Determining the impact of carrion decomposition on soil microbial activity levels and community composition

By

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Abstract

The ubiquitous nature of microorganisms and their specificity to certain locations make them potentially useful for forensic investigators. Advances in microbial profiling techniques have made it possible to compare microbial community profiles obtained from evidence or crime scenes to individuals and vice versa. Profiling microbial communities associated with cadaver decomposition may provide useful information concerning post-mortem intervals and aid in the identification of clandestine graves. Four experiments using pigs as human decomposition analogues were performed over the course of 2011 and 2012 in southern Ontario to document changes in soil microbiology following decomposition. Studies were conducted in the spring and summer to document the effect of environmental conditions on the decomposition process and subsequent changes in gravesoil microbiology. Microbial activity was measured using a fluorescein diacetate assay as a preliminary indicator of changes within the soil microbial population. Both decreases and increases in microbial activity were observed throughout each decomposition experiment indicating that the microbial response to decomposition is complex. It is believed that environmental conditions and decomposition rates play a role in determining how taphonomic events affect soil microbial activity. Fatty acid methyl esters (FAME) profiling was used document community level changes throughout decomposition. Shifts in FAMEs profiles were brought on by the onset of active decay and persisted through to the dry remains stage. The fatty acids 3OH 12:0, 12:0, 16:0 and 18:0 were frequently found in higher amounts in gravesoils and may prove useful as markers of cadaver decomposition. Metagenomic profiles of soil microbial communities were obtained using Illumina® sequencing. Decomposition was associated with changes

in microbial community composition. This allowed gravesoil samples to be differentiated from control samples for an extended period of time. Bacteria responsible for the shift in microbial profiles are those commonly associated with cadaver decomposition. Both sets of soil profiles indicated that weather had an effect on microbial community composition. Results highlight the need to document natural changes in microbial communities over seasons and years to establish normal microbial patterns to effectively use soil microbial profiles as post-mortem interval or clandestine grave indicators.

Keywords: Taphonomy, Soil microbiology, Decomposition, Post-mortem interval, FAME profiling, Illumina® sequencing, QIIME

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List of Abbreviations

16:1ω11c	11-hexadecanoic acid
*α*15:0	12-methyl tetradecanoic acid
*i*15:0	13-methyl tetradecanoic acid
*i*16:0	14-methyl pentadecanoic acid
2OH 12:0	2-hydroxy dodecanoic acid
2OH 16:0	2-hydroxy hexadecanoic acid
2OH 14:0	2-hydroxy tetradecanoic acid
3OH 12:0	3-hydroxy dodecanoic acid
3OH 14:0	3-hydroxy tetradecanoic acid
ADD	Accumulated degree days
ADONIS	Analysis of variance using distance matrices
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
α	Anteiso (fatty acid nomenclature)
CDI	Cadaver decomposition island
10:0	Capric acid
CO_2	Carbon dioxide
cm^3	Centimeters cubed
R	Correlation coefficient or R statistic (ANOSIM)
R^2	Coefficient of determination
cy17:0	Cyclopropane heptadecanoic acid
°C	Degree Celsius
DNA	Deoxyribonucleic acid
20:2	Eicosadienoic acid
22:1ω9	Erucic acid

FAMEs	Fatty acid methyl esters
FDA	Fluorescein diacetate assay
GC	Gas Chromatograph
GC-MS	Gas Chromatography-Mass Spectrometry
GIT	Gastro-intestinal tract
g	Gram
xg	Gravities
HCl	Hydrochloric acid
i	Iso confirmation (fatty acid nomenclature)
12:0	Lauric acid (Dodecanoic acid)
24:0	Lignoceric acid (Tetracosanoic acid)
18:2ω6	Linoleic acid
18:3ω3	Linolenic acid
17:0	Margaric acid (Heptadecanoic acid)
M	Molar
m/z	Mass to charge ratio
MeOH	Methanol
MTBE	Methyl-tert butyl ether
µg	Micro-gram
mm	Millimeter
mL	Milliliter
14:0	Myristic acid (Tetradecanoic acid)
14:1	Myritoleic acid
nm	Nanometer
nM	Nanomolar
NGS	Next-generation sequencing
N	Nitrogen

19:0	Nonadecanoic acid
18:1ω7c	Oleic acid *cis*
18:1ω9t	Oleic acid *trans*
OTU	Operational taxonomic unit
16:0	Palmitic acid
16:1 ω9c	Palmitoleic acid
15:0	Pentadecanoic acid
perMANOVA	Permutational multivariate analysis of variance
PLFA	Phospholipid fatty acid
PCR	Polymerase chain reaction
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PMI	Post-mortem interval
QIIME	Quantitative insights into microbial ecology
RM-ANOVA	Repeated measures analysis of variance
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
Na_2CO_3	Sodium carbonate
NaOH	Sodium hydroxide
Pb	Soil bulk density
Pd	Soil particle density
18:0	Stearic acid
13:0	Tridecanoic acid
WHC	Water holding capacity
11:0	Undecanoic acid
UV-VIS	Ultraviolet visible (spectrophotometry)

CHAPTER 1
Introduction

Cadaver decomposition is known to involve large amounts of microorganisms, yet decomposition microbiology remains poorly understood as it pertains to cadavers and carrion. Through putrefactive processes microorganisms present within the body are believed to actively break down the bodily structures (Janaway et al., 2009). Putrefactive microorganisms eventually leach out of the body into the surrounding environment and are believed to alter the soil microbial profile (Metcalf et al., 2013). Saprophytic soil microorganisms will also become involved in the decomposition process, further changing the soil microbial profile within gravesoils.

The changes that occur within soil microbial populations during and following decomposition could potentially be characterized and used as a means of estimating post-mortem interval (PMI) or aid in the location of clandestine graves. This research investigated the changes that occur within soil microbial communities associated with the decomposition of pig carcasses on the soil surface. The characterization of soil microbial communities throughout decomposition and across different seasons and years will provide novel information concerning soil microbial dynamics during cadaver decomposition and the potential use of microbial analyses in forensic investigations.

1.1 Cadaver decomposition

1.1.1 Stages of decomposition

During decomposition, a body will undergo breakdown via two major processes: autolysis, an intrinsic breakdown of cellular components and putrefaction, the active breakdown of tissues by microorganisms (Evans, 1963). During autolysis, the loss of cellular activity sets off a chain of chemical reactions which cause the pH to shift and the loss of membrane structures (Gill-King, 1997). The loss of structural integrity causes the release of hydrolytic enzymes capable of attacking the remaining cell structures (Gill-King, 1997). Autolysis occurs at the cellular level and thus cannot be as easily visualized. The appearance of fluid filled blisters and slippage of the skin are typically the first signs that autolysis is underway (Knight, 2004). The loss of cellular structures that occurs during autolysis will release nutrient rich liquids within the body. Bacteria will use the nutrients to flourish and spread throughout the body, triggering the process of putrefaction (Vass et al., 2002). The first signs of putrefaction are usually the discolorations of the body and bloating (Janaway, 1996; Gunn, 2009). Colour changes vary between shades of green, blue, red or black depending on where the changes are observed and how far along within the decomposition process the observation is made (Gill-King, 1997). Discoloration is due to the release of bile pigments following the enzymatic attack of the liver, gallbladder and pancreas and the release of haemoglobin breakdown products, i.e. sulf-haemoglobin (Janaway, 1996).

Five stages of decomposition described by Payne (1965) and adapted by Anderson and VanLaerhoven (1996) are commonly used in forensic taphonomy to aid in the description of cadavers or carrion. These stages are 1) fresh, 2) bloat, 3) active decay,

4) advanced decay and 5) dry remains. Examples of pig remains in each of these stages are presented in Figure 1.

Figure 1. Stages of decomposition of a pig carcass (*Sus scrofa*) in southern Ontario: a) fresh, b) bloat, c) active decay, d) advanced decay and e) dry remains.

The fresh stage is usually short and encompasses autolysis. Once the heart stops, blood is no longer pumped throughout the body, limiting the supply of oxygen to the body's tissues and halting the biosynthetic activities required to keep cells alive (Gill-King, 1997). The oxygen remaining in the body is rapidly used by the aerobic bacteria and an anaerobic environment is created within the body (Janaway, 1996). Proliferation of anaerobic bacteria from the gut will cause the body to become distended due to gas production and accumulation. This indicates the end of the fresh stage and the beginning of the bloat stage (Vass et al., 2002). Bloating of the body subsides shortly after it is observed, due to a purging of liquids and gases from the body via natural orifices (i.e. the mouth and anus). The pressure from the bloat may cause ruptures which also allow for purging (Knight, 2004).

When bloat comes to an end, the body remains in the putrefactive stage of chemical decomposition and enters active decay. Putrefaction leads to the loss of skin and soft tissues and will eventually lead to their disappearance. This limits the available substrate for microorganisms causing microbial activity to gradually slow down. Once active decay reaches its later stage, the body will begin to dry out transitioning into the advanced decomposition stage. By this point, the majority of the soft tissue has disappeared leaving only tendons, cartilage, nails, hair and the skeleton (Gunn, 2006). The dried out remains will continue to slowly disintegrate eventually leaving only bone (Janaway, 1996).

1.1.2 Factors affecting decomposition

Many factors influence the decomposition process including microbial, entomological and scavenging activities. Variables affecting decomposition that relate to the cadaver itself are referred to here as intrinsic factors and those pertaining to the decomposition environment are referred to as extrinsic factors.

1.1.2.1 Intrinsic factors

Each individual will present a different rate of decomposition based on body composition. Cadavers with a small body mass generally decompose more quickly than larger cadavers due to the more rapid cooling of the body and onset of early post-mortem changes (Goff, 2009; Sutherland et al., 2013). The cause of death or state of health of the deceased at time of death is also known to influence the rate of decomposition. Trauma producing open wounds, such as stabbing or gunshots, generally aid decomposition by providing attractive sites for flies to oviposit (Goff, 2009). Cases where individuals pass away due to severe infections have also been shown to increase the rate of putrefaction (Zhou et al., 2011). Clothing and other coverings will also affect how attractive the body is to insects and scavengers (DeVault et al., 2003).

1.1.2.2 Extrinsic factors

Temperature is the most important factor influencing rates of decomposition (Gill-King, 1997). As previously described, the first stage of decomposition, autolysis, is the result of various chemical reactions driven by the enzymes present in the body.

Increased temperatures tend to favor enzymatic activity while cooler temperatures slow down chemical processes. Microbial activity and subsequent putrefactive processes are affected by temperature in the same way (Campobasso et al., 2001). Overall, temperature can greatly affect the onset and rate of decomposition (Gill-King, 1997).

Temperature also influences the rate at which moisture is removed from the body through evaporation. Moisture is required for the breakdown of tissues to occur through the process of hydrolysis during the autolysis stage (Gill-King, 1997). Water is also necessary for microbial growth and proliferation throughout the body after death (Gill-King, 1997). When a body is drained of blood the moisture content of the body is greatly affected and decomposition halted as is observed in embalming practices (Mayer, 2005). Moisture from the decomposition environment will also influence the decomposition process. Where relative humidity is high, decomposition can be slowed down by saturation of tissues with water (Campobasso et al., 2001) or increased by promoting microbial activity and insect activity (Mann et al., 1990). Precipitation can influence both the moisture of the body and the surrounding environment and slow the drying process, rehydrating dried remains and increasing microbial activity (Archer, 2004).

Rates of decomposition have been shown to be greatly affected by extreme temperatures or humidity levels resulting in distinct decomposition or preservation trends. Arid and warm climates have been shown to favor the process of mummification whereas arid and cool climates promote the preservation of tissues through sublimation (Janaway, 1996). Extremely moist environments have also been shown to promote the production of adipocere, which can enhance preservation of the body (Forbes, 2008).

When decomposition occurs outdoors the presence of insects is a factor that can greatly influence the rate of decay and can subsequently impact the surrounding environment. A cadaver represents the ideal location for flies to oviposit as the tissue is a great source of nutrition for feeding larvae (Goff, 2009). Exclusion studies have shown that the absence of maggots will result in a much slower decomposition rate (Payne, 1965; Simmons et al., 2010b). Maggot masses associated with a decomposing body can weigh up to many kilograms and are known to increase the temperature at the site of feeding by up to 5°C above ambient temperature (Simmons et al., 2010a). Burrowing maggots may disturb the first few centimeters of soil where a body is deposited by burrowing (Bornemissza, 1957). The majority of the readily available energy and nutrients entering soil as a result of decomposition will mainly occur following maggot migration (Vass et al., 1992). This is likely the result of maggots breaking down the soft tissues into smaller components making leaching into the ground possible.

Scavenging of the body by larger animals can also influence rates of decomposition. Carnivorous animals can consume large amounts of soft tissue whether buried or placed on the soil surface (DeVault et al., 2003). Scavengers are also able to dismember and scatter remains often causing post-mortem trauma to the bone such as teeth marks (Moraitis and Spiliopoulou, 2010). Scavenging of a cadaver will depend on the rate of decomposition at the time of discovery as microorganisms can render a cadaver toxic to animal consumption (Janzen, 1977). The ability of animals to locate and gain access to the body can also influence scavenging potential (DeVault et al., 2003).

1.1.3 Decomposition Microbiology

Bacteria and fungi are responsible for the majority of cadaver decomposition processes, yet precisely which microorganisms take an active part in decomposition and how they evolve as a population over time remains unclear. Many regions of the human body are colonized by a highly varied micro-flora. These include the skin, mouth, upper respiratory tract, urinary tract and most importantly the gastro intestinal tract (GIT) (Wilson, 2005). Microorganisms that comprise the body's microflora are harmless to their hosts as human bodies possess a multitude of barriers and defence methods that prevent infection. Examples of these are the secretion of toxic substances and the attack of cells attempting to spread to defenceless areas of the body (Wilson, 2005).

When a person dies, the mechanisms and barriers that helped control the microflora are no longer functional. As such, microorganisms are able to proliferate throughout the body and begin the putrefactive process. Bacteria from the gut of the deceased will play a major role in decomposition processes (Janaway, 1996) as these represent the densest microbial load of the body at approximately 10^4 microorganisms per milliliter (Wilson, 2005).

Since oxygen is still present in the body immediately after death, aerobic bacteria flourish during the initial hours of decomposition. Shortly after, the body becomes an anaerobic environment ideal for the proliferation of the GIT bacteria. These microorganisms will migrate from the gut to other regions of the body using the lymphatic system and blood vessels (Janaway, 1996). The propagation of anaerobic bacteria within the body is accompanied by the production of gases which are formed

through bacterial fermentation processes (Vass et al., 2002). Gases accumulate within the bodily cavities causing the torso to distend as it enters the bloat stage of decomposition.

Once bloat occurs, pressure builds up within the body and this is relieved by purging of gases and liquids via the body's orifices (Knight, 2004). As decomposition progresses, decomposition products will leach into the environment surrounding the cadaver. The discharge from a cadaver is thought to represent an important novel source of nutrients for microorganisms in the soil (Towne, 2000).

Bacteria and fungi from the surrounding soil environment are also believed to play an important role in the decomposition process (Carter et al., 2006). Fungi are often observed on cadavers (Ishii et al., 2006). Ammonia-fungi and post-putrefactive fungi are the two major groups commonly associated with cadaver decomposition (Tibbett and Carter, 2003). Studying the fruiting patterns of cadaver associated fungi has even been proposed as a means of estimating PMI (Carter and Tibbett, 2003; Hitosugi et al., 2006).

1.2 Effects of cadaver decomposition on soil

1.2.1 Soil nutrient concentration

Body decomposition results in an important increase in the elemental nutrient concentrations at the site of decomposition including nitrogen, phosphorus and sodium (Vass, 1992; Parmenter and MacMahon, 2009; Benninger et al., 2008). The altered chemical composition of the soil can have lasting effects that will be seen years after a body or carrion has completely disappeared (Towne, 2000). Increased soil nutrients are known to affect surrounding vegetation (Bornemissza, 1957; Towne, 2000). Although

vegetation immediately below a cadaver will typically die off, increased soil nutrients can favor the growth of surrounding vegetation providing a useful indicator to locate clandestine graves (Hunter and Cox, 2005). Fluctuations in soil nutrients will alter soil microbial communities yet specific effects remain unclear (Carter et al., 2007; Hopkins et al., 2000; Parkinson et al., 2009).

The rate at which decomposition products can enter the soil will play an important role in the changes observed in microorganisms and vegetation. This rate is dependent on the vegetation already present, soil type and climatic conditions, notably precipitation. The actual amount of leachate leaving a body is thought to be extremely low and independent of carrion activity although the presence of flies results in considerably higher rates of liquefaction (Putman, 1978).

1.2.2 Soil pH

The effect of decomposition on the pH of soil remains unclear although most recent studies point to soil alkalinisation as a product of decomposition (Carter & Tibbett, 2006; Carter et al., 2008; Haslam & Tibbett 2009). Acidification of soil has also been observed in later stages of decomposition (Vass et al., 1992). Initial soil pH is likely, in part, responsible for the pH shifts that are observed as a result of decomposition. Previous taphonomic studies have shown that initial soil pH will determine the initial soil microbial community composition and that this will in turn dictate how the community responds to the influx of nutrients observed during decomposition (Haslam & Tibbett 2009). Effects of decomposition on soil pH have been shown to endure in soils that see repeated decomposition activity over the course of many years (Damann et al., 2012).

1.2.3 Soil moisture

The potential effects of cadaver decomposition on soil moisture have not been studied in depth. The purging of fluids into the decomposition environment produces what is often referred to as a cadaver decomposition island (CDI) (Carter et al., 2007). It can be hypothesized that the accumulation of decomposition fluids in the surrounding soil environment will produce an increase in soil moisture within a CDI, yet there is little evidence to confirm this. In a study investigating the impact of pig remains decomposition on soil biochemistry, Benninger et al. (2008) found that decomposition had no significant impact on soil moisture content. A study investigating the long term effects of cadaver decomposition by Damann et al. (2012) showed that soil moisture was generally higher in gravesoils than control soils. However, this was believed to be in part due to characteristics of the different sampling sites. The major factors likely to influence soil moisture during the decomposition process are soil drainage potential and permeability.

1.2.4 Soil microbial communities

A limited number of studies within the field of forensic taphonomy have investigated the impact that cadaver decomposition may have on soil microbiology. Increases in microbial biomass and microbial activity are often associated with the presence of decomposing carrion (Wilson et al., 2007; Carter et al., 2010). The presence of enteric bacteria in gravesoils has been reported by Hopkins et al. (2000) and Parkinson et al. (2009) confirming that microorganisms originating from the cadaver can find their way into the soil environment and survive there for prolonged periods of time. The

presence of cadaver microbiota in the environment is thought to drive away native soil microorganisms during decomposition with the original soil microbial community reestablishing itself over time (Parkinson, 2009; Howard et al. 2011).

The effects of decomposition on biochemical properties are also likely to influence the native soil microbial community. Changes to soil pH alone can greatly affect a microbial community as most bacteria are typically adapted to survive within a specific pH range (Rosso et al., 1995). A small change in pH can alter the availability of nutrients in the environment by affecting their solubility (Osman, 2013). Many biochemical processes carried out by microorganisms are also limited by the physiochemical characteristics of the surrounding environment such as pH (Torsvik and Ovreas, 2008). Soil moisture is another variable known to influence microbial activity and survival rates. When soil moisture is high, the concentration of certain nutrients may decrease through dilution limiting their availability (Stark and Firestone, 1995). Too little moisture can cause nutrients to become bound to soil particles and unavailable to microbes. Increased water content in the soil may create an anaerobic environment thus making conditions more favorable for the proliferation of anaerobic bacteria.

Soil microbial communities also change as a result of the influx of new nutrients into the environment observed during decomposition. A study by Howard et al. (2010) highlights several changes which might occur within a soil microbial community as a result of decomposition. In this study, a swine carcass was left to decompose on a plot of soil located in the southern United-States during the months of September to December. The authors studied changes in lipolytic bacteria and proteolytic bacteria within the soil. Proteolytic bacteria could be found in larger numbers during the initial weeks of

decomposition and then decreased considerably for the following remaining weeks. Lipolytic bacteria were present in low amounts but increased considerably after a few weeks and remained stable for the remainder of the trial.

More recently a study by Pechal et al. (2013) investigated the changes of the microbial communities from the mouth and skin of swine carrion during the decomposition process. Using pyrosequencing they found that they were able to associate several specific families of bacteria with the different stages of decomposition. Metcalf et al. (2013) used Illumina® sequencing to investigate the changes that occur within microbial communities following the decomposition of mice. Results indicated that changes in microbial profiles of the abdominal cavity, skin and surrounding soil were consistent across multiple replicates. Changes in microbial communities could also be associated with the visual changes brought on by decomposition. Both of these studies provide strong evidence that high-throughput sequencing of microbial communities could be used to establish timelines within forensic investigations.

1.3 Soil as evidence in forensic investigations

1.3.1 Estimating post-mortem interval

Establishing post-mortem interval is one of the most important yet difficult tasks required of forensic investigators. Within the first 72 hours following death it is possible to establish post-mortem interval based on observations of early post-mortem changes such as rigor mortis, the stiffening of muscles; livor mortis, the pooling of blood in the body; or algor mortis, the cooling of the body to ambient temperature (DiMaio and Dana 2006). Other methods commonly used by pathologists include gastric content analysis,

measuring potassium contents of the vitreous humor and establishing levels of DNA degradation in rib bones (Knight, 2004). Methods have been researched over the years, notably for establishing PMI based on potassium in the vitreous humor, allowing medical examiners to estimate PMI easily and accurately using simple formulae (Madea et al., 1990).

Correctly estimating PMI once putrefactive processes have begun is often very challenging. The rate of cadaver decomposition is subject to many intrinsic factors as described above. The field of forensic taphonomy which studies decomposition processes has greatly increased our knowledge of cadaver decomposition under a variety of conditions. New methods for estimating post-mortem continue to be proposed and are gradually replacing older methods.

Anthropological and entomological observations are often required to establish PMI once putrefaction has begun. Anthropologists are able to use observations of the different stages of cadaver decomposition and correlate these with temperatures to establish PMIs (Megyesi et al., 2005). Forensic entomologists are capable of providing estimated timelines as the reproductive cycles of carrion insects are well known and can be easily measured (Higley and Haskell 2001). The order in which insects colonize cadavers has also been studied for many urban and rural areas of the world allowing entomologists to estimate PMI much more accurately when insects are present (Wells and Lamotte, 2009).

Using soil based evidence to establish PMI is gaining interest within the field of forensic taphonomy. In 1992, Vass et al. published a study that examined the chemical

characteristics of gravesoils and their potential use in establishing PMIs. Findings from this study were limited in their application due to the lack of replicates and the use of a location with a micro-climate. A study by Benninger et al. (2008) indicated that changes in gravesoil pH, nitrogen and phosphorus were significant at different postmortem periods reinforcing that soil analysis could prove useful in establishing timelines within forensic casework. Fungi specifically associated with decomposition processes, known as post-putrefactive fungi, can also provide another means of estimating PMI using soil based evidence (Carter and Tibbett, 2003). These fungi have specific reproductive cycles that can be used to estimate the time since burial on larger timescales. More recently, ninhydrin-reactive nitrogen, which is released from a cadaver during the decomposition process, has been shown to be a potential indicator for PMI with its rate of release being related to body mass (Spicka et al., 2011).

The presence of microorganisms within a decomposing body and the soil environment, as well as recent advances in complex microbial community analysis have resulted in soil microorganisms being considered as a potential tool for PMI estimation. The analysis of soil microbial communities over the course of the decomposition process is expected to reveal specific changes within the soil community, which can be linked to decomposition events thus helping to establish a postmortem timeline.

1.3.2 Locating transit and clandestine graves

Any grave used by an offender to conceal a body is referred to as a clandestine grave. During a homicide investigation it is not uncommon for perpetrators to place the

body of their victim in one location before returning to move the body to a secondary location. The initial deposition site is often referred to as a transit grave. The identification of a transit grave is difficult due to limited evidence confirming that there once was a cadaver at a given site. The repeated disturbance of a site can also affect potential grave indicators. Clandestine graves can be easier to locate as cadaver decomposition will result in a soil mound or depression as well as changes in soil coloration and surrounding vegetation (Hunter and Cox, 2005). Confirming transit or clandestine grave locations as well as the amount of time a body has been deposited there may be crucial in establishing timelines.

The potential of collecting soil as a source of evidence to confirm the presence of a body and to aid in establishing PMI is promising as many methods are non-invasive and do not require that the body still be present. Multiple taphonomic studies over the past decades have characterized the changes in gravesoils with the intention of developing new forensic tools (e.g. Vass et al., 1992; Carter and Tibbett, 2003; Benninger et al., 2008; Spicka et al., 2011).

Currently the best means of locating a clandestine grave is the use of archaeological and geophysical survey methods such as foot searches, ground penetrating radar, and aerial imagery (Hunter and Cox, 2005). These methods are not always feasible based on search locations and often require considerable manpower. The sampling and testing of soils across a site of interest could be done with limited resources and often with little consequence to the property. This requires that the effects of decomposition on soils under various conditions be better understood. Since bacteria and fungi play an

important role in the decomposition process, the detection of marker microorganisms in soils could prove to be useful indicators of gravesites.

1.4 Studying soil microbial communities

1.4.1 Soil microbial activity

Measuring microbial biomass, respiration and enzyme levels are all means of assessing the soil microbial community. Microbial biomass is typically assessed through carbon measurements according to the fumigation-extraction method described by Vance et al. (1987). Measures of soil respiration and enzyme levels are often preferred over the time consuming fumigation method as they can also be used to estimate soil microbial biomass.

Microbial respiration, which refers to the production of carbon dioxide (CO_2) by soil microorganisms, can be measured by trapping and measuring CO_2 in sodium hydroxide (Isermeyer, 1952) or through more advanced automated systems which monitor CO_2 production through infrared gas analysis (Heinemeyer et al., 1989) or gas-chromatography (Brooks and Paul, 1987). Although more time consuming, trapping CO_2 protocols have been simplified (Rodella and Saboya, 1999) and do not require the purchase of advanced laboratory equipment.

Soil microbial activity is often evaluated using measures of specific enzyme activity according to the study design. It is sometimes difficult to appropriately interpret data collected in enzyme based assays due to the nature of enzymes themselves (Burns, 1982). Part of the issue stems from the fact that enzymes are not only found within active

cells but can also be found outside the cell environment. Enzymes can be secreted by active cells or released following cell death and become associated with components in the environment through adsorption or the formation of complexes (Burns, 1982). Nonetheless, the abundance of soil enzymes can be good indicators of a soil's quality and biochemical potential when observing changes over time (Taylor et al., 2002)

Multiple protocols are now well established for measuring levels of soil microbial enzymes such as proteases, lipases and esterases. Fluorescein diacetate hydrolysis (FDA) is frequently used in studies investigating the effects of different treatments on soil microbial enzymatic activity (e.g. Zelles et al., 1991; Iovieno et al., 2009; Piotrowska and Długosz 2012). Fluorescein diacetate is easily broken down into fluorescein by most enzymes present in microorganisms notably esterases, proteases and lipases which all take part in the assimilation of new organic matter by microorganisms (Schnurer and Rosswall, 1982). Fluorescein produces a green coloration allowing colorimetric measurement (Friedel et al., 1994). The FDA protocol it is particularly preferable for analyzing large numbers of samples to measure levels of microbial activity.

1.4.2 Soil microbial community profiles

The biodiversity of microorganisms in soil is tremendous yet only approximately 1% of the microorganisms present in a soil sample can be studied using traditional culture-based methods (Torsvik et al., 1990). In recent years, the development of culture-independent techniques for studying complex microbial communities has allowed the study of soil microbial ecology to grow considerably. The most commonly used methods

for obtaining soil microbial community profiles can be divided into two major groups: whole-cell fatty acid profiling, based on the analysis of structural sub-units of microbial cells; and nucleic acid based profiling, which analyses DNA or RNA sequences extracted from microorganisms. These techniques have allowed the discovery of new microbial species and given new insights into microbial community compositions and the factors that affect the dynamics of such communities (Hugenholtz et al., 1998). The use of these analytical methods within the field of forensic taphonomy is generating novel data concerning the changes that occur within soil microbial communities as a result of cadaver decomposition.

1.4.2.1 Whole cell fatty acid profiles

One of the most basic tools to differentiate bacteria is Gram staining which allows bacteria to be divided into Gram positive and Gram negative species. The distinction is possible due to differences in cell wall structure and molecular composition (Prescott et al., 2005). Differentiation can become precise when specific fatty acids which comprise the membranes as well as other cellular components are analyzed. This is due to each species having its own distinctive fatty acid signature (Osterhout et al., 1991). Microbial communities can be studied by analyzing these fatty acids and their variations over time. Over the past decade, whole cell fatty acid extractions of soil samples to obtain microbial profiles have become common in microbial ecology. Two major types of analyses fall within this category and are based on analysing either phospholipid fatty acids (PLFAs) or fatty acid methyl esters (FAMEs).

Phospholipid fatty acids are structural compounds of the cytoplasmic membrane of bacteria and can be extracted and used to study microbial biomass within soils (Dunfield, 2008). PFLA studies allow for only the viable microbial cells to be taken into account and thus are considered to be a good measure of total microbial biomass (Zelles et al., 1995*)*. Furthermore, specific PLFAs can be used as biomarkers for specific groups of microorganisms (Zelles et al., 1999). The extraction of PLFAs is a somewhat lengthy method which requires large amounts of soil to produce enough extract for analysis.

Fatty acid methyl ester profiles have proven to be a reliable soil profiling method over the years. Cavigelli et al. (1995) found that samples taken from the same soil profile had FAME profiles which were highly reproducible. Ibekwe and Kennedy (1995) studied FAME profiles from soils that had undergone various agricultural and plant treatments. They were able to differentiate soils subjected to different agricultural treatments based on their FAME profiles. They also noted that qualitative information could be gained concerning the types of bacteria present in a given soil sample based on the amounts of specific fatty acids detected. FAME profiling has also shown to be as useful as some nucleic acid based methods (Ritchie et al., 2000). More recently, Fernandes et al. (2013) investigated the source of the fatty acids detected during FAME profiling and found that a fraction of the fatty acids did in fact originate from plant based materials which must be taken into account when using FAME profiles. Compared to PLFA profiling, FAME extraction is a rapid method that requires smaller amounts of soil to obtain highly reproducible community profiles.

The choice between extracting PLFAs or FAMEs to obtain soil community profiles depends on the experimental hypothesis. PLFAs only account for living cells

which in certain cases is an advantage over the FAME method. On the other hand, the extraction of PLFAs is a lengthier process which may not suit a study with large numbers of samples to be analyzed. FAME profiling was selected for this study based on the large number of samples that were to be analysed (over 1200). The feasibility of using FAME profiling in any laboratory able to conduct GC-MS analyses means there is potential for this technique to routinely be used when soils are received as evidence in forensic investigations.

1.4.2.2 Nucleic acid based soil microbial community profiling

Commonly used methods to study microbial communities by obtaining DNA fingerprints include denaturing gradient gel electrophoresis (DGGE), terminal- restriction fragment length polymorphism (T-RFLP) and ribosomal intergenic spacer analysis (RISA). Each method will produce a fingerprint of a microbial community through different processes which include steps for DNA extraction and amplification, separation of DNA fragments and imaging. Methods generally differ based on their means for differentiating and separating the DNA fragments. The resulting fingerprints can be compared between samples to observe differences in community composition. T-RFLP has been used to characterize and compare soil communities for potential use in forensic investigations (Horswell et al., 2002; Heath and Saunders, 2006).

More recently, next-generation sequencing (NGS) has become the method of choice for scientists wishing to study complex microbial communities. The most common NGS technologies are 454™ (pyrosequencing), Illumina® and SOLiD ™. Each method begins by producing a library of DNA fragments by annealing linkers to the blunt-end of

fragments obtained directly from the DNA source of interest (Mardis, 2008). During the sequencing process, sequence reads are mass produced in parallel allowing for hundreds of thousands to millions of reads. During synthesis, the incorporation of the different nucleotides is recorded through fluorescent signals. Each recorded sequence undergoes a screening process to ensure the quality of the output data.

The development of next-generation sequencing has led to the expansion of metagenomics. A metagenome represents the whole genetic material obtained from an environmental sample which can be studied as a whole allowing a microbial community to be studied *in-situ*. Data-sets obtained through metagenomics studies have prompted the development of new bioinformatics tools that allow researchers to accurately analyze their results. These include open source pipelines such as Mothur, developed at the University of Michigan, and QIIME (Quantitative Insights into Microbial Ecology) developed at the University of Colorado, Boulder. The growing access to next-generation sequencing, the low cost associated with such analyses and the development of novel analytical tools has revolutionized studies in soil microbial ecology.

1.5 Research aims and objectives

The overall objective of the research presented in this thesis was to investigate the impact of cadaver decomposition on soil microbial communities, including their activity and composition. Seasonality and year to year variation was assessed to document effects on decomposition and microbial community over time. Results were anticipated to provide novel information concerning both decomposition rates and the associated soil microbiology in the southern region of Ontario, Canada. Research was divided into two types of studies: microcosm studies conducted in the laboratory and outdoor decomposition trials.

Two microcosm studies were undertaken during the course of this research. These studies investigated the effect that temperature or soil moisture have on animal tissue decomposition and soil microbial activity. Temperature is known to influence the decomposition process, but it is also a factor that greatly influences the survival and proliferation of microorganisms. As the outdoor trials in this study were going to span several months and different seasons, it was known that soil samples would be collected under varied temperatures. Soil moisture was the second parameter to be investigated as part of a microcosm study. It was expected that over the course of the outdoor trials, a range of soil moisture levels would be observed. Spring and fall months in Ontario are cooler and precipitation is frequent, which results in moist soil conditions. Summer months see less rainfall and higher temperatures and evaporation rates rendering soils very dry. Isolated rainfall events also have the potential to influence microbial activity. It was believed that the liquefaction of cadaver tissues might also influence soil moisture levels and potentially affect soil microbial dynamics.

Four large scale outdoor decomposition experiments were carried out as part of this research project. Experiments were spread out over two seasons and two years to document intra-year and inter-year differences in rates of decomposition of pig carrion (*Sus scrofa*) and the effects on associated soil microbial communities. Measures of soil microbial activity as well as profiles of microbial communities were obtained throughout the decomposition process up to 97 days post-mortem. Two methods were used to obtain soil microbial community profiles: FAME profiling and Illumina® sequencing of community DNA. Both profiling approaches were used to compare the potential use of either method as a forensic tool to establish post-mortem interval or locate clandestine graves.

CHAPTER 2
Microcosm studies to assess the effect of temperature and soil moisture on decomposition activity in soil

2.1 Introduction

Microcosms are small scale, controlled environments that allow scientists to make inferences about larger scale processes or phenomena in nature. Microcosm-based studies have often been used in forensic taphonomy to simulate the decomposition process in simplified environments. Such studies have produced novel data concerning decomposition rates under various conditions such as different temperatures (Carter and Tibbett 2006); soil pH (Haslam and Tibbett, 2009) or different soil moisture levels (Carter et al. 2010) as well as in lake water (Ueland et al. 2013). Other studies have investigated the impact that various treatments may have on decomposing tissue, notably the freezing of tissue (Stokes et al., 2009) and the application of lime (Schotsmans et al., 2012).

Microcosm studies generally rely on the use of various animal tissues to act as analogues for decomposing human tissue. Most common are the use of piglets or porcine tissues, ovine tissues as well as rat and mice carcasses. The validity of using analogues rather than human tissue in taphonomic research is often a point for debate among researchers. Stokes et al. (2013) showed that animal tissues generally have the same effect as human tissues on the surrounding soil environment. The use of animal tissues also has the advantage of allowing researchers to obtain sufficient replicates for generating meaningful data.

Microcosm studies can be useful in understanding the microbiological changes that occur during the decomposition process. Carter et al. (2008) observed that soil associated with decomposition showed greater CO_2 production and increased microbial biomass carbon. These differences were found to be most significant at higher temperatures. This same study revealed that protease and phosphodiesterase activities were higher in the presence of decomposition. The increase in enzyme activity occurred earlier in environments at higher incubation temperatures.

A second study by Carter et al. (2010) investigated the impact of soil moisture on cadaver decomposition and found that decomposition was typically more rapid in wetter soils, although it is possible to exceed optimal moisture content for aerobic decomposition. Cadaver burial produced significant increases in enzyme activities and an increase in soil microbial biomass carbon. CO_2 evolution was increased in the presence of decomposition and was affected by moisture levels. Overall this study indicated that soil microorganisms played an important role in early cadaver decomposition.

The microcosm studies presented in this chapter were conducted to better understand the microbial response to the addition of decomposing substrate at different temperatures and different levels of soil moisture. Temperature and moisture were chosen as they were believed to be the two major factors that would influence microbial activity during outdoor decomposition trials. The aim of the experiments presented in this section was to document the changes in a soil that would be representative of the soil used in subsequent outdoor experiments. Tissue was also deposited on the soil surface rather than buried to replicate soil surface decomposition.

Based on earlier published data presented above it was expected that decomposition rates, soil microbial activity and respiration may be higher at higher incubation temperatures. The presence of decomposing tissue was expected to increase microbial activity and respiration with a more significant increase occurring at higher temperatures. The projected effects of soil moisture on microbial dynamics during surface decomposition were not as clear. It was expected that of the different levels of moisture studied, an optimal range for microbial activity would be determined. It was hypothesized that the driest and wettest soils would produce the lowest levels of microbial activity.

2.2 Methods

2.2.1 Experimental design

Two microcosm experiments were conducted for this study, the first to investigate the impact of temperature on soil microbial activity during decomposition, the second to study the impact of soil moisture. Both microcosm experiments were carried out over a period of 8 weeks. Treatment microcosms consisted of soil and pieces of pork belly tissue as the decomposing substrate. Pork belly was considered the best choice of tissue as it is composed of skin, fat and muscle. Porcine tissue also resembles human tissue due to its lack of heavy fur. Pork tissue was also chosen as the decomposing substrate due to pig carcasses being used during the outdoor trials. Pieces of the pork belly measuring 5 x 5 x 4 cm (width x length x height) were cut and weighed before being placed skin down on the soil surface within each of the treatment microcosms. A set of control microcosms containing only soil was created for each sampling week and used to measure microbial

activity and microbial respiration. These are referred to as soil controls. A second set of controls containing only pieces of tissue was created to measure microbial respiration rates from tissue only and are referred to as tissue controls.

Microcosms were created using 1L glass mason jars fitted with metallic lids which were autoclaved prior to use. Lids from the jars were pierced to allow the exchanges of gases. Microcosms were filled with soil obtained from Hard Co. in Whitby, Ontario which consisted of sifted backfill removed from construction sites in the Durham region of southern Ontario. This soil was chosen at it was representative of soil found at the treatment facility where outdoor experiments were to take place. Soil was sieved using a 5mm sieve to remove larger debris prior to its use.

Eight sets of treatment microcosms and control microcosms were set-out in triplicate to allow for destructive sampling every week. At each sampling time tissue from the treatment microcosms was removed to gain access to the soil below. Soil samples were collected using a sterile scoopula and transferred to glass vials for storage. Soil samples were used to measure soil microbial activity immediately after sampling.

2.2.1.1 Specifications – Impact of temperature on soil microbial activity during decomposition

Treatment and control microcosms during the temperature experiment were maintained at either 5°C or 20°C. These temperatures represented approximate average daily temperatures from both cooler and warmer days that would be observed during the outdoor experiments. Microcosms at 5°C were incubated in a refrigerator while those at 20°C were stored in a fume cupboard. Temperatures for each incubation environment

were logged on a daily basis. Soil moisture was adjusted to 50% water holding capacity (WHC) to ensure consistency across all microcosms. Microcosms were aired on a daily basis to ensure oxygen levels were maintained. Soil was sprayed with sterile deionized water on a weekly basis to maintain soil moisture and counteract evaporation.

2.2.1.2 Specifications - Impact of soil moisture on soil microbial activity during decomposition

For the soil moisture experiment, treatment and control microcosms were adjusted to 20%, 40%, 60% or 80% WHC to observe microbial activity across a gradient of soil moistures. Average soil WHC prior to any manipulation of the soil was at 40% WHC. Soil required for microcosms with a 60% or 80% WHC required the addition of sterilized deionized water to the soil until the desired WHC was achieved. For microcosms with a 20% WHC, soil was air dried to a constant weight and water subsequently added until the desired water holding capacity was reached. Calculations to determine WHC used the formulae presented in the following section. Microcosms were aired on a daily basis to ensure oxygen levels were maintained. The soil was sprayed with sterile deionized water on a weekly basis to maintain soil moisture and counteract evaporation.

2.2.2 Measures of soil parameters

2.2.2.1 Soil moisture

Soil water holding capacity was used as a measure of soil moisture content. This measure allows for soil matrix characteristics to be taken into consideration by expressing

soil moisture as a percent of the full water content a soil sample could theoretically contain. This method requires that soil porosity, particle density and bulk density of the soil be determined.

Porosity is the amount of space in the soil available for air and water. Porosity is obtained by measuring a soil's bulk density and particle density. Bulk density is measured by weighing a known volume of soil and using the formula:

$$Pb = \frac{M_{soil}}{V_{soil}}$$

Particle density for most soils will range between 2.5 and 2.65 g/cm^3 and can be calculated by placing a known amount of soil in a volumetric flask which is then filled with water to the line and weighed. The following formula is then used to obtain particle density of the soil:

$$Pd = \frac{M_{soil}}{100ml - ((M_{water+soil} - M_{soil}) \times 1)}$$

This formula suggests that the flask used has a total volume of 100ml and that the water is at a temperature where its density is equivalent to 1 g/ml. Once bulk density and particle density have been measured, porosity is measured using:

$$Porosity(\%) = (1 - (\frac{\mathrm{Pd}}{\mathrm{Pb}})) \times 100$$

Once porosity is calculated, measuring how much water the soil must contain to have a given water holding capacity is straightforward. For example, 200g of dry soil with a porosity of 50% (or 100cm^3) will require 50 cm^3 of water to be added in order to reach 50% water holding capacity.

Since soil naturally contains water, determining the initial water holding capacity of a soil was necessary before any adjustments could be made. A sample of soil when moist (Wm) was weighed and then dried at 105°C until a constant weight was obtained (Wd). The values are subtracted to determine the weight of the water that was contained in the soil. The full weight of water that can be contained in the soil ($W_{100\%}$) is then determined using the previously measured porosity and the dry weight.

$$W_{100\%} = W_d \times Porosity$$

Initial water holding capacity is measured using:

$$WHC = \frac{W_m - W_d}{W_{100\%}} \times 100$$

For example, a soil that weighs 5g when moist and 4g when dry with a known porosity of 50% would thus be at 50% of its water holding capacity. If the desired water holding capacity were 55%, this would simply require that the water holding capacity be augmented by 5% by adding 0.1 g or 0.1 ml of water per 5 g.

