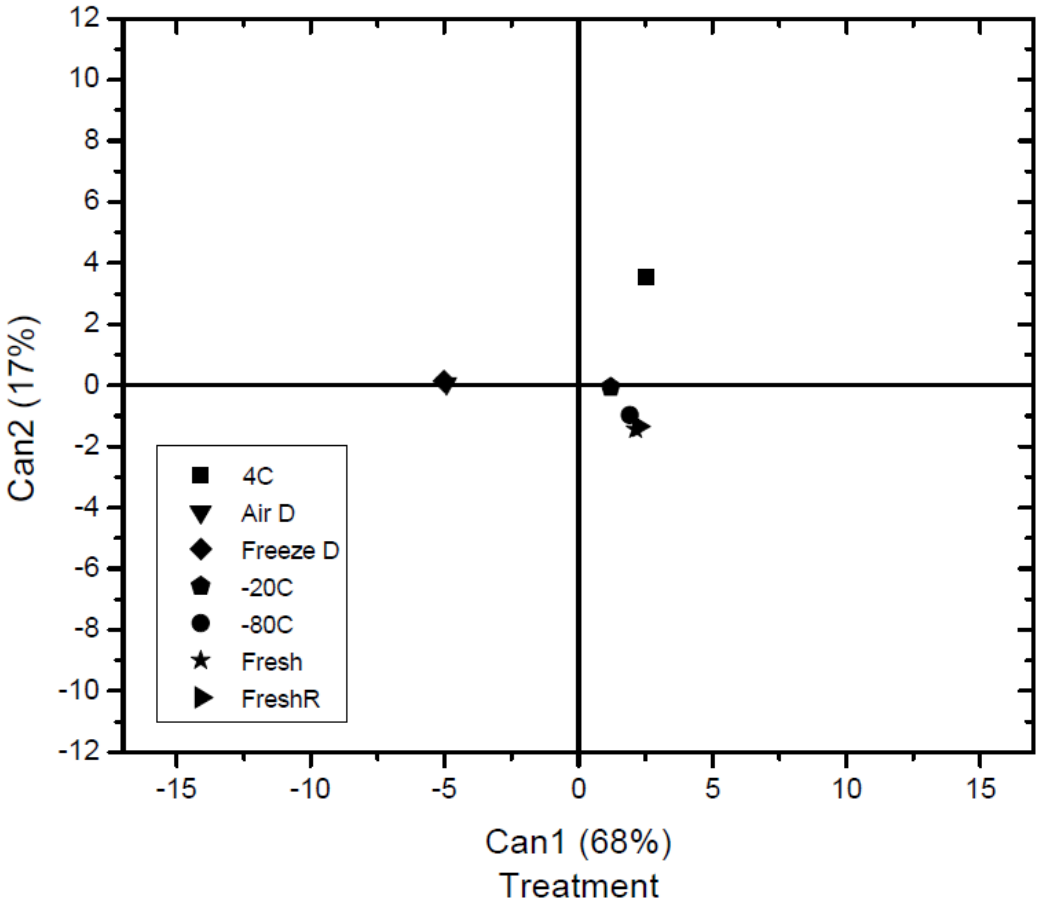

Figure 5a. Canonical correlation analysis class means for storage treatment over both season and collection site.

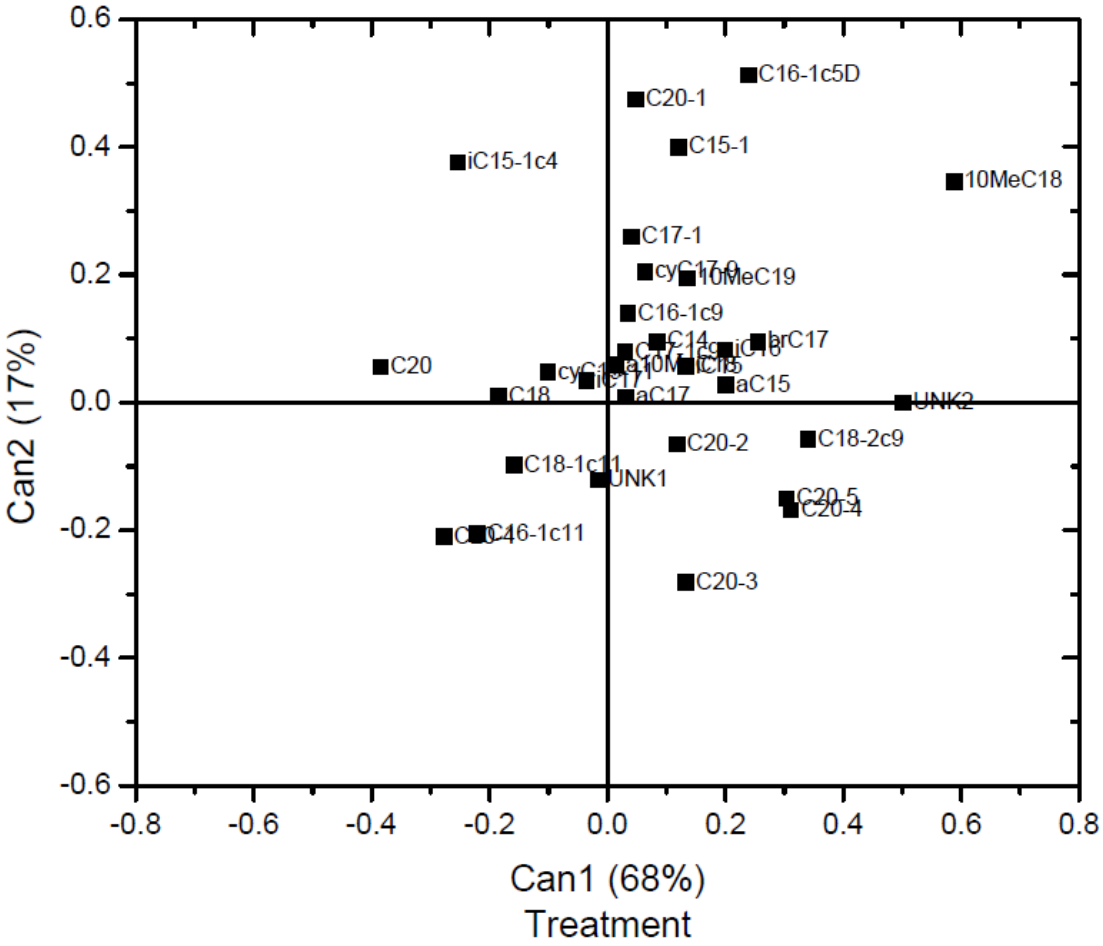

Figure 5b. Canonical correlation analysis with discriminate fatty acids of treatment over both season and soil collection site.

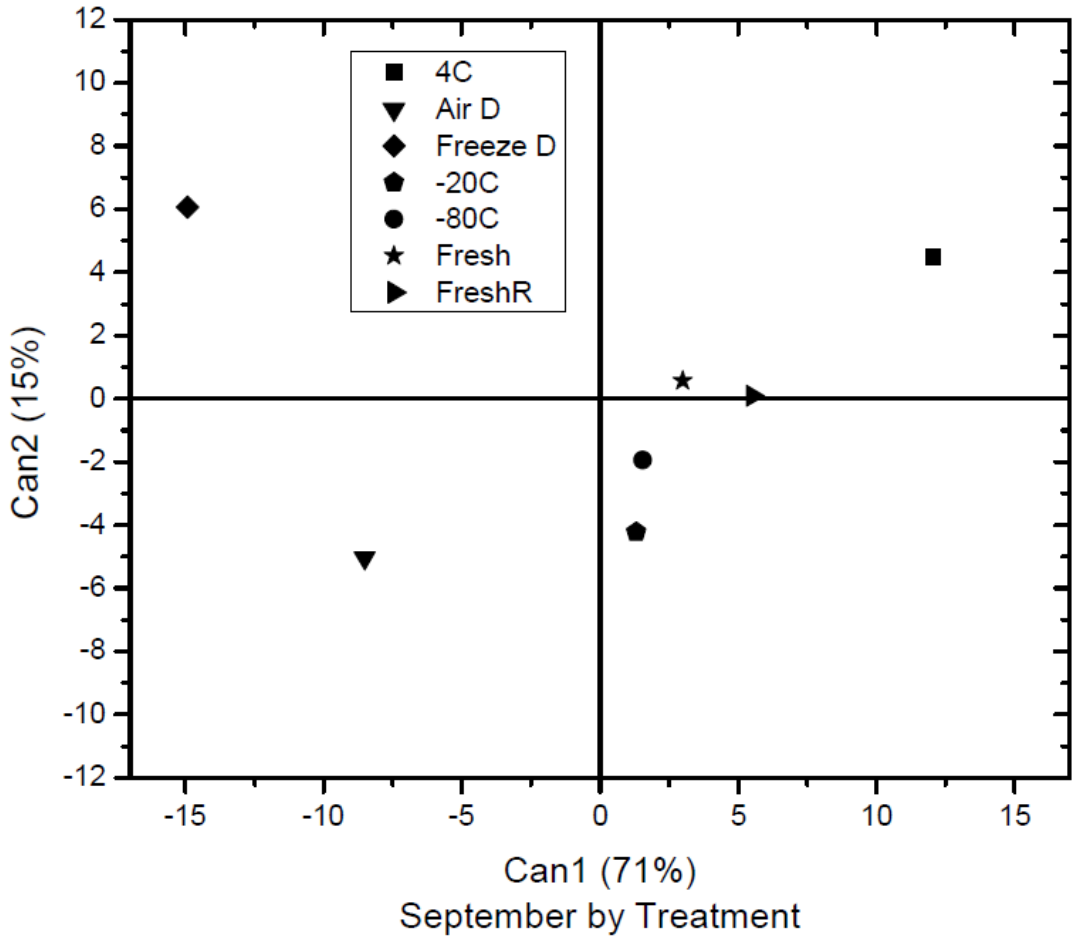

Figure 5c. Canonical correlation analysis class means of September 2010 sampling season by storage treatment.

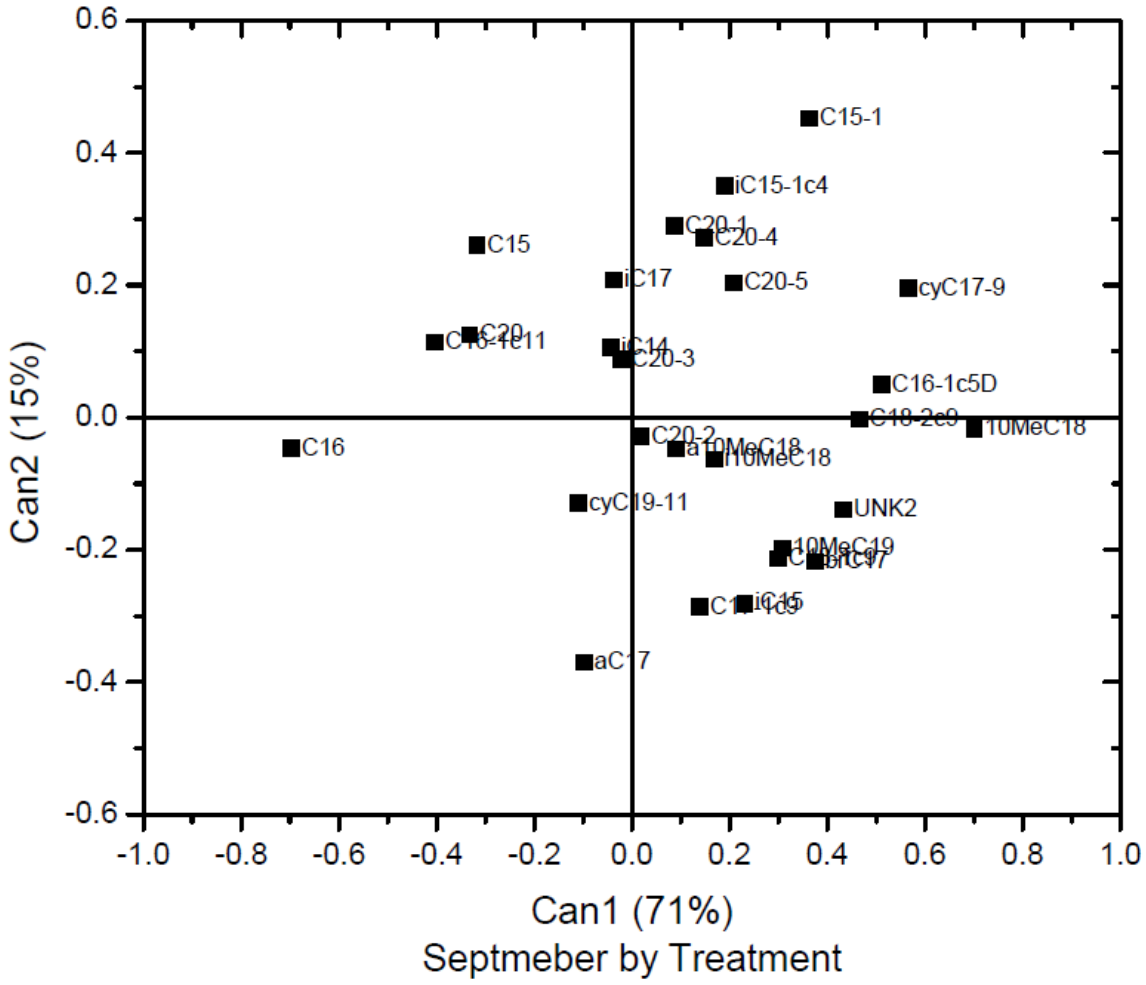

Figure 5d. Canonical correlation analysis of discriminating fatty acids for the September 2010 sampling season by storage treatment.