2.2.3 Soil total microbial activity

Microbial activity levels in soil samples were measured using a fluorescein diacetate assay (FDA) protocol adapted from Green et al. (2006). This method has been shown to measure microbial activity by measuring the hydrolysis of fluorescein diacetate by many enzymes including esterases, proteases and lipases (Schnurer and Rosswall, 1982). FDA measures were taken immediately after sampling.

For each treatment and control sample, 2g of sieved soil were weighed and placed in a 50 ml Falcon tube to which 15 ml of a potassium phosphate buffer (pH 7.6) and 200 µl of fluorescein diacetate stock solution prepared in acetone were added. The tubes were vortexed and heated in a water bath at 30°C for 20 minutes. After incubation, 20 ml of a 2:1 chloroform: methanol solution was added to each tube to inhibit further breakdown of fluorescein. Tubes were centrifuged at 800 xg for 3 minutes. A 2 ml aliquot of the top phase containing the fluorescein product was filtered using Whatman filter paper no. 42. The absorbance of the final product was measured using a Genesys 10S UV-VIS spectrophotometer (Thermo Scientific, Canada) at λ = 490 nm. A blank was produced for each set of samples analyzed and consisted of buffer and fluorescein stock solution only.

2.2.4 Microbial respiration

Measuring levels of respiration occurring within the microcosms during decomposition can be achieved using a conductimetric method of measuring carbon dioxide (CO_2) production (method taken from Rodella & Saboya, 1999). The method is based on the principle that when CO_2 is produced within the microcosms it can become absorbed if the appropriate substrate is available, in this case a solution of sodium

hydroxide (Isermeyer, 1952). Within the solution, the OH ions are consumed and replaced with CO_3. The exchange results in reduced electrical conductivity of the solution. The change in conductivity can be measured using a conductivity meter and compared to a standard curve. The standard curve is produced using solutions made of a sodium carbonate (Na_2CO_3) solution and sodium hydroxide (NaOH) added in different ratios to reproduce different levels of CO_2 absorption. CO_2 production was measured on a daily basis and averaged to give an average daily respiration rate for each week.

CO_2 traps were placed in all control and treatment microcosms reserved for week 8 as these would remain untouched until the final sampling date. For each trap 20ml of a 1M NaOH was aliquoted into an open glass vial. Vials were attached to the side of the microcosm using string allowing them to hang in the upper airspace of the microcosms. The vials were removed every 24 hours and conductivity measured using a S47-K Seven Multi^{TM} conductivity meter (Mettler Toledo, Columbus, OH) coupled with an InLab 731 conductivity probe (Mettler Toledo, Columbus, OH).

2.2.5 Statistical analysis

Average measures of soil microbial activity and microbial respiration were analyzed for overall significant differences between treatments using repeated m RM-ANOVA. Where normality failed a RM-ANOVA on ranks was performed. Differences between treatments on individual days were assessed using Student's t-test. Where data did not pass the normality test a Mann-Whitney rank sum test was performed. Analyses were performed using the SigmaPlot^{TM} 12.0 software package.

2.3 Results

2.3.1 Impact of temperature on soil microbial activity during decomposition

Microcosms maintained at 5°C showed little change during the first two weeks of the experiment. After this point tissue in the treatment microcosms began to change in color. The muscle fraction of the pork belly pieces changed from pink to slightly more white. There was little change in the physical appearance from week 5 onwards. After week 3 a change in smell consistent with rancid meat became noticeable from the jars.

Tissue in microcosms maintained at 20°C showed signs of liquefaction of the top layer within the first week. The presence of bubbles within the top layer was a sign of elevated rates of microbial respiration. Muscle tissue changed from a light pink to a darker pink or red color while the fatty layers of the tissue became darker and yellower (see Figure 2). There was a strong smell of hydrogen sulfide when jars were opened.

Fungal mycelia were present in many of the treatment microcosms. The growths occurred at both temperatures but were most noticeable in microcosms incubated at 20°C. The occurrence of mycelia was random across the different microcosms. Fungi from this treatment trial were white to gray in color and highly floccose.

Figure 2. Treatment microcosms maintained at 20°C for 6 weeks

The trends for average values of microbial activity are presented in Figure 3. Measures of microbial activity between control microcosms and treatment microcosms at 5°C and 20°C were compared for significant differences each week. Results of these analyses are summarized in Appendix A. For microcosms at 5°C, microbial activity was significantly higher in treatment soils for week 3, 4, 5 and 7. For the microcosms maintained at 20°C microbial activity was significantly higher in treatment soils for weeks 4 to 7. Measures of soil microbial activity of both the control samples and treatment samples maintained at 20°C fluctuated in the same way over the course of the experiment (Figure 3). Microbial activity increased between weeks 2 and 3 but subsequently decreased. After week 7, microbial activity increased once again, peaking at

week 6 and remaining relatively constant thereafter. Microbial activity measures of microcosms containing significant fungal growths were compared to those of microcosms with little to no fungal growths yet there was no significant difference between the presence and absence of fungi (Appendix A – Table 42).

RM-ANOVAs were used to determine if treatment or temperatures produced significant differences overall. Results of these analyses are presented in Table 1. The only significant difference was observed between temperatures for treatment microcosms.

Table 1. Statistical summary table of RM-ANOVA and RM-ANOVA on ranks (*) (X^2 are given) performed on average weekly measures of microbial activity between control microcosms and treatment microcosms at 5°C and 20°C and between control microcosms and treatment microcosms at the same temperature. Significant differences (α = 0.05) are highlighted in bold.

	Treatment 5°C		**Treatment 20°C**		**Soil control 20°C**	
	F	***p***	***F***	***p***	***F***	***P***
Treatment 5°C	--	--	**17.75**	**0.006**	--	--
Soil control 5°C	0.50*	0.727	--	--	2.00	0.289
Soil control 20°C	--	--	4.50*	0.070	--	--

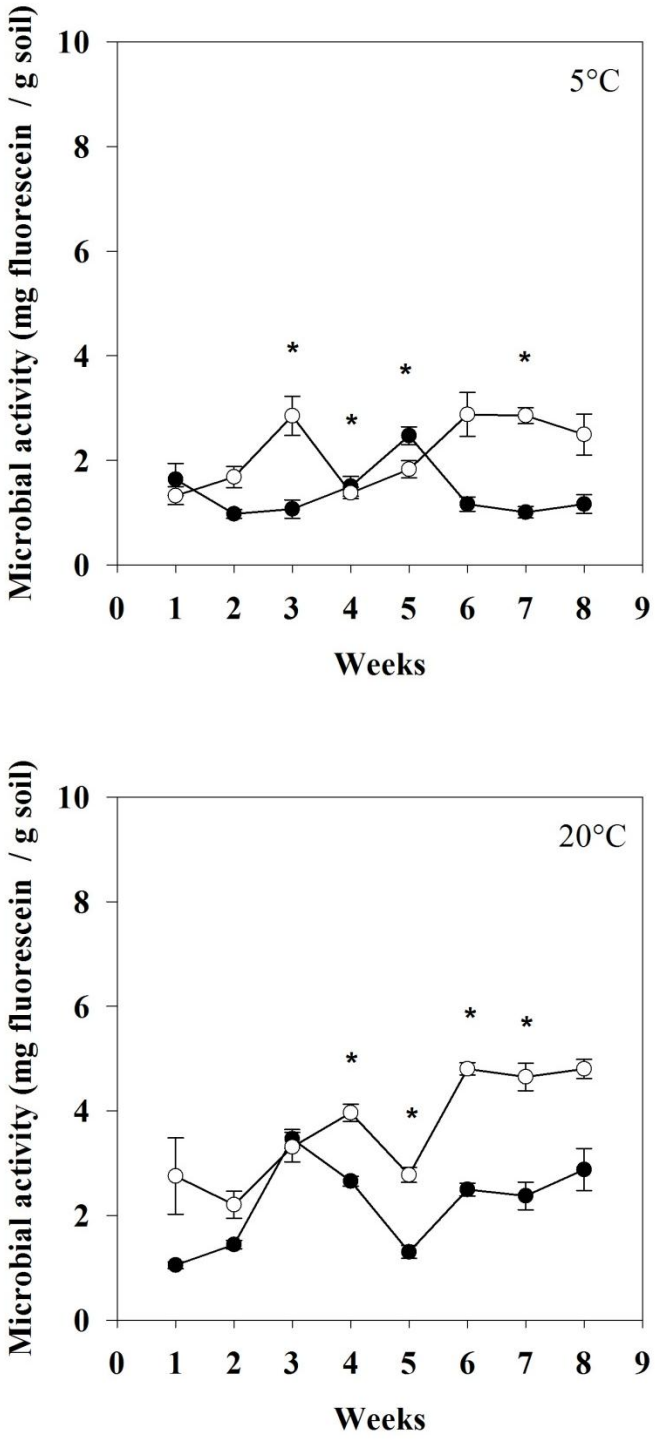


Figure 3. Average microbial activity measures for soils controls (-●-) and treatment (-○-) microcosms at 5°C and 20°C. Significant differences ($p < 0.05$) are marked with an *. Decomposing tissue slightly increased microbial activity in treatment microcosms versus control microcosms.

Weekly average measures of microbial CO_2 production at both temperatures for all microcosms are presented in Figure 4. Controls containing only soil indicated similar rates of respiration at both 5°C and 20°C. Microbial respiration in these microcosms decreased slightly over the course of the experiment. This may be due to microbial loss over time resulting from lack of nutrients and moisture influxes in a closed environment such as these microcosms.

At 5°C control and treatment microcosms showed a similar pattern of microbial respiration throughout the experiment. CO_2 production increased over the course of the first 3 weeks before reaching a plateau during weeks 4 and 5. At week 6, microbial respiration dropped. In the microcosms containing only tissue, respiration rates increased for the final two weeks of the experiment. This overall trend follows a normal microbial growth curve (Figure 4).

Respiration rates for the treatment microcosms maintained at 20°C appeared constant throughout the experiment. This does not reflect actual CO_2 production due to the traps having likely reached their maximum absorption capacity. CO_2 traps with a greater absorption capacity would have been required to accurately measure microbial respiration for these microcosms. Controls containing only tissue fluctuated slightly over the course of the 8 weeks. A drop in CO_2 production was noted at week 3 but was followed by a gradual increase during weeks 4 and 5. After week 5 respiration rates decreased slightly until the end of the experiment.

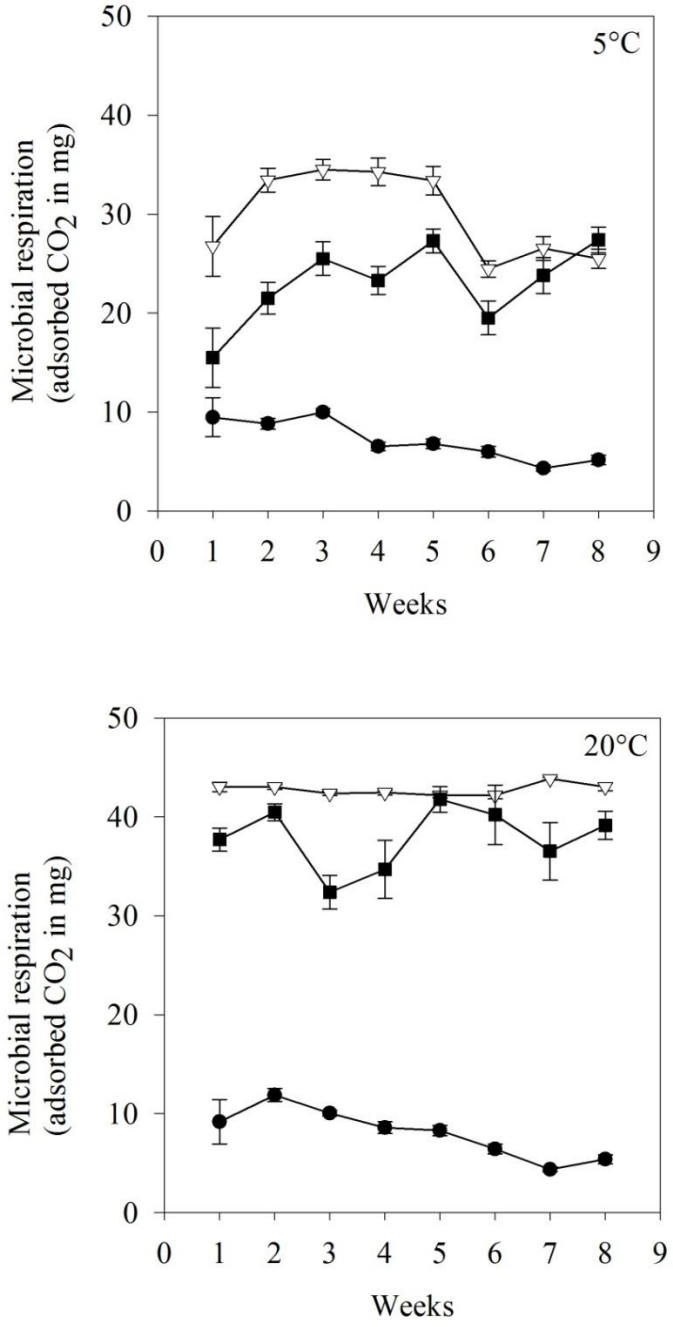


Figure 4. Figure 4. Average daily CO_2 production per week for soil control (-●-), tissue control (-■-) and treatment (-○-) microcosms at 5°C and 20°C. CO_2 production was greater in experimental microcosms versus control microcosms at both temperatures. Tissue controls indicated high levels of CO_2 are produced by microorganisms within decomposing tissue.

Results of statistical analyses to determine significant differences between treatments and temperatures are presented in Table 2. Differences between temperatures were significant for CO_2 production of treatment microcosms and jars containing only tissue. Differences between treatments were significant at both temperatures. There was no significant difference for CO_2 production between temperatures for the control microcosms.

Table 2. Summary of RM-ANOVA results between average daily CO_2 production per week for control microcosms, treatment microcosms and tissue only microcosms at 5°C and 20°C. Significant differences ($p < 0.05$) are highlighted in bold.

	Control 5°C		**Treatment 20°C**		**Tissue 5°C**	
	F	***p***	***F***	***p***	***F***	***p***
Treatment 5°C	**288.1**	**< 0.001**	**17584**	**< 0.001**	--	--
Control 20°C	4.495	0.072	**1274**	**< 0.001**	--	--
Tissue 20°C	--	--	--	--	**63.58**	**< 0.001**

2.3.2 Impact of soil moisture on soil microbial activity during decomposition

The variable levels of soil moisture produced varying decomposition trends. Microcosm images for each soil moisture level after 6 weeks is presented in Figure 5. Tissue in the treatment microcosms at 20% WHC appeared to dry out over the course of the experiment. Fungal mycelia quickly developed on the surface of both soil and tissue. After 2 weeks a few jars contained mycelia which were greater than 2.5 cm in thickness. At 40% WHC the surface of the tissue appeared glossy. Treatment microcosms at this moisture level also showed important fungal growth, but these were less significant than those observed at 20% WHC. At both 20% and 40% WHC fungal mycelia were highly

floccose. At 60% WHC tissue showed liquefaction of the muscle fraction. Fungal growths were still present at this moisture level, but these were smaller and contained to soil and tissue surface. Treatment microcosms at 80% WHC showed a similar degree of liquefaction to those at 60% WHC. Limited fungal growth appeared in the later weeks of the experiment. A strong smell of hydrogen sulfide was present when these microcosms were opened. There were no changes to the appearance of the soil-only treatments at any of the soil moisture levels. Microcosms containing only tissue showed similar levels of liquefaction to that observed in microcosms at 60% and 80% WHC.

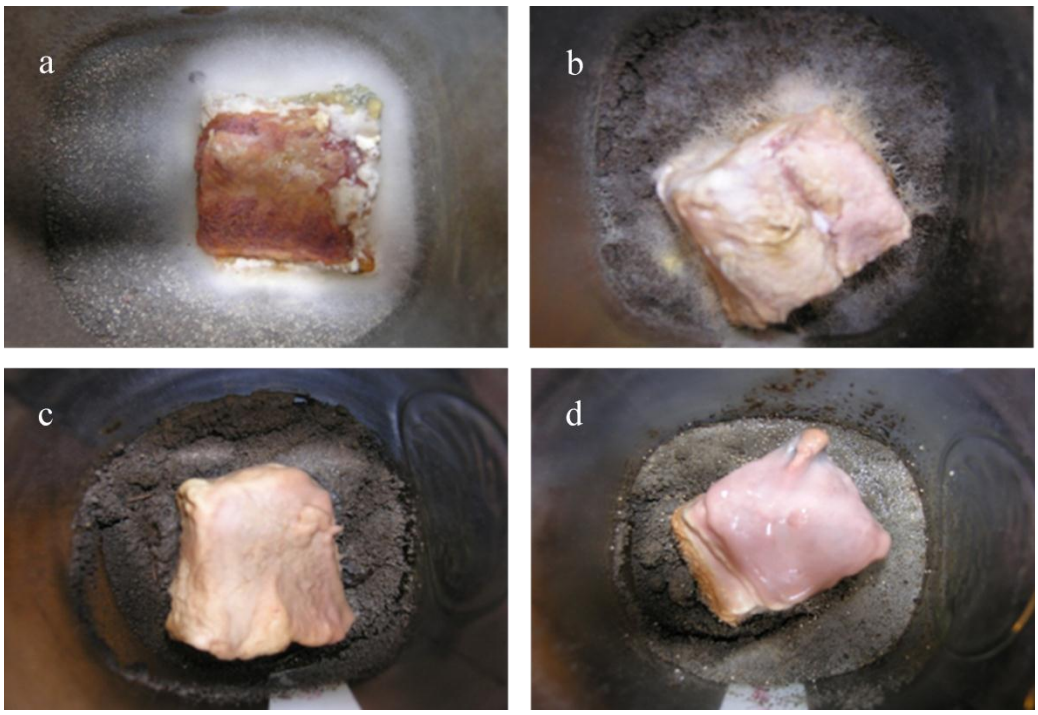


Figure 5. Images of treatment microcosms at a) 20%, b) 40%, c) 60% and d) 80% WHC after 6 weeks of decomposition. Important fungal growths were observed in jars maintained at 20% and 40% WHC. Tissue took on a different appearance for each soil moisture level.

Average microbial activity values for control and treatment microcosms at different soil moisture levels are presented in Figure 6. A summary of statistics comparing treatment microcosms for each week and soil moisture level is presented in Appendix A. At 20% WHC microbial activity was significantly higher in treatment samples than in control samples at weeks 1, 2, 5, 6 and 7 (Figure 6a). For weeks 3 and 4 microbial activity of treatment samples dropped below the average measures for control samples. At 40% soil microbial activity of treatment microcosms appeared slightly higher than that of control microcosms. The difference between control and treatment microcosms was significant at weeks 1, 2, 3, 4 and 7 (Figure 6b). Control microcosms at 60% WHC indicated that soil microbial activity levels in both control and treatment samples were very close and fluctuated throughout the experiment (Figure 6c). A small spike in the microbial activity of treatment samples at week 7 was the only time where a significant difference was observed. Results from the microcosms maintained at 80% WHC indicated that soil microbial activity for control samples was slightly higher than treatment samples throughout the experiment (Figure 6d). This difference was significant at weeks 1, 2 and 5. At this level of soil moisture microbial activity of treatment microcosms was relatively constant throughout the experiment. Microbial activity of soil samples from control microcosms decreased at weeks 3 and 6 but increased thereafter.

Overall averages of microbial activity for control microcosms indicated that microbial activity was highest at 40% and 60% WHC. Greater (80%WHC) and lesser (20%WHC) moisture resulted in lower measures of microbial activity. Overall microbial activity was highest in treatment microcosms at 40% WHC followed by 60% and 20% WHC with soils at 80% WHC showing the lowest measures of microbial activity.

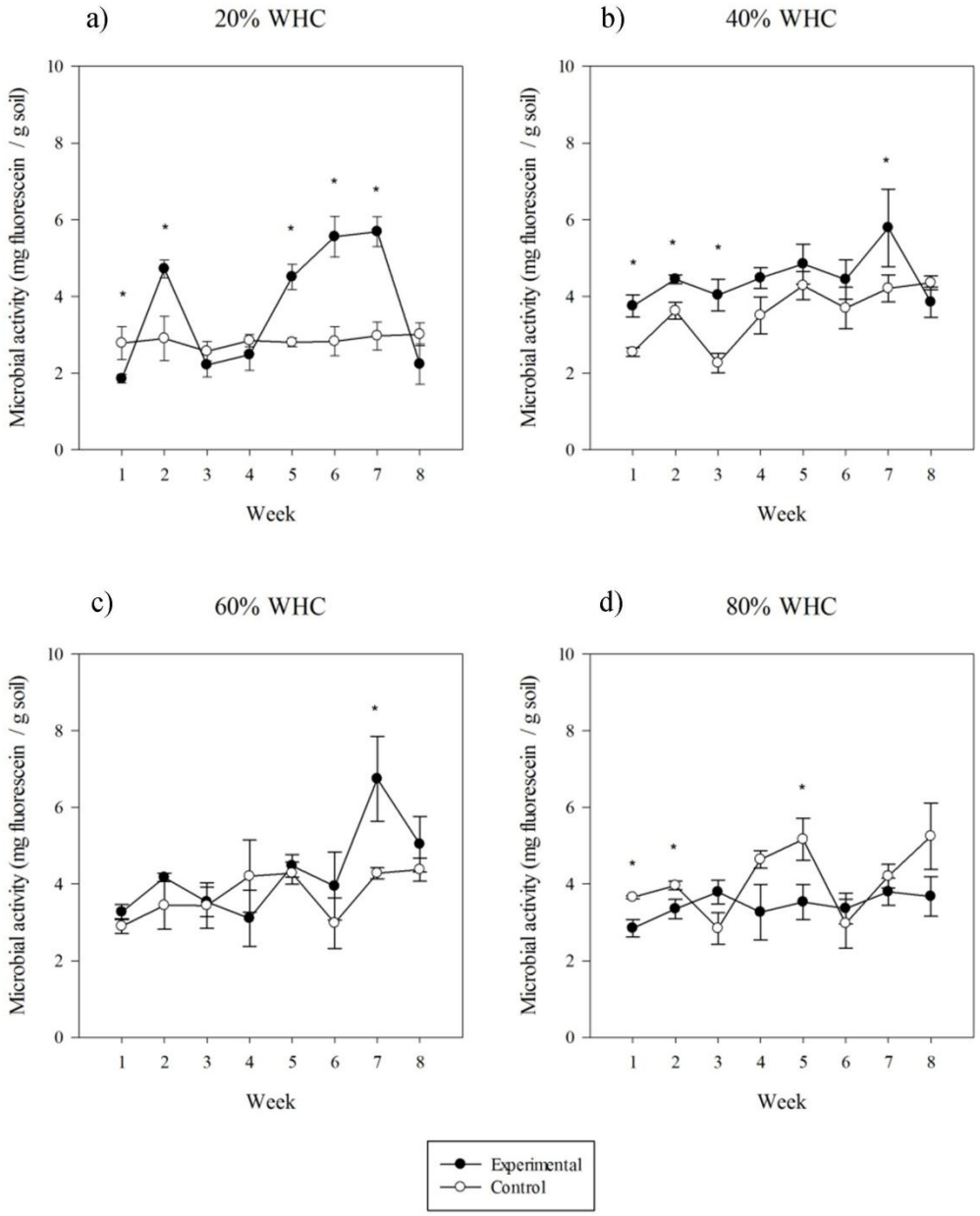


Figure 6. Average measures of microbial activity for control and experimental samples at a) 20%, b) 40%, c) 60% and d) 80% WHC. Significant differences ($p < 0.05$) are marked with an *. The presence of decomposing tissue favored microbial activity at both 20% and 40% WHC on multiple occasions. Microbial activity was lower in experimental microcosms in comparison to control microcosms when maintained at 80% WHC.

Microbial activity values in control and experimental microcosms were compared at each moisture level to determine overall differences based on treatment. Results of the statistical analyses are presented in Table 3. There was no significant difference between control and treatment values or microbial activity at 20%, 60% and 80% WHC. The difference between treatments was significant at 40% WHC. Microbial activity measures of experimental microcosms containing significant fungal growths were compared to those of microcosms with little to no fungal growths at for each soil moisture levels. There was no significant difference between the presence and absence of fungi (Appendix A – Table 42).

Table 3. Summary of RM-ANOVA or RM-ANOVA on ranks (*) results on average weekly measures of soil microbial activity between control microcosms and treatment microcosms at 20%, 40%, 60% and 80% WHC. Significant differences ($p < 0.05$) are highlighted in bold.

	Control vs. Treatment	
	F	***p***
20%	0.00*	1.00
40%	**13.004**	**0.009**
60%	2.401	0.165
80%	3.963	0.096

Average measures of microbial activity were used to compare the control and treatment values between different levels of soil moisture. The results of these analyses are presented in Tables 4 and 5. Control microcosms at 20% WHC presented significantly lower levels of microbial activity than control microcosms at 40%, 60% and 80% WHC. Statistical analyses indicated that treatment microcosms at 40% and 80% WHC were significantly different.

Table 4. Summary of RM-ANOVA or RM-ANOVA on ranks (*) results between average weekly measures of soil microbial activity between control microcosms at 20%, 40%, 60% and 80% WHC. Significant differences ($p < 0.05$) are highlighted in bold.

	40%		**60%**		**80%**	
	F	***p***	***F***	***p***	***F***	***P***
20%	**9.049**	**0.20**	**20.241**	**0.003**	**17.816**	**0.004**
40%	--	--	0.760	0.412	5.377	0.053
60%	--	--	--	--	3.435	0.106

Table 5. Summary of RM-ANOVA or RM-ANOVA on ranks (*) results between average weekly measures of soil microbial activity for experimental microcosms at 20%, 40%, 60% and 80% WHC. Significant differences ($p < 0.05$) are highlighted in bold.

	40%		**60%**		**80%**	
	F	***p***	***F***	***p***	***F***	***P***
20%	3.613	0.099	0.500	0.727	0.00*	1.00
40%	--	--	0.331	0.583	**8.00***	**0.008**
60%	--	--	--	--	5.408	0.053

Average respiration rates for all controls and microcosms are presented in Figure 7. Values for the treatment microcosms at all moisture levels appeared to be fairly constant throughout the experiment. Unfortunately, CO_2 production in these microcosms caused the traps to become saturated at just over 40-mg of CO_2. It is still possible to see that respiration rates increased slightly during the first few weeks of the experiment for all soil moisture levels. At week 1 there was no difference between CO_2 produced in the treatment microcosms and the tissue only jars. This indicates that the presence of the tissue did not have an impact on soil microbial activity after one week. Respiration rates for the tissue only jars decreased slightly between weeks 1 and 4 yet respiration in all treatment microcosms increased during this same period. This suggests that the presence of decomposing tissue had a favourable impact on soil microbial activity after week 1.

Respiration rates mirrored the trends observed for soil-only microbial activity (see Figure 6). Controls at 20% WHC indicated a gradual increase in respiration over the course of the experiment (Figure 7a). For this set of microcosms, soil was air dried before the soil moisture could be adjusted to 20%WHC. The drying of the soil may have resulted in the loss of a fraction of the microbial community within the soil. Drying may have also favored those microorganisms that require minimal moisture to survive. The addition of water to increase moisture to 20% WHC may have had a negative impact on the microorganisms present in the soil at the beginning of the experiment. Over time dormant microorganisms were likely to have been revived.

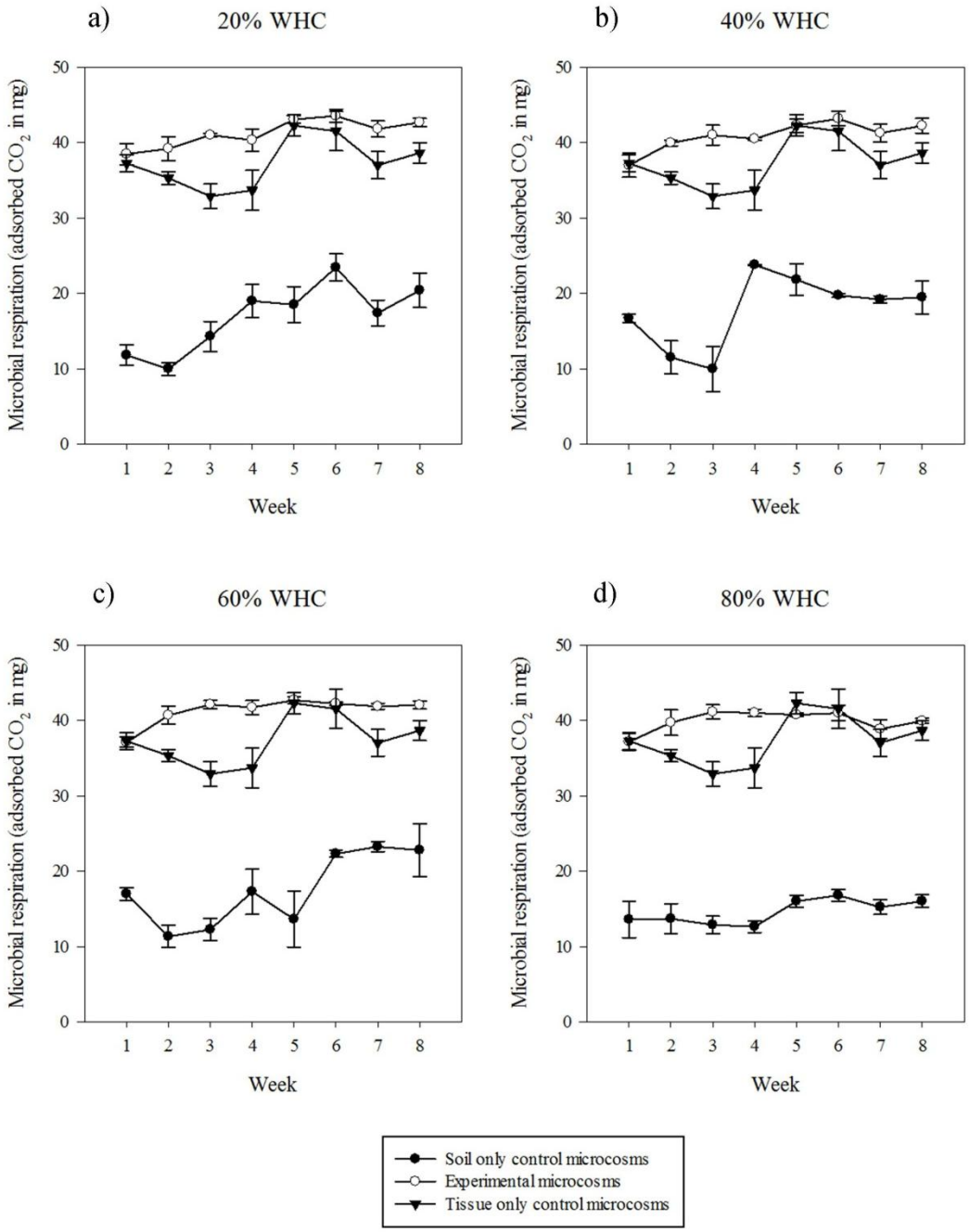


Figure 7. Average CO_2 production per week for control microcosms containing only soil, and experimental microcosms at a) 20%, b) 40%, c) 60% and d) 80% WHC and control microcosms containing only tissue. CO_2 production in tissue only controls was considerably greater than in soil only controls. CO_2 production in experimental microcosms caused CO_2 traps to reach their saturation points on multiple weeks and at all soil moisture levels.

Controls at 40% WHC did not undergo any manipulation to adjust soil moisture. A decrease in respiration was nonetheless observed during the first 3 weeks of the experiment (Figure 7b). It is possible that transferring the soil to a small contained environment affected the soil microbial dynamic negatively. Respiration rates spiked at week 4 and plateaued thereafter. Microorganisms better adapted to their new environment may have proliferated at week 4 and growth rates stabilised in the subsequent weeks. A similar trend was observed for soil controls at 60% WHC (Figure 7c). Respiration rates for controls containing only soil at 80% WHC were fairly constant over the course of the entire experiment (Figure 7d). Average CO_2 production in treatment microcosms was lowest at this level of soil moisture.

Average respiration measures were used to compare control and treatment values between different levels of soil moisture. The results of these analyses are presented in Tables 6 and 7. There were no significant differences between levels of CO_2 production across control microcosms. CO_2 production was significantly greater in treatment microcosms at 60% WHC compared to 80% WHC.

Table 6. Summary of RM-ANOVA or RM-ANOVA on ranks (*) results between average daily measures of respiration per week for control microcosms at 20%, 40%, 60% and 80% WHC. Significant differences ($p < 0.05$) are highlighted in bold.

	40%		60%		80%	
	F	***p***	***F***	***p***	***F***	***P***
20%	0.528	0.491	2.00*	0.289	0.214	0.657
40%	--	--	0.500*	0.727	4.133	0.082
60%	--	--	--	--	3.871	0.090

Table 7. Summary of RM-ANOVA or RM-ANOVA on ranks (*) results between average daily measures of respiration per week for treatment microcosms at 20%, 40%, 60% and 80% WHC. Significant differences ($p < 0.05$) are highlighted in bold.

	40%		60%		80%	
	F	***p***	***F***	***p***	***F***	***P***
20%	2.047	0.196	0.000*	1.00	0.500*	0.727
40%	--	--	2.00	0.200	0.500*	0.727
60%	--	--	--	--	**16.498**	**0.005**

2.4 Discussion

Using microcosms to study the effects of temperature on decomposition supported the prediction that decomposition would be slower at low temperatures and increase at higher temperatures. Tissue maintained at 5°C showed limited decomposition throughout the experiment whereas tissue with and without soil at 20°C showed signs of liquefaction and became discolored. Different levels of soil moisture produced different rates of

decomposition. Higher levels of soil moisture likely resulted in greater levels of humidity within the microcosms as a result of soil water gradually evaporating. The increased humidity may have maintained moisture levels within the tissue and favored microbial activity. Humidity and temperature have been highlighted as factors that favor putrefactive processes (Zhou et al., 2011).

Fungal mycelia were present in multiple treatment microcosms in both experiments. In the study investigating the impact of soil moisture, fungi were dominant in treatment microcosms at 20% WHC but their presence decreased as moisture increased. The proliferation of fungi in soils with extremely low soil moisture content is not uncommon (Griffin, 1972; Treseder et al., 2010). At lower levels of soil moisture fungi are able to grow and search out the necessary nutrients for their survival through hyphae extension (Griffin, 1969). In contrast, under low moisture conditions bacteria are mired as they depend on the movements of water in soil to bring nutrients and remove waste (Wong and Griffin, 1976).

Control microcosms containing only soil showed similar levels of microbial activity and CO_2 production under both incubation temperatures. It was anticipated that control microcosms at 20°C would have higher levels of soil microbial activity than control microcosms at 5°C. It has long been known that temperature has a considerable impact on soil microbial activity (Lindegardh, 1927; Ellert and Bettany, 1992), though other limiting factors can confound results. Fluctuations in microbial activity in relation to temperature may in fact reflect changes in the availability of nutrients within the soil matrix (MacDonald et al., 1995). Available carbon was also identified as the limiting factor for microbial activity at varying temperatures (Knapp et al., 1983). As the

microcosms used in this study represented closed environments, changes in nutrient concentrations could not occur through natural fluctuations in environmental conditions. For this reason soil-nutrient availability would have been comparable across all treatments for both temperatures.

Soil microbial activity in control microcosms generally increase as soil moisture increased between 20% and 60% WHC. This is consistent with the finding of many studies that have shown that when soil is drier microbial biomass and microbial respiration are diminished, whereas increased soil moisture has the opposite effect (Orchard and Cook, 1983; Stott et al., 1986). Microbial activity in control soils at 80% WHC was lowest overall. This is in agreement with findings of Doran et al. (1988) and Torbett and Wood (1992) who observed that microbial respiration rates were optimal within ranges of 55 to 61% WHC but that these rates dropped significantly at higher levels of soil moisture content.

The addition of tissue to the treatment microcosms resulted in increased soil microbial activity on multiple occasions at both temperatures and across different soil moisture levels. The increase in microbial activity was significant at 20°C and for soils at 40% WHC. The increased soil microbial activity may have occurred through the proliferation of soil microbes using the tissue as a source of nutrients and carbon substrate and/or through the colonization of bacteria from the tissue to the soil. Increased microbial activity as a result of increased nutrients in soil following decomposition is consistent with the findings of Orchard and Cook (1983) and Quemada and Cabrera (1997) who observed that the addition of new sources of organic carbon favoured bacterial growth in soils. To determine if the increased microbial activity is the result of

bacterial migration from the tissue source would require phylogenetic profiling of soil microbial communities.

Respiration data indicated that the addition of tissue to the treatment microcosms of both experiments significantly increased CO_2 production in comparison to soil control microcosms. The difference between amounts of CO_2 produced in the treatment microcosms and that produced in tissue controls was often equivalent. As such it is difficult to determine the extent to which the addition of tissue increased soil microbial respiration rates. A similar study by Tibbett et al. (2004) (see also Carter and Tibbett 2006) found that the biodegradation of ovine tissue was associated with a significant increase in microbial CO_2 production. Tumer et al. (2013) observed that CO_2 production was significantly higher in gravesoils composed of organic soil during the first three months of decomposition.

Though the increased production of CO_2 suggests considerable aerobic metabolism the noted smell of hydrogen-sulfide in many of the microcosms also suggested there was proliferation of anaerobic microorganisms (Dent et al., 2004). It is likely that between instances where microcosms were opened to refresh oxygen levels the jars became slightly anaerobic. Soil below the tissue and areas within the tissue were also expected to be deprived of oxygen. Multiple anaerobes and facultative anaerobes such as *Clostridia, Escherichia, Shigella* and *Salmonella* have long been known to take part in the rotting of meat (Janzen, 1977). Anaerobic bacteria are also recognized as making up the majority of microorganisms involved in cadaver putrefactive processes (Janaway et al., 2009). More recently, anaerobic microorganisms such as *Fusobacteria* and members

of the Firmicutes, including Clostridiae and Lactobacillae were identified as important members of necrotic microbial populations (Pechal et al., 2013; Metcalf et al., 2013).

The microcosm studies presented in this chapter were only carried out on one type of soil chosen to reflect soil used during larger outdoor decomposition trials. It has been shown that soil type influences the rate of decomposition (Carter et al., 2010; Tumer et al., 2013). Soil type will also influence microbial community responses to decomposition as a result of different soil water holding capacities, aggregate formation potentials, and buffering capacities. As such, the results presented here are likely only applicable to similar soils with comparable edaphic characteristics.

2.5 Conclusions

The microcosm studies confirmed that decomposition rates are affected by temperature, which in turn increases soil microbial activity levels. Increased soil moisture was found to favour decomposition of the pork tissue. This was likely a result of increased humidity within the microcosms which maintained microbial activity within the tissue. These observations highlight the relationship between soil moisture and relative humidity following evaporation demonstrating that humidity can promote decomposition. In environments where rainfall is frequent and temperatures favour the evaporation of soil moisture, decomposition rates may be influenced by increased humidity particularly in proximity to the soil surface.

Microbial activity at 5°C and 20°C did not vary considerably in the absence of decomposition. The presence of decomposing tissue was shown to produce significantly

higher levels of microbial activity on four occasions at both temperatures. These differences were observed a few weeks after experiments had begun. It remains unclear to what extent the increased microbial activity in treatment soils occurs through the use of the newly available nutrients by soil microorganisms compared to the migration of microorganisms from the tissue into the soil. Obtaining profiles of the soil microbial community throughout the decomposition process is required to fully understand the dynamics between soil microbial communities and those associated with decomposing matter.

CHAPTER 3
Outdoor trials: Environmental conditions, soils parameters and microbial activity

3.1 Introduction

The application of soil science in forensic casework mainly consists of chemical analyses or particle comparisons to link evidence, suspects or victims and locations. Taphonomic processes often take place in terrestrial environments and soil analyses within this field are routine. Cadaver decomposition in terrestrial environments is known to produce an influx of nutrients, notably nitrogen, phosphorus and sodium, which alter the chemical composition of soil (Parmenter and MacMahon, 2009). This change is known to affect surrounding vegetation (Bornemissza, 1957; Towne, 2000) and soil microbial communities (Carter et al., 2007; Hopkins et al., 2000; Parkinson et al., 2009). Decomposition is also believed to introduce a new microbial inoculum into the soil environment (Moreno et al., 2011). The possibility of monitoring and documenting the changes that soil microbial communities undergo as a result of decomposition suggests that alterations of the soil microbial profile can be used as indicators of clandestine graves or to aid in the estimation of post-mortem intervals (Carter et al., 2007; Metcalf et al., 2013).

Studies investigating the changes to soil microbial communities as a result of cadaver decomposition have mainly been conducted within the laboratory where environmental variables are controlled (Haslam and Tibbett, 2009; Carter and Tibbett, 2006). Although such studies provide an insight to the effects on soil during decomposition, they are not representative of the conditions typically observed in

forensic casework. When factors such as natural variation in ambient temperature, rainfall, insect activity and scavenging are not taken into consideration it is difficult to apply laboratory results to casework.

During decomposition a body will undergo breakdown via two major processes: autolysis and putrefaction (Evans, 1963). These processes lead to the liquefaction of soft tissue and the production of decomposition fluids. These fluids are high in microbial content, mainly originating from the gastro-intestinal tract and are purged from the body through orifices and ruptures following bloat (Knight, 2004). Native soil microbial communities are thought to react to the presence of a cadaver within the first 24 hours of deposition (Carter et al., 2008) and increases in soil microbial activity have been reported in the area immediately surrounding the cadaver following purging of fluids (Carter and Tibbett, 2008; Carter et al., 2010). Over time cadaver decomposition is believed to increase fertility within the decomposition island created around the body (Towne, 2000; Carter et al., 2007).

Although the microbial load in soil where decomposition takes place may increase as a result of a new source of inoculum, soil microbial communities may also be hindered by the presence of a decomposing substrate. Decomposition is known to be associated with large influxes of ammonia into the soil environment (Hopkins et al., 2000) which may be toxic to some microorganisms. Taphonomic processes are also known to discolor the soil, cause vegetation death and displace the natural soil fauna (Bornemissza, 1957; Towne, 2000). These events suggest that decomposition has a harmful effect on organisms within close proximity of decomposing carcasses or cadavers.