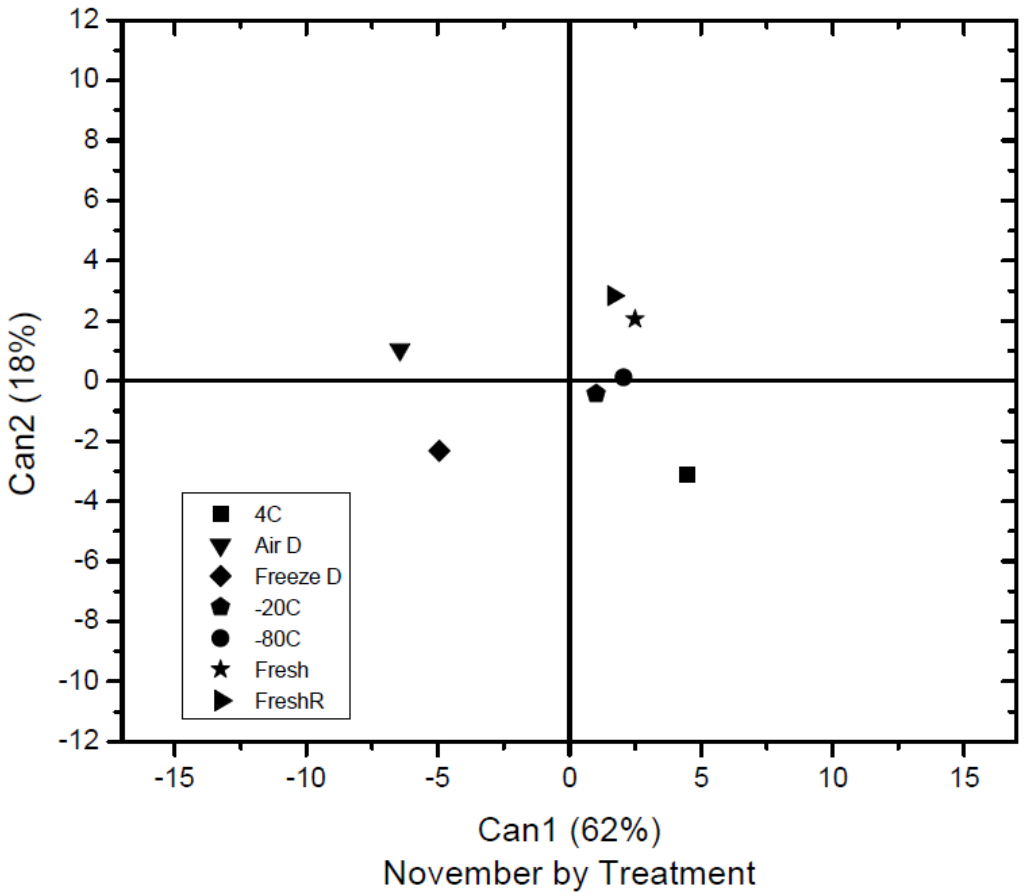

Figure 5e. Canonical correlation analysis class means for November 2010 collection season by storage treatment.

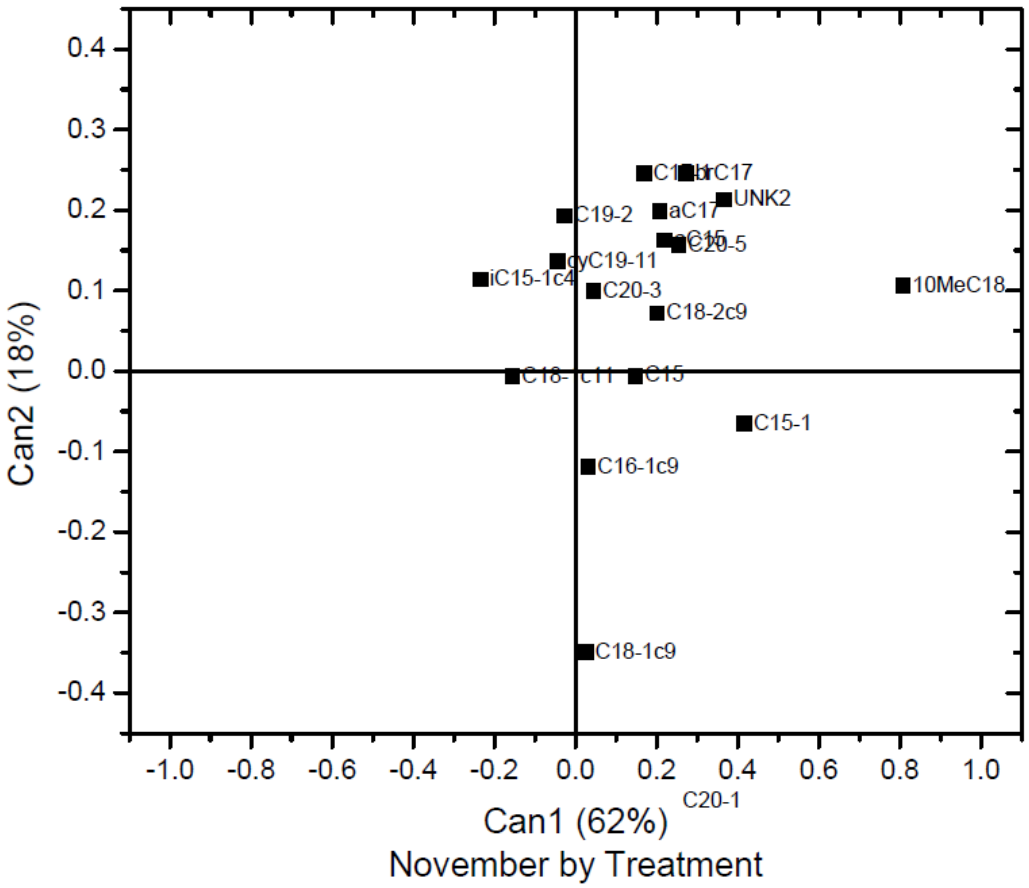

Figure 5f. Canonical correlation analysis discriminating fatty acids for the November 2010 collection season by storage treatment.