Soil pH fluctuations as a result of cadaver decomposition have been well documented. Most studies report the alkalinisation of the soil following decomposition (Vass et al., 1992; Towne, 2000; Hopkins et al., 2000; Carter and Tibbett, 2006; Carter et al., 2008; Haslam and Tibbett, 2009). This effect has been shown to endure in soils that see repeated decomposition activity over the course of many years (Damann et al., 2012). Chemical processes associated with decomposition will likely alter soil pH, either increasing acidity or alkalinity. As such, soil pH must be taken into account when attempting to understand soil microbial dynamics. Microorganisms are typically well adapted to survive within a specific range of pH values. A small change in pH can alter the availability of nutrients in the environment as well as the microorganisms' ability to utilize these nutrients (Bååth and Arnebrant, 1994; Aciego-Pietri and Brookes, 2008).

Soil moisture content can also alter microbial activity and survival rates. Too little moisture causes soil nutrients to become bound to soil particles and unavailable to microbes while too much water may create an anoxic environment, causing a shift from aerobic to anaerobic microbial metabolism. As decomposition is often associated with high levels of moisture and the seeping of fluids into the surrounding environment, moisture content in the soil may change when decomposition takes place. If such a change occurs rapidly, a decrease in microbial activity may ensue and long lasting effects on soil microbial community composition may be observed (Schimel et al., 1999).

The experimental design of the studies presented here allowed natural fluctuations in environmental variables (i.e. ambient temperature and rainfall) and their impact on decomposition and soil parameters to be investigated. Soil microbial activity was measured throughout decomposition to determine whether the presence of a cadaver

increased or decreased microbial activity. A fluorescein diacetate assay was used to measure soil total microbial activity. This method has proven useful in characterizing changes in soil microbial activity in a variety of soils (Schnürer and Rosswall, 1982) and is commonly used to characterize the effects of various soil treatments. Soil pH and soil moisture were measured to understand how these variables influenced soil microbial activity in the presence or absence of decomposition.

3.2 Method

3.2.1 Experimental design

To study the effects of decomposition on soil from the fresh stage through to the dry remains stage a total of 4 trials were conducted over 2011 and 2012. Each year, one trial was started in the late spring and a second trial started in the summer. All trials were carried out at the University of Ontario Institute of Technology decomposition facility located in Oshawa, Ontario, Canada (43.948 °N, 78.900°W). The location of the facility is shown in Figure 8a. Soil at the facility has been characterized as a gravely sandy loam (pH 7.78 ± 0.26) by the University of Guelph Agriculture and Food Laboratory. Ambient temperature and precipitations within the facility were monitored using a Hoboware® weather station (Onset, Cape Cod, USA).

For each experiment three pig carcasses (*Sus scrofa*) weighing approximately 23kg were used as human cadaver analogues. Each pig ingested the same diet as they were reared together on the same farm. The pigs were killed at a local abattoir according to the guidelines set out by the Ontario Ministry of Agriculture, Food and Rural Affairs

on the morning of each trial (day 0) and immediately transported to the decomposition facility. The pig carcasses were deposited on the soil surface and covered with wire cages to prevent scavenging.

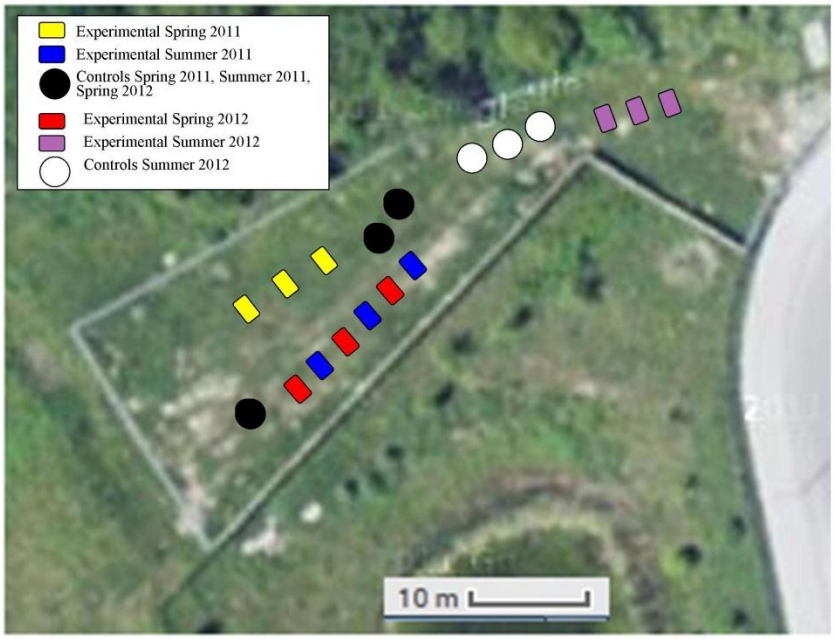
A schematic of the experimental set-up within the facility for each trial is presented in Figure 8b. The location of experimental sites for each trial was chosen according to the known history of the facility and the need to select sites that had not been previously used to deposit decomposing tissues. The spacing between each carcass during each experiment was a minimum of 2 meters to ensure that there would be no cross contamination between sites. Experimental sites were also maintained a minimum of 5 meters away from control sites.

Soil samples at experimental sites were collected from below the head, torso and hind quarters of all the pigs during each trials for a total of 9 experimental samples per sampling day. Three sites located within the facility but having had no contact with decomposing carcasses were used as controls. Control sites measured approximately 2 meters by 2 meters. Each control site was sampled in triplicate on each day for a total of 9 control samples per sampling day. Triplicate samples were collected randomly within an area of approximately 20cm by 20cm within the control site. The area within the control plots where triplicate samples were collected on each day was continuously alternated along a spiral sampling pattern to ensure that the same area was not repeatedly sampled. All soil samples were obtained using a sterilized stainless steel scoopula that was inserted 3 cm into the soil and used to produce soil cores which were stored in glass scintillation vials fitted with Teflon lined caps. Sample collection occurred on days 0, 2, 4, 6, 8, 11, 14, 17, 20, 27, 34, 41, 48, 62 and 90. Samples were immediately transported to the

laboratory where analyses requiring fresh soil (i.e. measures of microbial activity) were carried out. Remaining soils were stored at -20°C.

The decomposition stages described by Payne (1965) and adapted by Anderson and VanLaerhoven (1996) were used to categorize the stage carcasses had reached at each sampling day. These stages are: fresh, where the body appears the same as before death with some slight discoloration; bloat, during which the body becomes distended due to the proliferation of gut bacteria and the accumulation of gases within the body; active decay, during which the majority of soft tissue will be broken-down; advanced decay, typically observed when the rate of soft tissue breakdown is slowed and the body becomes weathered; dry remains; at which point all that remains are bones, hair and dried tissue.

(a)



(b)

Figure 8. (a) UOIT decomposition facility located near Founders Drive, north of Conlin Road in Oshawa, Ontario, Canada (b) Schematic of the site layout for all experimental and control sites from the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 trials. Positioning of sites on the diagram is approximate.

Accumulated degree days (ADD) were calculated using the temperature data to compare rates of decomposition between trials based on ambient temperatures or heat units. ADD is calculated by obtaining daily average temperatures for each experimental day up to a given time point and calculating the sum of these temperatures (Edwards et al., 1987).

3.2.2 Soil pH and soil moisture

Soil pH was measured from 1:5 w/v suspensions of soil in distilled water (pH 7.5). Soil samples and water were placed in 20 ml vials, shaken vigorously and left to settle for at least 30 minutes before pH measurements were taken using an UltraBasic Benchtop pH-meter (Denver Instruments, Bohemia, NY, USA) coupled with an Accumet double junction gel filled pH electrode (Cole Palmer, Montreal, Canada).

Soil moisture measures were obtained using the methods described in Chapter 2. Soil samples were taken across the experimental facility to determine average values for soil bulk density, particle density and porosity at this site.

3.2.3 Soil total microbial activity

Soil total microbial activity was measured using the fluorescein diacetate assay protocol described in Chapter 2. Samples were collected in the field and immediately transported to the laboratory to obtain measures of microbial activity.

3.2.4 Statistical analyses

Data was tested for normal distribution by the Shapiro-Wilk's test and equal variance by F-test prior to analysis. Student's t-test was used to determine if significant differences existed between daily measures of soil pH, soil moisture and microbial activity from control samples and experimental samples. When the normality test failed, a Mann-Whitney Rank sum test was performed. Statistically significant differences among controls and experimental treatments for each trial were analyzed by one way repeated measure analysis of variation on ranks. To determine if soil pH, soil moisture or ambient temperature could be correlated with the measures obtained for microbial activity, Pearson product-moment correlations were conducted. All data was analyzed using the SigmaPlot^{TM} 12.0 software package.

3.3 Results and Discussion

It was hypothesized that experiments conducted in the spring would produce slower rates of decomposition and that rainfall might affect levels of soil moisture in both control and experimental sites. Trials undertaken during the summer were expected to produce faster rates of decomposition due to higher ambient temperatures. Since precipitation is more sporadic in the summer months, it was expected that soil at the experimental site would become intermittently dry. It was also anticipated that soil in contact with decomposing carcasses would be subjected to an increase in moisture as a result of tissue liquefaction and purging of decomposition fluids. Despite the varying effects of season on decomposition rates, an overall increase in microbial activity at sites

where decomposition occurred was anticipated due to both increased nutrient release and the influx of microorganisms from the carcasses during decomposition.

3.3.1 Environmental conditions and stages of decomposition

Average air temperatures measures for spring trials were 21.0°C (2011) and 20.6°C (2012). As is consistent with the seasonal changes observed in Southern Ontario the temperature gradually increased over the course of both spring trials reaching a maximum daily average temperature on day 49 (30.3°C) in 2011 and day 46 (30.2°C) in 2012 (see Figure 9). Both summer trials commenced during the warmest period of the season and temperatures steadily decreased overtime as the seasons changed from summer to fall. Overall average temperatures for trials conducted in the summer were 20.7°C (2011) and 14.0°C (2012). The fall of 2012 was cool with daily average temperatures at the end of the experiment nearing 0°C.

Spring 2011 was characterized by high precipitation levels during the first week of the experiment and a gap in precipitation between days 30 and 50. The lack of rainfall caused drought like conditions in the region where the experiment took place. Rainfall was sporadic after day 40 of this trial which coincided with the early days of the Summer 2011 trial. Spring 2012 saw below normal temperatures during the first few experimental days with temperatures averaging 15°C rather than the seasonal average of 20°C. After day 10, seasonal temperatures were observed. Precipitation was recorded on a regular basis during the spring and summer 2012 trials.

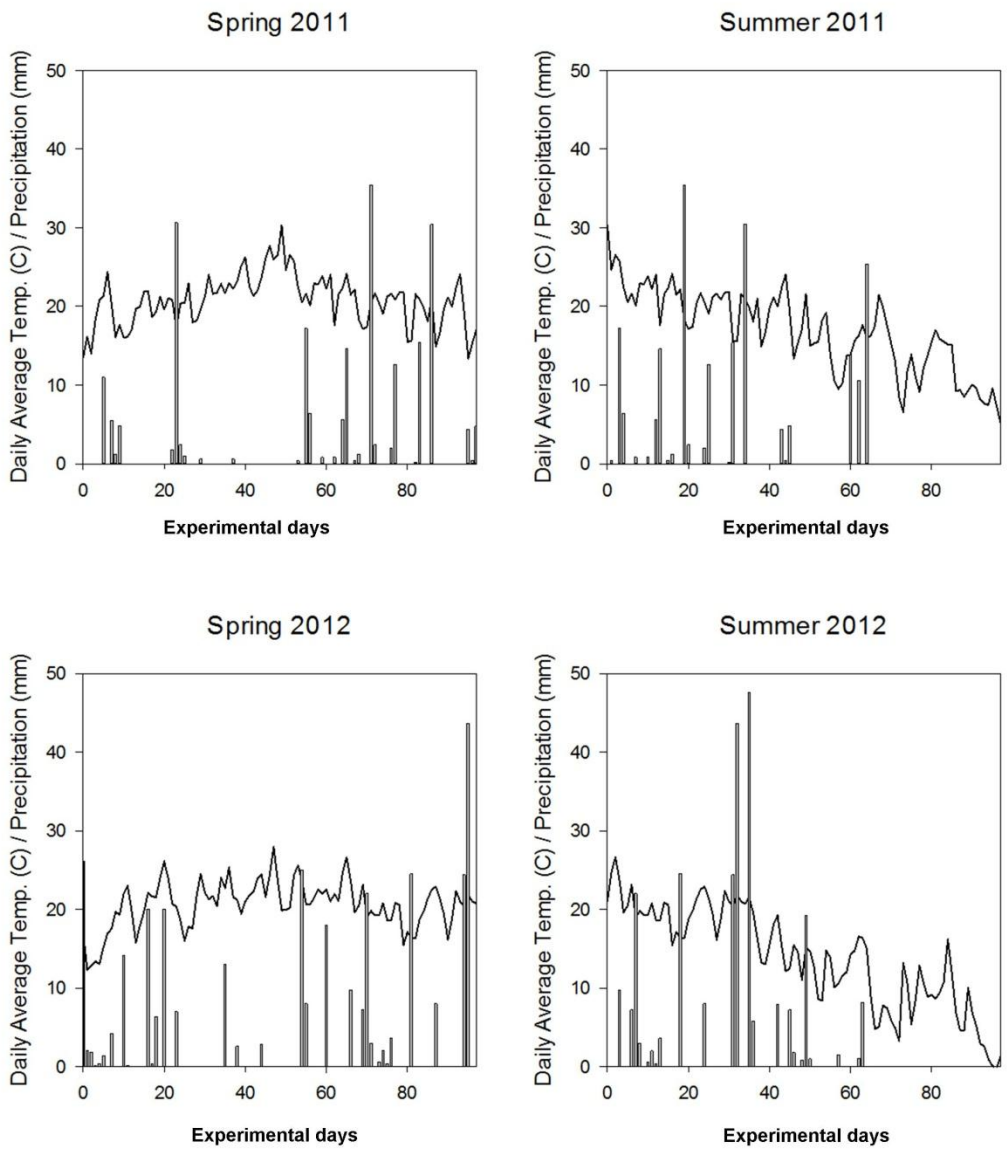


Figure 9. Average daily temperatures (°C) and precipitation (mm) for the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 experimental trials. Average daily temperatures gradually increased over the course of both spring trials as spring changed to summer and subsequently decreased as summer changed to fall. Summer trials were launched during periods of peak daily average temperatures for the summer and gradually decreased as summer changed to fall. Varying trends of precipitations were observed in each trial.

Each trial was subjected to a unique set of temperatures and precipitation, which likely contributed to varied rates of decomposition. To allow decomposition stages to be compared between trials, the length of each stage per trial was expressed in accumulated degree days or heat units (see Figure 10). In all trials the fresh stage was observed on days 0 and 1. When accumulated degree days were calculated, the end of the fresh stage and beginning of bloat was noted to begin at 74.4 ADD (Spring 2011), 52.2 ADD (Summer 2011), 20.0 ADD (Spring 2012) and 51.3 ADD (Summer 2012). Bloat was observed by day 2 in all cases although the classification of bloat was more ambiguous for the Spring 2012 trial with full bloat not being recorded until day 6. Active decay was characterized by maggot masses covering the carcasses and deflation of the torso. The pig carcasses were characterized as being in active decay on day 6 at 128.4 ADD (Spring 2011), day 4 at 150.2 ADD (Summer 2011), day 8 at 179.3 ADD (Spring 2012) and day 4 at 115.6 ADD (Summer 2012). The stage of advanced decay was distinguished by the migration of maggots away from the carcasses. During the Spring 2011 trial, all maggots present on the pig carcasses migrated away from the bodies on day 8, earlier than was expected based on previous experiments. Soft tissue consumption by the larvae was limited resulting in much of the tissue still being present on the carcass. It is believed that higher than normal amounts of precipitation during the first few days of decomposition may have been responsible for initiating early maggot migration. A relationship between rain and maggot dispersal has previously been suggested by Lewis and Benbow (2011). The saturation of tissues with water is believed to deter the maggots from remaining present on carrion. Based on the criteria used to distinguish decomposition stages, carcasses from the Spring 2011 trial were considered as being in the advanced decay

stage on day 8 at 164.6 ADD. The onset of advanced decay for the other trials occurred on day 14 at 346.8 ADD (Summer 2011), day 12 at 221.9 ADD (Spring 2012) and day 14 at 315.4 ADD (Summer 2012). The carcasses were characterized as dry remains by day 41 at 699.2 ADD (Spring 2011), day 49 at 384.3 ADD (Summer 2011), day 17 at 76 ADD (Spring 2012) and day 27 at 269.7 ADD (Summer 2012).

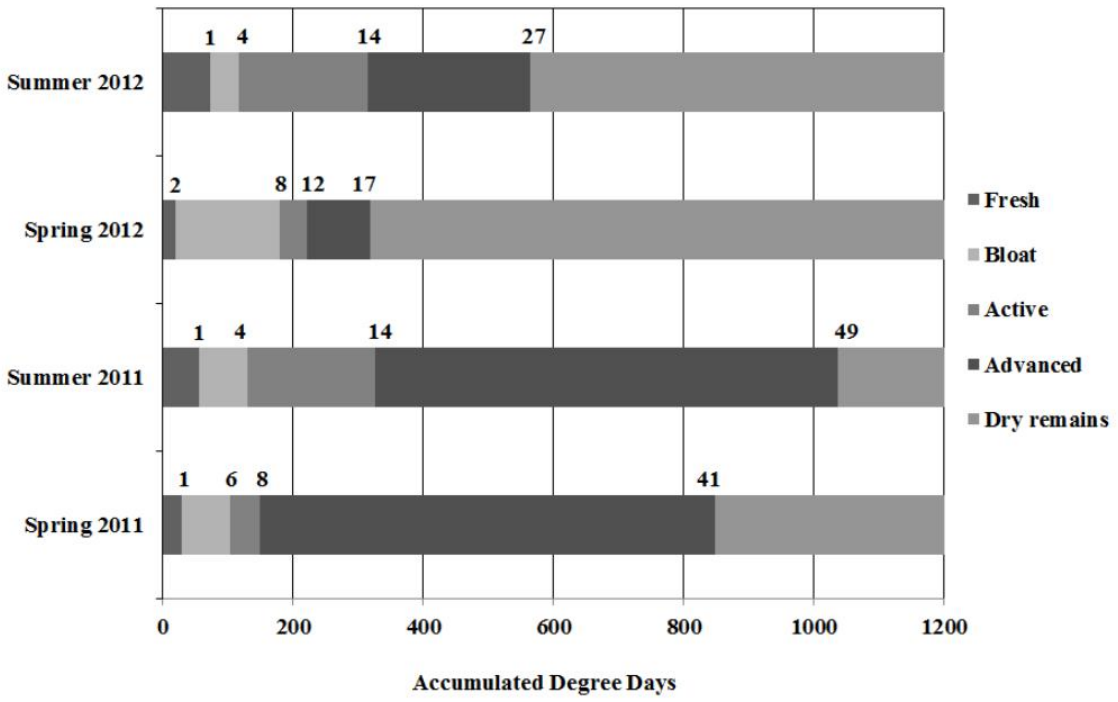


Figure 10. Decomposition stages for the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 experimental trials expressed in accumulated degree days (ADD). The length of each stage in experimental days is shown above the bar.

The onset of the first 3 stages of decomposition (i.e. fresh, bloat, active decay) occurred below 200 ADD in all four trials. The onset of advanced decay and dry remains varied greatly between trials with the distinction between the two stages often being difficult to determine. Pig carcasses from the spring and summer 2011 trials had significant amounts of soft tissue remaining once they had reached the stage of advanced decay (see Figure 11). The presence of residual soft tissue at this later stage of decomposition made it difficult to determine the beginning of the dry remains stage for these two trials. Remaining soft tissue became rehydrated following rainfall making it appear that the carcasses had not yet reached the dry remains stage. Soft tissue was efficiently removed from the carcasses in both trials carried out in 2012 and skeletonisation was more pronounced. The beginning of the dry remains stage was observed earlier for both of these trials.

Throughout this study larvae were a major factor of decomposition and were responsible for the majority of soft tissue removal. Increased rates of decomposition as a result of maggot activity have been well documented by Simmons et al. (2010 a & b). The early dispersal of larvae in the spring 2011 trial greatly affected the progress of decomposition. During this study, early maggot migration was thought to explain the difference in decomposition rates observed between the two spring trials. Maggots migrated on day 8 in the Spring 2011 trial versus day 12 in the Spring 2012 trial. Both summer trials produced similar decomposition rates and the length of each decomposition stage when measured in ADD was comparable. These results were in agreement with the hypothesis that temperature will dictate the progression of decomposition and the transition from one stage of decomposition to the next. Slight differences between larval

colonization rates across triplicates were observed. These variations appeared to be a result of differences in the opening of the mouth and eyes as well as the presence or absence of feces. Although pig carcasses were chosen based on their similarity in weight, some carcasses were smaller than others. Soft tissue from smaller carcasses was removed slightly more rapidly than that of larger carcasses. Body constitution is known to influence maggot activity (Campobasso et al.2001).

The onset of the advanced stage was associated with the formation of a crust on the soil surface in all of the trials conducted. It is believed that this crust forms through the mixing of decomposition fluids, remnants of broken down tissue and soil particles. This phenomenon was previously reported in entomological studies (Bornemissza, 1957; Forbes and Dadour, 2010) suggesting it may be a common phenomenon in cases where maggots are present on the decomposing body. In order to obtain soil samples it was necessary for the crust to be broken or lifted to gain access to the soil beneath. It was also noted that the presence of the crust caused water to pool on the surface of experimental sites after rainfall events. Consequently, soil below the crust remained slightly drier than surrounding soil due to the barrier created by the crust on the soil surface.

Figure 11. Carcass decomposition on experimental day 42 of the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 trials. Significant amounts of tissue were still present on carcasses from both 2011 trials after 42 days. Carcasses from both 2012 trials were showed greater skeletonisation by day 42 and remaining tissue was dry.

3.3.2 Soil pH

Average soil pH values of control and experimental sites were compared on each sampling day (see Figure 12). Soil pH values were only significantly different ($p < 0.05$) on a few days during each trial; specifically days 14, 20 and 62 in Spring 2011; days 2, 6 and 34 in Summer 2011; days 48 and 62 in Spring 2012; and days 0, 6, 8, 11 and 62 in Summer 2012. No relationship could be found between the occurrence of these pH shifts and decomposition stages. Soil pH values obtained over the course of each trial were compared for overall significant differences between control and experimental samples but no significant difference was identified (see Table 8).

Table 8. Summary of repeated measures ANOVA on ranks used to determine overall significant differences ($p < 0.05$) (highlighted in bold) between control measures and experimental measures of microbial activity, soil moisture and soil pH for the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 trials.

	Spring 2011		**Summer 2011**		**Spring 2012**		**Summer 2012**	
	χ^2	**P**	χ^2	**P**	χ^2	**P**	χ^2	**P**
Soil pH	17.30	0.240	17.90	0.211	23.20	0.057	18.804	0.173
Soil moisture	22.60	0.067	21.49	0.064	23.650	0.051	**25.961**	**0.026**
Microbial activity	14.00	0.442	**23.78**	**0.049**	13.05	0.523	14.10	0.442

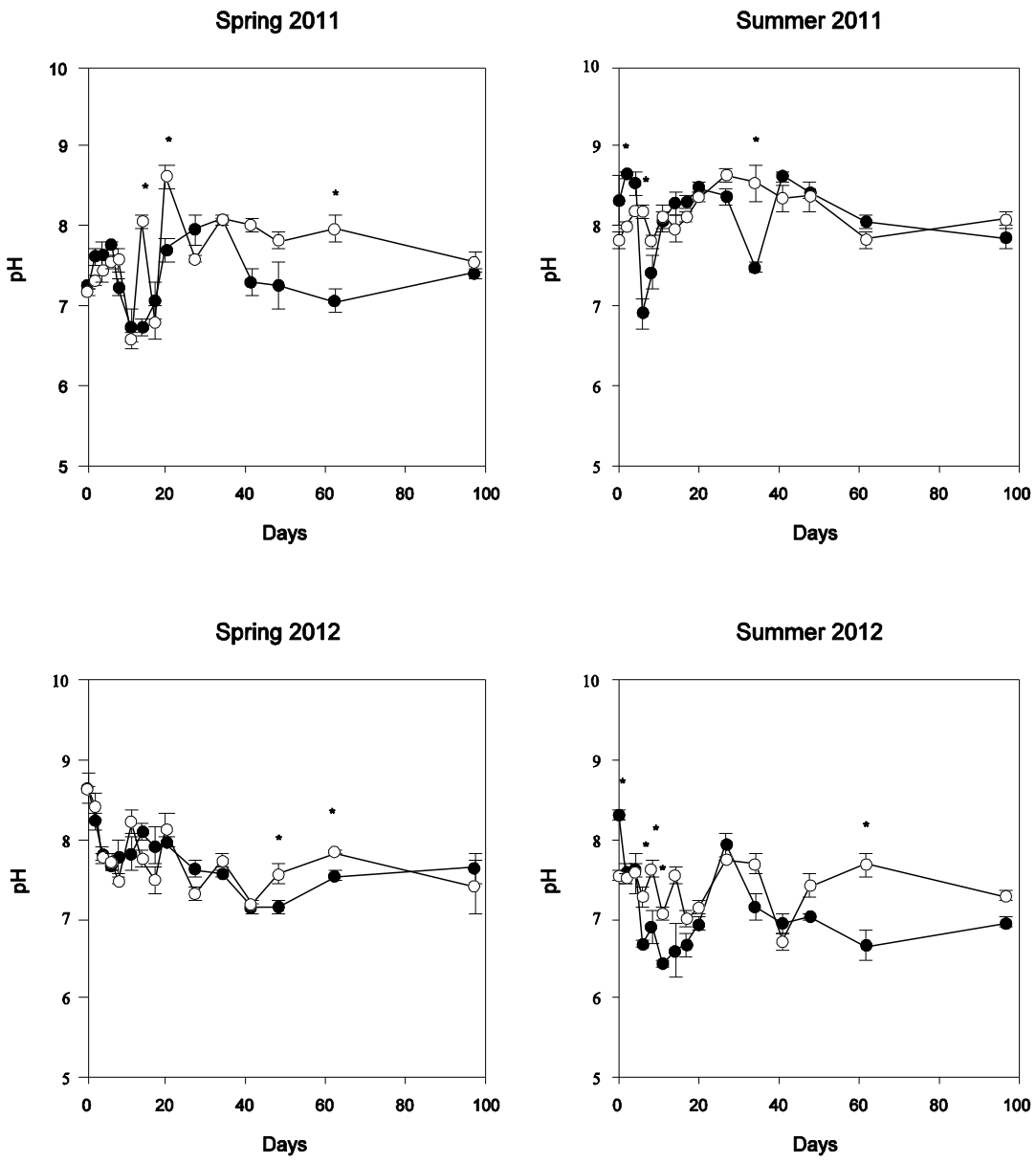


Figure 12. Soil pH measurements for control (-○-) and experimental (-●-) samples collected on each sampling day during the Spring 2011, Summer 2011, Spring 2012, and Summer 2012 trials. Significant differences ($p < 0.05$) between experimental and control samples are marked with an *. Values of soil pH can be seen to fluctuate in a similar way for both control sites and experimental sites during each trial. A slight decrease in soil pH was observed during the first few weeks of each trial.

Published studies investigating the changes that occur in soil following taphonomic events indicate that a localized increase in pH can be expected as a result of cadaver decomposition (Vass et al., 1992; Towne, 2000; Hopkins et al., 2000; Carter and Tibbett, 2006; Carter et al., 2008; Haslam and Tibbett, 2009). This was not observed in any of the four trials conducted in this study and correlates with studies conducted by Van Belle et al. (2009) in the same environment. The acidification of the soil observed in all experiments coincides with rainfall and increases in soil moisture, suggesting that rain may be the cause for the observed changes in soil pH. In most experiments, it is possible that increases in alkalinity as a result of decomposition were masked by the effects of rain. Soil pH at the experimental facility is naturally alkaline with high buffering potential, which may explain why soil did not become more alkaline with decomposition. Initial soil pH has been shown to influence the soil pH changes observed following decomposition (Haslam and Tibbett, 2009).

Acidification of the soil later in the decomposition process has been reported by Vass et al. (1992) and by Carter and Tibbett (2008). This change is thought to result from cations being released following bone decomposition. However the decrease in pH observed in this study occurred in the early stages of decomposition. Although the changes in soil moisture were more pronounced during the spring, rainfall was logged on multiple days during the first weeks of all four trials. It is possible that the acidification observed is a result of rainfall which is generally known to have an acidic pH of approximately 5.6 and can become even more acidic in urban areas (Charlson and Rhode, 1982).

3.3.3 Soil moisture

Seasonality appeared to play an important role in soil moisture levels. An increase in soil moisture was observed in both spring studies following extended periods of rain. Experiments which commenced in the summer months were subjected to drier soil conditions during the first few weeks with soil moisture gradually increasing as the trial progressed (Figure 13). Temperature was found to be negatively correlated with soil moisture in all four trials. Increases in temperature corresponded with decreases in soil moisture suggesting rates of evaporation played an important role in regulating soil moisture levels.

During the Spring 2011 trial, soil moisture was significantly higher ($p < 0.05$) in control soils on days 2 and 4 but significantly higher in experimental soils on days 6, 14, 34, 41 and 48. For the Summer 2011 trial, soil moisture was significantly higher in control soil on days 20 and 27. The Spring 2012 trial showed that soil moisture was significantly higher ($p < 0.05$) in experimental soil on days 27 and 34. During Summer 2012, soil moisture was significantly higher ($p < 0.05$) in experimental soils on days 20 and 27. Summer 2012 was the only trial to show a significant difference between soil moisture values for control sites and experimental sites overall (see Table 8).

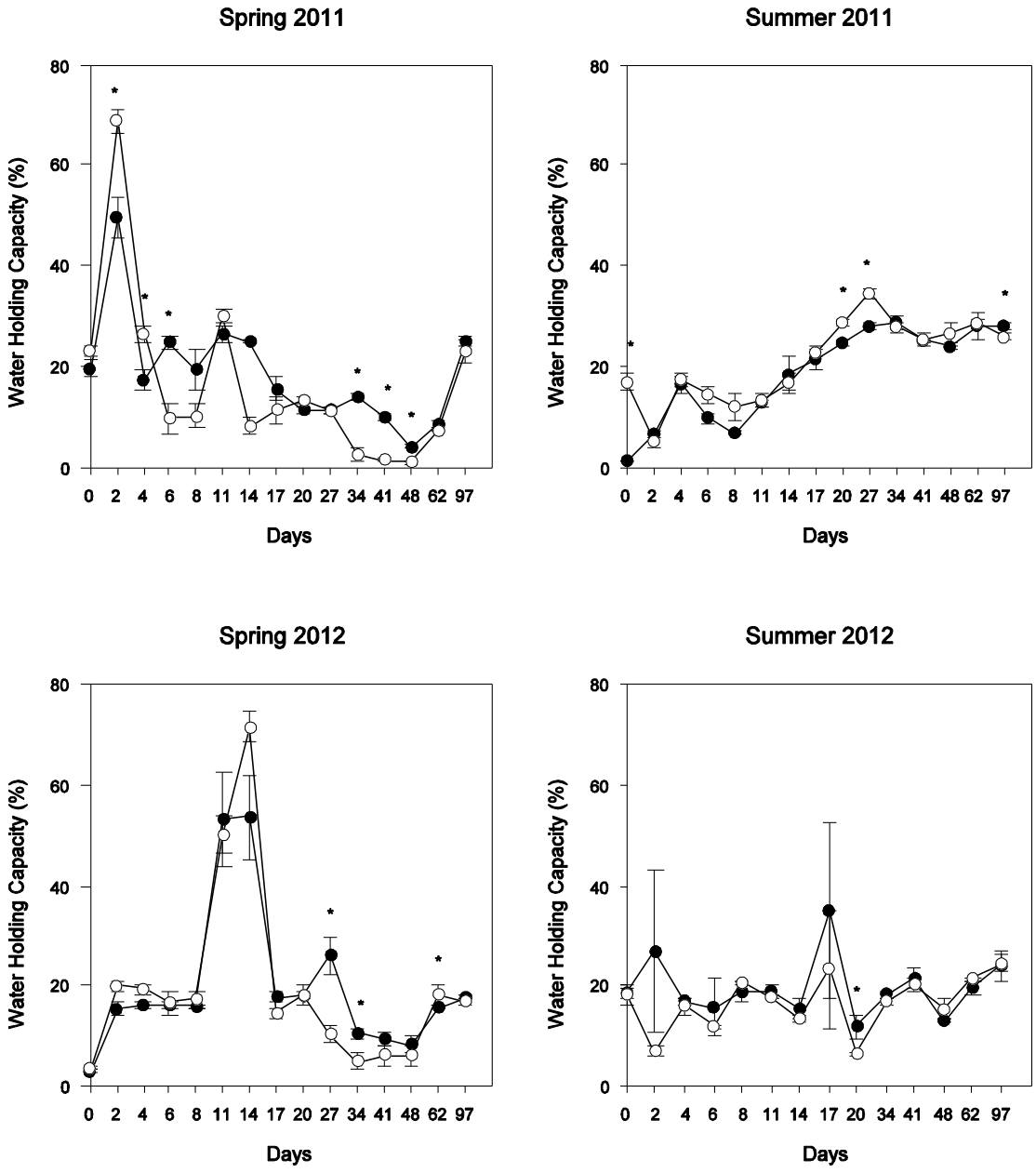


Figure 13. Soil water holding capacity for control (-○-) and experimental (-●-) soil samples collected on each sampling day during the Spring 2011, Summer 2011, Spring 2012, and Summer 2012 trials. Significant differences ($p < 0.05$) between experimental and control samples are marked with an *. Spikes in soil moisture were observed at different times during both spring trials. Moisture trends for both summer trials indicated that soil moisture content remained mostly constant throughout these experiments. Soil moisture of both control and experimental sites were seen to fluctuate in a similar manner for all trials.

Throughout this study, it was observed that when rainfall occurred, an increase in soil moisture would follow and was typically more pronounced in control soils than at experimental sites. Soils below the pig carcasses were likely protected from rainfall either by the pigs and their remains or by the crust formed by decomposition products later in the decomposition process.

Soil moisture levels were expected to fluctuate in the experimental soils as a result of decomposition fluids being purged from the body and the liquefaction of soft tissue. This was expected to occur immediately following bloat and at the beginning of active decay when purging and liquefaction are most noticeable. A significant ($p < 0.05$) increase in experimental soil moisture that could be associated with these decomposition events was only observed on day 6 of the Spring 2011 trial.

Soil moisture was significantly higher overall in experimental samples versus control samples during the Summer 2012 trial. This is in accordance with the hypothesis that decomposition increases moisture at the site of decay. During the Summer 2012 trial, the pigs were deposited on the soil surface in the western portion of the decomposition facility. Although all experimental sites were cleared of vegetation prior to the deposition of the carcasses to facilitate sampling, vegetation in this area was observed to be denser than elsewhere in the facility. It is believed that this difference in vegetation may have resulted in higher water retention and increased soil fertility.

3.3.4 Soil total microbial activity

Average microbial activity for control and experimental sites on each sampling day of the four trials can be seen in Figure 14. Statistical analyses of these results are presented in Table 2. During Spring 2011, significantly higher microbial activity levels were observed for the decomposition sites on eight of the fourteen sampling days (2, 6, 11, 14, 17, 34, 41 and 48). These days fell within three different stages of decomposition: bloat, advanced decay and dry remains, however the increase during the bloat stage is unlikely to be related to decomposition processes. The Summer 2011 trial produced five instances where experimental soils showed significantly higher microbial activity levels. These occurrences fell within the bloat stage (day 2), active decay stage (day 8) and advanced decay stage (days 27, 34 and 41). During the Spring 2012 trial, microbial activity was found to be significantly higher in experimental soils during the bloat stage (days 6), active stage (day 11) and dry remains stage (days 27 and 62). During the Summer 2012 trial, microbial activity was significantly higher in experimental soils during the fresh stage (day 0). It is unlikely that this increase related to decomposition activity as no changes at the soil level were observed at the time of deposition. Furthermore, microbial activity of experimental soils was significantly lower than control soils on most days of the summer 2012 with the exception of days 0, 2, 4, 62 and 97. Comparison of microbial activity for control and experimental samples for each trial overall, demonstrated a significant difference in the Summer 2011 trial only (see Table 8).

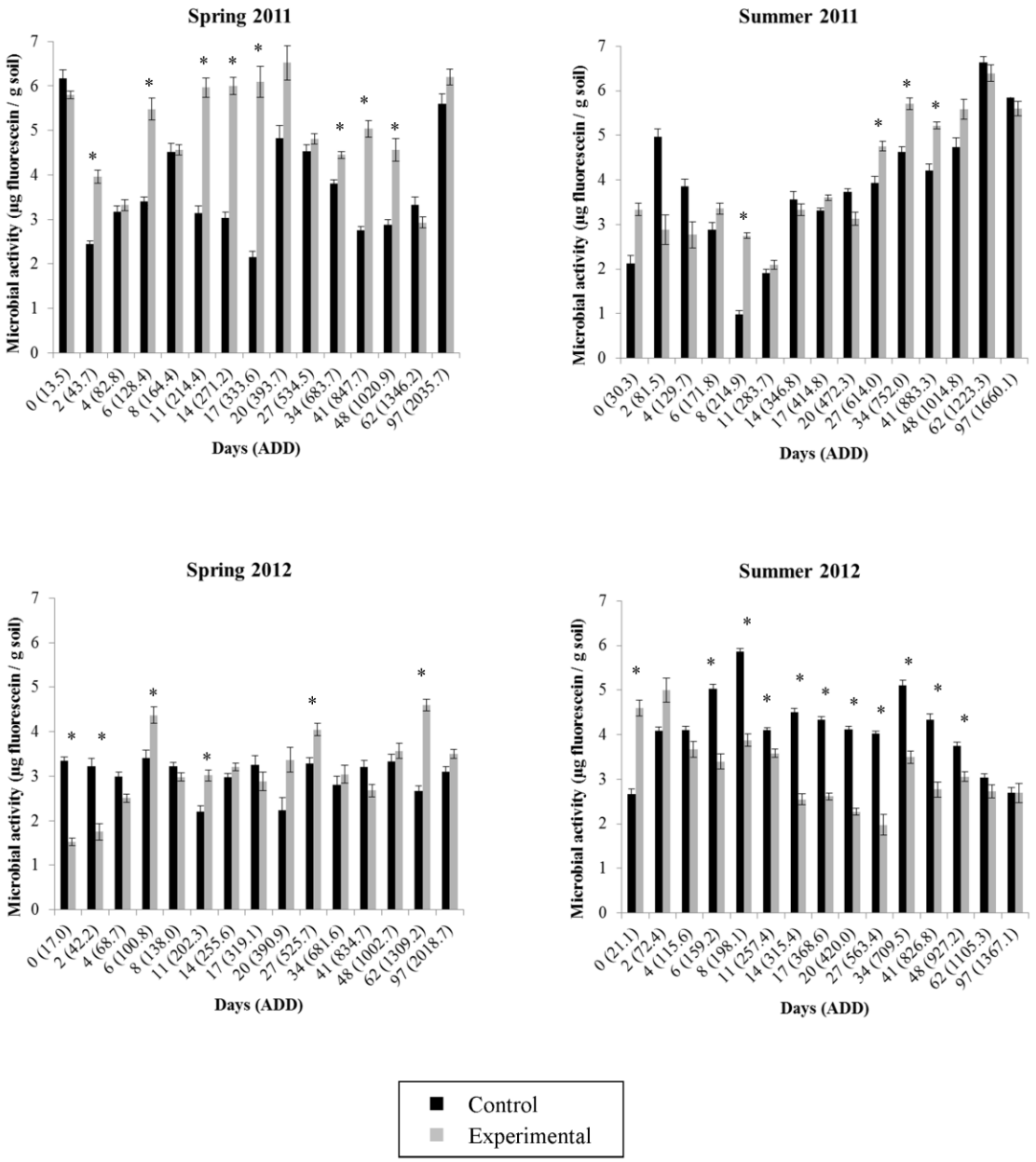


Figure 14. Average measures of total microbial activity for control sites and experimental sites collected on each sampling day during the Spring 2011, Summer 2011, Spring 2012, and Summer 2012 trials. Significant differences ($p < 0.05$) between experimental and control samples are marked with an *. Microbial activity fluctuated in a similar way for control and experimental sites during both 2011 trials. Multiple days from the Spring 2011, Summer 2011 and Spring 2012 trials produced significantly higher microbial activity within experimental sites following decomposition. Summer 2011 microbial activity levels were significantly lower in experimental sites on multiple days following the onset of decomposition.

Table 9. Summary of t-tests or Mann-Whitney rank sum tests (*) used to determine significant differences ($p < 0.05$) (highlighted in bold) between average microbial activity levels of control samples and experimental samples for each day of the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 trials.

	Spring 2011		Summer 2011		Spring 2012		Summer 2012	
Day	**t**	**P**	**t**	**P**	**T**	**P**	**t**	**P**
0	-0.898	0.382	**-1.199**	**0.017**	**4.913**	**< 0.001**	**-4.528**	**< 0.001**
2	**4.742**	**< 0.001**	**2.812**	**0.013**	**2.151**	**0.047**	63.00*	0.052
4	0.421	0.680	1.610	0.127	1.287	0.216	1.125	0.277
6	**3.887**	**0.001**	-1.184	0.254	**-2.150**	**0.047**	**120.0***	**0.003**
8	0.107	0.916	**-8.864**	**< 0.001**	0.807	0.431	**6.383**	**< 0.001**
11	**5.283**	**< 0.001**	-0.721	0.481	**-3.660**	**0.002**	**2473**	**0.025**
14	**6.360**	**< 0.001**	0.516	0.613	-1.106	0.285	**6.749**	**< 0.001**
17	**5.419**	**< 0.001**	-1.798	0.091	0.772	0.451	**8.565**	**< 0.001**
20	1.767	0.096	103.0*	0.133	-1.828	0.086	**8.460**	**< 0.001**
27	0.737	0.472	**-2.201**	**0.043**	**-2.225**	**0.041**	**4.385**	**< 0.001**
34	**2.895**	**0.011**	**-3.100**	**0.007**	-0.480	0.638	**4.653**	**< 0.001**
41	**5.662**	**< 0.001**	**-3.070**	**0.007**	1.261	0.225	**3.752**	**0.002**
48	**3.026**	**0.008**	-1.353	0.195	-0.462	0.651	**2.429**	**0.027**
62	0.925	0.369	0.522	0.609	**-4.528**	**< 0.001**	0.907	0.381
97	1.068	0.301	0.464	0.649	-1.211	0.244	0.007	0.995

The results suggest that decomposition can result in increased soil microbial activity although the effect differed between seasons and years. During the Spring 2011 trial, early dispersal of maggots was observed which in turn meant that soft tissue removal was slow and the amount of liquefaction was reduced. Summer 2011 and Spring

2012 data showed several days where microbial activity appeared to increase as a result of decomposition while Summer 2012 data pointed to decomposition having an inhibiting effect on microbial activity. During the Summer 2011, Spring 2012 and Summer 2012 experiments, maggot masses completely covered the carcasses, soft tissue removal was accelerated and products of liquefaction leached into the decomposition sites. The varied rate of decomposition across all trials coupled with the seasonal climatic differences is thought to have produced varying rates of entry of decomposition products into the soil. When decomposition products were abundant, the accumulation of toxic products and the formation of an anoxic environment may have negatively impacted soil microbial communities.