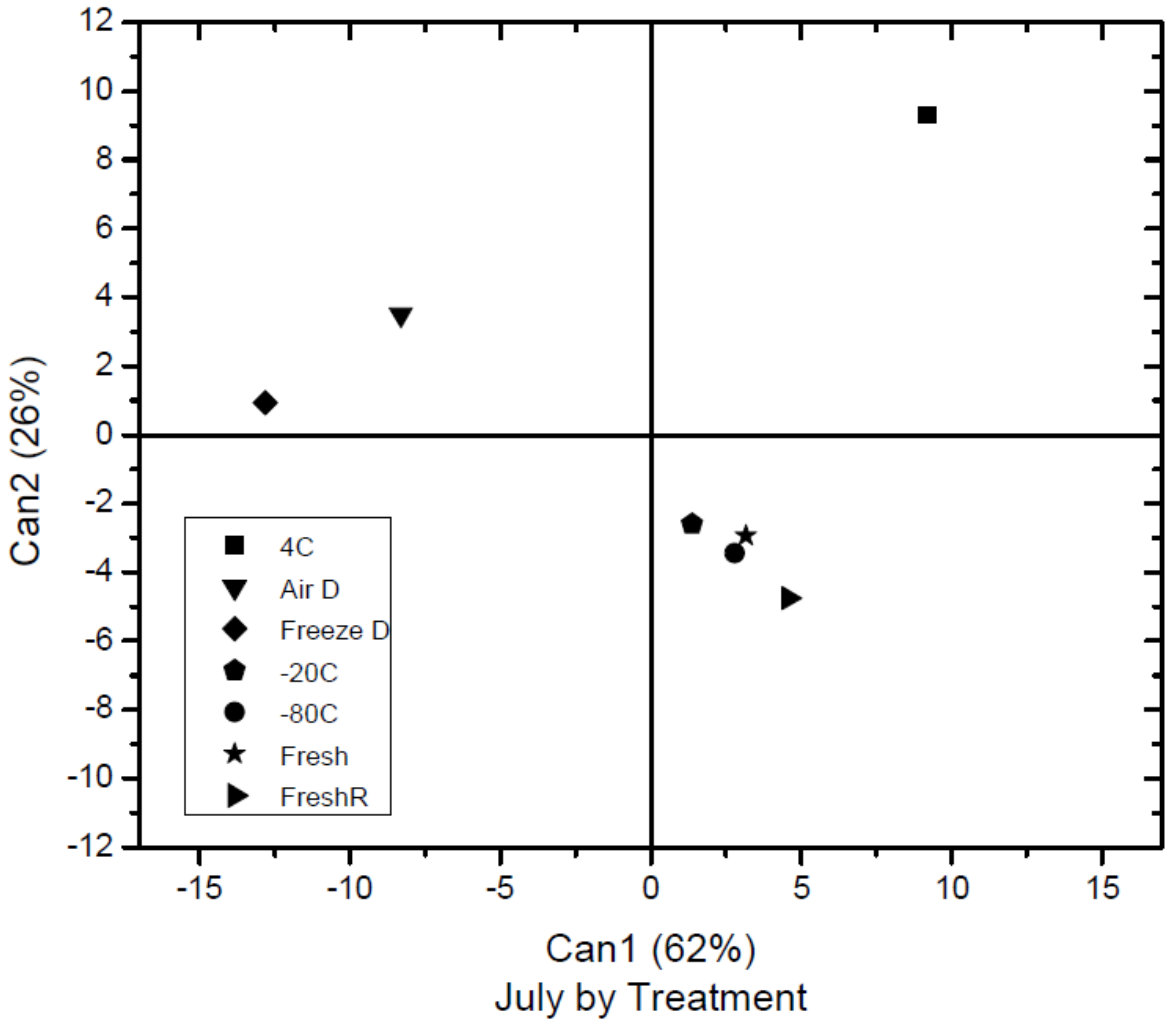

Figure 5g. Canonical correlation analysis class means for July 2011 collection season by storage treatments.

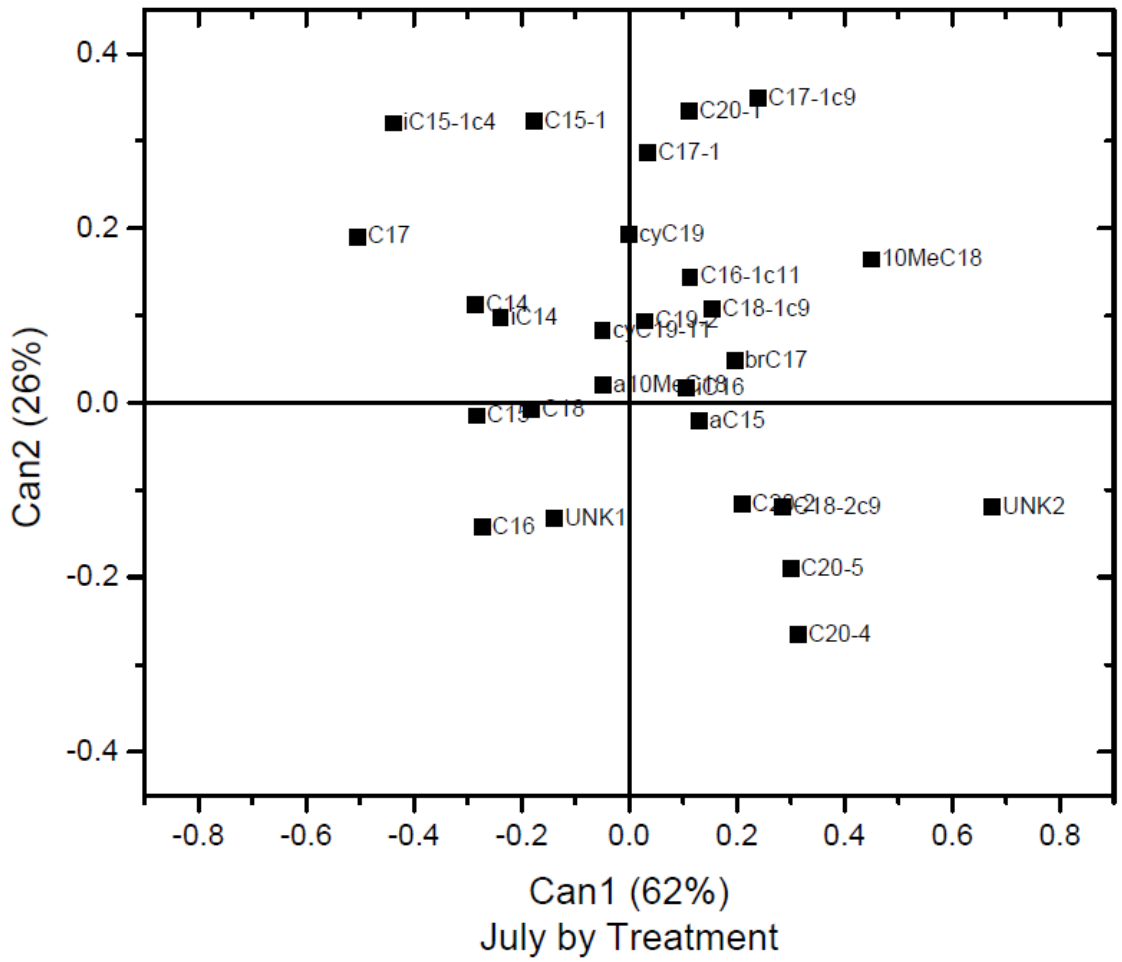

Figure 5h. Canonical correlation analysis of discriminating fatty acids for July 2011 soil collection season by storage treatment.

Table 6. Squared Mahalanobis distance P values for storage treatment compared to their respective fresh sample for overall treatment and for individual seasons.

Class Means Treatment	Fresh
-80	0.004
-20	<.0001
4	<.0001
Air Dried	<.0001
Freeze Dried	<.0001
Fresh Revisit	0.1313
September by Treatment	
-80	<.0001
-20	<.0001
4	<.0001
Air Dried	<.0001
Freeze Dried	<.0001
Fresh Revisit	<.0001
November by Treatment	
-80	0.0097
-20	<.0001
4	<.0001
Air Dried	<.0001
Freeze Dried	<.0001
Fresh Revisit	0.0018
July by Treatment	
-80	0.9994
-20	0.0006
4	<.0001
Air Dried	<.0001
Freeze Dried	<.0001
Fresh Revisit	<.0001

Table 7. Soil microbial biomass nanomoles lipid-phosphate per gram soil.

	September	November	July
Soil 1			
Fresh	333.90	317.53	449.97
-80	274.30	307.30	457.30
-20	274.10	315.80	472.20
4	266.47	324.57	438.13
Air Dried	244.77	320.20	399.93
Freeze Dried	277.77	356.57	454.57
Fresh Revisit	294.57	325.27	344.37
Soil 2			
Fresh	369.50	462.17	451.93
-80	294.60	421.73	439.73
-20	409.40	415.60	423.70
4	385.87	448.73	422.00
Air Dried	367.23	436.23	385.17
Freeze Dried	450.77	488.20	474.43
Fresh Revisit	357.83	343.67	314.17
Soil 3			
Fresh	374.23	447.23	647.53
-80	355.09	423.69	526.88
-20	370.20	430.53	516.03
4	367.53	441.13	511.43
Air Dried	282.20	401.37	516.00
Freeze Dried	352.33	472.27	638.33
Fresh Revisit	346.83	323.20	437.50
Soil 4			
Fresh	296.73	329.10	262.17
-80	244.43	326.97	297.53
-20	228.27	305.80	290.50
4	221.53	306.40	266.77
Air Dried	205.70	312.63	289.17
Freeze Dried	257.27	358.30	373.33
Fresh Revisit	232.43	251.17	200.13

	Den DF	F Value	P
Season	78.50	144.05	<0.0001
Storage	84.70	25.69	<0.0001
Soil	85.00	217.20	<0.0001
Season + Storage	78.10	4.30	<0.0001
Storage + Soil	84.10	1.69	0.0582
Season + Storage + Soil	77.00	4.04	<0.0001

Conclusions

Although soil texture is a driver of soil microbial diversity, other characteristics that are known to have a role in soil microbial community structure, such as geographic location (Meyers and Foran, 2008) and land management (Drijber et al., 2000), which are different between the soil collection sites (Table 1). These slight differences should allow for unique microbial community profiles for each collection site.

When analyzing the electropherograms it is important to have the software correctly identify the peaks of the size standard. LIZ600 was used for the size standard as it has multiple peaks in our region of interest for a more robust size determination. When running LIZ600 at a lower temperature and with CAP polymer the peaks shift and the software does not label all peaks correctly. To correct this, a size standard was set using the Genemapper software that labeled the LIZ600 from 80 bp to 320 bp.

The use of CE-SSCP allows for both the forward and reverse primers to give different profiles due to sequence differences in the primers that may cause secondary folding differences of the single stranded DNA. The forward and reverse profiles were analyzed for a more robust output. In Figure 5 the comparison of the forward (Figures 5a – 5c) versus the reverse (5d – 5f) primers illustrates this difference in profiles. This difference in profiles is also clear on the electropherograms from the 3130 genetic analyzer. Figure 5a – 5f also illustrates there is a difference for each season of collection as well as differences in the soil were the samples were collected. The difference for each season can be correlated with the differences in precipitation and temperature for the different

seasons. The September 2010 collection season had nine days with precipitation for a total of 9.4 cm of rain. The temperature ranged from highs of 32.7˚C to 14.4˚C and lows of 18.8˚C to 6.1˚C. The November 2010 collection season precipitation had a total of 4 days with rain for a total 5 cm of water. The temperature had the greatest ranges out of all three seasons with highs of 23.3˚C to -2.2˚C and lows from 7.7˚C to -11.1˚C. The July / August 2011 season had 15 days with precipitation for a total of 11.1 cm of rain. The temperature highs were 40.0˚C to 26.1˚C and the lows were from 13.3˚C to 25.2˚C.

The significant differences in the combined soil section between fresh vs fresh revisited shows that there is a significant difference over the four soil types during the two week sampling period. This is important because it demonstrates that the CE-SSCP technique can be used to discriminate one soil from another. Also, Fresh vs 4C and Fresh vs -80C in the combined were significantly different during the November collection season for both forward and reverse primers. This shows that there is a difference between fresh and 4C or fresh and -80C between the four soil types. Thus, the combined table illustrates that different soils react differently to contrasting storage treatments. Similar observations were seen in a storage method study by Tzeneva et al. (2009). They observed that soil type was a stronger influence on microbial community profile than storage itself. When each soil was examined individually only the overall sample generated significant differences.

The current data also illustrate that storage treatment for examining the DNA profile does not play a major role in the microbial profile. This could be because the microbes are

generally encased in aggregates and they were protected from degradation within those soil aggregates. With the use of both the forward and reverse primers some of the samples are significantly different for both primers and some are only significantly different for one primer. This allows for determining the very robust significant changes to the microbial community and identifying the unique changes to the community as well.

The CE-SSCP method is extremely sensitive and thus storage of the soil does not alter the soil microbial DNA profile when examining one soil type at a time. When multiple soils are compared to each other there is a significant difference in their individually microbial community's reactions to storage. Thus thoughtful collection of soils must be done to have a realistic reference sample when using this method in environmental applications. Another area of further validation is the potential for variability between samples caused by technicians. The reproducibility has been examined by other laboratories, but may need to be tested in every lab for complete validation of users (Zinger et al. 2007).

Fatty Acid Analysis

When accounting for differences in the microbial community, collection site was the biggest discriminating factor, followed by season and storage treatment (Tzeneva et al., 2008). The four soils used were all classified as loam texture, but still had unique microbial communities (Singh et al., 2007). This may stem from differing geographic locations, land management, and concentrations of silt, sand, and clay (Table 1). These

differences are enough to give a unique microbial community profile to each soil (Drijber et al., 2000; Bradley et al., 2006).

Oven drying samples was initially meant to be a negative control, knowing that the fatty acids from the soil microorganism would be significantly degraded. Including the oven-dried samples in the analysis shifted the discriminant analysis, thus altering the significance of the other storage and handling methods. For this reason we removed all oven dried samples from the analysis, knowing that oven drying for storage of samples is not suitable for examining the soil microbial profile.

Air Dried samples tended to group with freeze-dried samples in the canonical correlation plots, although, there is a significant difference between the two samples in all comparisons (data not shown). This illustrates that the two methods have changes within their microbial community that are different, but the changes in both of the storage treatments, increase or decrease in similar FAMEs. Samples stored at 4C most often grouped with the frozen samples and fresh samples, however when the soils were examined by soil collection site, at soil4 4C grouped closely to air-dried and freeze dried. This variability shows that 4C is not the best storage option for soil samples to preserve the microbial community.

The frozen samples, -20C and -80C, were most often the closest storage treatment to the fresh sample. Tables 4 - 6 have samples that were not significantly different from the fresh sample, meaning the microbial community did not significantly change over the 5

week storage period (thus preserving the integrity of the sample) highlighted in yellow. Although both -20C and -80C grouped fairly close to the fresh in the Canonical correlation plots, -80C was more often not significantly different from the fresh sample than -20C. Therefore -80C was the best storage method for soils when examining the microbial community via FAME, followed by -20C. Wallenius et al. (2010) came to a similar conclusion when storing soils.

Not only was change of the soil microbial community during the storage of soil examined, but also within the soil at the collection site as well. This is what the fresh revisited sample emulates, as it was collected two weeks after the fresh sample. The fresh revisited sample always grouped near the fresh sample in the Canonical correlation plots. For the July season samples the fresh revisited sample was significantly different from the fresh sample (Table 6) meaning the microbial community within the soil at the collection site was significantly altered over a two-week period. During the September sampling season all samples were significantly different from the fresh sample. One explanation is the changes that occur from the plant reproductive stage alters the soil microbial community (Grigera et al., 2007). This change could also be due to precipitation over the collection time. Precipitation patterns were similar for September and November, with a large rain early in the collection season and a few smaller rains scattered throughout the collection season. In July the precipitation pattern was different in that there was often precipitation, but in smaller amounts. Precipitation plays a key role in activity of microorganisms within the soil (Orchard and Cook, 1983). The water allows for the movement of nutrients as well as the movement of the microorganism themselves.