The presence of larvae may also have an impact on the microbial population within the carcass and in the soil due to anti-microbial activities. As maggots feed on a cadaver, consumed tissues pass through the digestive system of the larvae and are effectively disinfected. This is thought to occur through the production of antibacterial peptides (Bexfield et al. 2004) and the alkaline pH of the maggots' secretions (Mumcuoglu et al. 1998). When maggot masses are substantial, it may be possible that the majority of the microbial load originating from the cadaver or carcass never makes its way into the surrounding environment.

Data was analyzed to see if correlations existed between microbial activity and soil pH, soil moisture or ambient temperature (see Table 10). A significant negative correlation was identified in the Spring 2011 trial between microbial activity and soil moisture. The correlation existed for both control and experimental samples when considered separately. Although soil moisture was not statistically correlated to microbial

activity in each trial, spikes in soil moisture were often associated with decreases in microbial activity. In these instances, soil became saturated with water perhaps creating anoxic conditions which were unfavorable to soil microbial communities. Previous ecological studies have shown that changes to soil moisture, i.e. from very dry to very moist, can significantly affect soil microbial activity and alter soil microbial communities for prolonged periods of time (Schimel et al.1999). During the Summer 2011 trial, a negative correlation between soil pH and microbial activity was observed. During this trial, soil pH in both control and experimental soils became slightly alkaline during which time a decrease in microbial activity was observed. When pH values returned to their initial state, microbial activity increased which is in agreement with the well-known effect pH can have on soil microbial properties (Aciego-Pietri and Brookes, 2008).

Table 10. Summary of Pearson product moment correlation analyses used to determine significant correlations ($p < 0.05$) (highlighted in bold) between soil microbial activity measures and soil pH, soil moisture and ambient temperature during the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 trials.

	Environmental parameter					
	Soil pH		**Soil moisture**		**Ambient temperature**	
	r	***P***	***r***	***P***	***R***	***P***
Spring 2011	0.300	0.107	**-0.390**	**0.033**	0.267	0.153
Summer 2011	**-0.459**	**0.014**	-0.075	0.697	0.116	0.550
Spring 2012	0.384	0.0360	-0.0329	0.863	0.456	0.113
Summer 2012	-0.304	0.116	-0.420	0.261	0.271	0.164

3.4 Conclusion

As was predicted, the rates of decomposition observed in the spring were slightly slower than those observed in the summer. This can be attributed to the higher temperatures to which the carcasses are exposed during the summer, which will favor microbial activity as well as larval development. Both 2012 trials demonstrated an increase in soft tissue removal when compared to the 2011 trials which may be due to increased colonization of the carcasses by carrion insects.

Larvae were responsible for soft tissue removal throughout this study with their absence clearly slowing down the decomposition process. Observations made over the course of all four trials indicate that larvae may also play a role in the microbial response observed in soil following decomposition. The presence of larvae accelerated soft-tissue removal and liquefaction of the carcass, thus influencing the rate at which decomposition products entered the surrounding environment. When the pulse of decomposition products is strongest, soil microorganisms may have difficulty adapting to the extreme change in their environmental conditions. If an influx of microorganisms originating from the body does enter the soil, the influence of this new microbial load may be counterbalanced by the loss of the original soil microbial community.

The trials conducted as part of this study demonstrated an increase in microbial activity as a result of decomposition in some instances. The Spring 2011 trial produced multiple days where microbial activity was significantly higher in experimental soils when compared to control soils. This coincided with limited maggot activity and slow removal of soft tissue. The rate of decomposition likely affects the soil microbial response. Where decomposition is slower, microorganisms are able to better adapt and

utilize the nutrients which are slowly entering the soil environment. When decomposition is accelerated and liquefaction is rapid, the influx of decomposition products into the environment may be overwhelming and potentially toxic for soil microorganisms resulting in decreased soil microbial activity. The prolonged effect of decomposition on soil microorganisms remains to be investigated.

During this study, soil pH did not become more alkaline following decomposition. Changes in soil pH such as a slight acidification were observed over the course of the experiments and appear to be related to environmental changes rather than decomposition events. Rainfall and vegetation cycles may play an important role in the regulation of soil pH and need to be accounted for when attempting to understand the impact that decomposition may have on soil pH and soil microbial communities.

Decomposition was expected to create an influx of moisture into the decomposition environment yet this was not consistently observed during this study. Soil moisture in both control samples and experimental samples appeared to fluctuate comparably for the majority of all four trials indicating that rainfall, as well as rates of evaporation, were the major factors that affected soil moisture. Soil texture is also believed to play a role in the effect of decomposition on soil microbial communities. The soil used during this study consisted of sandy loam. This soil type has relatively low water holding capacity, allowing good drainage of excess water. This may explain why decomposition did not appear to increase soil moisture within the decomposition site. This soil type may also have been favorable for the formation of the crust that was observed on the surface of the soil beneath the carcasses.

Data generated during this study showed no clear trends in soil microbial activity or environmental parameters. Although soil moisture and soil pH could not be related to fluctuations in microbial activity in experimental soil samples, correlations did exist in control soil samples. It appears that the microbial response to decomposition is a complex process with multiple factors requiring further consideration in order to fully understand microbial dynamics. Total organic content may be a better predictor for microbial activity measures throughout the decomposition process and should be considered in future studies.

CHAPTER 4
Outdoor trials – Fatty Acid Methyl Ester Community Profiles

4.1 Introduction

Soil lipids are chemically diverse and originate from plant, animal and microbial cells (Jeannotte et al., 2008). The phospholipid fatty acids present in microbial membranes make up a portion of soil fatty acids and can be extracted and analysed to profile soil microbial communities (Zelles, 1999; Ibekwe and Kennedy, 1999; Shutter and Dick, 2001; Pankhurst et al., 2001). There are two main methods used to profile soil microbial lipids: fatty acid methyl ester (FAME) profiling, which considers all fatty acids extracted from a sample, and phospholipid fatty acid (PLFA) profiling, which selects only for microbial fatty acids (Marschner, 2007).

Through the use of FAME and PLFA profiles across multiple studies, marker fatty acids have been identified for various groups of soil microorganisms. These markers can be used to characterize changes in microbial community composition and identify specific groups of microorganisms that may be of interest. A compilation of marker fatty acids from the literature are presented in Table 11. Principal component analysis (PCA) or other multivariate analyses are typically used to investigate the relationships that exist between a set of soil profiles.

Table 11. Fatty acid markers and their microbial origin as compiled from the literature

Marker	Origin	Reference
15:0, 16:1ω9, 17:0	Bacteria (general)	Bossio and Scow, 1998, Mechri et al., 2007
16:1ω7t, 16:1ω7c, 17:0cy, 17:1ω9c, 18:1ω7c, 18:1ω9c, 19:0cy 30H fatty acids	Gram negative bacteria	Hinojosa et al., 2005; McKinley et al., 2005; Potthoff et al., 2006
12:1, *i*14:0, *a*14:0; *i*15:0, *a*15:0, *i*16:0, *a*16:0, *i*17:0, *a*17:0, *i*18:0, *i*19:0, *a*19:0	Gram positive bacteria	Hinojosa et al., 2005; Potthoff et al., 2006
16:1ω5c, 18:1ω9t, 18:2ω6c, 18:2ω9c, 18:3ω6c, 18:3ω9c, 18:3ω12c 15:0, 16:0, and 18:1ω7 18:1ω9*t*	Fungi Plant	Zelles, 1999; Buyer et al., 2002; McKinley et al., 2005; Potthoff et al., 2006; Mechri et al., 2007; Findlay, 2004
16:1ω5c	Arbuscular mycorrhizae Bacteria	Hinojosa et al., 2005; Bossio and Scow, 1998
cy17:0, cy19:0	Anaerobic bacteria	Vestal and White, 1989
12:0, 16:1ω7, 18:2ω9c, 18:2ω12c, 18:1ω9	Eucaryotes	Erwin, 1973
14:0, 16:0, 18:0	All organisms	Cavigelli et al., 1995

Soil microbial profiles from whole cell fatty acids have proven useful in distinguishing soil responses to various treatments such as different agricultural practices (Bossio et al., 1998; Steenwerth et al., 2003; Marschner et al., 2003; Larkin, 2003) and comparing microbial communities from different geographical regions (Polymenakou et al., 2005; Sun et al., 2013). Climate, specifically periods of rain or drought, vegetation and site history have all been shown to have a significant influence on microbial community dynamics (Buckley and Schmidt, 2001; Cregger et al., 2012; Huesco et al., 2012).

Only a few studies have investigated the changes in microbial community composition as a result of cadaver decomposition (Parkinson et al., 2009; Howard et al., 2010; Moreno et al., 2011). These studies have shown that the groups of microorganisms present in the soil vary according to the stages of decomposition and environmental conditions. There is also strong evidence that microorganisms originating from cadavers or carcasses can been detected in grave soils during or after decomposition (Hopkins et al., 2000; Parkinson et al., 2009; Howard et al., 2011).

PLFA and FAME profiles have been used in numerous studies to determine the changes in soil microbial communities under varying soil treatments, vegetation types and climatic conditions (Frostegard et al., 1992; Zogg et al., 1997; Bossio et al., 1998; Klamer and Bååth, 1998; Marschner et al., 2003; Steenwerth et al., 2005; Potthoff et al., 2006; Sagova-Marekova et al., 2011; Hueso et al., 2012; Sun et al., 2013). Such studies make it possible to anticipate and interpret the changes in soil microbial communities observed during decomposition.

Profiling fatty acids present in soils has also been done to characterise the chemical changes that may occur in soil as a result of decomposition. During the breakdown of adipose tissue fatty acids are released into the surrounding environment (Dent et al., 2004). As such, lipid content of soils throughout the decomposition process has been studied in a variety of forensic and taphonomic studies as potential PMI and grave indicators (Vass et al., 1992; Benninger et al., 2008; Swann et al., 2009). A group of short-chain fatty acids, 3:0, *i*4:0, 4:0, *i*5:0 and 5:0, have been detected in decomposition soils from the onset of bloat to the dry remains stage and are thought to be produced through anaerobic fermentation (Vass et al., 1992). These same compounds were also found in decomposition fluids from pig carcasses (Swann et al., 2009). A decomposition study investigating fatty acids in soils following pig carcass decomposition in southeastern Ontario found that levels of 14:0, 16:0, 16:1, 18:0 and 18:1 were significantly higher in experimental soils versus control soils once leaching of decomposition products into the soil began (Larizza, 2010).

Fatty acid content of soils has also been studied in regards to the formation of adipocere, a waxy substance found on cadavers in anaerobic conditions (Forbes et al., 2003; Fiedler et al., 2004). These studies demonstrated that certain fatty acids could still be detected in gravesoils years after decomposition. Fatty acid content of soils and tissues collected from mass graves of carcasses from the Foot and Mouth epidemic in 1967 have also been characterized (Vane and Trick, 2005).

Obtaining FAME profiles from decomposition associated soils may prove useful from a microbial and chemical perspective. The impact that decomposition has on soil suggests that microbial communities are forced to adapt to changes in nutrients, pH,

vegetation, etc. A fraction of the fatty acids present in FAME profiles may originate from animal or plant tissue suggesting the method may also allow changes in the chemical composition of soil to be detected and correlated to decomposition events.

This chapter presents results for FAME profiles obtained from soil collected during decomposition experiments undertaken at the University of Ontario Institute of Technology (Oshawa, Ontario) decomposition facility. Over the course of 2011 and 2012 four experiments were undertaken during which pig carcasses were allowed to decompose on the soil surface. Soil samples were taken from below the carcasses throughout the decomposition process and characterised using FAME profiling. Control samples were also profiled to document natural changes in microbial communities over the course of the experiments. Soil samples from decomposition sites and those from control sites were compared to characterise the changes in soil profiles resulting from decomposition. It was hypothesised that characterising soil microbial communities over the course of the decomposition process would reveal patterns that could be used as a PMI estimation tool and that specific marker fatty acids may be recognized as indicators of cadaver decomposition.

4.2 Method

FAME profiles were obtained for all samples collected as part of the four outdoor trials described in Chapter 3. Samples were stored at -20°C after collection until they were used for FAME extraction.

4.2.1 Extraction of FAMEs from soil samples

Fatty acid methyl esters (FAMEs) were obtained from experimental soil samples using the standard protocol developed for the Sherlock Microbial Identification System (Sherlock Microbial Identification System, 1996) and method described by Shutter and Dick (2000). The process comprises four main steps: 1) saponification, 2) methylation, 3) extraction and 4) base wash.

The first stage of the extraction is a saponification reaction allowing for the microbial cells in the soil to be broken down through the action of a methanolic base compound combined with heating. For the samples used in this study, 2.5 g of dried soil was found to give optimal FAME yields. Each sample was placed in a 15ml Pyrex tube to which 2.5 ml of a 1.125 M NaOH solution is added. The solution was prepared using a 1:1 solution of methanol and water as solvent. Tubes are then heated to 100°C for 30 minutes and cooled in a water bath at room temperature.

The second stage is a methylation reaction that converts the sodium salts into FAMEs. This stage results in an increased volatility of the fatty acids making them suitable for GC analysis. To complete this stage 5 ml of an HCl: MeOH solution is added to all tubes and heated to 80°C for 10 minutes.

To remove the newly obtained FAMEs from the acidic phase of the solution they were transferred to an organic phase though a liquid-liquid extraction. A solution of equal parts methyl-tert butyl ether (MTBE) and methanol was prepared and 1.5 ml added to all samples before being vortexed. Samples were then centrifuged at 900G for 5 minutes.

Finally, extracts were washed to remove any unwanted residue before analysis using gas-chromatography mass-spectrometry (GC-MS). FAME extracts found in the

upper layer following centrifugation were pipetted into clean tubes and 3ml of a 0.27M solution NaOH added.

Extracts were removed from the test tubes and placed in gas chromatography vials fitted with 400 µl glass insets. A 200µl extract was use for each analysis. An internal standard of nonadecanoic acid (C19:0) with a final concentration of 95ppm was added to all extracts.

4.2.2 GC-MS analysis of soil FAME content

FAME extracts from all soil samples were analyzed using the Varian 450 Gas Chromatograph (GC) coupled with a Varian 240 Ion Trap Mass Spectrometer (MS). Samples were run on an Agilent HP-5 (5% diphenyl, 95% dimethyl polysiloxane) low bleed chromatography column (30m x 0.25mm ID x 0.25µm film). Four pre-injection washings were performed using 5.0 µl of hexane, followed by 3 pre-injection flushes using the sample. 1µL of the sample was injected and 4 post-injection washes were performed to ensure that no cross contamination would occur between samples. The column oven temperature began at 135 °C and was increased at a rate of 4°C per minute until the final temperature of 250°C was reached and held for 10 minutes. A full scan was performed with an emission current of 30uAmp and a scan time of 0.50 sec/scan with a scan range of 50-450 m/z.

The GC-MS output provided the peak area for the compounds of interest, a measure of the abundance of the compound. Total content ratios for each compound were determined by dividing the peak area of the given compound by the total peak area

for the entire sample. Retention times for the fatty acids of interest were determined using the bacterial fatty acid methyl ester standard (BAME) from Sigma Aldrich Canada.

4.2.3 Statistical Analysis

Average proportions of fatty acids were obtained for all controls and treatment samples on each sampling day and used to create plots representing the total proportion of individual fatty acids from total FAME extracts. Graphs were produced using Microsoft Excel 2010. To determine if levels of specific fatty acids were significantly different between control and decomposition samples, Student's t-tests or Mann-Whitney rank sum tests were performed using SigmaPlot^{TM} 12.0 software package.

Principal component analyses (PCA) was used to characterize soil FAME profiles and compare their similarities among control and treatment samples. PCAs were performed for each of the five stages of decomposition across all experimental trials. Analyses were carried out using PAST Version 2.16 statistical software. Each principal component (PC) or axis represents a proportion of the variation within the original data, where PC1 represents the greatest amount of variation. PC axis scores for PC1 and PC2 were analyzed for correlations with specific fatty acids using linear regression. Linear regressions were performed in SigmaPlot^{TM} 12.0. Only regression models with $R^2 > 0.7$ ($p < 0.05$) were judged strong enough to be reported.

The effect of soil pH and soil moisture on FAMEs was investigated using Pearson product moment correlations between individual fatty acid values for each soil profile and the corresponding values of soil pH and soil moisture. To determine the impact of soil pH and soil moisture on FAME profiles during each experimental trial a new set of

PCAs was analyzed which included soil pH and soil moisture data. PC axis scores for PC1 and PC2 were analyzed for correlations with either soil parameter using linear regressions. Where significant correlations were found, loading plots for FAMEs were produced to show the relationship between PC1 and PC2 and individual FAMEs present in soil samples.

Data from all four experiments were compared to determine if FAMEs showed significant variance according to season and year. Two-way ANOVAs were performed on major fatty acids commonly found across all trials with season and year as the major factors.

4.3 Results

4.3.1 Fatty acid composition

Average distributions of fatty acids within control samples and experimental samples on each sampling day of the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 experimental trials are presented in Figures 15 to 18. Results from the Pearson product moment correlations between fatty acid levels and soil pH or soil moisture are presented in Tables 16 to 19.

4.3.1.1 Spring 2011

Spring 2011 fatty acid distribution data indicated that FAME soil profiles were similar in control and experimental samples during the first 6 days of the experiment (see Figure 15). A shift in FAME content was observed on day 8. The change in profiles was

visible until day 17. FAME profiles underwent two more pronounced shifts, one on day 20 and the other on day 48. FAME composition of control samples and experimental samples were similar once both changes took place. During days 20 to 41 a decrease in soil moisture and overall soil pH were observed. After day 48 soil moisture began to increase but there was little change in soil pH. Major fatty acids present in samples from days 48, 62 and 97 were similar to those present in samples collected within the first few days of the experiment. Profiles from samples collected on days 48, 62 and 97 showed a change in overall FAME distributions when compared to those from the previous days.

Fatty acid distribution patterns did not follow stages of decomposition. For this reason individual fatty acids were compared between control and experimental soils according to the shifts observed in FAME proportions rather than according to stages. The results of these analyses are presented in Table 12. Between days 0 and 6 the only fatty acid to show a significant difference based on treatment was 18:2ω6 which could be found in greater proportions in control samples. For days 8 to 17 there were significant differences for levels of *a*15:0 and 16:0 between treatments. Levels of *a*15:0 were higher in experimental samples for these days whereas 16:0 was found in higher proportions in control samples. Between days 20 and 41 there were significant differences between levels of 16:1ω11c, *i*15:0 and 18:1ω9*t* according to treatment. 16:1ω11c could be found in higher proportions in the control samples whereas *i*15:0 and 18:1ω9*t* was higher in experimental samples. For days 48 to 97 18:1ω9*t* and 16:1ω9*c* were found in higher proportions in control samples whereas levels of 3OH 12:0 and 10:0 were significantly greater in experimental samples.

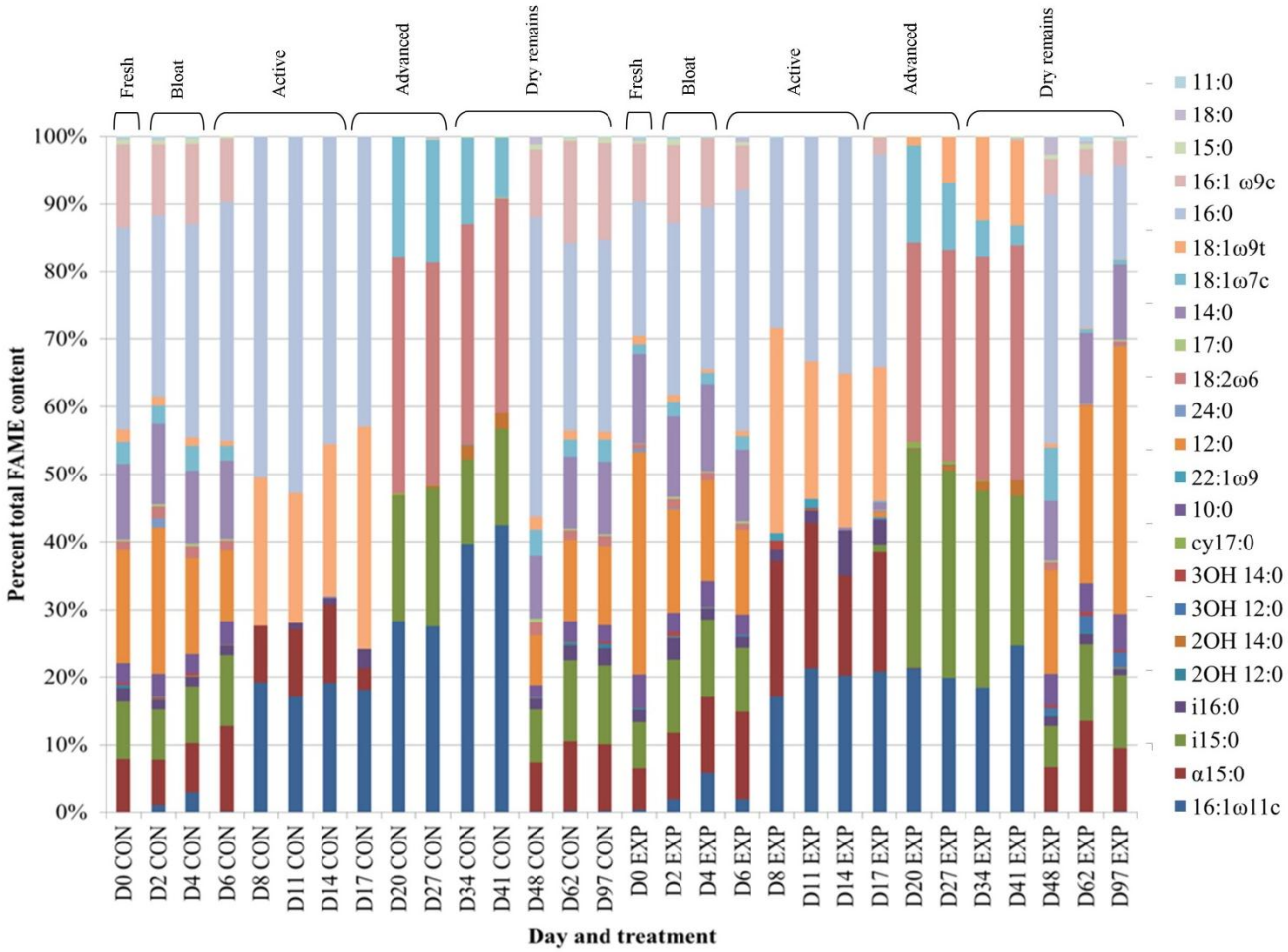


Figure 15. Average total fatty acid distribution of control samples and experimental samples for the Spring 2011 trial. Major shifts in FAME composition occurred at the same time in both control and experimental sites indicating environmental factors likely affected FAME profiles. Experimental profiles were characterised by increased proportions of: *α*15:0 (days 8 to 17), *i*15:0 and 18:1ω9*t* (days 20 to 34), 3OH 12:0 and 10:0 (days 48 to 97).

Table 12. Summary of t-tests or Mann-Whitney rank sum tests (*) between control and experimental samples for days 0 to 6, 8 to 17, 20 to 41 and 48 to 97. Significant differences (p < 0.05) are highlighted in bold.

	Days 0-6		Days 8-17		Days 20-41		Days 48-97	
	t	***p***	***t***	***p***	***t***	***p***	***T***	***p***
16:1ω11c	-1.106	0.311	-1.315	0.237	**26.00***	**0.029**	2.765	0.051
***α*15:0**	-0.708	0.506	**-4.310**	**0.005**	14.00*	0.343	-0.334	0.755
***i*15:0**	-0.827	0.440	-1.000	0.356	**-4.021**	**0.007**	13.00*	0.400
***i*16:0**	13.00*	0.200	-1.770	0.127	18.00*	1.000	2.452	0.070
2OH 12:0	17.00*	0.886	0.000	1.000	18.00*	1.000	-0.363	0.735
2OH 14:0	1.062	0.329	-1.000	0.356	0.095	0.927	0.029	0.978
3OH 12:0	-0.720	0.499	-1.000	0.356	14.00*	0.343	**-3.731**	**0.020**
3OH 14:0	-0.101	0.923	-1.195	0.277	18.00*	1.000	-1.901	0.130
cy17:0	-0.693	0.514	0.000	1.000	-1.296	0.243	-1.000	0.374
10:0	-0.966	0.371	-1.000	0.356	18.00*	1.000	**-5.209**	**0.006**
22:1ω9	16.00*	0.686	-2.188	0.071	20.00*	0.686	1.000	0.374
12:0	-0.031	0.573	-1.000	0.356	18.00*	1.000	-2.323	0.081
24:0	18.00*	1.000	-1.000	0.356	18.00*	1.000	-0.630	0.563
18:2ω6	**3.121**	**0.021**	-1.000	0.356	0.850	0.428	2.762	0.051
17:0	0.824	0.442	-1.000	0.356	20.00*	0.686	1.761	0.153
14:0	-1.269	0.251	-1.184	0.281	18.00*	1.000	-0.021	0.985
18:1ω7*c*	**0.011**	**0.024**	-1.000	0.356	1.856	0.113	0.074	0.945
18:1ω9*t*	1.477	0.190	0.233	0.823	**10.00***	**0.029**	**4.382**	**0.012**
16:0	1.221	0.268	**5.548**	**0.001**	18.00*	1.000	1.060	0.349
16:1 ω9*c*	1.432	0.202	-1.000	0.356	16.00*	0.686	**5.362**	**0.006**
15:0	0.513	0.626	-1.000	0.356	0.574	0.587	0.640	0.557
18:0	-1.069	0.326	0.000	1.000	20.00*	0.686	-0.657	0.547
11:0	-0.405	0.700	-1.000	0.356	18.00*	1.000	-1.344	0.250

4.3.1.2 Summer 2011

FAME distribution patterns for control and experimental samples for days 0, 2 and 4 of this trial were varied (Figure 16). On day 6, a change in the most abundant fatty acids present in both control and experimental samples was observed. Control samples and experimental samples indicated some degree of dissimilarity from day 6 onwards. During the Summer 2011 trial soil moisture fluctuated slightly during the first 8 days before gradually increasing over the remainder of the trial. These changes were consistent with the transition from summer weather, typically drier with higher daily average temperatures, to fall weather, which is characterized by lower daily average temperatures and more frequent precipitation. Soil pH of control samples remained within the range of 8.0 and 8.5 with experimental samples only decreasing below this on a few occasions. Overall FAME profiles were similar across the majority of this trial (days 8 to 97) reflecting the generally stable measures of soil moisture and pH.

Results of the analyses comparing the individual FAMEs between control and experimental samples per stage can be found in Table 13. There were no significant differences between any of the FAMEs based on treatment for the fresh and bloat stages or the dry remains stage. During active decay levels of 3OH12:0 and 16:0 were significantly higher in experimental samples. During advanced decay levels of 16:1ω11c were significantly higher in control samples while levels of 15:0 were higher in experimental samples.

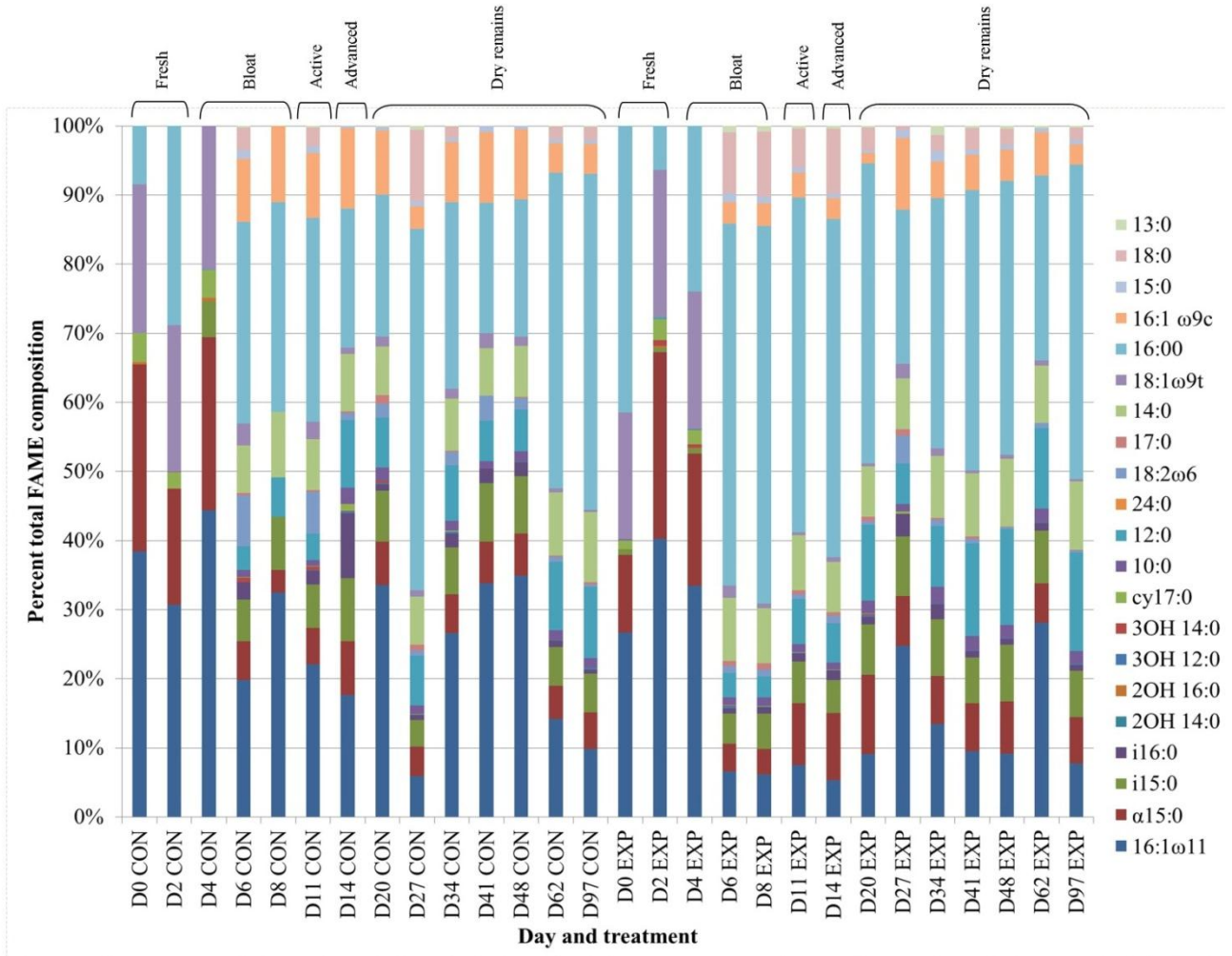


Figure 16. Average total fatty acid distribution of control samples and experimental samples for the Summer 2011 trial. Higher proportions of 3OH12:0 and 16:0 were observed in experimental samples during the active decay stage and may be introduced into the soil through decomposition activity. From the bloat stage onwards experimental samples differentiated from control samples due to increased proportions of 16:0 and 18:0 fatty acids.

Table 13. Summary of t-tests or Mann-Whitney rank sum tests (*) between control and experimental samples for days within the stages of fresh / bloat, active decay, advanced decay and dry remains for the Summer 2011 trial. Significant differences (p < 0.05) are highlighted in bold.

	Fresh / Bloat		**Active**		**Advanced**		**Dry remains**	
	t	***p***	***t***	***p***	***t***	***p***	***t***	***P***
16:1ω11c	0.511	0.627	1.002	0.422	**2.898**	**0.016**	-0.426	0.712
***α*15:0**	0.412	0.694	-1.612	0.248	-1.598	0.141	-0.776	0.519
***i*15:0**	18.00*	1.000	-2.689	0.115	0.076	0.941	-1.855	0.205
***i*16:0**	18.50*	0.886	-0.800	0.508	39.00*	1.000	-0.704	0.554
2OH 14:0	17.50*	0.886	-0.450	0.697	44.50*	0.394	6.00*	0.667
2OH 16:0	1.091	0.317	0.000	1.000	39.00*	1.000	0.000	1.000
3OH 12:0	16.00*	0.686	**-5.000**	**0.038**	0.780	0.454	-0.978	0.431
3OH 14:0	18.00*	1.000	1.000	0.423	43.00*	0.589	0.000	1.000
cy17:0	0.906	0.400	-2.125	0.168	39.50*	0.937	-1.000	0.423
10:0	15.00*	0.486	-3.707	0.066	-0.257	0.802	-3.679	0.067
12:0	16.50*	0.686	-1.731	0.226	-1.082	0.304	-1.080	0.393
24:0	18.00*	1.000	0.000	1.000	42.00*	0.699	5.00*	1.000
18:2ω6	15.50*	0.486	0.513	0.659	1.144	0.279	6.00*	0.667
17:0	17.50*	0.886	-3.549	0.071	-0.089	0.931	7.00*	0.333
14:0	17.50*	0.886	-4.201	0.052	-0.772	0.458	7.00*	0.333
18:1ω9t	21.00	0.486	0.187	0.869	1.957	0.079	5.00*	1.000
16:0	-1.286	0.252	**-5.909**	**0.028**	-1.973	0.077	1.668	0.237
16:1 ω9c	18.50*	0.886	0.293	0.797	1.998	0.074	0.168	0.870
15:0	17.50*	0.886	-1.960	0.189	**26.00***	**0.041**	0.038	0.973
18:0	17.50*	0.886	-3.042	0.093	27.00*	0.065	1.373	0.303
13:0	17.50*	0.886	-2.690	0.115	29.50*	0.132	-2.264	0.152

4.3.1.3 Spring 2012

FAME distribution data from the Spring 2012 trial indicated that control sample profiles remained similar throughout the entire experiment with a slight change occurring between days 11 and 20 (Figure 17). This change in experimental soil profiles occurred at the peak of the active decay stage, during which rapid liquefaction was observed. After day 20, the proportions of FAMEs in experimental samples were similar to those found in control samples. Between days 11 and 27, along with the aforementioned changes in experimental samples, the proportions of FAMEs in control samples were slightly different from what was observed on all other days. The timing of this variation corresponded with an increase in soil moisture from ~20% WHC to above 50% WHC on days 11 and 14. After day 27, FAME distributions in both control and experimental samples were comparable.

Results of the analyses comparing the individual FAMEs between control and experimental samples per stage can be found in Table 14. During the fresh and bloat stages *a*15:0, *i*15:0. 17:0 and 15:0 were significantly greater in control samples. There were no significant differences between FAMEs from samples collected during the active decay and advanced decay stages. During the dry remains stage 16:1ω11c and 18:1ω9t levels were significantly greater in control samples. The fatty acids 3OH 12:0 and 12:0 were significantly greater in experimental samples.

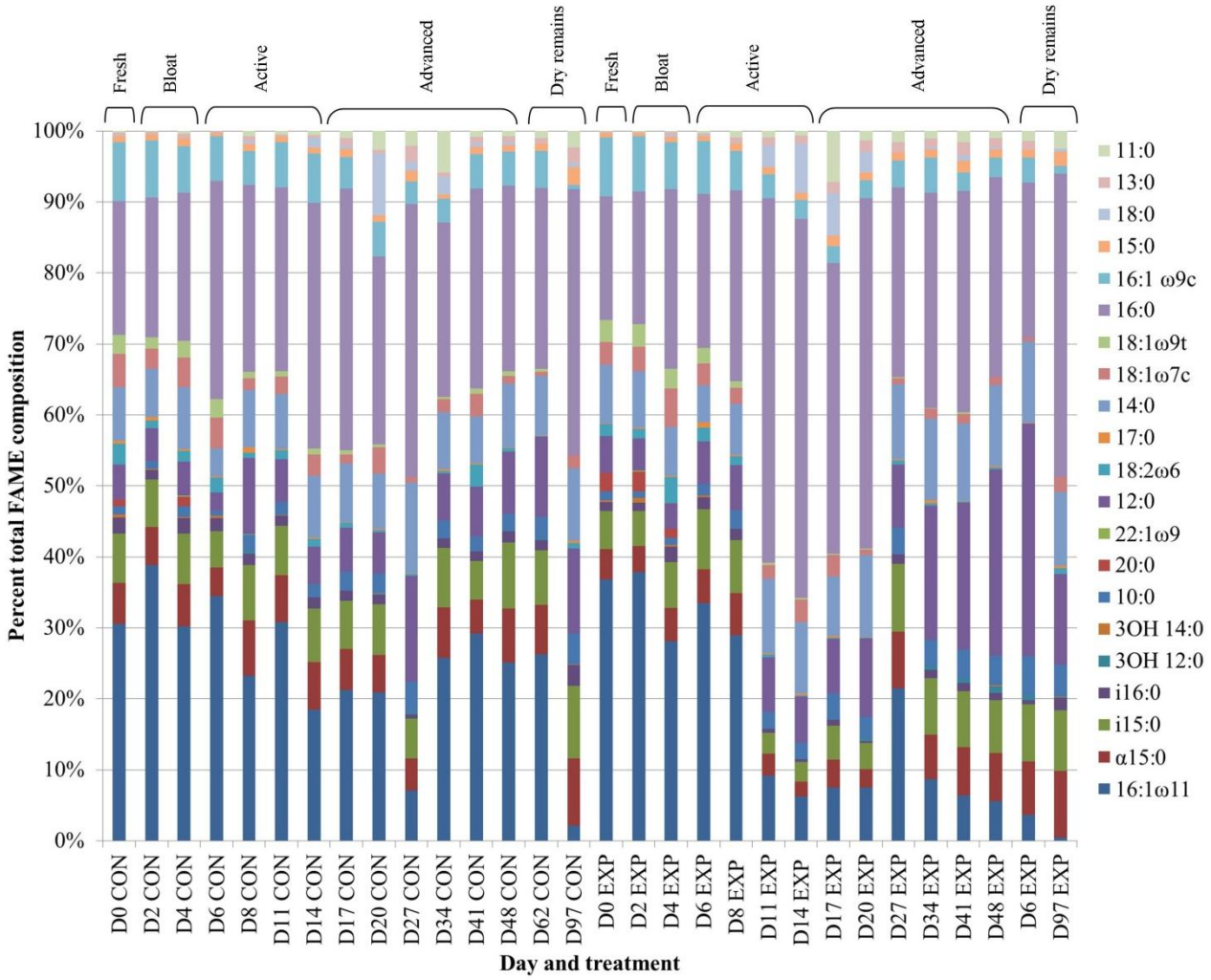


Figure 17. Average total fatty acid distribution of control samples and experimental samples for the Spring 2012 trial. In later days of the active stage and early advanced stage a change in FAME composition was observed in experimental samples and was due to increased proportions of 16:0 and 18:0 fatty acids.

Table 14. Summary of t-tests or Mann-Whitney rank sum tests (*) between control and experimental samples for days within the stages of fresh / bloat, active decay, advanced decay and dry remains for the Spring 2012 trial. Significant differences (p < 0.05) are highlighted in bold.

	Fresh / Bloat		Active		Advanced		Dry remains	
	t	***p***	***t***	***p***	***t***	***p***	***t***	***P***
16:1ω11c	-0.262	0.806	-0.046	0.965	0.434	0.687	**2.971**	**0.018**
***α*15:0**	**5.416**	**0.005***	0.648	0.541	-0.038	0.972	0.468	0.652
***i*15:0**	**4.529**	**0.011***	-0.071	0.945	-0.007	0.995	0.506	0.627
***i*16:0**	1.118	0.326	0.261	0.803	0.607	0.577	2.224	0.057
3OH 12:0	1.000	0.374	0.505	0.631	0.116	0.913	**-2.903**	**0.019**
3OH 14:0	-0.565	0.602	-0.359	0.732	0.859	0.439	-0.693	0.508
10:0	0.986	0.380	-1.905	0.105	-0.557	0.607	-1.628	0.142
20:2	-2.209	0.092	-1.000	0.356	0.000	1.000	0.000	1.000
22:1ω9	-0.160	0.880	0.865	0.420	1.000	0.374	0.000	1.000
12:0	0.461	0.669	-1.126	0.303	-0.205	0.847	**-2.564**	**0.033**
18:2ω6	-0.387	0.718	-0.141	0.892	0.502	0.642	1.488	0.175
17:0	**2.874**	**0.045**	-0.407	0.698	-2.303	0.083	-0.084	0.932
14:0	-0.064	0.952	-1.390	0.214	-0.487	0.652	-1.010	0.342
18:1ω7c	-0.085	0.956	-1.850	0.114	0.214	0.841	1.226	0.255
18:1ω9t	-2.176	0.095	-0.331	0.752	0.913	0.413	**2.370**	**0.045**
16:0	-0.257	0.810	-1.766	0.128	-0.900	0.419	0.091	0.930
16:1 ω9c	0.024	0.982	-0.231	0.825	1.673	0.170	0.540	0.604
15:0	**3.435**	**0.026***	-2.028	0.089	-0.492	0.649	-0.310	0.765
18:0	-0.621	0.568	-1.363	0.222	0.109	0.919	0.991	0.351
13:0	2.152	0.101	-1.955	0.098	-0.615	0.572	-0.581	0.577
11:0	0.949	0.397	-1.690	0.142	-0.810	0.463	0.856	0.417

4.3.1.4 Summer 2012

The distribution plot for the Summer 2012 soils indicated that FAME proportions in control samples and experimental samples for days 0 and 2 were very different (Figure 18). As the experiment progressed, FAMEs in control and experimental samples showed similar proportions. Samples from the advanced decay stage and early days of the dry remains stage (days 14 to 48) indicated comparable FAME distributions in both control and experimental soils. Samples collected on days 62 and 97 of this experiment showed a different FAME composition from all other samples collected during this trial.

Results of the analyses comparing the individual FAMEs between control and experimental samples per stage can be found in Table 15. There was no significant difference between FAMEs present in control and experimental samples during the fresh and bloat stages of decomposition. During the active decay stage there was a significant difference between levels of *a*15:0, *i*15:0, 3OH 12:0, 18:1ω9t, 16:0, 16:1ω9c and 18:0. The fatty acids *a*15:0, *i*15:0, 18:1ω9t and 16:1ω9c were found in higher proportions in control samples whereas 3OH 12:0, 16:0, and 18:0 were found in higher levels in experimental samples. During the advanced decay stage there was a significant difference between levels of *a*15:0, *i*15:0, 3OH 12:0, 12:0, 18:1ω9t and 18:0 according to treatments. The FAMEs *i*15:0, 18:2ω6 and 18:1ω9t showed higher levels in control soils. FAMEs *a*15:0, 3OH 12:0, 12:0 and 18:0 were found in higher amounts in experimental soils. During the dry remains stage, levels of *a*15:0, *i*16:0, 3OH 12:0, 3OH 14:0 and 12:0 showed significant differences between treatments. These fatty acids were all found in greater proportions in the experimental samples.