When examining the differences between seasons three fatty acids were noted: C:17, C:17-1c9, and iC:17. All three are bacterial markers. This would indicate that in September C:17 and C:17-1c9 concentrations of these bacteria were higher than in the other two seasons, and vise versa with iC:17. This may be due to the ambient temperatures during the seasons. September had highs from 32.7˚C to 14.4˚C and lows from 18.8˚C to 6.1˚C. July temperatures had the narrowest range of the three seasons with highs of 40.0˚C to 26.1˚C and lows of 25.5˚C to 13.3˚C. November had the largest range in temperature with highs of 23.3˚C to -2.2˚C and lows of 7.7˚C to -11.1˚C. The differences in temperature could account for activity of different microbes, thus a different FAME profile for each season.

The process of utilizing the FAME method to extract evidentiary value in a criminal case has been demonstrated as a viable option here. The ability of the FAME method and statistically analysis to distinguish between and among soil samples was illustrated at a high level discrimination. Ultimately we found that to preserve the soil microbial community, the soil must be stored at -80˚C. It is understood that not all laboratories have access to such equipment so the second best storage method is -20˚C.

To have the most robust use for forensic science a soil microbial community profile database would be ideal. The potential for such database is viable as soil itself is the number one discriminating factor. Soils that have been archived could be used and new collections would need to be made. But a database would provide the ability to reliably

add a probability percentage that would be similar to human DNA, thus a more concrete evidentiary value.

Appendix A. Soil Collection Site

The four soil collection sites were set up in late August of 2010. Dr. Mark Zulia helped in identifying soils with different textures using the USDA soil map on a Nebraska game and parks reserve. Dr. Drijber suggested a location near Raymond Nebraska for a soil with a higher sand content. The plots at each collection site were marked by orange flags placed in the ground. Soil2 had the flags removed in between the November 2010 and July / August 2011 collection. Thus it is possible the exact same plots were not used, but would have been extremely close to the original plots with the aid of GPS coordinates. Before and during the July / August 2011 collection season cow / calf pairs were added to soil1 and soil2 sites. The compaction and feces from the animals may have caused some changes to the microbial profile. The soil samples were sent to Ward Laboratories, Inc. for physicochemical analysis.

Table A1. Complete physicochemical characteristics for all four collection soils and two control soils from Ward Lab, Inc, Kearney, Nebraska, USA.

	Soil 1	**Soil 2**	**Soil 3**	**Soil 4**	Standard	Standard
1:1 Soil pH	6.3	6.3	6.3	6.1	6.1	6.1
Modified WDRF BpH	6.9	6.8	6.9	6.9	6.8	6.7
Soluble Salts 1:1, mmho/cm	0.16	0.28	0.37	0.32	0.39	0.39
Excess Lime Rating	None	None	None	None	none	none
Organic Matter LOI, %	2.9	6	4.9	2.8	3.2	3.3
Nitrate-N KCl, ppm N	3.3	14.5	18.4	13.6	4.3	3.9
Nitrate-N, lbs N / Acre	2	9	11	8	3	2
Ammonium-N, KCl ppm N	7.1	19.1	16.5	9.6	19	17.1
Ammonium-N, lbs N / Acre	4	11	10	6	11	10
Phosphorus Bray P1, ppm P	2	8	20	6	22	22
Potassium NH4OAc, ppm K	208	412	376	325	451	429
Sulfate Ca-P, ppm S	9	5	8	7	39	37
Zinc DTPA, ppm Zn	0.92	1.83	1.43	0.43	5.64	3.9
Iron DRPA, ppm Fe	21.9	26.1	48.4	15.8	60.7	52.2
Manganese DTPA, ppm Mn	6.1	8.6	6.6	10.3	78.7	71.8
Copper DTPA, ppm Cu	2.67	1.29	1.54	1.47	13.23	8.65
Calcium NH4OAc, ppm Ca	1633	1640	1855	1842	1920	1813
Mangesium NH4OAc, ppm Mg	358	436	261	468	372	352
Sodium NH4OAc, ppm Na	16	19	18	16	19	22
Total Carbon, % C	2.03	2.93	2.43	1.45	2.12	2.16
Total N, ppm N	1727	2886	2583	1585	2349	2370

Table A2. Examination of specific elements within all four soils and two control soils from Ward Laboratories, Inc., Kearney, Nebraska, USA.

Soil Collection	Sum of Cations	% Saturation				
	me/100g	**H**	**K**	**Ca**	**Mg**	**Na**
Soil 1	13.2	11	4	61	23	1
Soil 2	14.5	10	7	57	25	1
Soil 3	13.7	9	7	67	16	1
Soil 4	14.7	5	6	62	27	0
Standard	15.8	16	7	57	19	1
Standard	15.8	12	7	60	20	1

Table A3. Soil texture for all soils as determined at Ward Laboratories Inc. Kearney, Nebraska, USA

Soil Collection	**% Sand**	**% Silt**	**% Clay**
Soil 1	48	36	16
Soil 2	30	50	20
Soil 3	32	50	18
Soil 4	36	42	22