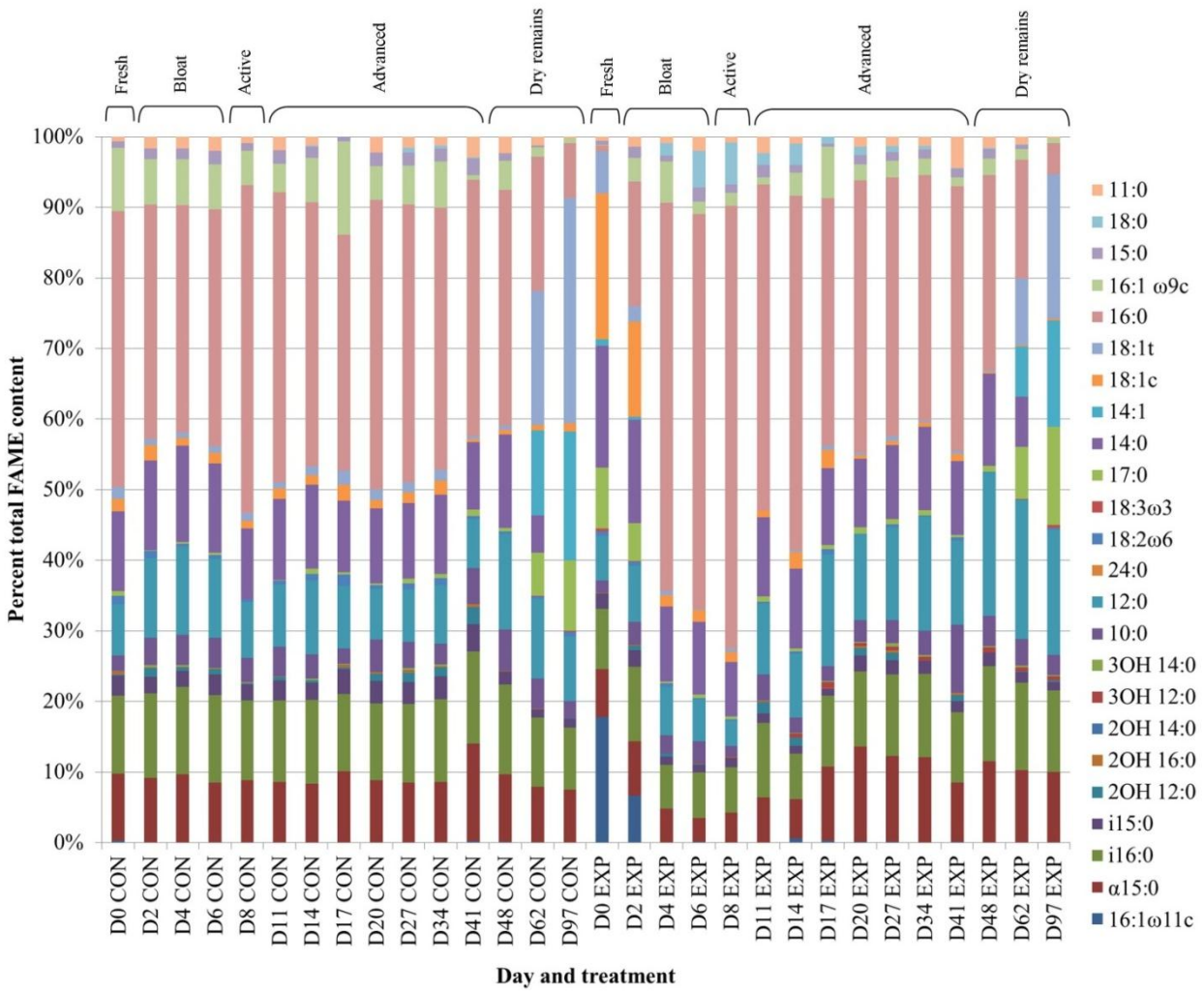


Figure 18. Average total fatty acid distribution of control samples and experimental samples for the Summer 2012 trial. A shift in FAME composition was observed in experimental sites beginning in the late bloat and active decay stages. Proportions of 3OH 12:0, 16:0, and 18:0 were significantly higher in experimental samples than control samples during these days.

Table 15. Summary of t-tests or Mann-Whitney rank sum tests (*) between control and experimental samples for days within the stages of fresh / bloat, active decay, advanced decay and dry remains for the Summer 2012 trial. Significant differences (p < 0.05) are highlighted in bold.

	Fresh / Bloat		Active		Advanced		Dry remains	
	t	***p***	***t***	***p***	***t***	***p***	***t***	***p***
16:1ω11c	-1.484	0.212	12.00*	0.114	-0.701	0.499	1.381	0.301
***α*15:0**	0.043	0.968	**2.505**	**0.046**	**-2.589**	**0.027**	**-10.60**	**0.009**
***i*16:0**	-0.110	0.918	1.477	0.190	-0.707	0.496	**-16.22**	**0.004**
***i*15:0**	-0.378	0.725	**3.586**	**0.012**	**55.00***	**0.009**	-1.087	0.391
2OH 12:0	0.490	0.650	-0.954	0.377	1.040	0.323	-1.472	0.279
2OH 16:0	0.256	0.811	0.662	0.532	0.654	0.528	-0.394	0.732
2OH 14:0	1.000	0.374	18.00*	1.000	1.166	0.271	5.00*	1.000
3OH 12:0	-2.384	0.075	**10.00***	**0.029**	**26.00***	**0.041**	**-13.62**	**0.005**
3OH 14:0	2.474	0.068	0.980	0.365	-1.020	0.332	**-24.43**	**0.001**
10:0	0.084	0.936	0.774	0.468	-0.470	0.648	0.0694	0.951
12:0	0.242	0.821	0.313	0.765	**24.00***	**0.015**	**-10.21**	**0.009**
24:0	1.000	0.374	12.00*	0.114	36.00*	0.699	5.00*	1.000
18:2ω6	-0.245	0.819	1.568	0.168	1.880	0.089	1.587	0.253
18:3ω3	-1.287	0.267	18.00*	1.000	45.00*	0.394	-0.718	0.547
17:0	-1.766	0.152	-1.591	0.163	-1.600	0.141	-0.629	0.594
14:0	-1.669	0.170	-0.717	0.501	-1.049	0.319	-0.204	0.857
14:1	-1.526	0.202	18.00*	1.000	39.00*	1.000	0.719	0.547
18:1ω7c	-1.815	0.144	-1.587	0.164	0.710	0.494	3.297	0.081
18:1ω9t	-1.302	0.263	**3.991**	**0.007**	**3.154**	**0.010**	1.112	0.382
16:0	0.037	0.972	**-5.544**	**0.001**	-0.835	0.423	0.352	0.759
16:1 ω9c	1.219	0.290	**2.695**	**0.036**	47.00*	0.240	-0.068	0.952
15:0	-0.023	0.983	-0.773	0.469	48.00	0.180	-0.544	0.641
18:0	-1.033	0.360	**-3.832**	**0.009**	**-2.285**	**0.045**	-1.000	0.423
11:0	0.128	0.905	-0.693	0.514	-0.117	0.909	0.117	0.918

4.3.2 Characterization of FAME profiles per decomposition stage

4.3.2.1 Spring 2011

During the fresh stage, plots from both spring trials showed that FAME profiles from control samples and experimental samples were diverse and little clustering of samples was observed (Figure 19a). Samples within proximity to one another were found to originate from the same sampling site. PCA for the fresh stage of both spring trials indicated a high level of diversity within sampling sites and across the various sites regardless of the presence or absence of a carcass (Figures 19a and 21a). These results indicated little change in soil profiles since the fresh stage. Little clustering according to control and experimental sites was observed during the bloat stage (Figure 19b)

Control and experimental samples collected during the active decay stage of the Spring 2011 trial clustered separately (Figure 19c). PC1 from this analysis explained 56% of the variation. This component showed a strong linear regression with levels of 3OH 14:0 (R^2 = 0.985, $p < 0.001$) suggesting this fatty acid was responsible for differences observed between the majority of samples. PC2 explained 12% of the variation and did not show a strong linear regression with individual fatty acids.

The advanced decay stage for the Spring 2011 trial included 6 days of sampling. Due to the large amount of data, the PCA results were difficult to interpret (Figure 19d). Data from each day within this stage was analyzed separately to facilitate the interpretation of results. Additional PCA plots are presented in Appendix B. Control samples were distinct from the experimental samples on days 17 and 27. This trend was less apparent on the remaining days of this stage of decomposition. PC1 and PC2 from

days 17 through 27 were investigated for associations with specific fatty acids but no significant linear regressions were found.

The PCA for the dry remains stage indicated some degree of separation between control and experimental samples (Figure 19e). There was no distinction within control samples or experimental samples according to their collection date. PC1 explained 24% of the variation and showed a strong linear regression with levels of 3OH 14:0 (R^2 =0.714, $p < 0.001$). PC2 explained 16% of the variation and showed a strong linear regression with 16:0 ($R^2 = 0.776$, $p < 0.001$).

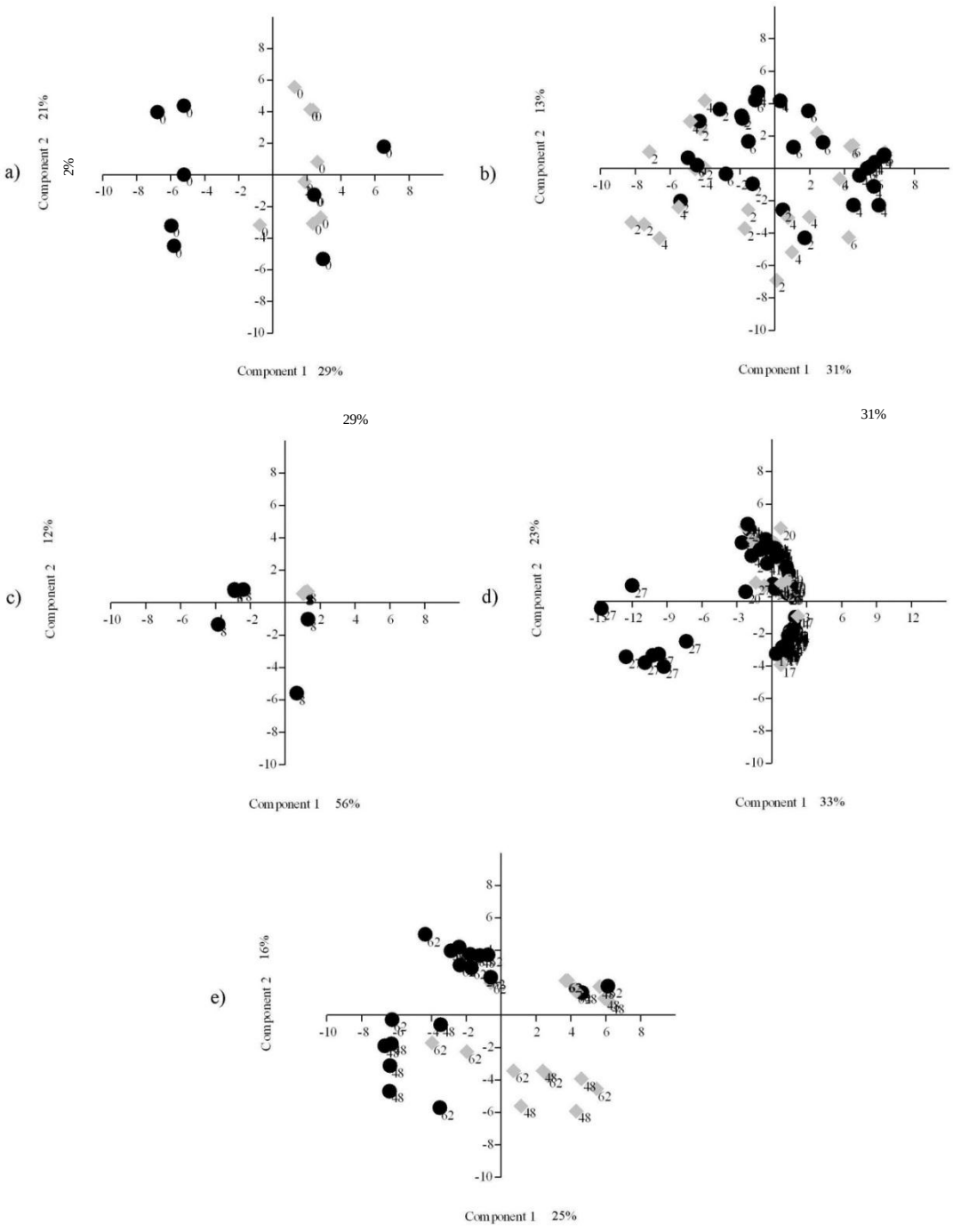


Figure 19. PCA plot of control samples (♦) and experimental samples (●) for the a) fresh, b) bloat, c) active decay, d) advanced decay and e) dry remains stages of the Spring 2011 trial. PC1 of the active decay stage (c) and that of the dry remains stage (e) showed strong linear regressions with levels of 3OH 14:0. Distinguishing between treatments was possible for each stage of decomposition when samples were compared according to sampling day.

4.3.2.2 Summer 2011

Profiles from the fresh stage of the Summer 2011 trial indicated that control samples and experimental samples were distinct (Figure 20a). Control samples were loosely grouped together at one end of PC1 with experimental samples tightly clustered at the other. The PC1 of the Summer 2011 PCA plot explained 72% of the variation and four fatty acids showed a strong linear regression with this component: 18:1ω9t (R^2= 0.983, $p < 0.001$), *a*15:0 ($R^2 = 0.953$, $p < 0.001$), 15:0 ($R^2 = 0.850$, $p < 0.001$) and 16:1ω11c ($R^2 = 0.752$, $p < 0.001$). It was noted that 15:0 was found in all control samples but was not detected in experimental samples. The fatty acid 16:1ω11c was found in a few of the controls but could be found in all experimental samples. PC2 explained 10% of the variation and did not show a strong relationship with any individual fatty acid.

The PCA for the bloat stage showed clustering of experimental samples and control samples in relation to their collection date (Figure 20b). It should be noted that control samples and experimental samples which overlap in the upper right-hand corner of the PCA are from different sampling days. PC1 from this analysis explained 62% of the variation and showed a strong linear regression with *a*15:0 (R^2=0.940, $p < 0.001$), 16:1ω11c (R^2=0.879, $p < 0.001$), 18:1ω9t (R^2= 0.841, $p < 0.001$) and 16:0 ($R^2 = 0.768$, $p < 0.001$). PC2 explained 17% of the variation and did not show a strong relationship with any individual fatty acid.

The Summer 2011 active stage showed separation between control samples and experimental samples yet this had also been observed in the previous stages (Figure 20c). Experimental samples from this trial were closely clustered together indicating the soil profiles across the three sites of decomposition remained similar. Control samples were

clustered into three distinct groups which corresponded to the three control sites sampled. This indicates similar soil profiles could be obtained within a given control site but all three sites were distinct. PC1 from this analysis explained 65% of the variation. A strong linear relationship was observed for 18:1ω7c (R^2=0.928, $p < 0.001$) and 18:2ω6 (R^2=0.846, $p < 0.001$). PC2 explained 8% of the variation and was not strongly associated with any specific fatty acid.

The advanced stage of decomposition for the Summer 2011 included 6 days of sampling. For this reason, the PCA plot presented in Figure 20d included a large number of data points and was difficult to interpret. Data from each day was analyzed separately to facilitate the interpretation of results. Additional PCA plots are presented in Appendix B. Individual PCAs indicated that soil profiles from controls sites were different from profiles of the experimental sites on all days. On day 14 specifically, experimental soil samples were found to cluster tightly together. For the remaining days (17 to 48) although experimental soil profiles were distinct from control soil profiles they were more scattered indicating a greater diversity as decomposition progressed. PC1 and PC2 from days 17 through 48 were investigated for associations with specific fatty acids but no significant linear regressions were found.

FAME profiles for the dry remains stage that all soil profiles grouped together irrespective of treatment (Figure 20e). Distinguishing samples based on treatment (control or experimental) or collection day was not possible for this stage. Components 1 and 2 showed no strong relationship with any individual fatty acid.

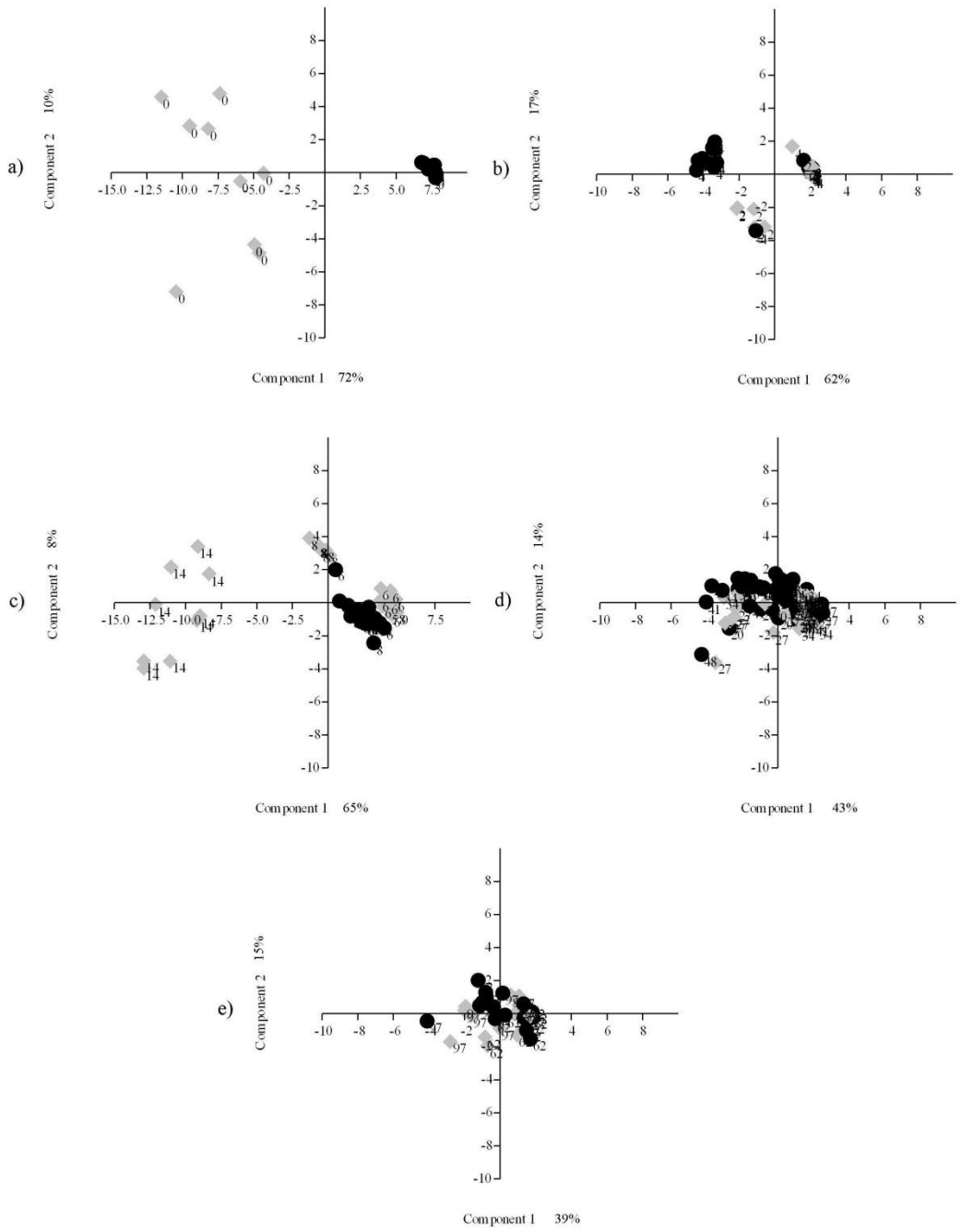


Figure 20. PCA plot of control samples (♦) and experimental samples (●) during the a) fresh, b) bloat, c) active decay, d) advanced decay and e) dry remains stages of the Summer 2011 trial. Distinguishing between treatments was possible during the fresh (a) and bloat (b) stages. PC1 for both these stages showed a strong linear regression with multiple fatty acids including 18:1ω9t, *a*15:0 and 16:1ω11c. Distinguishing between treatments was possible through to the active decay stage (c) and advanced decay stage (see supplementary PCAs in Appendix B). Samples from the dry remains stage clustered together regardless of treatment.

4.3.2.3 Spring 2012

As previously reported, the fresh stage from both spring trials showed that FAME profiles from control samples and experimental samples were diverse (Figure 19a and Figure 21a). Once again, samples within proximity to one another were found to originate from the same sampling site. The PCA of samples collected during the bloat stage indicated that FAME profiles were diverse as little clustering could be observed (Figure 21b). This was true for samples taken within a specific site and across the different site locations regardless of treatment. Soil profiles did not change between the fresh and bloat stages during Spring 2012. Samples from control and experimental sites remain scattered showing a similar trend to that observed during the fresh and bloat stages. This indicated that although decomposition had begun, at the time of sampling a distinct change between control and experimental FAME profile could not be observed.

Profiles from the Spring 2012 active decay stage were loosely grouped according to treatment (control vs. experimental) (Figure 21c). Experimental samples were also roughly grouped according to the date on which they were collected. This suggests that experimental soils underwent changes between sampling days. Both axes for this PCA influenced sample distribution evenly. PC1 from this plot explained 23% of the variation and PC2 explained 16% of the variation. Individual fatty acids were not significantly correlated to either component.

FAME profiles for days falling within the advanced decay stage were spread out and overlap between control samples and experimental samples could be observed (Figure 21d). The majority of control samples could be found in the upper quadrants and experimental samples in the lower quadrants. There was no differentiation of samples

based on sampling date. PC1 explained 21% of the variation and PC2 explained 20% of the variation. PC1 was strongly correlated with values of 18:1ω9t ($R^2 = 0.776$, $p < 0.001$) and 18:2ω6 ($R^2 = 0.727$, $p < 0.001$) while PC2 showed a strong linear regression with values of 17:0 ($R^2 = 0.813$, $p < 0.001$).

The dry remains stage for this trial included days 20 to 97. For this reason, the PCA plot presented in Figure 21e included a large number of data points and was difficult to interpret. Data from each day was analyzed separately to facilitate the interpretation of results. Additional PCA plots are presented in Appendix B. Within PCA plots obtained for days 20, 27, 34, 41, 48 and 62 a separation between control soil samples and experimental samples can be observed however control and experimental samples are not seen to cluster tightly based on treatment or according to sampling site. As control samples and experimental samples could be distinguished in each PCA, scores for the PC1 and PC2 were analyzed to determine if they correlated with specific fatty acids. The only significant linear regression was for PC1 of the day 27 plot and levels of 16:1 acid ($R^2 = 0.791$, $p < 0.001$). On day 97, the distinction between control and experimental samples was no longer visible as clustering did not occur.

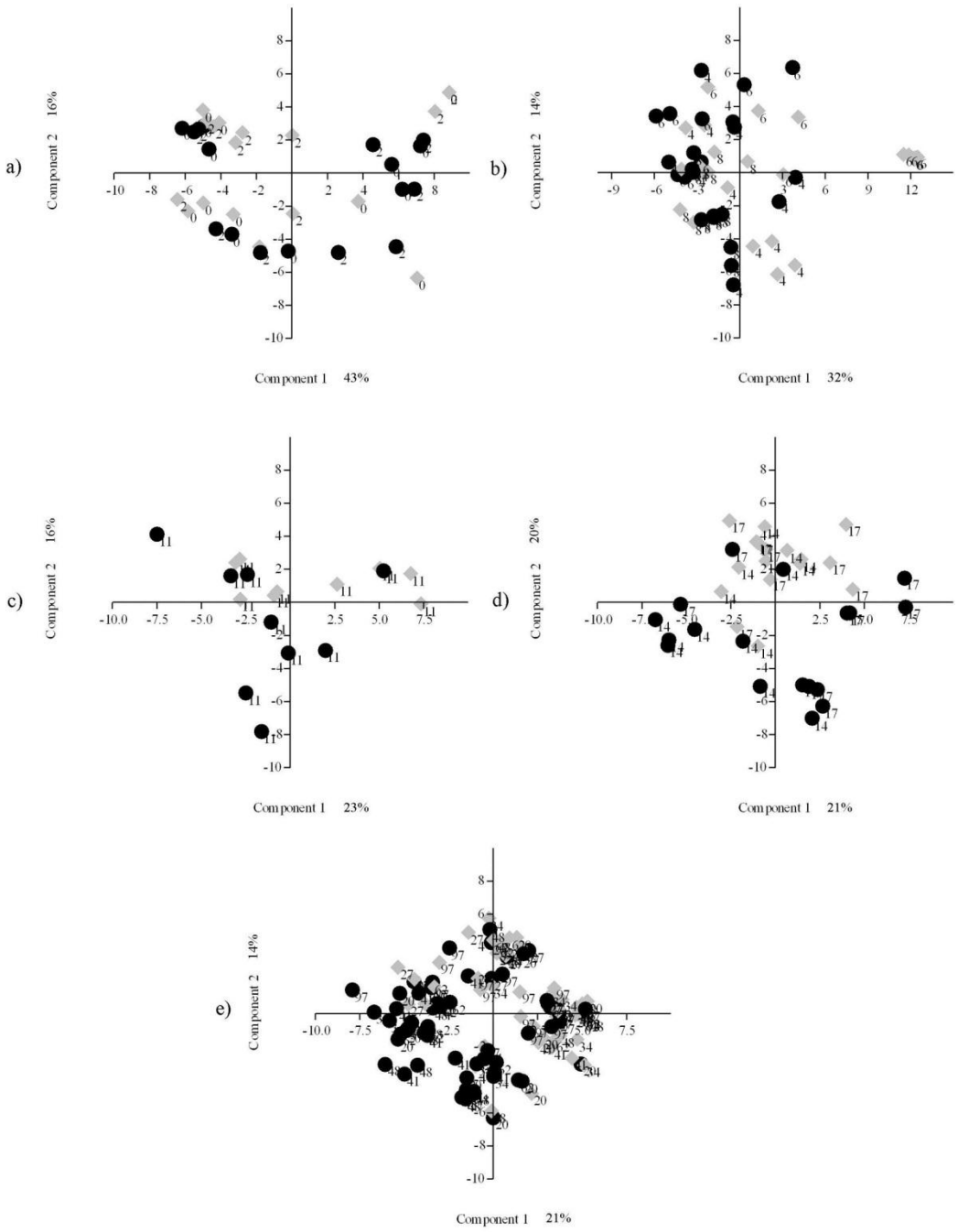


Figure 21. PCA plot of control samples (♦) and experimental samples (●) during the a) fresh, b) bloat, c) active decay, d) advanced decay and e) dry remains stages of the Spring 2012 trial. Clustering of samples according to treatments was not observed during the fresh (a) or bloat (b) stages. FAME profiles of samples from the active decay stage (c) were loosely grouped according to treatment and day. Distinguishing between treatments was partly possible during the advanced decay stage (d). PC1 for this stage showed a strong relationship with levels of 18:2ω6 while PC2 showed a strong relationship with levels of 17:0. Dry remains stage samples were analysed per day and supplementary figures can be found in Appendix B.

4.3.2.4 Summer 2012

PCA results for the Summer 2012 fresh stage indicated that control samples and experimental samples were distinct (Figure 22a). The same distribution of samples observed in the Summer 2011 fresh stage was seen for this trial (Figure 5d). PC1 of the Summer 2012 plot explained 68% of the variation between samples. PC1 and values for 18:1ω7c were a perfect fit (R^2 = 1.000, $p < 0.001$). PC1 was also found to strongly correlate with values for 16:1ω11c (R^2 = 0.937, $p < 0.001$), 16:0 (R^2 = 0.935, $p < 0.001$), 17:0 (R^2 = 0.834, $p < 0.001$) and 18:1ω9t (R^2 = 0.925, $p < 0.001$). PC2 from this analysis explained 9% of the variation and did not show a strong linear regression with any individual fatty acid.

The Summer 2012 bloat stage PCA indicated little change within soil microbial communities had occurred between the fresh stage and bloat stage (Figure 22b). Almost all samples clustered together yet control and experimental samples remained separate from each other. PC1 explained 45% of the variation and showed a strong linear regression with values of 16:0 (R^2 = 0.924, $p < 0.001$) and 18:1ω9t (R^2 = 0.804, $p < 0.001$). PC2 explained 12% of the variation and did not show a linear regression with any individual fatty acid.

Control samples from the Summer 2012 active decay stage were clustered tightly together indicating a change occurred between the bloat stage and active decay stage causing profiles to become similar (Figure 22c). The majority of the experimental sample profiles were seen to group together but were distinct from the control samples. PC1 explained 29% of the variation while PC2 explained 12% of the variation observed.

Strong regressions between scores for PC1 or PC2 and measures of specific fatty acids were not found.

PCA results for the Summer 2012 advanced decay stage indicated that samples profiles were more spread-out than the active decay stage. Control samples and experimental samples were distinct from each other with the separation of samples occurring mainly along PC1 (Figure 22d). Diversity within the group of control samples and experimental samples occurred along PC2. PC1 explained 20% of the variation and showed a strong regression with 3OH 14:0 (R^2=0.749, $p < 0.001$). PC2 explained 18% of the variation but did not show a relationship with any specific fatty acid.

The PCA for the dry remains stage of the Summer 2012 showed that samples for days 34, 41, 48 and 62 clustered together on the left hand side of the plot whereas samples from day 97 clustered together on the right hand side of the plot (Figure 22e). Within the two clusters of samples a distinction between control samples and experimental samples was observed. PC1 explained 56% of the variation and showed a strong relationship with 18:1ω9t (R^2=0.959 $p < 0.001$), 14:1 (R^2=0.957, $p < 0.001$), 14:0 (R^2=0.940, $p < 0.001$), 17:0 (R^2=0.932, $p < 0.001$) and 16:0 (R^2=0.857, $p < 0.001$). PC2 explained 8% of the variation and showed a relationship with 18:2ω6 (R^2=0.702, $p < 0.001$).

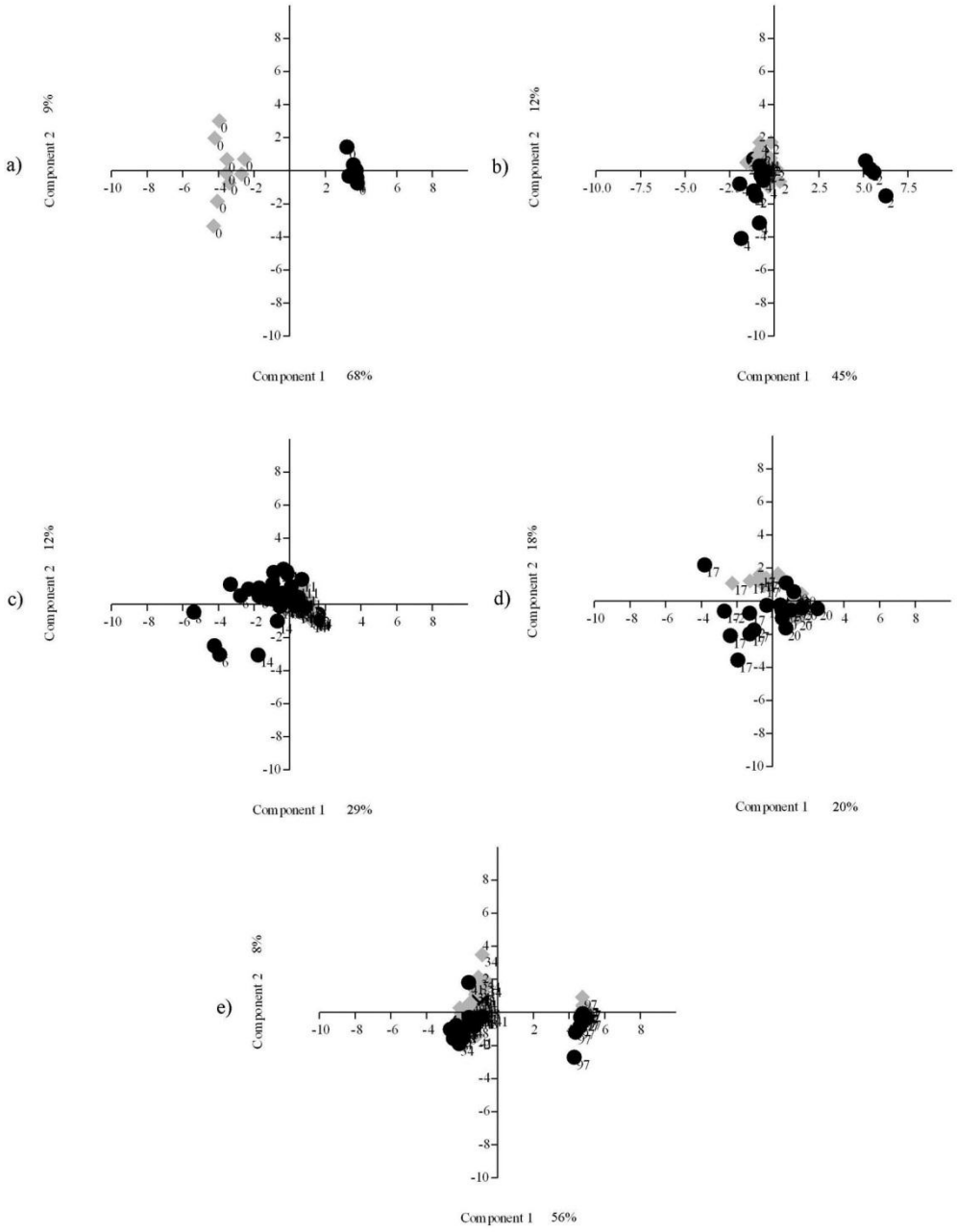


Figure 22. PCA plot of control samples (♦) and experimental samples (●) during the a) fresh, b) bloat, c) active decay, d) advanced decay and e) dry remains stages of the Summer 2012 trial. There was a clear distinction between treatment sites during the fresh stage (a). PC1 from this stage showed a strong linear regression with levels of 18:1ω7c, 16:1ω11c, 16:0, 17:0 and 18:1ω9t. Profiles from all sites clustered together during the bloat and active stage though distinguishing between treatments remained possible. Differentiating between treatments during the advanced decay stage (d) was possible on separate days. Separation occurred mainly along PC1 which showed a strong relationship with levels of 3OH 14:0. During the dry remains stage (e) samples clustered on the left were collected on days 34 through 62 and those on the right on day 97. Distinguishing between treatments remained possible during this stage.

4.3.3 Influence of soil pH and soil moisture on FAME profiles

4.3.3.1 Spring 2011

Spring 2011 correlation results indicated that soil pH was negatively correlated to four fatty acids, *i*15:0, 2OH 14:0, cy17:0, 18:1ω7c; and positively correlated to two fatty acids, *a*15:0 and 16:0 (Table 16).). These fatty acids are associated with various groups of microorganisms, aerobes to anaerobes, Gram-positive bacteria and Gram-negative bacteria as well as fungi (see Table 11). A clear trend between soil pH and specific groups of microorganisms could not be established. Soil moisture was inversely correlated to 16 of the 22 identified fatty acids indicating that soil moisture was an important predictor of soil FAME content. When correlation results were compared to FAME distributions it was noted that when soil moisture was highest, FAME content was more diversified whereas when soil moistures decreased, the number of distinct fatty acids was reduced although their proportions within samples increased.

PCA of all samples from the Spring 2011 indicated that PC1 explained 75% of the variation and was strongly correlated with soil moisture ($R^2 = 0.998$, $p < 0.001$). A strong loading for soil pH was not observed. PCA results did not indicate that soil pH has a significant effect on overall FAME profiles. As PC1 showed a strong linear regression with values of soil moisture a loadings plot for FAMEs according to PC1 and PC2 was produced and is shown in Figure 23. Where we consider that PC1 is analogous with soil moisture, loadings indicated that higher concentrations of 2OH 12:0 and 2OH 14:0 could be found in samples with higher moisture content. Low soil moisture was associated with greater levels of 3OH 12:0, 11:0 and 18:1ω9t. All hydroxyl fatty acids and 18:1ω9t are associated with Gram-negative bacteria indicating that changes in soil moisture may have

had an impact on this fraction of the microbial population. The FAME 3OH 12:0 and 2OH 12:0 are associated with aerobic bacteria whereas 2OH 14:0, 11:0 and 18:1ω9t are markers for facultative aerobic bacteria. That aerobic bacteria would be favored in low soil moisture soils suggests these soils were more aerobic than soils where soil moisture was greater.

Table 16. Summary of Pearson product moment correlation results for values of soil pH and soil moisture and the corresponding measures of fatty acids obtained from FAME profiles during the Spring 2011 trial. Significant correlations ($p < 0.05$) are highlighted in bold.

	Soil pH		Soil moisture	
	R	**p**	**r**	**p**
16:1ω11c	0.053	0.414	**-0.262**	**0.000**
***α*15:0**	**0.271**	**0.000**	**-0.290**	**0.000**
***i*15:0**	**-0.321**	**0.000**	-0.025	0.705
***i*16:0**	0.117	0.070	**-0.233**	**0.000**
2OH 12:0	0.037	0.566	**0.158**	**0.014**
2OH 14:0	**-0.205**	**0.001**	0.105	0.106
3OH 12:0	0.033	0.610	**-0.250**	**0.000**
3OH 14:0	0.060	0.359	**-0.139**	**0.031**
cy17:0	**-0.183**	**0.005**	-0.136	0.036
10:0	-0.036	0.583	**-0.209**	**0.001**
22:1ω9	0.057	0.380	0.015	0.818
12:0	-0.046	0.478	**-0.166**	**0.010**
24:0	0.002	0.972	**-0.296**	**0.000**
18:2ω6	-0.089	0.168	**-0.168**	**0.009**
17:0	-0.041	0.525	**-0.135**	**0.037**
14:0	-0.027	0.681	**-0.176**	**0.006**
18:1ω7*c*	**-0.237**	**0.000**	-0.060	0.356
18:1ω9*t*	0.045	0.485	**-0.286**	**0.000**
16:0	**0.338**	**0.000**	**-0.385**	**0.000**
16:1 ω9*c*	-0.053	0.418	-0.112	0.085
15:0	-0.031	0.638	**-0.154**	**0.018**
18:0	-0.026	0.688	-0.003	0.959
11:0	0.033	0.608	**-0.286**	**0.000**

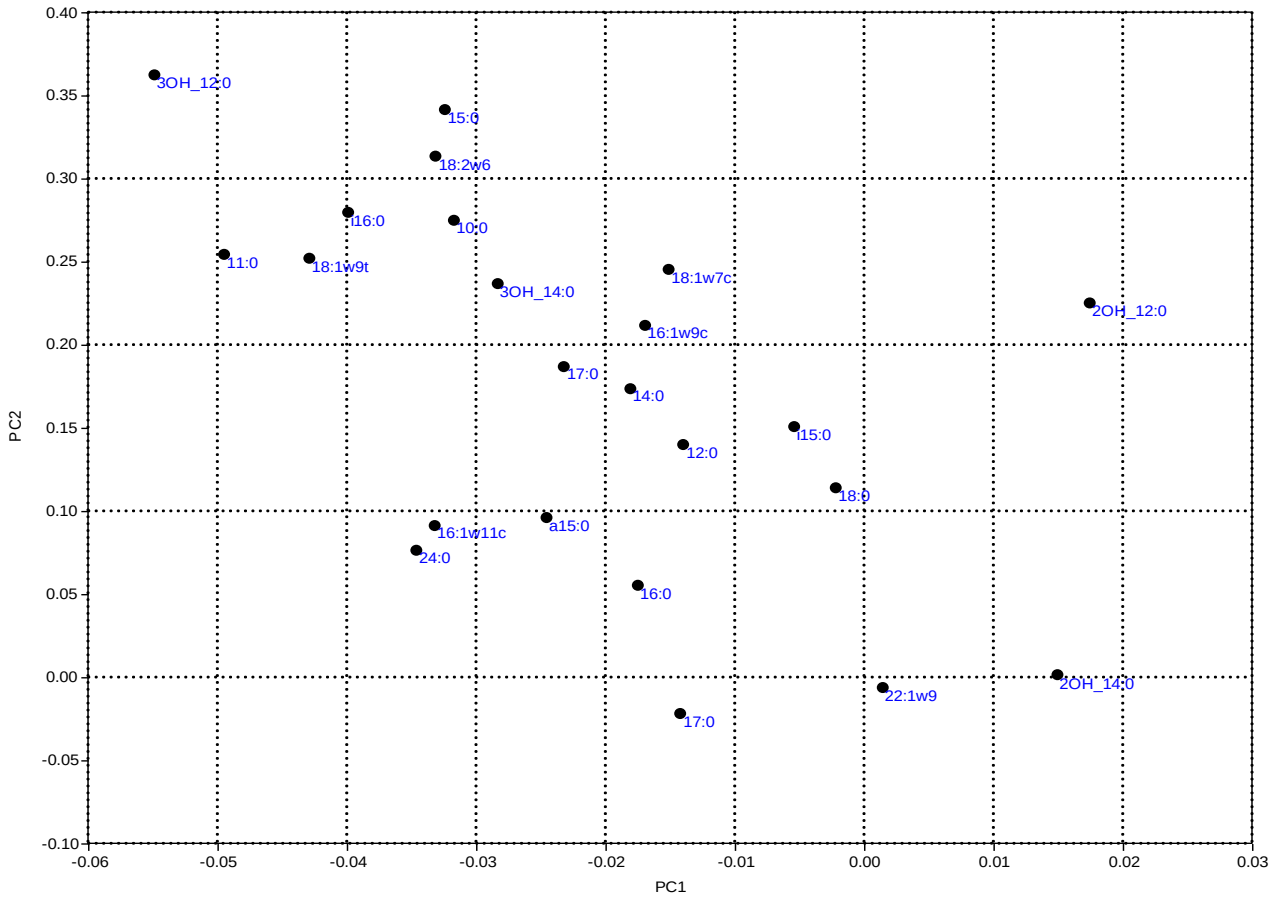


Figure 23. Loadings of the individual FAMEs from the principal component analysis of the Spring 2011 FAMEs according to PC1 and PC2. PC1 and soil moisture showed a strong linear regression indicating PC1 can be interpreted as measures of soil moisture. Fatty acids to the right are more common in low moisture soils while those to the left indicate those associated with high moisture soils.

4.3.3.2 Summer 2011

Correlation results from Summer 2011 data showed that soil pH was positively correlated with six fatty acids: *a*15:0, *i*15:0, 12:0, 18:1ω9t, 16:1ω9c; and negatively correlated to one fatty acid, 18:0 (Table 17). Fatty acids *a*15:0, *i*15:0 and 18:1ω9t indicate a potential change in Gram-positive bacteria and fungi present in soils according to soil pH.

Soil moisture was positively correlated with seven different fatty acids: *i*15:0, 2OH 16:0, 10:0, 12:0, 14:0, 16:0, 16:1ω9c; and negatively correlated to one fatty acid, 18:1ω9t (Table 17). All FAMEs positively correlated to soil moisture are associated with the bacterial population present in soil indicating this portion of the microbial population was favored by increases in soil moisture. The relationship between levels 18:1ω9t and soil moisture indicates that this parameter had an adverse effect on the fungal fraction of the microbial community.

The PCA performed for all samples from the Summer 2011 including soil pH and soil moisture indicated that PC1 explained 76% of the variation and was strongly correlated with soil moisture ($R^2 = 0.999$, $p < 0.001$). PCA results did not indicate that soil pH has a significant effect on overall FAME profiles. As PC1 showed a strong linear regression with values of soil moisture a loadings plot for FAMEs according to PC1 and PC2 was produced and is shown in Figure 24. The loadings plot for this trial showed that 12:0, 14:0, *i*15:0, 16:1 and 16:0 were most common in samples with higher measures of soil moisture within the trial. With the exception of 12:0 these fatty acids are bacterial markers. As previously mentioned, 12:0 is typically found in eukaryotic cells. The only

FAME to be commonly found in soils of low moisture was 18:1ω9t which has been accepted as a fungal marker.

Table 17. Summary of Pearson product moment correlation results for values of microbial activity, soil pH and soil moisture and the corresponding measures of fatty acids obtained from FAME profiles during the Summer 2011 trial. Significant correlations (p < 0.05) are highlighted in bold.

	Soil pH		Soil moisture	
	R	**p**	**r**	**p**
16:1ω11c	0.229	0.001	0.007	0.916
***α*15:0**	**0.281**	**0.000**	-0.004	0.956
***i*15:0**	**0.186**	**0.005**	**0.394**	**0.000**
***i*16:0**	0.113	0.092	0.050	0.454
2OH 14:0	0.109	0.105	0.023	0.729
2OH 16:0	-0.043	0.522	**0.219**	**0.001**
3OH 12:0	0.049	0.468	0.042	0.537
3OH 14:0	0.044	0.514	0.094	0.163
cy17:0	0.157	0.019	-0.088	0.193
10:0	0.097	0.148	**0.230**	**0.001**
12:0	**0.203**	**0.002**	**0.512**	**0.000**
24:0	-0.030	0.652	0.028	0.681
18:2ω6	0.120	0.074	0.012	0.859
17:0	0.115	0.086	0.062	0.358
14:0	0.098	0.144	**0.427**	**0.000**
18:1ω9t	**0.134**	**0.046**	**-0.303**	**0.000**
16:0	0.044	0.513	**0.225**	**0.001**
16:1 ω9c	0.127	0.059	**0.398**	**0.000**
15:0	**0.138**	**0.039**	-0.019	0.775
18:0	**-0.140**	**0.037**	-0.045	0.504
13:0	**0.136**	**0.042**	-0.024	0.717

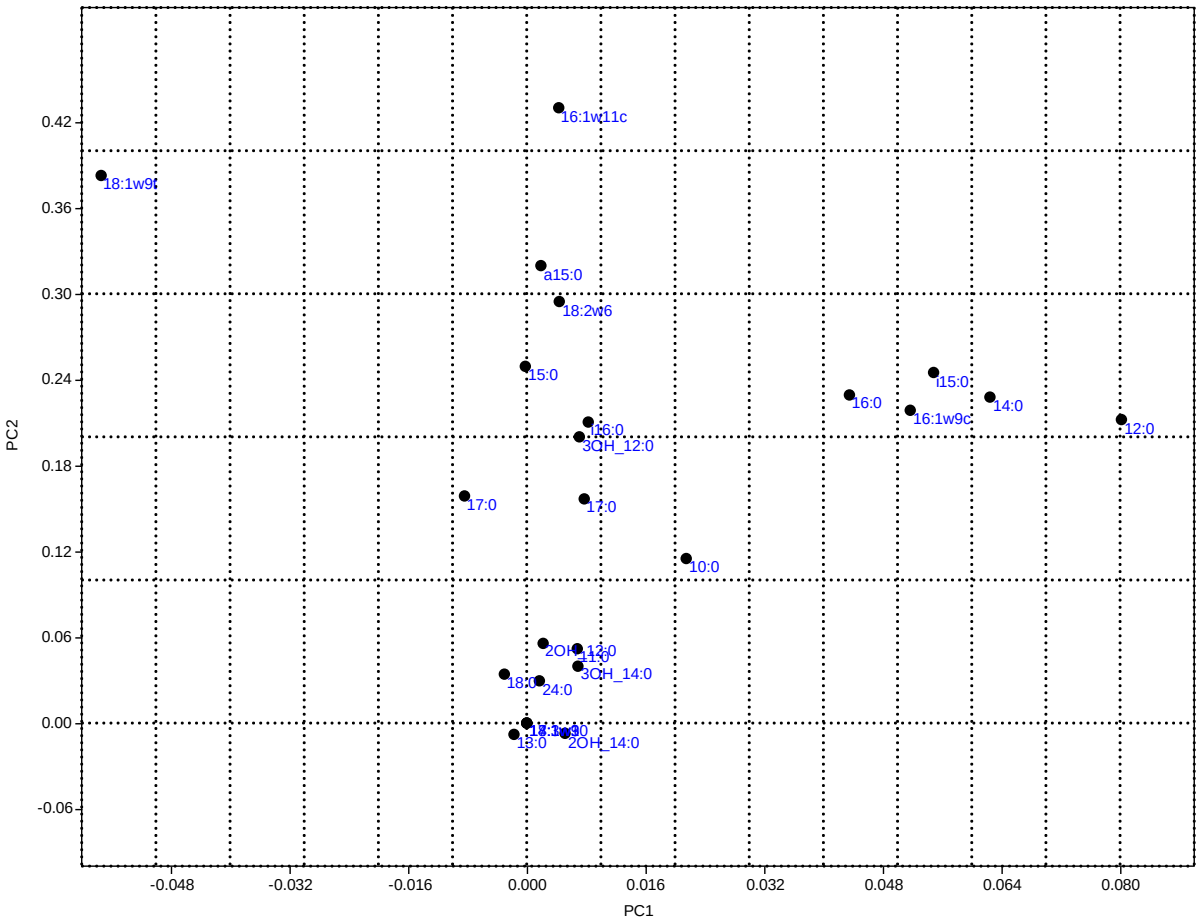


Figure 24. Loadings of the individual FAMEs from the principal component analysis of the Summer 2011 FAMEs data according to PC1 and PC2. PC1 and soil moisture showed a strong linear regression indicating PC1 can be interpreted as measures of soil moisture. Fatty acids to the right are more common in low moisture soils while those to the left indicate those associated with high moisture soil.

4.3.3.3 Spring 2012

Correlation analyses indicated that soil pH was positively correlated to four fatty acids: 16:1ω11c, 3OH 12:0, 15:0, 11:0; and negatively correlated to five fatty acids: 10:0, 12:0, 22:1ω9, 20:2 and 18:1ω9t (Table 18). Positively correlated fatty acids are bacterial in origin whereas negatively correlated fatty acids are commonly found in eukaryotic organisms notably plants (12:0, 22:1ω9, 20:2) and fungi (18:1ω9t).

Soil moisture was positively correlated to two fatty acids: 3OH 14:0, 16:0; and negatively correlated to three fatty acids: *i*15:0, 18:2ω6 and 15:0 (Table 18). Fatty acids positively correlated with soil moisture included a general biomarker (16:0) and a marker for Gram-negative bacteria (3OH 14:0). The compounds negatively correlated with soil moisture were of bacterial (*i*15:0, 15:0) and fungal (18:2ω6) origin.

The PCA performed for all samples from the Spring 2012 including soil pH and soil moisture indicated that PC1 explained 83% of the variation and was strongly correlated with soil moisture ($R^2 = 1.00$, $p < 0.001$). PCA results did not indicate that soil pH has a significant effect on overall FAME profiles. As PC1 showed a strong linear regression with values of soil moisture a loadings plot for FAMEs according to PC1 and PC2 was produced and is shown in Figure 25. The loadings plot for PC1 and PC2 indicated that 18:2ω6 was most common in soils with low moisture content and 3OH 14:0 was present in higher amounts in soil with high moisture content.

Table 18. Summary of Pearson product moment correlation results for values of soil pH and soil moisture and the corresponding measures of fatty acids obtained from FAME profiles during the Spring 2012 trial. Significant correlations ($p < 0.05$) are highlighted in bold.

	Soil pH		Soil moisture	
	R	**p**	**r**	**p**
16:1ω11c	**0.166**	**0.007**	-0.034	0.590
***α*15:0**	-0.039	0.529	-0.084	0.176
***i*15:0**	-0.092	0.141	**-0.122**	**0.049**
***i*16:0**	0.062	0.322	-0.108	0.082
3OH 12:0	**0.127**	**0.041**	0.056	0.373
3OH 14:0	-0.061	0.328	**0.140**	**0.024**
10:0	**-0.204**	**0.001**	-0.058	0.349
20:2	**-0.165**	**0.008**	0.094	0.130
22:1ω9	**-0.123**	**0.048**	0.020	0.753
12:0	**-0.170**	**0.006**	-0.090	0.146
18:2ω6	0.014	0.823	**-0.202**	**0.001**
17:0	-0.087	0.163	-0.018	0.776
14:0	-0.075	0.229	0.038	0.546
18:1ω7c	0.078	0.211	-0.031	0.615
18:1ω9t	**-0.158**	**0.011**	-0.083	0.182
16:0	0.043	0.490	**0.176**	**0.004**
16:1 ω9c	0.085	0.173	-0.022	0.730
15:0	**0.130**	**0.036**	**-0.133**	**0.033**
18:0	0.064	0.304	-0.092	0.141
13:0	0.005	0.931	0.010	0.868
11:0	**0.182**	**0.003**	-0.113	0.070

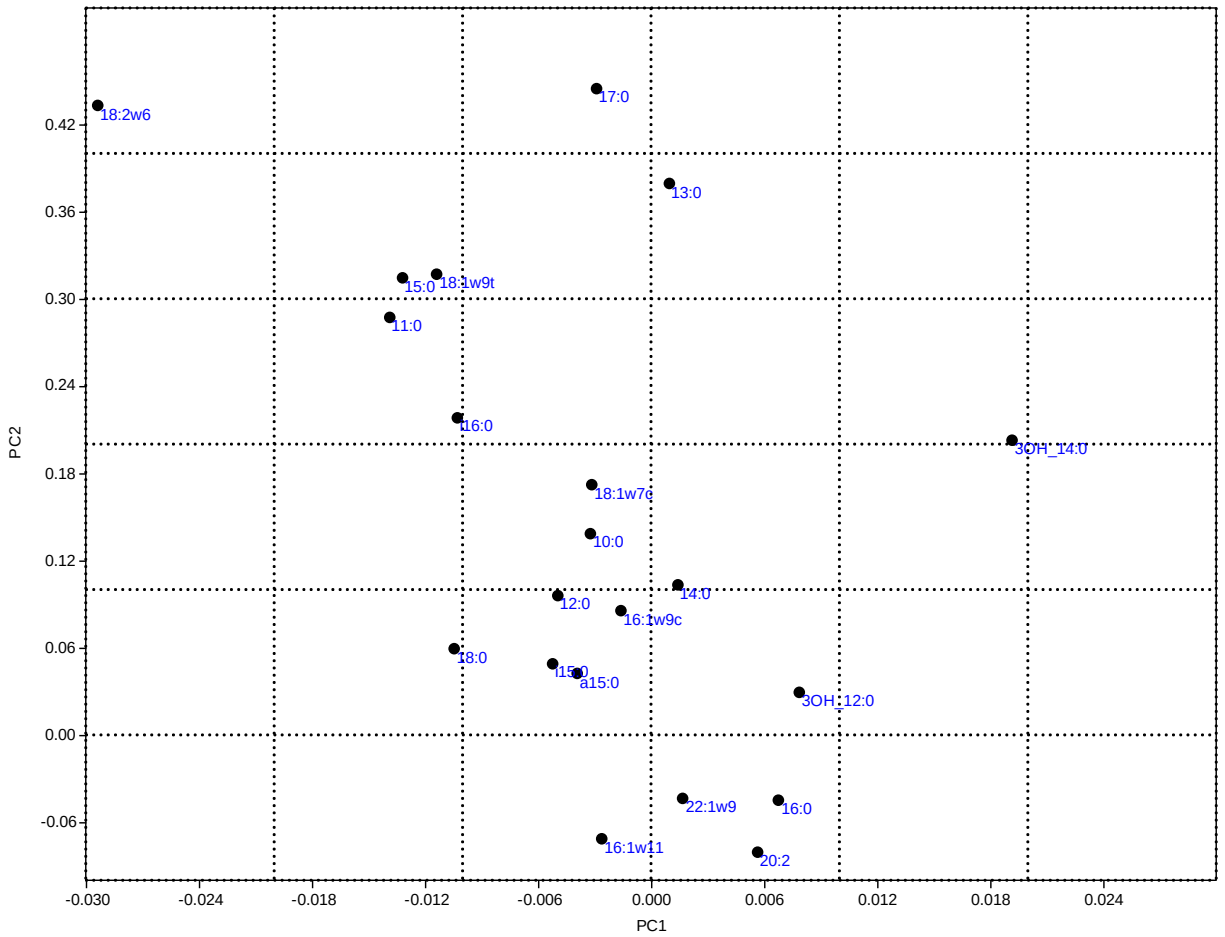


Figure 25. Loadings of the individual FAMEs from the principal component analysis of the Spring 2012 FAMEs data according to PC1 and PC2. PC1 and soil moisture showed a strong linear regression indicating PC1 can be interpreted as measures of soil moisture. Fatty acids to the right are more common in low moisture soils while those to the left indicate those associated with high moisture soils.

4.3.3.4 Summer 2012

Results for the Summer 2012 showed that soil pH was positively correlated with seven fatty acids: 16:1ω6c, *i*15:0, 3OH 12:0, 24:0, 17:0, 18:1ω7c, 18:1ω9t; and negatively correlated with four fatty acids: 12:0, 16:0, 18:0 and 11:0 (Table 19). Soil moisture was found to be negatively correlated with levels of four fatty acids: *i*15:0, 3OH 14:0, 10:0 and 15:0. With the exception of 10:0 which is a common animal fat (Beare-Rogers et al., 2001), *i*15:0, 3OH 14:0 and 15:0 are common microbial markers.

PCA performed for all samples from the Summer 2012 including soil pH and soil moisture indicated that PC1 explained 91% of the variation and was strongly correlated with soil moisture ($R^2 = 1.00$, $p < 0.001$). Although pH was correlated to many individual FAMEs PCA results did not indicate that soil pH has a significant effect on overall soil profiles. As PC1 showed a strong linear regression with values of soil moisture a loadings plot for FAMEs according to PC1 and PC2 was produced and is shown in Figure 26. The loadings plot for PC1 and PC2 indicated that multiple FAMEs were prevalent in soils with higher moisture content. Compounds most affected by increased soil moisture were 14:1, 12:0, 17:0 and 15:0. Soils with lower moisture content showed increased levels of 3OH 14:0, *i*15:0 and 15:0. It is interesting to note that during the Spring 2012 trial, 3OH 14:0 was found in soils with greater moisture content.

Table 19. Summary of Pearson product moment correlation results for values of soil pH and soil moisture and the corresponding measures of fatty acids obtained from FAME profiles during the Summer 2012 trial. Significant correlations ($p < 0.05$) are highlighted in bold.

	Soil pH		Soil moisture	
	R	**P**	**r**	**p**
16:1ω11c	**0.299**	**0.000**	-0.072	0.279
α15:0	0.120	0.071	0.067	0.315
i16:0	0.060	0.364	0.011	0.864
i15:0	**0.226**	**0.001**	**-0.196**	**0.003**
2OH 12:0	0.008	0.910	-0.108	0.104
2OH 16:0	0.100	0.130	-0.089	0.179
2OH 14:0	-0.040	0.552	0.060	0.370
3OH 12:0	**0.245**	**0.000**	-0.031	0.639
3OH 14:0	0.096	0.146	**-0.281**	**0.000**
10:0	-0.077	0.247	**-0.131**	**0.048**
12:0	-0.151	0.022	0.117	0.078
24:0	**0.151**	**0.022**	-0.078	0.237
18:2ω6	-0.111	0.093	-0.053	0.429
18:3ω3	-0.096	0.149	0.003	0.959
17:0	**0.227**	**0.001**	0.016	0.809
14:0	0.047	0.476	-0.054	0.418
14:1	-0.023	0.731	0.061	0.362
18:1ω7c	**0.162**	**0.014**	0.064	0.337
18:1ω9t	**0.138**	**0.037**	-0.026	0.692
16:0	**-0.342**	**0.000**	-0.037	0.583
16:1 ω9c	0.010	0.877	0.026	0.701
15:0	-0.115	0.084	**-0.318**	**0.000**
18:0	**-0.282**	**0.000**	0.011	0.867
11:0	**-0.263**	**0.000**	-0.050	0.450

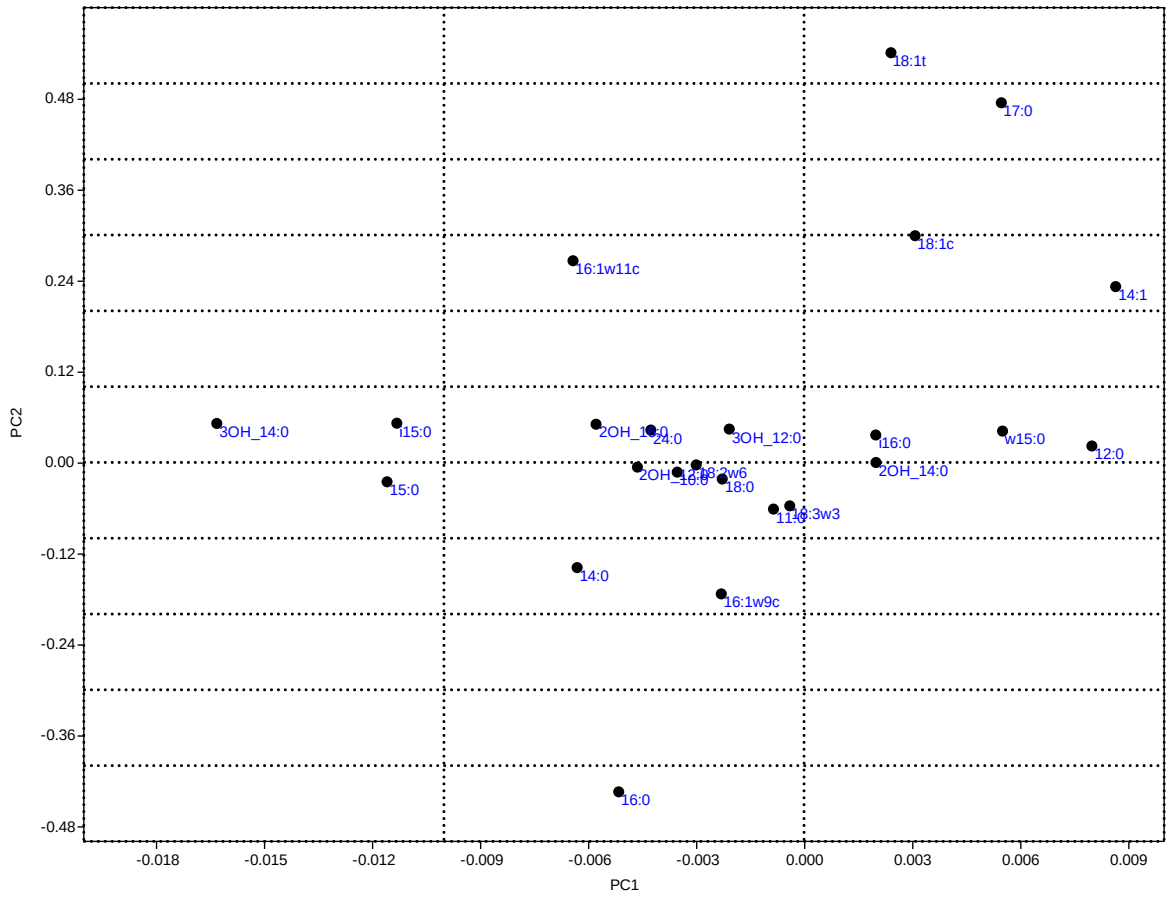


Figure 26. Loadings of the individual FAMEs from the principal component analysis of the Summer 2012 FAMEs data according to PC1 and PC2. PC1 and soil moisture showed a strong linear regression indicating PC1 can be interpreted as measures of soil moisture. Fatty acids to the right are more common in low moisture soils while those to the left indicate those associated with high moisture soils.

4.3.4 Influence of season and year on FAME profiles

Data for major fatty acids common across all experimental trials was pooled to assess the effects of season and year. Two-way ANOVAs on each fatty acid were performed per decomposition stage using season as the first factor and year as the second factor. Results of these analyses are presented in Table 20. Results of the individual effects of season and year are presented in Appendix C - Tables 43 to 47.

Data from the fresh stage indicated that only 6 of the 17 FAMEs varied significantly according to seasons and years suggesting a certain degree of similarity between proportions of FAMEs across the different trials at the start of the experiments. The majority of fatty acids were found to be significantly different according to season when this factor was measured on its own (Appendix B – Table 42). This is believed to reflect both temperatures and vegetation which were comparable for the first few days of both spring and both summer trials.

During the bloat stage, 14 FAMEs showed significant difference between seasons and year. The effects of season and year when considered independently were mostly even (Appendix B – Table 43). This suggests that profiles were altered in distinct ways within each experiment. This may reflect the different trends in soil moisture observed across the four trials during the first week of the experiments (see Chapter 3 – Figures 9 and 13). Variability between FAME profiles obtained during the bloat stage may also indicate the onset of decomposition produced distinct changes within experimental profiles for each trial. These changes would be the result of purging of feces and early signs of liquefaction due to larval activity observed during bloat.

During the active decay stage 9 out of 17 FAMEs indicated an effect based on the interaction of season and year. These fatty acids were the same which varied across different trials during the bloat stage with the exception of 16:1ω11c, *α*15:0, *i*16:0, 17:0 and 16:0. Of these FAMEs, 16:0 was identified as a fatty acid which could be found in greater proportions in experimental samples. Reduced variance of FAMEs according to season or year during the active stage may be due to the increased similarity between experimental samples. Decomposition is thought to produce similar shifts in FAME profiles at this stage making gravesoil profiles more alike.

Two-way ANOVA results for the advanced decay stage indicate a similar trend to what could be observed during the bloat stage with 14 of the 17 fatty acids varying significantly according to season and year. This may reflect the prolonged influence that distinctive weather conditions and decomposition rates may have on soil microbial community composition.

During the dry remains stage only 4 out of 17 FAMEs varied according to both season and year. This suggests that profiles were comparable across all experiments similarly to what was observed during the fresh stage. Sampling days for the dry remains stages of all experiments fell within the late summer and fall. Changes in weather, notably cooler temperatures, are believed to have altered microbial community composition in a similar way across all experiments. The convergence of samples during the later days of the experiments is in accordance with the changes in FAME composition and the relationship between samples outlined in sections 4.3.1 and 4.3.2.

Table 20. Two-way ANOVA results for FAMEs common to all four experimental trials per decomposition stage using season and year as the main factors. Significant differences (p < 0.05) are highlighted in bold.

	Fresh		Bloat		Active		Advanced		Dry remains	
	F	***p***	***F***	***p***	***F***	***p***	***F***	***p***	***F***	***p***
16:1ω11c	**99.94**	**<0.001**	**156.0**	**<0.001**	0.404	0.559	**7.935**	**0.048**	24.63	0.008
***α*15:0**	3.942	0.118	**12.21**	**0.025**	1.132	0.347	**919**	**<0.001**	2.66	0.178
***i*15:0**	5.496	0.079	**57.18**	**0.001**	**32.99**	**0.005**	**1262**	**<0.001**	0.119	0.755
***i*16:0**	7.528	0.052	**44.90**	**0.003**	1.723	0.269	**44.42**	**0.003**	0.150	0.718
3OH 12:0	**49.168**	**0.002**	**9.305**	**0.038**	**20.11**	**0.011**	3.155	0.150	0.893	0.398
3OH 14:0	0.244	0.647	0.726	0.442	0.726	0.442	**1184**	**<0.001**	4.908	0.091
10:0	**7.863**	**0.049**	**$2.1^{E}4$**	**<0.001**	**58.59**	**0.002**	**29.29**	**0.006**	0.025	0.881
12:0	6.907	0.048	**$3.9^{E}4$**	**<0.001**	**54.30**	**0.002**	**9.298**	**0.038**	0.000	0.998
18:2ω6	0.830	0.414	6.47	0.063	5.093	0.087	4.941	0.090	2.616	0.181
17:0	0.540	0.503	**41.71**	**0.003**	4.671	0.097	**9.236**	**0.038**	4.712	0.096
14:0	**29.195**	**0.006**	**20.24**	**0.011**	**19.28**	**0.012**	**49.15**	**0.002**	**21.23**	**0.010**
18:1ω7c	0.198	0.679	**344.3**	**<0.001**	**120.5**	**<0.001**	**1037**	**<0.001**	**51.78**	**0.002**
18:1ω9t	**153.23**	**<0.001**	**226.3**	**<0.001**	**37.06**	**0.004**	4.087	0.113	0.296	0616
16:0	0.694	0.452	**59.96**	**0.001**	0.763	0.432	**37.93**	**0.004**	**10.34**	**0.032**
16:1ω9c	**4.485**	**0.102**	**74.31**	**<0.001**	**15.84**	**0.016**	**49.02**	**0.002**	0.397	0.562
15:0	0.650	0.465	**5785**	**<0.001**	**30.67**	**0.005**	**36.98**	**0.004**	**140.8**	**<0.001**
18:0	4.633	0.098	3.654	0.1285	2.67	0.178	**61.85**	**0.001**	3.529	0.134

Results of two-way ANOVAs from the advanced decay stage indicated that 14 FAMEs showed an effect from the interaction of season and year. With the exception of 2 fatty acids (3OH14:0 and 18:0) results were similar to what was observed during the bloat stage. The advanced decay stage was typically the longest of the decomposition stages encompassing multiple sampling days which were spread-out.

During the dry remains stage only 3 fatty acids were significantly different according to season and year. Similarly to the fresh stage FAME profiles from across the different trials were very similar. The 3 FAMEs which differed between trials were 14:0, 18:1ω7c, 16:1ω9c and 15:0.

4.4 Discussion

It was hypothesized that cadaver decomposition would alter soil microbial profiles and that the change would be detectable for an undetermined amount of time after decomposition had taken place. The experiments conducted as part of this study indicated that shifts in FAME composition of experimental soil profiles mainly during the active decay stage allowed experimental soil samples to be differentiated from control soil samples. After active decay, experimental soil sample FAME profiles gradually became similar to those of control samples. Thus the original hypothesis was accepted.

When all four experimental trials were compared, four marker fatty acids were found in significantly greater amounts in experimental soils collected after active decay had begun. These fatty acids were: 3OH 12:0, 12:0, 16:0 and 18:0. The recurrent increase of these fatty acids in experimental samples suggests they may be potential indicators of

decomposition or of shifts in the microbial population as a result of decomposition processes.

The FAMEs 16:0 and 18:0 are general markers for microorganisms and cannot be associated to specific groups of microorganisms (see Table 11). Both these fatty acids have been reported as some of the most abundant fatty acids in soils collected below decomposing pig carcasses in a similar decomposition study (Larizza, 2010). Palmitic acid (16:0) was also consistently detected in the decomposition fluids of decomposing pig carcasses in the absence of soil (Swann et al., 2009). It is possible that a fraction of the 16:0 and 18:0 detected in experimental samples was of animal origin as these fatty acids are commonly found in porcine fat (Vizcarrondo et al., 1988). Palmitic acid (16:0) may also originate from the breakdown of plant products (Klamer and Bååth, 2004). Stearic acid may originate from fungi but is rarer in plant tissues (Gunstone, 1996).

The fatty acid 12:0 is commonly reported in PLFA or FAME based studies yet it is generally considered as an unspecific marker sometimes associated with eukaryotic microorganisms (see Table 11). Pinpointing the origin of this fatty acid, whether animal, plant or microbial, is difficult as it may be a breakdown product of longer chain fatty acids (Amir et al., 2008).

The fatty acid 3OH 12:0 is a common fatty acid marker for Gram-negative bacteria (see Table 11). Its increased presence in experimental samples suggests that the growth of Gram-negative bacteria was favored in decomposition associated soil or that decomposition products introduced new Gram-negative bacteria into the soil environment. Based on the data available it is not possible to determine the cause of this

increase. Profiling soils using a method capable of identifying specific groups of microorganisms is the next step to understanding the changes observed in these soils.

PCAs for all samples across each trial which included soil pH and soil moisture data indicated that soil moisture explained over 75% of the variation between soil profiles in all four trials. Different groups of FAMEs were identified as being most susceptible to soils of high or low soil moisture content in each trial. Low soil moisture appeared to favor fungal growth. During both 2011 trials 18:1ω9t was found in soils with lower soil moisture contents. This fatty acid has been reported as a marker for fungi (Bååth, 2003) and facultative aerobic bacteria (Quezada et al., 2007). Spring 2012 profiles indicated that the fungal biomarker 18:2ω6 was predominantly found in soil samples with lower soil moisture content. Other marker fatty acids dominant in low soil moisture samples included 11:0 and 3OH 12:0 (Spring 2011) as well as 3OH 14:0, *i*15:0 and 15:0 (Summer 2012). Both 3OH fatty acids are markers for Gram-negative bacteria. A negative correlation between fatty acids associated with Gram-negative bacteria and soil moisture was reported by Brockett et al. (2012) in a study of forest soil profiles across various regions of Canada. This indicates that the Gram-negative bacteria from these soils may be more sensitive to an increase in soil moisture and favored in drier conditions.

When soil moisture was factored in to soil profile distributions its impact greatly surpassed the effect of individual fatty acid levels. Soil moisture has frequently been shown to have a significant impact on soil microbial community composition (Fierer et al., 2003; Baldrian et al., 2010; Huesco et al., 2012). Different microbial responses to wetting have also been observed in a number of studies (Bååth et al., 1998; Fierer et al., 2003; Burger et al., 2005). Site history and plant composition are believed to be

responsible for the varied reactions of different soil microbial communities to changes in soil moisture (Buckley and Schmidt, 2001; Steenwerth et al., 2005). During the advanced decay and dry remains stages experimental profiles were similar to control samples. Despite having been exposed to the same weather, a distinction between control and experimental soils was possible in these later stages. The presence of carcasses and insects as well as the vegetation death observed following decomposition are thought to have altered the microbial response to wetting or drying of the experimental sites.

Moisture content of soils varies primarily according to rainfall and different precipitation regimes are known to have varying effects on soil microbial community composition (Angel et al., 2009; Cregger et al., 2012). However, soil microorganisms are thought to be well adapted to natural fluctuations in soil moisture according to typical seasonal rainfall for their geographical region (Steenwerth et al., 2005). Droughts or prolonged periods of rain may cause shifts in microbial communities (Hueso et al., 2012). More severe changes in soil moisture content can cause the death of certain groups of bacteria and fungi or favor those microorganisms capable of physiological modifications to ensure their survival under stress (Griffiths et al., 2003; Huesco et al., 2012). Spring 2011 showed both periods of rain and drought and has been highlighted as the experiment with the most pronounced changes in soil FAME profiles.

With the exception of a short period of drought during the Spring 2011, both experiments conducted in the spring were subject to similar temperatures and precipitations. FAME profiles at the beginning of both spring trials were diverse but became similar as spring changed to summer indicating that seasonal weather variations produced similar changes in soil FAME profiles between years. Daily average

temperature measures and precipitation patterns were also similar for both summer trials. FAME profiles for both summer trials paralleled changes in daily average temperatures and soil moisture. Profiles from days 62 and 97 were most different when compared to profiles for all other experimental days. These sampling days fell within the late summer and early fall respectively and were subject to different weather conditions than other samples collected earlier in the trial. Samples collected on the final days of both spring trials indicate that seasonal changes influenced soil microbial community profiles in a similar way between years.

Seasonal changes have been shown to have a greater influence on soil microbial profiles than different soil treatments (Bossio et al., 1998). A recent study by Lauber et al. (2013) showed that seasonal changes significantly affected soil microbial community diversity but soil treatment, specifically when relating to varying types and growths of vegetation, had the greatest impact on the soil microbial community composition. Profiles from control samples changed over the course of the experiment and this was believed to represent the natural changes in microbial communities resulting from seasonal changes in temperature and vegetation growth. Changes observed in experimental soil profiles over time were similar to those observed in the control samples. A minor difference between control and experimental samples after decomposition was generally still noticeable. This indicated that although experimental soil microbes were affected by changes in weather the effects of decomposition still allowed for the differentiation of gravesoils from controls.

Measures of soil pH from experimental sites followed the same patterns as soil pH from control sites but decreased to a neutral pH on several occasions. Although certain

deviations in soil pH loosely coincided with shifts in soil FAME profiles no clear relationship was observed. Statistical analysis did not indicate that soil pH had a significant impact on FAME profile distributions across each trial. Certain fatty acids were found to be sensitive to changes in soil pH for each individual trial. Levels of *i*15:0, 12:0 and 18:1ω9t were correlated to soil pH across multiple experiments. Lauric acid (12:0) is a marker for eukaryotic organisms suggesting it may originate from fungi, vegetation or porcine tissues. *i*15:0 is indicative of Gram-positive bacteria and 18:1ω9t may derive from fungi or bacteria. Soil pH did not appear to influence one particular group of microorganisms in individual trials or across the entire study.

A large number of studies documenting soil microbial profiles under various conditions have shown a strong correlation between soil pH and microbial community composition (Frostegard et al., 1993; Bååth and Anderson, 2003; Fierer and Jackson 2006; Wu et al., 2009). The current study did not indicate that soil pH influenced soil profiles. The majority of fluctuations in soil pH observed during each trial occurred within 1 pH unit. Most microorganisms are shown to have optimal growth within a range of 3 to 4 pH units (Rosso et al., 1995). The diversity of soil bacteria has been shown to be unaffected by changes in pH which fall within the range of 6.8 and 8.0 (Rousk et al., 2010). Soil pH values observed across all four trials remained between 6.12 and 8.65 suggesting soil pH fluctuations were not significant enough to influence FAME profile distributions.

4.5 Conclusion

The role of soil moisture in predicting the relationship between samples collected throughout each trial highlights the importance of taking this soil parameter into consideration when attempting to profile soil microbial communities. Periods of rainfall or drought are known to change soil microbial composition and FAME profiles from this study demonstrated the microbial sensitivity to important changes in soil moisture levels. The relationship between soil moisture content and rainfall as well as temperature or evaporation rates is evident. Obtaining accurate environmental data for the weeks or months prior to obtaining soil samples will prove essential in establishing site history and correctly interpreting data from soil profiles. Vegetation is also known to influence the soil response to the addition of water. As such, the homogeneity or heterogeneity of vegetation across an experimental site or potential crime scene would need to be accounted for before profiling soils in regions where precipitations are frequent.

Differences between control and experimental profiles could be consistently observed during the active decay stage. The FAMEs 3OH12:0, 12:0, 16:0 and 18:0 were frequently found in significantly higher amounts in experimental soil samples. These fatty acids may indicate changes in the microbial community, notably Gram-negative bacteria, in decomposition soils or may be detected as a product of adipose tissue degradation. Future studies investigating changes in soil FAME profiles as a result of decomposition should include measures of these fatty acids to determine whether they can be consistently detected in various geographical regions and under varied environmental conditions.

CHAPTER 5
Outdoor trials – Soil Metagenomes

5.1 Introduction

The ubiquitous nature of microbes and their specificity to different environments make them highly suitable for use as forensic evidence. Until recently, the application of microbiological analyses to the field of forensics had been mainly for purposes of examining evidence from bioterrorism acts or biocrimes (Schutzer et al., 2011). Novel microbial profiling technologies have recently been used in forensic studies to include of exclude potential relationships between evidence and individuals and the results are promising. Fierer et al. (2010) published a study showing the potential for using bacterial community profiles from fingertips to associate suspects and objects. Goga (2012) subsequently showed that the microbial communities present on the feet of an individual could be matched up to footwear providing a novel means of obtaining forensic footwear evidence.

Soil based evidence is also of interest to the forensic community due the prevalence and transferability of soil. In the past decade there have been attempts at using soil microbial communities to support the potential relationships between soil samples collected on evidence and a specific crime scenes or vice versa (Horswell et al., 2002; Heath and Saunders, 2006, 2008; Moreno et al., 2006; Macdonald et al., 2008; Lenz and Foran, 2010). Such studies confirmed the potential use of soil microbial profiles in forensic investigations. In 2009, Sensabaugh emphasized that the limitations to using microbial community profiling in forensics was due to the fact that it had not been demonstrated that microbial population variability was highly specific and the lack of

analytical tools to allow communities to be reliably compared. The growing use of next-generation sequencing (NGS) to profile microbial genomic-DNA in soils coupled with the development of novel bio-informatics approaches has highlighted NGS as a potential tool in forensic science. Furthermore, with the development of collaborative works such as the Earth Microbiome Project (www.eathmicrobiome.org; Gilbert et al., 2010) an extensive database of microbial community profiles across the globe is being generated.

The potential of using highly specific microbial profiling methods is of particular interest within the field of forensic taphonomy. Although decomposition is well known to involve a multitude of microorganisms there has been reticence to study these microbial communities due to their complexity (Vass, 2000). New technologies have made it possible for microorganisms involved in the decomposition process to be studied over time, potentially providing microbial timelines which can be used as PMI indicators. In two recent studies, Pechal et al. (2013) and Metcalf et al. (2013) studied the microbial communities associated with decomposing swine and mouse carcasses in the attempts of establishing such microbial timelines. Pechal et al. (2013) focused on the microorganisms present within carcasses over the course of decomposition. They found that decomposition affected microbial richness and abundance and specific changes to the microbial community could be observed for each decomposition stage. From this they proposed a framework for establishing physiological time for a cadaver based on microbial community profiles. This study only included one trial conducted in late summer during which decomposition was deemed complete within 5 days. Similar studies are required to determine if the microbial changes observed in this study are reproducible. Metcalf et al., (2013) showed that changes in the presence and abundance

of bacteria and eukaryotes in abdominal, skin and soil samples were specific enough to associate them to the visual changes observed during decomposition. Community profiles obtained from tissues of the mice carcasses enabled PMI to be estimated within a few days. As this experiment was conducted within the laboratory under controlled environmental conditions there remains a need to conduct experiments in the field to produce results representative of what is observed during forensic investigations and confirm the feasibility of using forensic microbial timelines.

The current chapter presents results from the Illumina® sequencing of soil microbial DNA extracts from samples collected during the four experimental trials described in the previous chapters. Illumina® sequencing is based on the principle of sequencing by synthesis which allows the bases of a DNA fragment to be identified by the signal they emit as they are added to the new DNA strand produced by the sequencing process (Illumina, 2010). The technology can be used to sequence whole genomes including the total genetic material obtained from ecological samples referred to as metagenomes. When studying microbial populations the 16S region of the rRNA gene is targeted as it is present in almost all bacteria and is highly conserved (Woese, 1987).

The decomposition trials which make up this study took place over the course of 2011 and 2012 and were conducted over two different seasons to allow for seasonal and yearly changes in environmental conditions. By obtaining microbial profiles from four different experiments it was possible to investigate both the potential impact of different weather conditions and that of decomposition on the microbial communities. Based on the literature (Parkinson et al., 2009; Metcalf et al., 2013) and results from the microbial FAME profiles described in Chapter 4 it was believed that differentiating control and

experimental samples would be possible once the active decay stage had begun. Soil moisture was also identified as a factor which influenced soil microbial activity and FAME profiles of samples collected during this study. It was expected that soil moisture would be associated with changes in community composition by favoring and hindering particular groups of microorganisms at various moisture levels.

5.2 Methods

Samples obtained from the outdoor experimental trials described in Chapter 3 were used for metagenomics profiling. A total of 1224 soil samples were collected over the course of the four experimental trials. To reduce the number of samples used for sequencing the triplicate soil samples obtained per site were combined. This reduced the 9 control and 9 experimental samples to 3 control and 3 experimental samples per sampling day. One gram of each triplicate soil sample was placed in a new vial and the soil mixed thoroughly. This mixture was then sampled and weighed to proceed with the DNA extraction stage described below. Soil moisture, soil pH and environmental data previously described in Chapter 3 were used for the analyses presented in this chapter.

5.2.1 DNA Extraction and Sequencing

DNA was extracted from soil samples using the Mo-Bio PowerSoil® DNA isolation kits (MoBio Laboratories, Carlsbad, CA, USA). The steps involved in this isolation process are summarized in Figure 27. Once DNA extracts were obtained, 50 µl of the each extract was loaded into a 96 well plate and stored at -20°C until they were

shipped to the University of Colorado – Boulder for amplification and sequencing. The remaining 50 µl of the extracts were stored at -20°C for future use.