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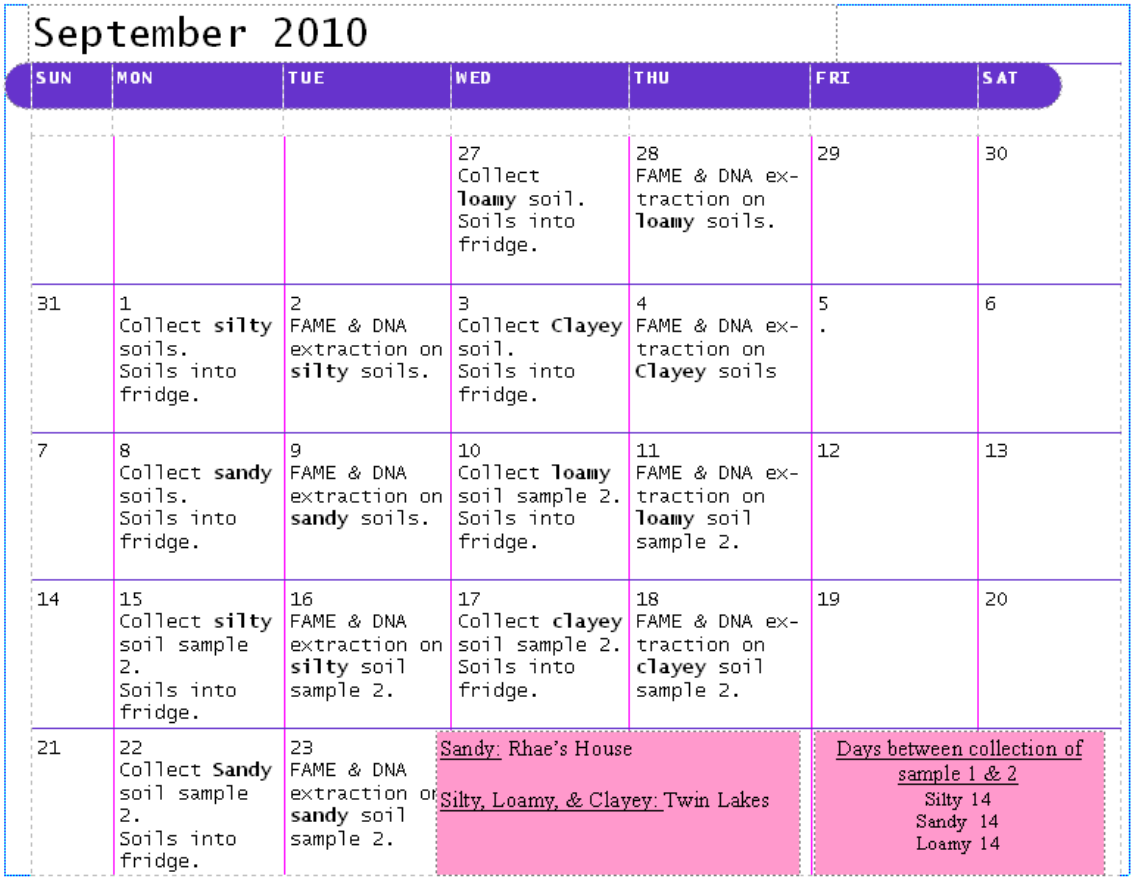
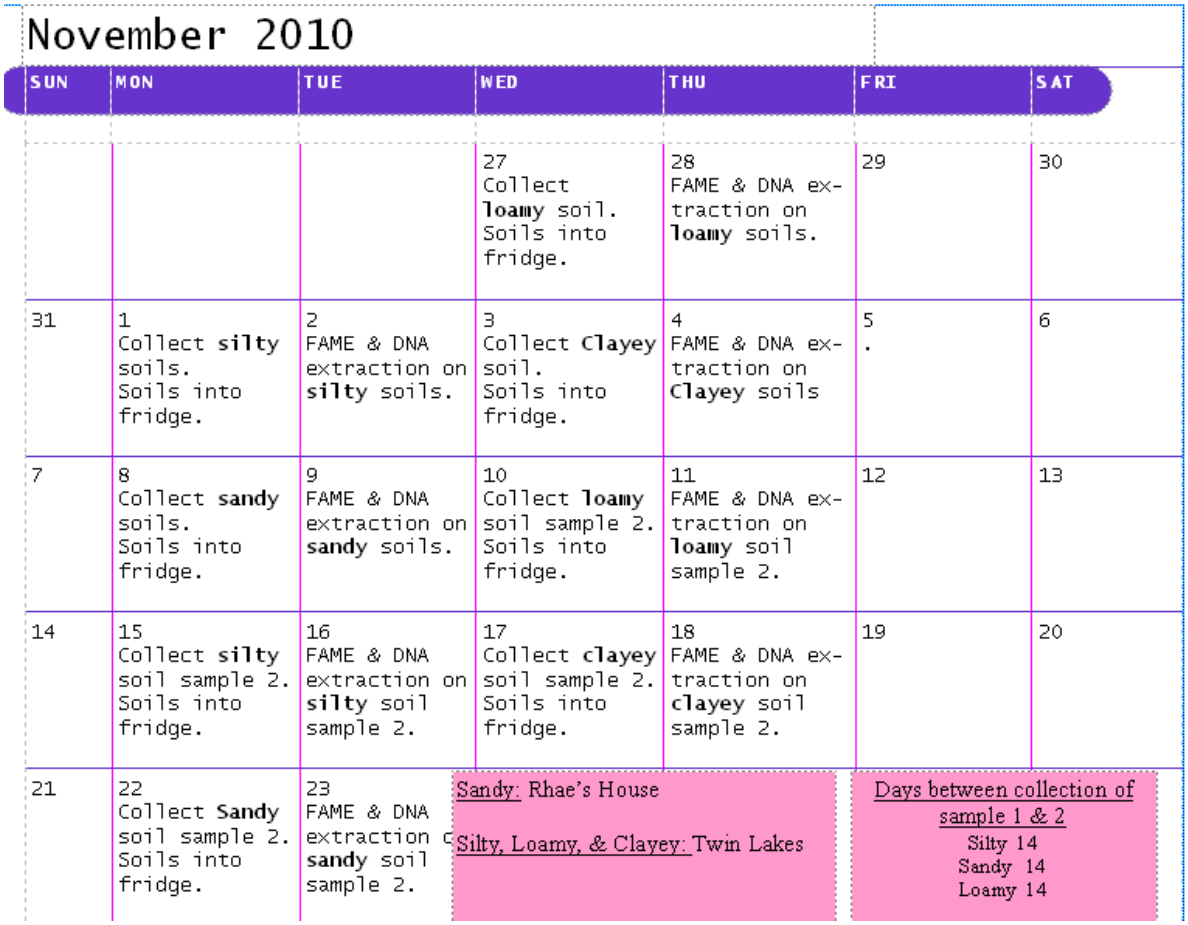


September 2010

SUN	MON	TUE	WED	THU	FRI	SAT
			27 Collect **loamy** soil. Soils into fridge.	28 FAME & DNA extraction on **loamy** soils.	29	30
31	1 Collect **silty** soils. Soils into fridge.	2 FAME & DNA extraction on **silty** soils.	3 Collect **Clayey** soil. Soils into fridge.	4 FAME & DNA extraction on **Clayey** soils	5 .	6
7	8 Collect **sandy** soils. Soils into fridge.	9 FAME & DNA extraction on **sandy** soils.	10 Collect **loamy** soil sample 2. Soils into fridge.	11 FAME & DNA extraction on **loamy** soil sample 2.	12	13
14	15 Collect **silty** soil sample 2. Soils into fridge.	16 FAME & DNA extraction on **silty** soil sample 2.	17 Collect **clayey** soil sample 2. Soils into fridge.	18 FAME & DNA extraction on **clayey** soil sample 2.	19	20
21	22 Collect **Sandy** soil sample 2. Soils into fridge.	23 FAME & DNA extraction or **sandy** soil sample 2.	Sandy: Rhae's House Silty, Loamy, & Clayey: Twin Lakes		Days between collection of sample 1 & 2 Silty 14 Sandy 14 Loamy 14	

Figure A1. Soil collection calendar for September 2010. (Loamy = soil1, clay = soil2, silty = soil3, and sandy = soil4)



November 2010

SUN	MON	TUE	WED	THU	FRI	SAT
			27 Collect **loamy** soil. Soils into fridge.	28 FAME & DNA extraction on **loamy** soils.	29	30
31	1 Collect **silty** soils. Soils into fridge.	2 FAME & DNA extraction on **silty** soils.	3 Collect **Clayey** soil. Soils into fridge.	4 FAME & DNA extraction on **Clayey** soils	5 .	6
7	8 Collect **sandy** soils. Soils into fridge.	9 FAME & DNA extraction on **sandy** soils.	10 Collect **loamy** soil sample 2. Soils into fridge.	11 FAME & DNA extraction on **loamy** soil sample 2.	12	13
14	15 Collect **silty** soil sample 2. Soils into fridge.	16 FAME & DNA extraction on **silty** soil sample 2.	17 Collect **clayey** soil sample 2. Soils into fridge.	18 FAME & DNA extraction on **clayey** soil sample 2.	19	20
21	22 Collect **Sandy** soil sample 2. Soils into fridge.	23 FAME & DNA extraction o **sandy** soil sample 2.	Sandy: Rhae's House Silty, Loamy, & Clayey: Twin Lakes		Days between collection of sample 1 & 2 Silty 14 Sandy 14 Loamy 14	