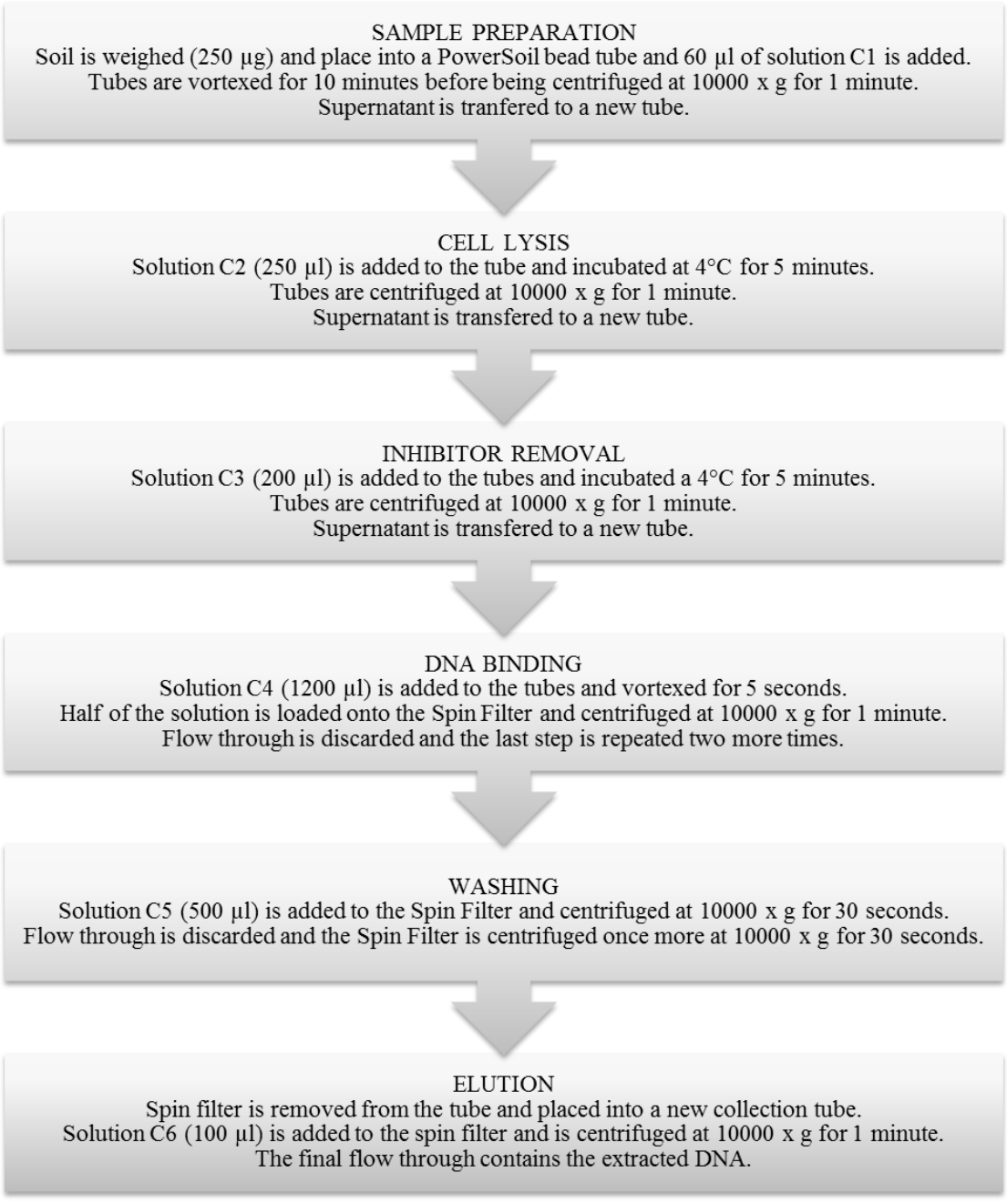


Figure 27. Outline of MoBio PowerSoil® DNA isolation stages taken from the manufacturer's instruction manual (MoBio, 2011)

Extracted DNA was PCR-amplified in triplicate using barcoded primers for the 16S rRNA gene (Bates et al., 2010). The primer set described by Caporaso et al. (2010) was used to amplify the targeted variable region 4 (V4) portion of the 16S rRNA gene from most bacteria and Archaea. Amplicons were quantified using Quant-It™ PicoGreen® kits according to the manufacturer's instructions (Molecular Probes Inc., 2008).

16S library pools were initially analyzed by Bioanalyzer (Agilent Technologies, Santa Clara, USA) using DNA1000 chips to ascertain library quality and average size distribution. The concentration of the pools was determined via Qubit (Invitrogen, Carlsbad, USA) using High Sensitivity reagents and the pools diluted to 2nM. Following NaOH denaturation, the libraries were applied to a v2.5 TruSeq Paired End HiSeq flow cell cluster kit (Illumina® Inc., San Diego, USA) at 4pM per manufacturer's instructions (Illumina, 2012). For clustering, sequencing of read 1, sequencing of the index read and sequencing of read 2, custom sequencing primers (IDT) were used at a final concentration of 500nM in Illumina's hybridization buffer (HT1). Sequencing on the HiSeq system was done according to manufacturer's instructions (Illumina, 2012). Application of the library pools resulted in approximately 340k clusters/mm2 and 38M reads pass-filter. Base calling was performed using CASAVA-1.7.0 (Illumina® Inc., San Diego, USA).

5.2.2 Analysis of community profiles

Unless specified the open-source software package QIIME v1.7.0 was used to process the sequences and conduct all statistical analyses. Reads were assigned to operational taxonomic units (OTUs) using an OTU picking protocol (Caporaso et al., 2010) where the UCLUST algorithm (Edgar, 2010) was applied to search sequences against a subset of the Greengenes database filtered at 97% identity. Reads were assigned to OTUs based on their best hit to this database at greater than or equal to 97% sequence identity. Reads that did not match a reference sequence were discarded as a likely sequencing error. Taxonomy was assigned to each read by accepting the best matching Greengenes sequence.

Beta diversity was used to assess differences in microbial community composition across all samples collected. Samples were rarified at 19,500 sequences per sample. Unweighted and weighted UniFrac distances were computed between all samples to produce sample distance matrices (Lozupone and Knight, 2005). The weighted UniFrac metric accounts for relative abundance of sequences while the unweighted UniFrac metric does not. UniFrac distances were also computed for the pooled data from all four trials. Principal coordinates analysis (PCoA) was applied to visualize the distance matrices using PAST Version 2.16. Analyses of variance using distance matrices (ADONIS) and permutational multivariate analyses of variance (perMANOVA) (Anderson, 2001) were used to determine if various categories explained the variation of samples distances. perMANOVAs carried out on the dataset comprising all four experiments also measured the effects of season and year. Analyses of similarity (ANOSIM) were performed to determine if there was a significant difference between

samples groups across the individual trials and within the pooled dataset. ANOSIM were also performed on the subsamples of control and experimental samples from all trials according to decomposition stage. It should be noted that "treatment" refers to the distinction between control and experimental samples while "decomposition stage" refers to the distinction between control samples and experimental samples from each of the decomposition stages.

Average proportions of OTUs present in control and experimental samples per sampling day were summarized in taxonomic summary plots at the phylum level. For visualization purposes, plots were created using the top 25 OTUs present in samples at this level. Taxonomic data were used to determine if a correlation existed between measures of soil moisture or soil pH and specific groups of microorganisms. Taxonomic data were also used to identify specific OTUs which showed significant differences in relative abundance according to decomposition stage using ANOVA. The same analysis was performed on taxonomic profiles obtained from the pooled dataset of all trials were used to determine if specific OTUs showed significant differences between control samples and experimental samples from the different stages of decomposition.

The Shannon index was used as an indicator of sample alpha-diversity as it is correlated with species richness and evenness (Hill et al., 2003). Based on indications that changes in microbial communities were most pronounced during the active and advanced stages of decomposition Shannon indices were used to compare control and experimental samples during these stages and between stages. Data was tested for normal distribution by the Shapiro-Wilk's test and equal variance by F-test prior to analysis. Student's t-test was used to determine significant differences between groups. When the normality test

failed, a Mann-Whitney Rank sum test was performed. Analyses were performed using PAST Version 2.16.

5.3 Results

5.3.1 Spring 2011

The relationship between samples based on weighted and unweighted UniFrac distances was visualized using PCoA plots for each trial. The unweighted data did not prove as useful as distinguishing samples based on treatment and decomposition stage. Unweighted plots for each trial can be found in Appendix C. Weighted UniFrac distances showed better groupings of samples based on treatment and decomposition stage. This suggested the overall abundance of taxa was affected during the decomposition process. The PCoA for samples based on weighted UniFrac distances for the Spring 2011 trial is presented in Figure 38. PCoA for this experiment indicated that soil samples were similar for both control and experimental sites up to and including the active stage. With the onset of advanced decomposition experimental samples began to differentiate from those collected on previous days. Certain control samples collected during the advanced decay stage also showed a degree of differentiation from samples collected on previous days. It is believed this change may have been due to changes in environmental conditions. It is possible that the changes observed in experimental samples may also have been influenced by environmental parameters.

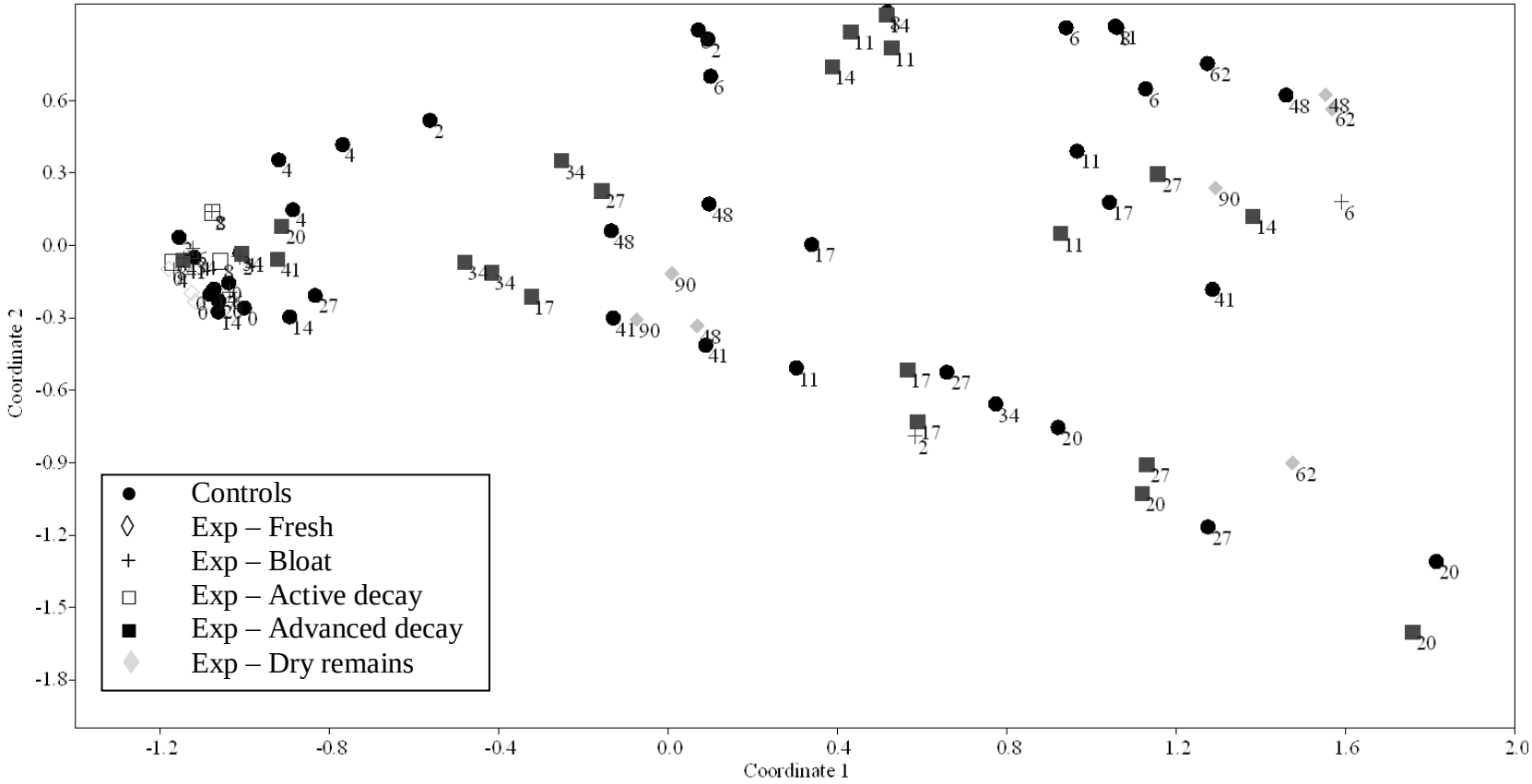


Figure 28. Weighted UniFrac distances of control samples and experimental samples per decomposition stage in Spring 2011 using principal coordinate analysis. Multiple control and experimental sample profiles indicated changes in community composition over the course of the experimental trial. Clustering of experimental samples according to decomposition stages was not observed. Clustering of samples according to treatments throughout the trial was also not observed.

The effect of soil moisture, soil pH and ambient temperature on weighted and unweighted UniFrac distances were determined using ADONIS and results are presented in Table 21. These variables did not have a significant effect on either weighted or unweighted UniFrac distances. The effect of soil treatment (control or experimental) and decomposition stages on sample distributions were assessed using perMANOVAs and results are presented in Table 22. Decomposition stage had a significant effect on sample distribution for both weighted and unweighted distances. Treatment was only found to have a significant impact on unweighted sample distributions.

Table 21. ADONIS results for soil moisture, soil pH and temperature on weighted and unweighted UniFrac distances of samples from the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 trials. Significant effects ($p < 0.05$) are highlighted in bold.

	Spring 2011		**Summer 2011**		**Spring 2012**		**Summer 2012**	
Weighted	R^2	p	R^2	p	R^2	p	R^2	p
Soil moist.	0.381	0.596	0.815	0.897	**0.978**	**0.002**	0.778	0.840
Soil pH	0.013	0.330	0.025	0.063	0.014	0.217	0.017	0.193
Temperature	0.012	0.435	**0.026**	**0.036**	**0.042**	**0.009**	0.015	0.241
Unweighted	R^2	p	R^2	p	R^2	p	R^2	p
Soil moist.	0.819	0.902	0.819	0.917	0.729	0.165	0.786	0.956
Soil pH	0.015	0.239	0.015	0.229	0.015	0.127	0.013	0.347
Temperature	0.021	0.074	0.021	0.087	**0.041**	**0.002**	0.017	0.187

Table 22. perMANOVA for treatment and decomposition stages on weighted and unweighted UniFrac distances of samples from the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 trials. Significant effects ($p < 0.05$) are highlighted in bold.

	Spring 2011		Summer 2011		Spring 2012		Summer 2012	
Weighted	*pseudo-F*	*p*	*pseudo-F*	*p*	*pseudo-F*	*p*	*pseudo-F*	*p*
Treatment	1.188	0.301	**1.887**	**0.049**	**26.65**	**0.001**	**7.688**	**0.001**
Stage	**1.734**	**0.018**	**1.903**	**0.003**	**15.25**	**0.001**	**3.1402**	**0.001**
Unweighted	*pseudo-F*	*p*	*pseudo-F*	*p*	*pseudo-F*	*p*	*pseudo-F*	*p*
Treatment	**2.144**	**0.026**	**2.144**	**0.019**	**16.01**	**0.001**	**3.680**	**0.006**
Stage	**1.853**	**0.004**	**1.853**	**0.003**	**7.450**	**0.001**	**1.666**	**0.034**

ANOSIM were used to determine if there was a significant difference between samples based on either treatment or decomposition stage. Results of these analyses are presented in Table 23. There was no significant difference overall between control and experimental samples for both the weighted and unweighted distances. When ANOSIM were performed for treatments within each stage of decomposition, control and experimental samples were only significantly different during the bloat stage (Table 24). The different stages of decomposition were found to produce significant differences between samples based on unweighted UniFrac distances.

Distributions of the main OTUs for controls and experimental samples per day are presented in Figure 29. Between days 6 and 14 an increase in proportions of Firmicutes and a drop in proportions of Actinobacteria was observed in control samples. Proteobacteria became more prominent in both control and experimental samples between days 20 and 41. There was no clear difference between taxa present in control

and experimental samples once active decay had begun. Changes in distributions over time are similar between control and experimental samples suggesting that environmental conditions may have played a large part in determining community composition.

Table 23. ANOSIM results between sample groups according to treatment and decomposition stages for weighted and unweighted UniFrac distances for the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 trials. Significant differences ($p < 0.05$) are highlighted in bold.

	Spring 2011		Summer 2011		Spring 2012		Summer 2012	
Weighted	***R***	***p***	***R***	***p***	***R***	***p***	***R***	***p***
Treatment	0.007	0.228	0.007	0.527	**0.377**	**0.001**	**0.149**	**0.001**
Stage	0.018	0.414	0.016	0.382	**0.683**	**0.001**	**0.276**	**0.001**
Unweighted								
Treatment	-0.007	0.507	-0.007	0.486	**0.332**	**0.001**	**0.075**	**0.012**
Stage	**0.153**	**0.016**	**0.153**	**0.012**	**0.593**	**0.001**	**0.085**	**0.038**

Table 24. Weighted ANOSIM results between control and experimental samples for each stage of decomposition for the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 trials. Significant differences ($p < 0.05$) are highlighted in bold.

	Fresh		Bloat		Active		Advanced		Dry remains	
	R	***p***	***R***	***p***	***R***	***P***	***R***	***p***	***R***	***p***
Spring 2011	0.556	0.100	**0.207**	**0.011**	1.000	0.101	0.016	0.225	-0.182	0.920
Summer 2011	0.425	0.240	**0.1778**	**0.027**	0.085	0.116	**0.219**	**0.001**	0.175	0.226
Spring 2012	0.182	0.113	0.086	0.072	0.998	**0.002**	**0.706**	**0.003**	**0.834**	**0.001**
Summer 2012	0.185	0.202	**0.296**	**0.002**	**0.215**	**0.003**	**0.218**	**0.015**	**0.125**	**0.010**

OTUs correlated to either soil moisture or soil pH are presented in Table 25. Acidobacteria, Chloroflexi, Proteobacteria were mostly found to be positively correlated with soil moisture. OTUs correlated to soil pH measures were for the most part negatively correlated to this variable. A drop in pH was observed between days 8 and 20 of the Spring 2011 trials (see Chapter 3 – Figure 12) which may have temporarily favored microorganisms better adapted to the change in pH. Bacteroidetes were positively correlated with soil pH.

OTUs indicating a significant difference between controls or experimental samples according to decomposition stage are presented in Table 26. Solirubrobacterales could be found in greater proportions in experimental samples from the active stage. All other orders appeared in greater proportions in experimental samples from the fresh and bloat and in lesser proportions during the active, advanced and dry remains stages.

Shannon indices for control and experimental samples from the active and advanced stages for the Spring 2011 trial are presented in Figure 30a. Alpha diversity of experimental samples was significantly different between the active decay stage and advanced stage ($U = 11$, $p < 0.08$) due to a decrease of diversity in samples from the advanced stage. Alpha-diversity of control samples across both stages did not change significantly ($U = 30$, $p = 0.935$) and there was no difference between control and experimental samples during the active decay stage ($U = 0.000$, $p = 0.100$) or the advanced stage ($U = 192$, $p = 0.481$).

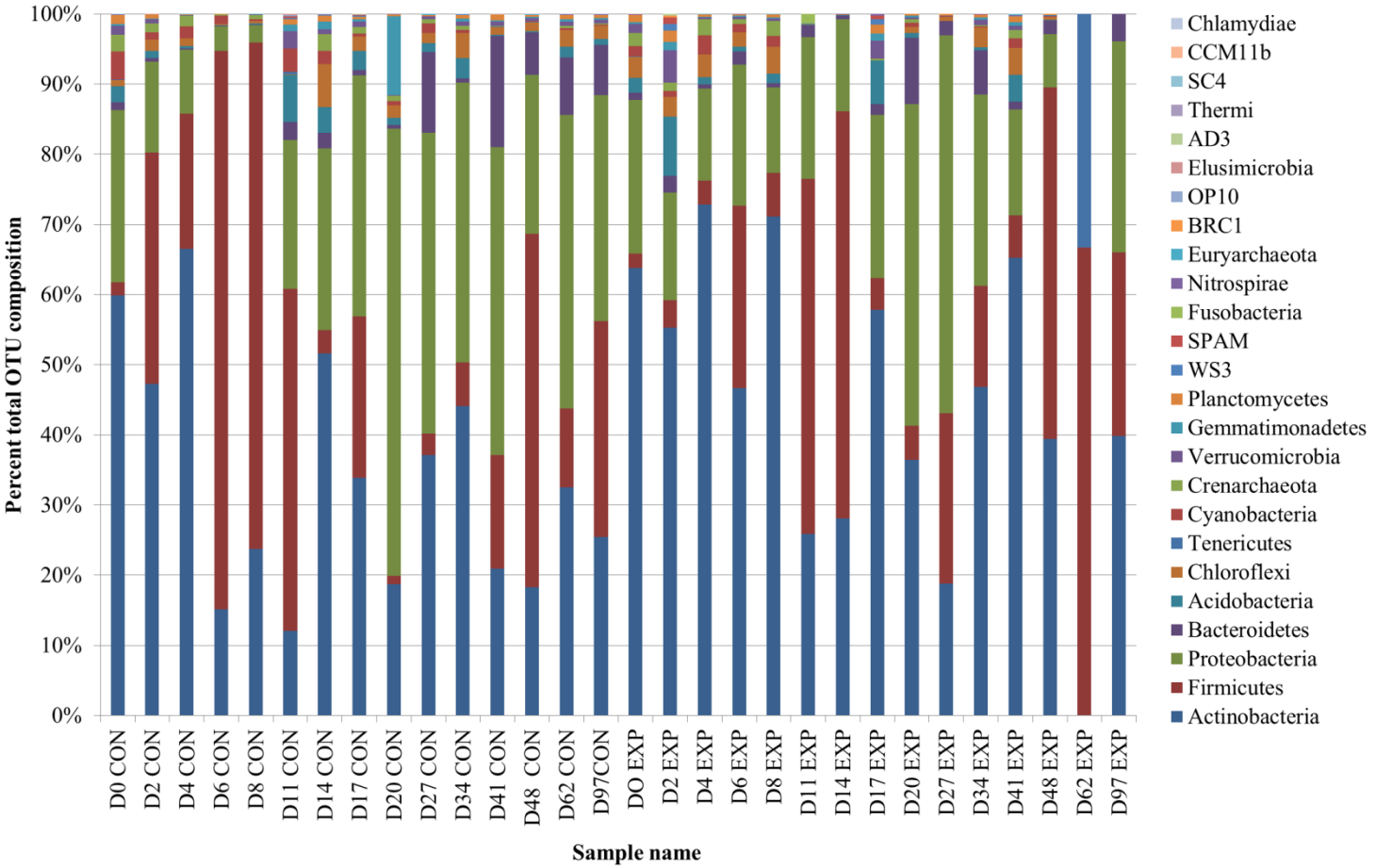


Figure 29. OTU distributions for the top 25 taxa at the phylum level for the Spring 2011 trial. An increase in proportions of Firmicutes within control samples at days 6 and 8 is likely to have been brought on by environmental changes. A similar increase in proportions of Firmicutes can be observed in experimental samples on days 11 and 14 and is likely due to the influx of decomposition bacteria into the soil environment. Similar changes were observed in control and experimental samples over the course of the trial suggesting environmental variables likely influences microbial community composition.

Table 25. OTUs showing significant correlations ($p < 0.05$) with soil moisture or soil pH for the Spring 2011 trial

OTU (Phylum, Class, Order)	*R*	*p*
Soil moisture		
Acidobacteria; Acidobacteria ; Acidobacteriales	0.229	0.037
Acidobacteria; Acidobacteria-5;	0.262	0.016
Acidobacteria; Solibacteres; Solibacterales	0.376	0.000
Actinobacteria; Actinobacteria; Solirubrobacterales	0.223	0.043
Chlamydiae; Chlamydiae; Chlamydiales	0.329	0.002
Chloroflexi; Anaerolineae;H39	0.340	0.002
Chloroflexi; TK17;	0.223	0.042
Planctomycetes; Phycisphaerae; Phycisphaerales	0.221	0.044
Proteobacteria; Betaproteobacteria; Rhodocyclales	0.298	0.006
Proteobacteria; Deltaproteobacteria;	0.228	0.038
Proteobacteria; Deltaproteobacteria; Syntrophobacterales	0.323	0.003
SPAM; n/a	0.307	0.005
Verrucomicrobia; Spartobacteria;	0.324	0.003
WS3; PRR-12;	0.338	0.002
Bacteroidetes; Flavobacteria;	-0.217	0.049
BRC1; PRR-11;	-0.228	0.038
Chloroflexi; Thermomicrobia; HN1-15	-0.259	0.018
Cyanobacteria; S15B-MN24;	-0.260	0.018
Proteobacteria; Gammaproteobacteria; Xanthomonadales	-0.226	0.039
Soil pH		
Bacteroidetes;	0.286	0.009
Bacteroidetes; Bacteroidia; Bacteroidales	0.241	0.028
Proteobacteria; Alphaproteobacteria; Rhodospirillales	0.283	0.009
SC4;	-0.265	0.015
Nitrospirae; Nitrospira ; Nitrospirales	-0.244	0.026
Acidobacteria; RB25;	-0.226	0.040
Planctomycetes; Phycisphaerae;	-0.218	0.047
Proteobacteria; Alphaproteobacteria;	-0.223	0.042
Proteobacteria; Gammaproteobacteria; Chromatiales	-0.215	0.050

Table 26. OTUs showing significant differences between controls and experimental samples from the different stages of decomposition for the Spring 2011 trial ($p < 0.05$ after Bonferroni correction)

		Mean total percent composition					
OTU (Phylum,; Class; Order)	***p***	**Control**	**Fresh**	**Bloat**	**Active**	**Adv.**	**Dry**
Actinobacteria; Actinobacteria; Euzebiales	0.000	0.02	0.11	0.02	0.01	0.00	0.00
Actinobacteria; Actinobacteria; Solirubrobacterales	0.000	7.70	18.00	21.00	30.00	1.50	9.20
Chloroflexi; Bljii12;	0.000	0.07	0.30	0.04	0.03	0.01	0.02
Chloroflexi; Thermomicrobia; Thermomicrobiales	0.000	0.03	0.15	0.04	0.03	0.01	0.01
Proteobacteria; Alphaproteobacteria; Rhodobacterales	0.000	0.76	1.60	1.70	1.60	0.24	0.33
Actinobacteria; Actinobacteria; Rubrobacterales	0.003	0.31	0.45	0.57	1.20	0.03	0.26
Proteobacteria; Deltaproteobacteria; MIZ46	0.003	0.03	0.11	0.05	0.04	0.00	0.01
Crenarchaeota; Thaumarchaeota; Nitrososphaerales	0.008	0.84	1.90	1.20	2.10	0.12	0.35

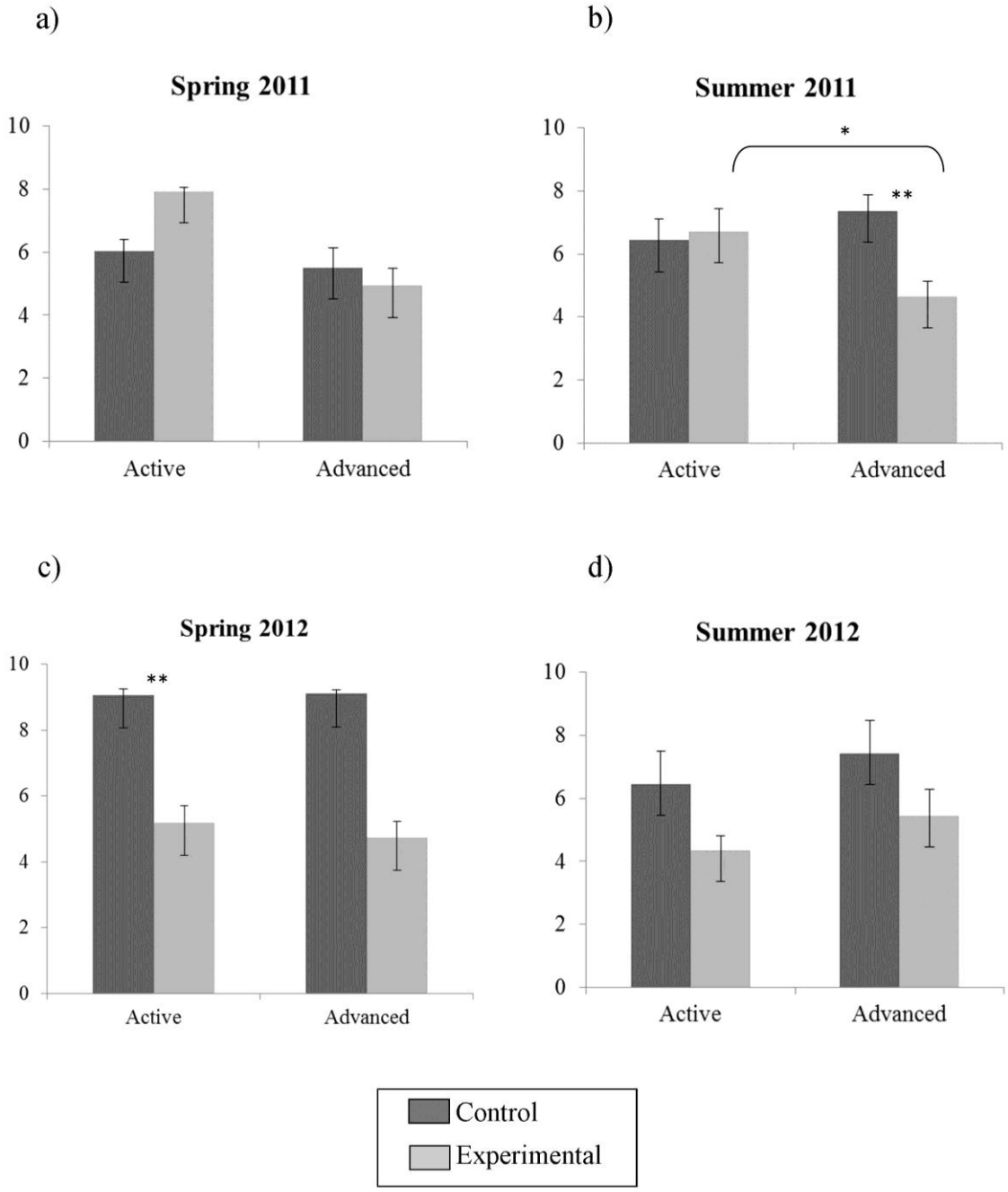


Figure 30. Average Shannon indices and standard errors for microbial communities of control and experimental samples collected during the active and advanced stages of decomposition for the a) Spring 2011 trial, b) Summer 2011 trial, c) Spring 2012 trial and d) Summer 2012 trial. Significant difference are indicated by ** for highly significant differences ($p < 0.001$) and * for significant differences ($p < 0.01$).

5.3.2 Summer 2011

The relationship between samples based on weighted UniFrac distances is presented on a PCoA plot in Figure 31. Control and experimental samples collected while the carcasses were in the stages of fresh and bloat are clustered together on the right-hand side of the plot. As decomposition progressed, distances between the original experimental samples and those collected later during decomposition increased. Many of the control samples collected at later days also show distancing from samples collected at the onset of the experiment. This suggests a common factor was responsible for the differentiation of control and experimental microbial communities overtime. There is very little grouping of the experimental samples during the stages of active decay, advanced decay and dry remains. Grouping of samples according to sampling day was not observed suggesting changes in microbial community at each experimental site were somewhat distinct.

ADONIS and perMANOVA results are presented in Tables 21 and 22. Unweighted results showed no effect related to soil moisture, soil pH or ambient temperature (Table 21). Weighted UniFrac distances were affected by temperature although the effect was not particularly strong. The effect was only significant on weighted distances showing a link between ambient temperature and relative abundance of OTUs in soil samples. Treatment and decomposition stage had a significant effect on weighted and unweighted UniFrac distances. ANOSIM results for the Summer 2011 trial are presented in Tables 23 and 24. Results indicated that for weighted UniFrac distances, treatment and decomposition stage groups were not significantly different overall (Table 23). Unweighted data showed that decomposition stages produced significantly different

sample groups overall. ANOSIM comparing control and experimental samples per stage indicated that there was significant difference based on treatment during the bloat and advanced decay stages (Table 24).

Average distributions of OTUs present in control and experimental samples during the Summer 2011 are presented in Figure 32. Similar to what was observed during the Spring 2011 there was an increase in the proportions of Firmicutes and a decrease in Actinobacteria although this change is most noticeable on days 8 and 11. Firmicutes present in samples during this stage included Lactobacillales and Clostridiales. Later in the experiment at days 20 and 28 proportions of Actinobacteria present in control samples increased while levels of Firmicutes were decreased considerably. Changes in distributions of OTUs in experimental samples can be observed overtime although changes between days are more subtle.

OTUs indicating a significant correlation with soil moisture or soil pH are presented in Table 27. The majority of OTUs found to be significantly correlated with soil moisture showed a negative correlation. Overall measures of soil moisture for samples collected during the Summer 2011 were seen to gradually increase as the experiment progressed (see Chapter 3 – Figure 13). Soil at the beginning of the experiment was dry due to lack of rainfall and high temperatures. This correlates with greater levels of Actinobacteria (Figure 32). As moisture increased proportions of Actinobacteria decreased. Five orders falling within the family of Actinobacteria were found to correlate negatively with soil moisture. Only one order of Proteobacteria and Cyanobacteria were found to positively correlate with soil moisture. The prevalence of Proteobacteria increased towards the last days of Summer 2011 when soil moisture was

highest. The change in proportions of Proteobacteria was likely in part due to the increase in bacteria from the order Oceanospirillales**.** Cyanobacteria are observed in greater proportions for samples from days 34, 41, 48 and 62 though there occurrences are independent of treatment.

OTUs indicating significant difference between controls or experimental samples according to decomposition stage are presented in Table 28. The average composition of Rhodospiralles, MC47 and SOGA31 (class) present in experimental samples decreased as decomposition progressed. A larger proportion of Caulobacterales was observed in experimental samples from the advanced stage. By the dry remains stage the average composition of Caulobacterales had returned to proportions similar to what was observed in earlier stages of decomposition.

Shannon indices of control and experimental samples for the active decay and advanced decay stages are presented in Figure 30b. During the active decay stage, alpha-diversity of control samples and experimental samples was similar ($U = 47$, $p = 0.651$). For the advanced decay stage a significant difference was observed between control and experimental samples ($t = 3.979$, $p < 0.001$). The difference between alpha-diversity of experimental samples from both stage was also significant ($t = 2.603$, $p = 0.015$). There was no difference in alpha-diversity of control samples between stages ($U = 55$, $p = 0.189$).

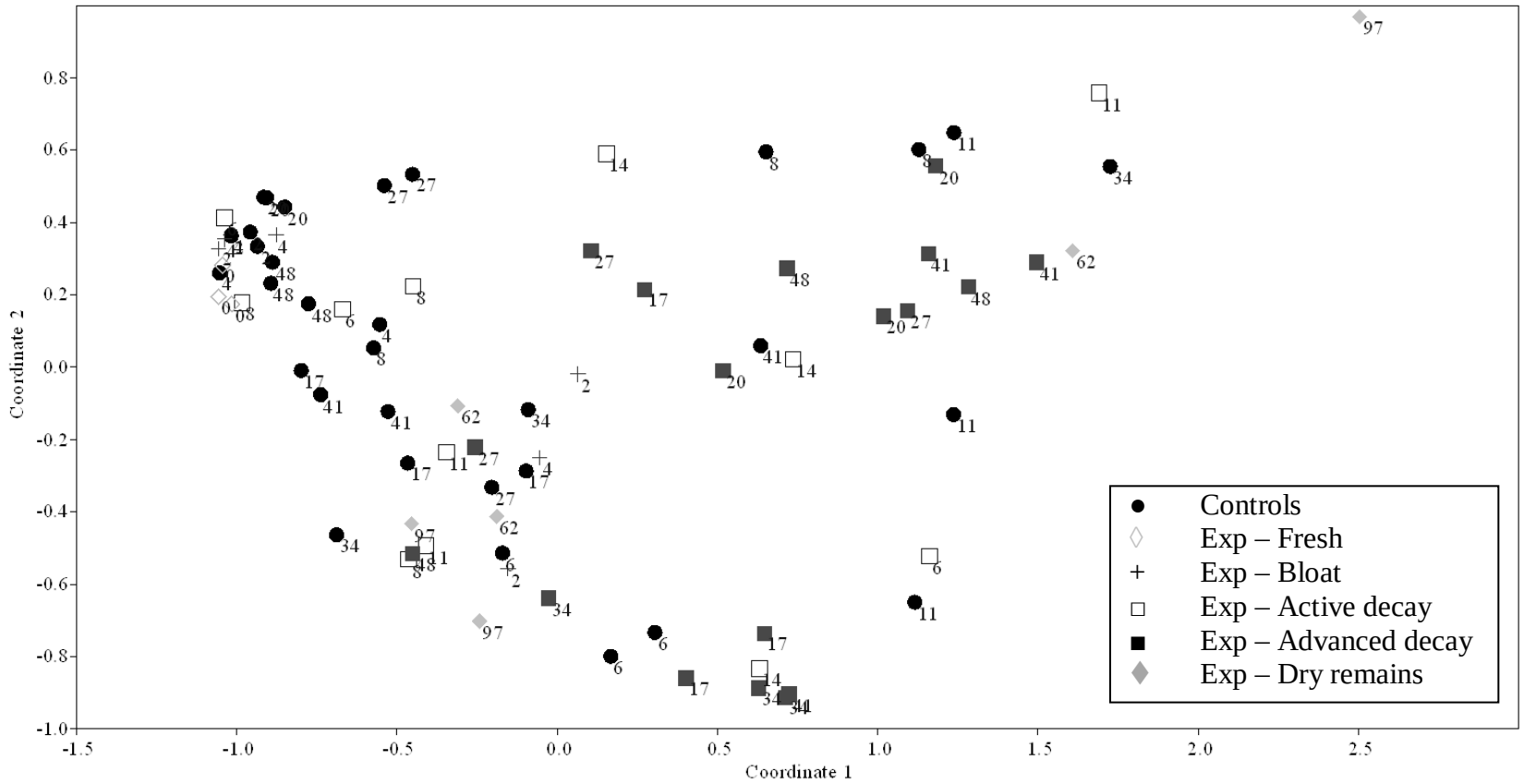


Figure 31. Weighted UniFrac distances of control samples and experimental samples per decomposition stage in Summer 2011 using principal coordinate analysis. The majority of control samples shared a degree of similarity and are seen to group on the left hand side of the plot. Experimental samples showed increased dissimilarity as decomposition progressed through the stages of active decay, advanced decay and dry remains. The overlap of multiple control and experimental samples made it difficult to discern between treatments.

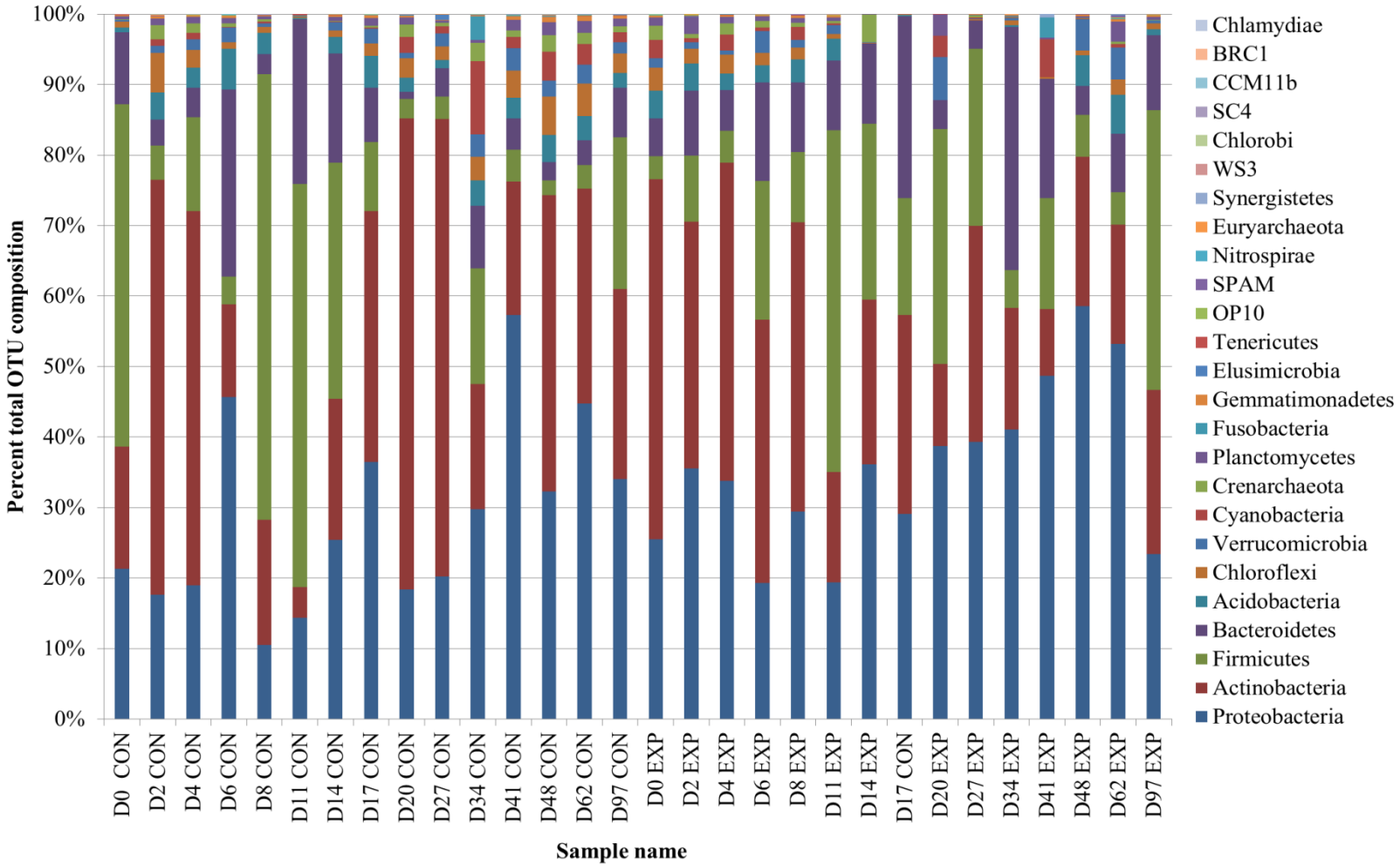


Figure 32. OTU distributions for the top 25 taxa at the phylum level for the Summer 2011 trial. An increase in the proportions of Firmicutes and a decrease in Actinobacteria was observed in control samples between days 8 and 14. This same change was observed on day 11 only in experimental samples. Firmicutes remained a major component of microbial communities of experimental samples following decomposition whereas Actinobacteria dominated control samples.