Figure A2. Soil collection calendar for November 2010 season. (Loamy = soil1, clay = soil2, silty = soil3, and sandy = soil4)

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
		19 **JULY 2011**	20 Collect 200	21 Extract 200	22	23
24	25 Collect 300	26 Extract 300	27 Collect 400	28 Extract 400	29	30
31	1 Collect 500 **AUGUST 2011**	2 Extract 500	3 Collect 200 revisit	4 Extract 200 revisit	5	6
7	8 Collect 300 revisit	9 Extract 300 revisit	10 Collect 400 revisit	11 Extract 400 revisit	12	13
14	15 Collect 500 revisit	16 Extract 500 revisit	17	18	19	20

Figure A3. Soil collection calendar for July / August 2011 collection season. (Loamy = soil1, clay = soil2, silty = soil3, and sandy = soil4)

Figure A4. Aerial view of the four soil collection sites. From left to right: Soil3, Soil4, Soil2, and Soil1.

APPENDIX B: DNA ANOSIM Results

ANOSIMs data is designed to identify if a sample is more similar to its group or more similar to an out group. An example would be is -20 sample from soil1, plot1 more similar to -20 sample from soil1, plot2 and plot3 or more similar to samples from soil2, soil3, or soil4? ANOSIM reports its results with an R value. This value is between +1 and -1. An R value equal to or close to 0 means no change is occurring, or rather the -20 sample from soil1, plot1 is most similar to -20 samples from soil1, plot2 and plot3. For each R value a P value can be obtained to demonstrate the confidence or significance in the R value. The P values are in Tables 2, 3, and 4 in Chapter 2. The R values from the ANOSIMs are located below in Tables 1, 2, and 3.

Table B1. ANOSIMs R values for storage samples compared to fresh samples with combined soil sites over three seasons.

	September	November	July/August
Forward Primer			
Combined			
Overall	0.0441	0.0141	0.0096
fresh vs. -20C	0.0043	-0.0298	-0.0611
fresh vs. 4C	0.0672	0.0208	-0.0320
fresh vs. -80C	0.0319	0.0354	-0.0458
fresh vs. air dried	-0.0230	0.0230	-0.0183
fresh vs. freeze dried	-0.0389	-0.0567	0.0193
fresh vs. fresh revisited	0.2602	0.0968	0.1531
Reverse Primer			
Combined			
Overall	0.0367	0.0389	-0.0023
fresh vs. -20	-0.0641	0.0075	-0.0467
fresh vs. 4C	-0.0318	0.0791	-0.0412
fresh vs. -80	0.0085	0.1268	-0.0826
fresh vs. air dried	-0.0676	-0.0035	-0.0418
fresh vs. freeze dried	-0.0671	0.0291	-0.0278
fresh vs. fresh revisited	0.1945	0.1600	0.1595

Table B2. ANOSIMs R values for forward primer for storage treatment compared to fresh sample for each soil site over three seasons.

	September	November	July/August
Soil1			
Overall	0.1348	0.0945	0.1177
fresh vs. -20C	0.2593	-0.2963	-0.1481
fresh vs. 4C	0.5926	-0.0741	0.1481
fresh vs. -80C	0.0741	0.0741	-0.1111
fresh vs. air dried	0.2222	0.2593	0.0741
fresh vs. freeze dried	0.0370	0.0370	0.3333
fresh vs. fresh revisited	0.4815	0.3333	0.4074
Soil2			
Overall	0.0950	0.0723	0.3656
fresh vs. -20C	-0.2222	0.1481	-0.2222
fresh vs. 4C	-0.0370	0.1852	-0.0370
fresh vs. -80C	-0.1481	0.0370	-0.0741
fresh vs. air dried	0.2963	-0.1481	0.0370
fresh vs. freeze dried	-0.2593	0.1852	0.0000
fresh vs. fresh revisited	0.0741	0.2593	0.3333
Soil3			
Overall	0.1736	0.1212	-0.0315
fresh vs. -20C	-0.0741	-0.1481	-0.3333
fresh vs. 4C	-0.3704	0.0370	-0.2593
fresh vs. -80C	0.1481	0.1481	-0.2222
fresh vs. air dried	-0.0370	0.1481	0.2222
fresh vs. freeze dried	0.3333	-0.0741	0.2222
fresh vs. fresh revisited	0.7778	0.1481	0.0000
Soil4			
Overall	0.1887	0.4512	0.0773
fresh vs. -20C	-0.3704	0.0000	-0.3333
fresh vs. 4C	0.7037	0.4444	0.1481
fresh vs. -80C	0.1111	0.8519	0.1111
fresh vs. air dried	0.3333	0.3333	0.0370
fresh vs. freeze dried	0.0370	1.0000	0.1852
fresh vs. fresh revisited	0.2222	0.8148	0.0741

Table B3. ANOSIMs R values for the reverse primers of storage treatment compared to fresh sample for four soil sites over three seasons.

	September	November	July/August
Soil1			
Overall	0.0990	0.0859	0.2240
fresh vs. -20	0.0000	-0.1481	-0.1111
fresh vs. 4C	0.5556	0.0370	0.0741
fresh vs. -80	0.1852	0.2963	-0.2222
fresh vs. air dried	0.3333	0.0741	-0.1111
fresh vs. freeze dried	0.0741	-0.0741	0.4074
fresh vs. fresh revisited	0.2222	0.2593	0.7037
Soil2			
Overall	0.1282	0.2744	0.3711
fresh vs. -20	-0.4074	0.4074	-0.2222
fresh vs. 4C	-0.4444	0.2222	0.1111
fresh vs. -80	-0.1111	0.3704	0.1111
fresh vs. air dried	0.3704	0.0000	0.1481
fresh vs. freeze dried	-0.4074	0.5185	0.1852
fresh vs. fresh revisited	0.3704	0.3333	0.9259
Soil3			
Overall	0.2600	0.1010	0.0396
fresh vs. -20	0.1852	0.1111	0.0370
fresh vs. 4C	-0.1111	0.1111	0.0370
fresh vs. -80	0.0000	-0.2963	0.0370
fresh vs. air dried	-0.1481	0.4815	-0.1111
fresh vs. freeze dried	0.3333	-0.2222	-0.2222
fresh vs. fresh revisited	0.7407	0.4815	-0.1481
Soil4			
Overall	0.2381	0.2769	0.0506
fresh vs. -20	0.0370	-0.1852	-0.0370
fresh vs. 4C	0.5556	0.2963	0.3333
fresh vs. -80	-0.0741	0.1852	0.1111
fresh vs. air dried	0.5556	-0.0370	0.2593
fresh vs. freeze dried	0.4074	0.2963	0.3333
fresh vs. fresh revisited	0.7778	1.0000	0.2963

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