Table 27. OTUs showing significant correlations ($p < 0.05$) with soil moisture or soil pH for the Summer 2011 trial

OTU (Phylum; Class; Order)	*R*	*p*
Soil moisture		
Acidobacteria;	-0.273	0.015
Acidobacteria; Acidobacteria; Acidobacteriales	-0.356	0.001
Acidobacteria; PAUC37f;	-0.299	0.008
Actinobacteria; Actinobacteria;	-0.394	0.000
Actinobacteria; Actinobacteria; 0319-7L14	-0.259	0.022
Actinobacteria; Actinobacteria; Acidimicrobiales	-0.224	0.049
Actinobacteria; Actinobacteria; koll13	-0.274	0.015
Actinobacteria; Actinobacteria; MC47	-0.415	0.000
CCM11b;	-0.257	0.023
Chloroflexi; Anaerolineae; A31	-0.240	0.035
Chloroflexi; Anaerolineae; Anaerolineales	-0.254	0.025
Chloroflexi; Anaerolineae; SJA-15	-0.371	0.001
Euryarchaeota; Methanobacteria; Methanobacteriales	-0.304	0.007
Euryarchaeota; Methanomicrobia; Methanosarcinales	-0.334	0.003
Firmicutes; Bacilli;	-0.365	0.001
Firmicutes; Bacilli; Bacillales	-0.231	0.042
OP10; SJA-176;	-0.316	0.005
Proteobacteria; Alphaproteobacteria; Rhodospirillales	-0.283	0.012
Proteobacteria; Deltaproteobacteria; NB1-j	-0.264	0.019
Proteobacteria; Deltaproteobacteria; Syntrophobacterales	-0.258	0.022
Proteobacteria; Gammaproteobacteria;	-0.232	0.041
Proteobacteria; Gammaproteobacteria; Xanthomonadales	-0.251	0.027
SC4; KD3-113;	-0.242	0.033
Cyanobacteria; S15B-MN24;	0.261	0.021
Proteobacteria; Gammaproteobacteria; Oceanospirillales	0.232	0.041
Soil pH		
Acidobacteria; Acidobacteria; Acidobacteriales	-0.233	0.040
Acidobacteria; iii1-8;	-0.257	0.023
Acidobacteria; PAUC37f;	-0.274	0.015
Actinobacteria; Actinobacteria; Actinomycetales	0.260	0.021
Actinobacteria; Actinobacteria; MC47	0.235	0.038
Bacteroidetes;	0.244	0.031
Firmicutes; Clostridia; OPB54	0.258	0.022
Proteobacteria; Gammaproteobacteria; Xanthomonadales	-0.438	0.000
SC4; KD3-113;	0.234	0.039

Table 28. OTUs significantly different between controls and experimental samples according to decomposition stage during the Summer 2011 trial ($p < 0.05$ after Bonferroni correction)

		Mean total percent composition					
OTU (Phylum,; Class; Order)	***p***	**Control**	**Fresh**	**Bloat**	**Active**	**Adv.**	**Dry**
Proteobacteria; Alphaproteobacteria; Rhodospirillales	0.000	2.33	5.77	4.86	2.68	0.39	0.19
Actinobacteria; Actinobacteria; MC47	0.006	5.09	9.28	5.24	2.67	0.08	0.87
Proteobacteria; Alphaproteobacteria; Caulobacterales	0.013	1.31	0.49	0.84	0.79	6.71	0.73
Chloroflexi; SOGA31;	0.019	1.66	2.08	1.90	0.77	0.05	0.63

5.3.3. Spring 2012

The relationship between samples based on weighted UniFrac distances is presented on a PCoA plot in Figure 33. Experimental samples from each decomposition stage appear to clearly separate from each other during this trial. Experimental samples collected during the fresh and bloat stage remained close to the group of control samples. Experimental samples from the active and advanced stages show the greatest degree of differentiation and are located on the far right of the plot. Samples from the dry remains stage indicate that once after the active and advanced stages of decay were over samples gradually changed becoming more similar to the control samples. Samples from the last days of the dry remains stage (48 to 97) are nonetheless distinct from the control samples collected on these days. Most control samples can be seen to cluster together on the left-hand side of the plot. Controls samples from days 41, 48 and 62 make up the majority of the control samples that are seen to differentiate from the group. The change in microbial community in control samples overtime is to be expected due to weather variations as spring turned into summer.

ADONIS and perMANOVA results for the Spring 2012 trial are presented in Tables 21 and 22. Soil moisture and ambient temperature both had a significant effect on the dissimilarity between samples based on weighted UniFrac distances. The effect of soil moisture was particularly strong. Temperature had a significant impact on weighted and unweighted data. Differences between groups according to treatment or stage of decomposition were also significant for both weighted and unweighted UniFrac distances. ANOSIM results for the Spring 2012 trial are presented in Tables 23 and 24. Results indicated that groups of samples based on treatment and decomposition stages

were significantly different for both weighted and unweighted data. The differences between control and experimental samples were significant during the active decay, advanced decay and dry remains stages.

Average distributions of OTUs present in control and experimental samples during the Spring 2012 trials are presented in Figure 34. Proportions of Proteobacteria increased in experimental samples from day 11 onwards. An important increase in proportions of Firmicutes and the disappearance of many other less abundant phyla is observed for experimental samples from days 11 and 14. Actinobacteria generally make up larger proportions in control samples overall. The phyla of Verrumicrobia and OP10 indicated similar proportions in control and experimental samples up until the onset of the active stage (day 11). After day 11, proportions of these phyla observed in experimental samples were considerably reduced.

The OTUs which were found to be significantly correlated to soil moisture or soil pH are presented in Table 29. For this trial, correlations with soil moisture produced too many orders showing a significant correlation. Data up to the class level is presented instead. Of all the classes which were correlated with measures of soil moisture only two showed a positive correlation, Firmicutes and Tenericutes. During this experimental trial a spike in soil moisture was observed at days 11 and 14 which corresponded with rain events (see Chapter 3, Figures 9 and 13). A surge in the proportions of Firmicutes and the loss or reduction of many other taxa in experimental samples was observed for these days (Figure 34). Increased moisture did not cause a surge in Firmicutes in control samples on days 11 and 14 suggesting that the change is also brought on by decomposition events. Chloroflexi and Planctomycetes were the major phyla to show correlations with soil pH.

Chloroflexi were positively correlated to pH while Planctomycetes were negatively correlated.

OTUs showing significant differences according to decomposition stage are presented in Table 30. Too many orders and classes were found to be significantly different across the different stage of decomposition. Results at the phylum level are presented instead. Firmicutes and Tenericutes showed and increased average composition during the active and advanced stages. Firmicutes showing a significant increase in experimental soils included different orders of Bacilli and Clostridia. Proteobacteria increased at the active stage and could be found in slightly higher proportions during the advanced decay and dry remains stages in comparison to the fresh and bloat stages. Other OTUs which were significantly different according to decomposition stage were found to decrease as decomposition progressed. Most noteworthy were the decreases in levels of Acidobacteria and Actinobacteria.

Shannon indices of control and experimental samples for the active decay and advanced decay stages are presented in Figure 30c. Alpha-diversity of control and experimental samples were significantly different during the active decay stage ($t = 7.569$, $p < 0.001$). Control and experimental values were not significantly different for the advanced decay stage ($U = 0.000$, $p = 0.100$). There was no significant difference between stages for control samples ($U = 7$, $p = 0.891$) or experimental samples ($U = 5$, $p = 0.556$).

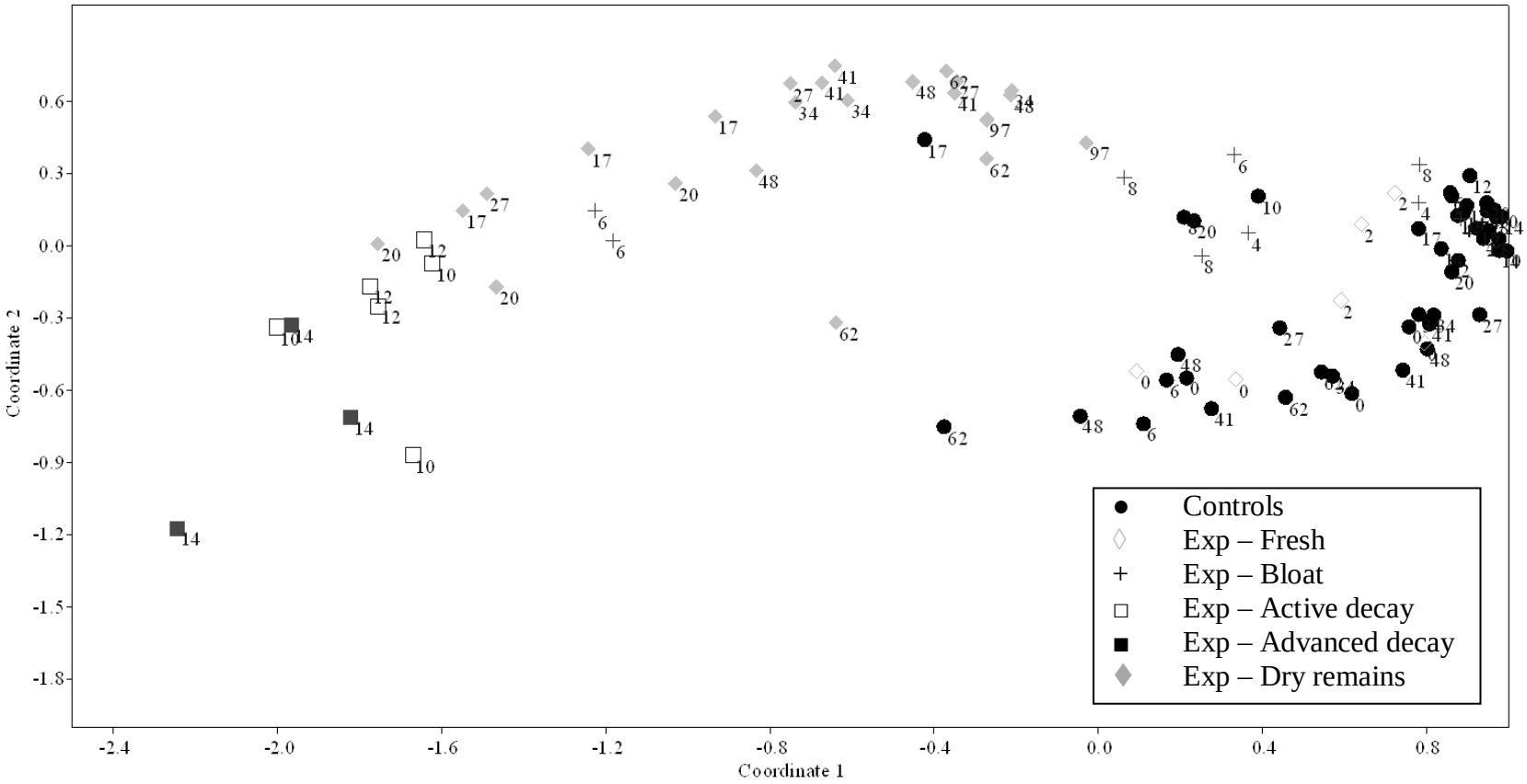


Figure 33. Weighted UniFrac distances of control samples and experimental samples per decomposition stage in Spring 2012 using principal coordinate analysis. Control samples grouped together on the right hand side of the plot indicating limited change in soil community composition over the course of the experiment. Experimental samples from the active and advanced decay stages showed the greatest degree of dissimilarity extending to the left of the plot. Samples collected during the dry remains stage gradually became more similar to those collected during the fresh and bloat stages as well as control samples as the trial progressed.

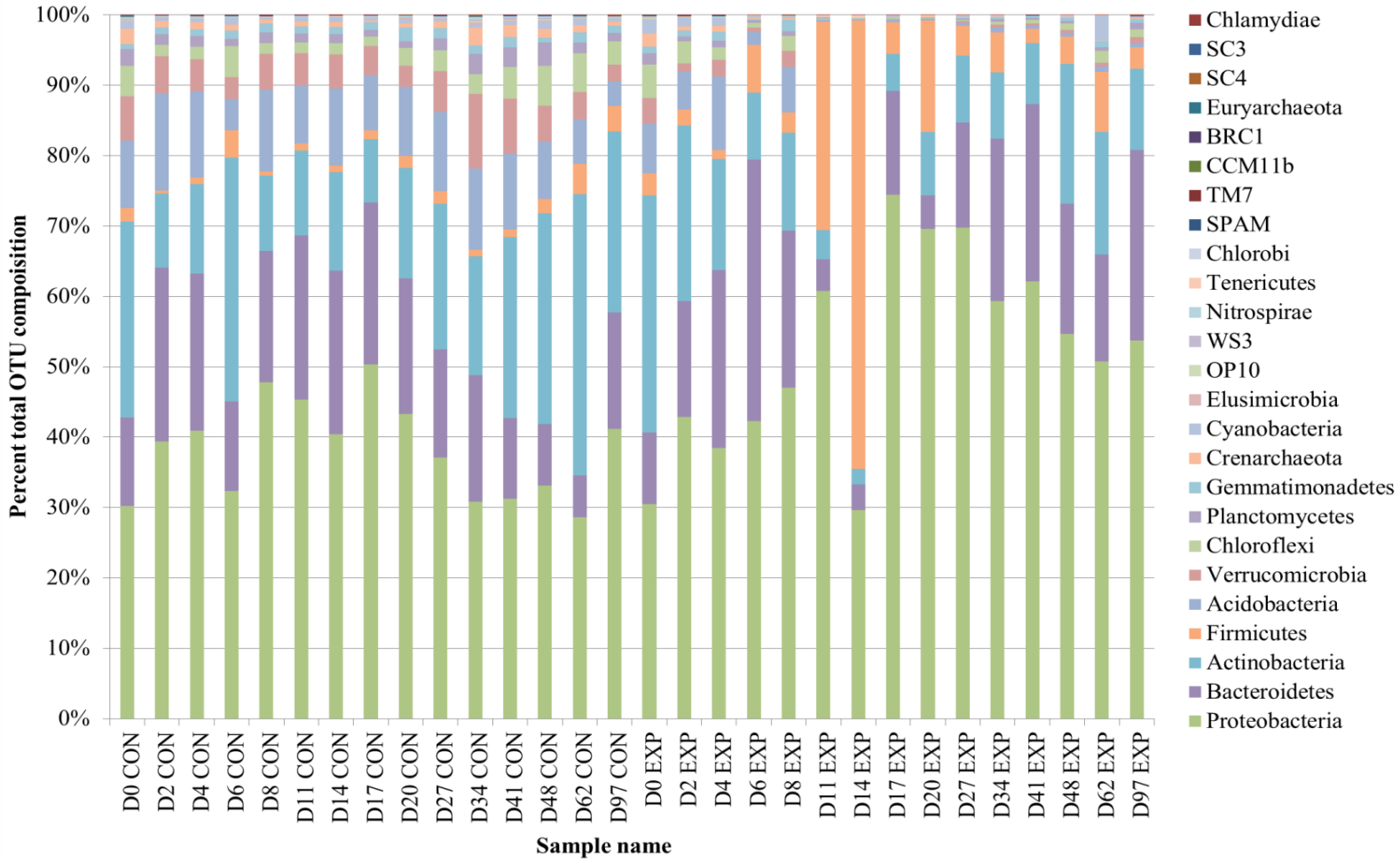


Figure 34. OTU distributions for the top 25 taxa at the phylum level for the Spring 2012 trial. Proportions of Proteobacteria increased in experimental samples from day 11 onwards. Proportions of Firmicutes increased considerably in experimental samples at days 11 and 14. Proportions of Verrumicrobia and OP10 decreased in experimental samples following the onset of active decay. Proteobacteria, Bacteroidetes and Actinobacteria dominated in control samples overall.

Table 29. OTUs showing significant correlations ($p < 0.05$) with soil moisture or soil pH for the Spring 2012 trial

OTU (Phylum; Class)	*R*	*p*
Soil moisture		
Acidobacteria;	-0.215	0.039
Acidobacteria; iii1-8	-0.235	0.024
Acidobacteria; Solibacteres	-0.231	0.026
Actinobacteria; Actinobacteria (class)	-0.363	0.000
CCM11b;	-0.258	0.013
Chlamydiae; Chlamydiae	-0.214	0.040
Chlorobi;	-0.279	0.007
Chloroflexi; Anaerolineae	-0.362	0.000
Chloroflexi; Bljii12	-0.238	0.022
Chloroflexi; Chloroflexi (class)	-0.251	0.015
Chloroflexi; SOGA31	-0.275	0.008
Chloroflexi; Thermomicrobia	-0.393	0.000
Chloroflexi; Thermomicrobia	-0.393	0.000
Crenarchaeota; Thaumarchaeota	-0.310	0.002
Cyanobacteria; mle1-12	-0.232	0.026
Euryarchaeota; Methanobacteria	-0.229	0.027
Euryarchaeota; Methanomicrobia	-0.216	0.037
OP10; 5B-18	-0.275	0.008
OP10; CH21	-0.260	0.012
OP10; S1a-1H	-0.224	0.031
OP10; SJA-22	-0.223	0.032
OP3;	-0.208	0.046
Planctomycetes; Planctomycea	-0.340	0.001
Proteobacteria; Gammaproteobacteria	0.207	0.046
SC4; KD3-113	-0.228	0.028
SPAM;	-0.240	0.02
TM7; TM7-1	-0.245	0.018
Verrucomicrobia; Opitutae	-0.292	0.005
Verrucomicrobia; Verrucomicrobiae	-0.205	0.048
Tenericutes; Erysipelotrichi	0.343	0.001
Firmicutes; Bacilli	0.405	0.000

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Soil pH		
CCM11b;	0.240	0.021
Chloroflexi; Anaerolineae	0.210	0.044
Chloroflexi; Chloroflexi (class)	0.236	0.023
Cyanobacteria;	0.210	0.043
Planctomycetes; agg27	-0.224	0.031
Planctomycetes; FFCH393	-0.258	0.012
Planctomycetes; PW285	-0.216	0.037
Planctomycetes; vadinHA49	-0.255	0.014
WS3; PRR-12	-0.226	0.029

Table 30. OTUs significantly different between controls and experimental samples according to decomposition stage during the Spring 2012 trial ($p < 0.05$ after Bonferroni correction)

		Mean total percent composition					
OTU (Phylum)	***p***	**Control**	**Fresh**	**Bloat**	**Active**	**Adv.**	**Dry**
Acidobacteria	0.000	9.60	6.20	6.20	0.22	0.22	0.52
Bacteroidetes	0.000	18.00	13.00	28.00	4.50	9.20	18.00
CCM11b	0.000	0.03	0.03	0.01	0.00	0.00	0.00
Chloroflexi	0.000	2.90	3.90	1.50	0.17	0.13	0.71
Elusimicrobia	0.000	0.12	0.05	0.02	0.00	0.00	0.00
Firmicutes	0.000	1.60	2.70	3.60	30.00	34.00	6.20
Gemmatimonadetes	0.000	1.20	0.90	1.00	0.06	0.05	0.35
OP10	0.000	0.10	0.15	0.05	0.00	0.00	0.01
Planctomycetes	0.000	1.70	1.10	0.64	0.08	0.04	0.36
Proteobacteria	0.000	38.00	37.00	43.00	61.00	52.00	60.00
SC3	0.000	0.01	0.01	0.00	0.00	0.00	0.00
SPAM	0.000	0.04	0.02	0.01	0.00	0.00	0.01
Tenericutes	0.000	0.01	0.02	0.01	0.29	0.21	0.08
TM7	0.000	0.02	0.05	0.03	0.00	0.00	0.01
Verrucomicrobia	0.000	5.30	2.40	1.80	0.05	0.05	0.39
Actinobacteria	0.001	20.00	29.00	13.00	4.10	3.80	12.00
WS3	0.001	0.12	0.03	0.01	0.00	0.00	0.00

5.3.4 Summer 2012

The relationship between samples based on weighted UniFrac distances is presented on a PCoA plot in Figure 35. Similarly to what was observed during the Spring 2012 trial, the majority of control samples are clustered closely together. Eighteen control samples show differentiation from the remaining control and are distributed randomly across the PCoA plot. These control samples are from various days throughout the experiment. Experimental samples from the fresh stage are clustered with the majority of the control samples. Experimental samples from all subsequent stages (bloat through to dry remains) show no clear clustering according to stage or days. That samples collected on the same day are not closely related suggests that changes to the microbial community composition were somewhat distinct for each decomposition site.

ADONIS and perMANOVA results for the Summer 2012 are presented in Tables 21 and 22. Soil moisture, soil pH and ambient temperature were not found to have a significant effect on sample dissimilarities observed during this experiment for either UniFrac distance matrices. Both treatment and stages of decomposition had a significant impact on weighted and unweighted UniFrac distances. Treatment and stage both had a significant effect on sample dissimilarities for both weighted and unweighted distances ANOSIM results for the Summer 2012 trial are presented in Table 23 and 24. Groups of samples according to treatment or decomposition stage were significantly different based on weighted and unweighted UniFrac distances.

Average distributions of OTUs present in control and experimental samples during the Spring 2012 trials are presented in Figure 36. Experimental samples on day 0 appeared to include a large proportion of Chloroflexi. From day 2 onwards the abundance

of Chloroflexi was considerably reduced while proportions of Firmicutes increased and remained a major constituent of experimental samples thereafter. The increased proportion of Firmicutes in experimental samples coincided with a smaller proportion of Actinobacteria. Actinobacteria made-up a large proportion of the microbial community of control samples throughout the entire experiment. Changes in proportions of Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes appear to drive the differentiation between samples from each day. No clear changes in OTU proportions can be correlated with stages of decomposition.

OTUs indicating a correlation with measures of soil moisture or soil pH are presented in Table 31. Fourteen OTUs showed a significant correlation with soil moisture, 9 of these were inversely correlated. Members of the phyla Proteobacteria were found to correlate positively and negatively to soil moisture. During the Summer 2012 it was noted that soil moisture was fairly constant and small increases in experimental samples were only observed on days 2 and 17 (see Chapter 3 – Figure 13). A distinct change in OTU distributions on these days was not observed. It appears that the slight changes in soil moisture may have affected certain groups of bacteria but that the changes to overall microbial community composition as a result of soil moisture were minor. Soil pH was positively correlated to 5 OTUs and negatively correlated to 4 OTUs. Orders of the Proteobacteria phylum were both positively and negatively correlated to soil pH. Surprisingly, the order RJB25 (Acidobacteria) was positively correlated to pH.

OTUs showing significant differences according to decomposition stage are presented in Table 32. Levels of Nitrosospherales, Myxococcales and Thermomicrobiales were higher in samples collected during the fresh stage but were not

found in comparable levels during any other stage or in control samples. Levels of Enterobacteriales, Pseudomonadales and Xanthomonadales were greatest during the bloat stage and remained higher through the active stage. All three showed a decline during the advanced and dry remains stages. Average composition of other selected OTUs decreased as decomposition progressed.

Shannon indices of control and experimental samples for the active decay and advanced decay stages are presented in Figure 30d. There were no significant differences between control and experimental samples for the active decay stage ($t = 1.839$, $p = 0.080$) or the advanced decay stage ($U = 12$, $p = 0.142$). There was no significant difference between stages for control samples ($t = -0.651$, $p = 0.524$) or experimental samples ($U = 16$, $p = 0.090$).

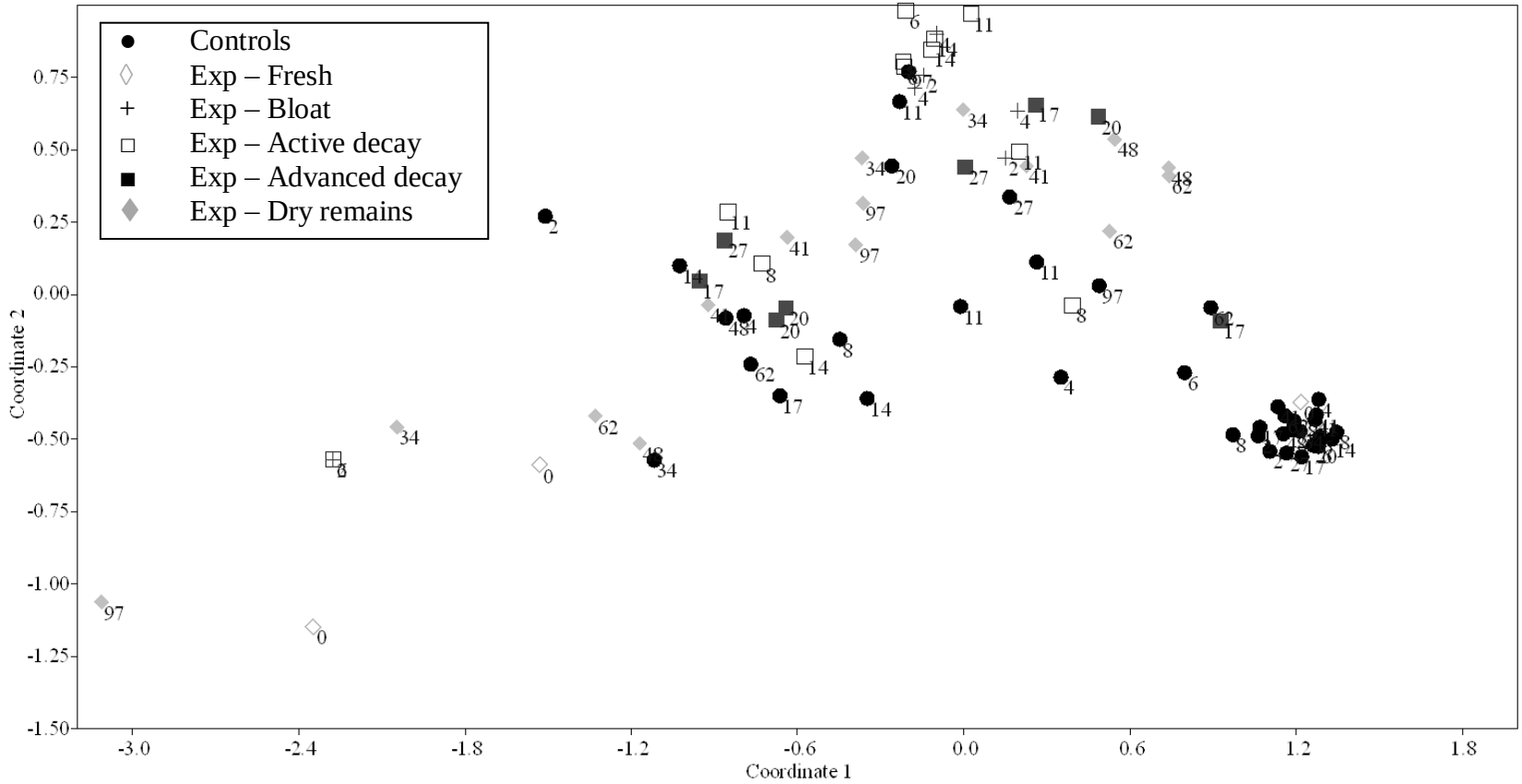

Figure 35. Weighted UniFrac distances of control samples and experimental samples per decomposition stage in Summer 2012 using principal coordinate analysis. The majority of control samples show a good degree of similarity and are grouped on the right hand side of the plot. A limited number of control samples indicated differentiation from the bulk of control samples and are scattered across the plot. Experimental samples from the active decay, advanced decay and dry remains stages show the greatest degree of dissimilarity. It is possible to distinguish between treatments on the days following the onset of active decay.

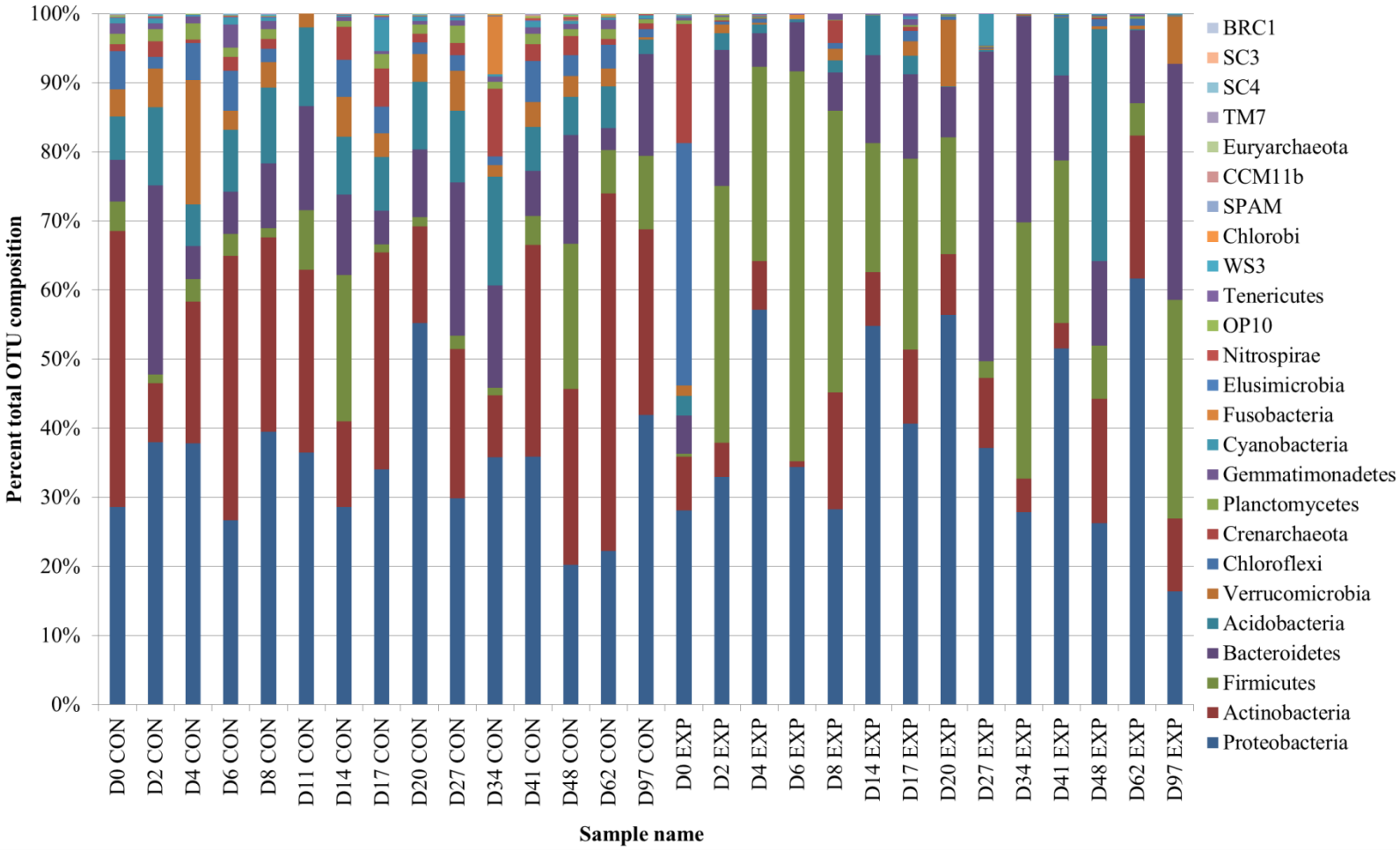

Figure 36. OTU distributions for the top 25 taxa at the phylum level for the Summer 2012 trial. Firmicutes increased and remained a major constituent of experimental samples from day 2. This change coincided with a decrease in proportion of Actinobacteria. Actinobacteria and Proteobacteria dominated microbial communities of control samples throughout.

Table 31. OTUs showing significant correlations ($p < 0.05$) with soil moisture or soil pH for the Summer 2012 trial

OTU (Phylum; Class; Order)	*R*	*p*
Soil moisture		
Chlorobi; SJA-28;	-0.231	0.035
Chloroflexi; Anaerolineae; H39	-0.244	0.026
OP10; SJA-22;	-0.242	0.027
Planctomycetes; agg27; CL500-15	-0.282	0.010
Planctomycetes; Planctomycea; Planctomycetales	-0.220	0.046
Planctomycetes; vadinHA49;	-0.276	0.011
Proteobacteria; Alphaproteobacteria; Rickettsiales	-0.269	0.014
Proteobacteria; Betaproteobacteria; Hydrogenophilales	-0.223	0.042
Proteobacteria; Deltaproteobacteria;	-0.235	0.033
Cyanobacteria; n/a ; Chroococcales	0.291	0.008
Proteobacteria; Alphaproteobacteria;	0.248	0.024
Proteobacteria; Alphaproteobacteria; Caulobacterales	0.223	0.043
Proteobacteria; Deltaproteobacteria; Syntrophobacterales	0.258	0.019
Tenericutes; Erysipelotrichi; Erysipelotrichales	0.282	0.010
Soil pH		
Acidobacteria; RB25;	0.283	0.010
Chlorobi; SJA-28;	0.274	0.012
Proteobacteria; Gammaproteobacteria; Aeromonadales	0.227	0.039
Proteobacteria; Gammaproteobacteria; Alteromonadales	0.296	0.007
WS3; PRR-12;	0.286	0.009
Chloroflexi; Thermomicrobia; HN1-15	-0.252	0.022
Firmicutes; Bacilli; Bacillales	-0.246	0.025
Proteobacteria; Betaproteobacteria; Methylophilales	-0.225	0.041
Proteobacteria; Betaproteobacteria; Nitrosomonadales	-0.229	0.037

Table 32. OTUs significantly different between controls and experimental samples according to decomposition stage during the Summer 2012 trial ($p < 0.05$ after Bonferroni correction)

		Mean total percent composition					
OTU (Phylum; Class; Order)	***p***	**Control**	**Fresh**	**Bloat**	**Active**	**Adv.**	**Dry**
Proteobacteria; Gammaproteobacteria; Enterobacteriales	0.000	0.22	0.18	3.37	0.62	0.00	0.03
Chloroflexi; Thermomicrobia; Thermomicrobiales	0.001	0.08	33.38	0.01	0.00	0.02	0.08
Proteobacteria; Deltaproteobacteria; Myxococcales	0.002	2.09	17.42	0.36	0.05	0.37	0.42
Proteobacteria; Gammaproteobacteria; Pseudomonadales	0.002	0.78	0.01	8.07	6.43	0.49	1.11
Crenarchaeota; Thaumarchaeota; Nitrososphaerales	0.005	1.74	17.25	0.14	0.93	0.23	0.06
Actinobacteria; Actinobacteria; Rubrobacterales	0.008	0.15	0.06	0.01	0.00	0.00	0.01
Chloroflexi; Chloroflexi; Roseiflexales	0.010	0.11	0.05	0.02	0.00	0.00	0.00
Chloroflexi; Anaerolineae; A4b	0.016	0.20	0.18	0.04	0.00	0.02	0.00
Planctomycetes; Planctomycea; Pirellulales	0.019	0.33	0.10	0.09	0.01	0.04	0.03
Proteobacteria; Deltaproteobacteria; Desulfuromonadales	0.020	0.15	0.04	0.02	0.00	0.01	0.01
Chloroflexi; Anaerolineae; Caldilineales	0.025	0.13	0.09	0.03	0.00	0.01	0.02
Proteobacteria; Gammaproteobacteria; Xanthomonadales	0.032	2.71	0.70	12.23	13.40	2.91	2.95
Proteobacteria; Deltaproteobacteria; Entotheonellales	0.040	0.04	0.05	0.01	0.00	0.00	0.00

5.3.4 Overall dataset

The relationship between samples from all four experimental trials based on weighted UniFrac distances is presented on a detrended PCoA plot in Figure 37. The majority of control samples from all trials cluster together. A few controls from each trial show some degree of differentiation from other control samples. These are typically the same control samples which show a degree of dissimilarity to other control samples in PCoA plot for individual experiments. Within the cluster of controls, samples were grouped according to trial with Spring 2011 and Summer 2011 samples overlapping. That controls are grouped together indicates that the microbial communities of soils which were not subject to carcass decomposition were similar across different seasons and between years. There was nonetheless an overall effect of season and year.

ADONIS results for pooled data from all four experiments are presented in Table 33. Soil moisture, soil pH and ambient temperature were not significant in determining either weighted or unweighted UniFrac distances. perMANOVA results for pooled data are presented in Table 34. Treatment, decomposition stage, season and year were all significant in determining weighted and unweighted UniFrac distances of samples from across all four experiment.

Groups of samples for all four experiments were compared according to treatment, decomposition stage, year and season to determine if these factors produced significant differences. Results of the ANOSIM are presented in Table 35. Groupings according to all four categories were all found to be significantly different for both the weighted and unweighted UniFrac matrices.

Control and experimental samples from all four experiments were grouped according to the decomposition stage during which they were collected to compare the different trials within each stage. ANOSIM were run on data from each stage to determine if groupings according to treatment, trial, season or year produced significant differences. Results are presented in Table 36.

During the fresh stage samples were not significantly different when compared according to treatment or season. Groupings according to trial and year were significantly different. This agrees with the relationship between samples observed in the PCoA plot for pooled data (Figure 37). Samples from the bloat stage also indicated no significant difference when grouped according to treatment or season but were significantly different between trials and years.

During the active stage there was a significant difference between the control and experimental samples suggesting that at this stage decomposition began to noticeably affect microbial composition. Active stage samples were also significantly different when grouped according to year but not by trial or season. That significant difference were observed between years but not seasons suggests that community composition may be different from year to year but remains somewhat similar through the spring and summer seasons.

Samples collected during the advanced decay stage and dry remains stage were all significantly different when grouped according to treatment, trial, season and year. The advanced and dry remains stages of each trial included a wide range of sampling dates and varied considerably in length. This may explain why differences between trials are

more pronounced at this stage. Nonetheless, the degree of dissimilarity between experimental samples collected during both active decay and dry remains seen in Figure 37 does suggest that, at these stages, microbial communities varied greatly between samples from a particular trial and across all experiments.

Taxonomic data was analyzed to determine if any OTUs were significantly different between control and experimental samples per decomposition stage. There were no OTUs found to be significantly different between control and experimental samples during the bloat stage. During the active stage, the order of Acidobacteriales were found in significantly greater proportions in control samples than in experimental samples ($p = 0.045$, after Bonferroni correction). The fresh stage and active stage both produced multiple OTUs which were significantly different between control and experimental samples. These OTUs and results of the ANOVAs are presented in Tables 37 and 38. The dry remains stage produced over 40 OTUs at the class level which were significantly different according to treatment groups. Results at the phylum levels are presented instead in Table 39. For all three stages with multiple OTUs showing significant difference according to treatment it was noted that all OTUs were present in greater proportions in control samples than in experimental samples. For the advanced decay and dry remains stage this indicates that decomposition may have reduced the abundance of many bacterial groups in experimental samples.

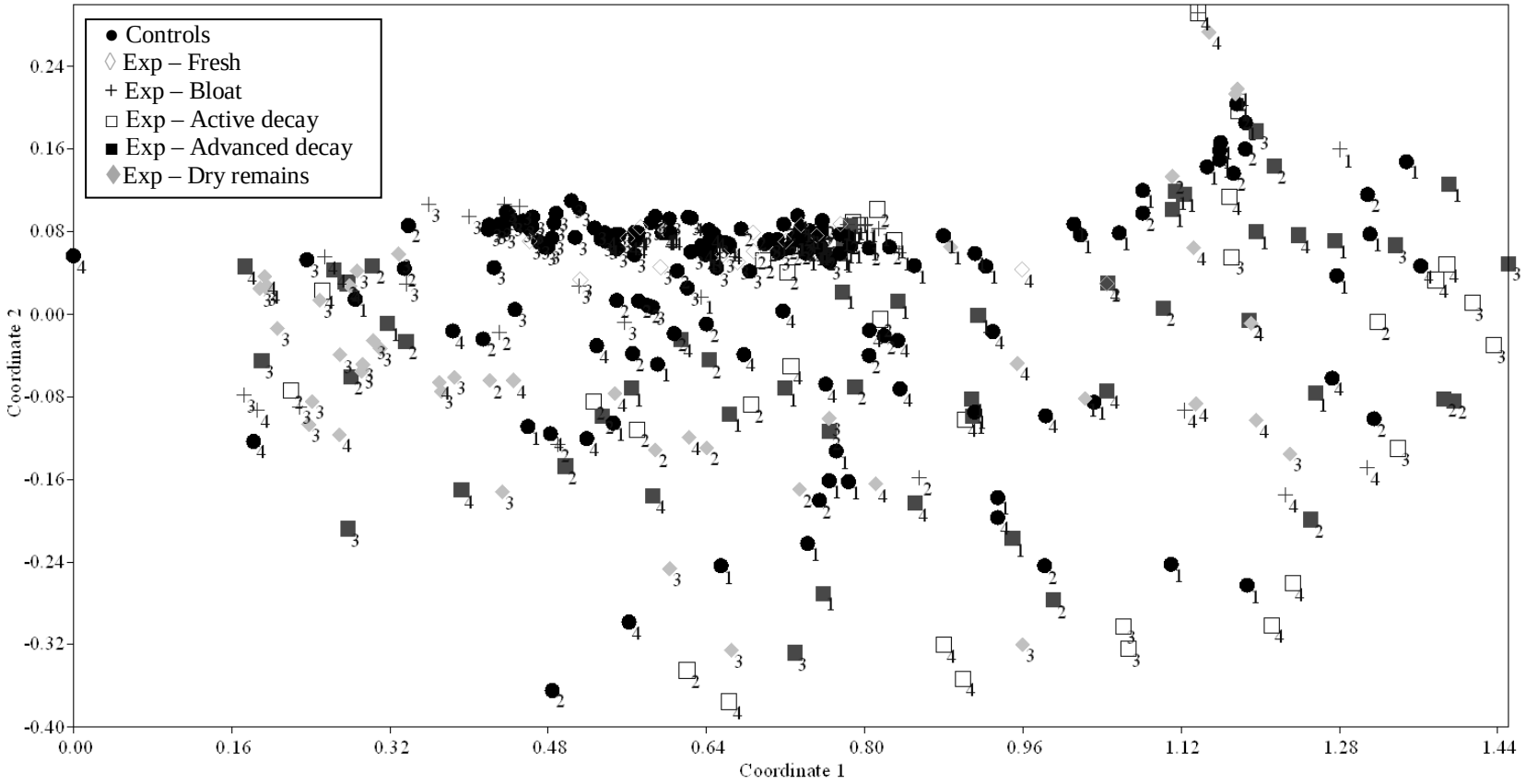

Figure 37. Weighted UniFrac distances of control samples and experimental samples per decomposition stage using principal coordinate analysis for the Spring 2011 (1), Summer 2011 (2), Spring 2012 (3) and Summer 2012 (4) trials. Samples are labeled according to experimental trial. Controls across all experimental trials show a degree of similarity though the distinction between trials remains possible. Samples from the active decay stages showed the greatest change in community composition and are seen to disperse to the extremities of the plot.

Table 33. ADONIS results for soil moisture, soil pH and daily average temperature on weighted and unweighted UniFrac distances of pooled samples from the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 trials. Significant effects ($p < 0.05$) are highlighted in bold.

	Weighted		Unweighted	
	R^2	*p*	R^2	*p*
Soil moist.	0.763	0.650	0.636	0.080
Soil pH	0.005	0.081	0.003	0.310
Temperature	0.003	0.502	0.003	0.202

Table 34. perMANOVA results for decomposition stage, year and season on weighted and unweighted UniFrac distances of pooled samples from the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 trials. Significant effects ($p < 0.05$) are highlighted in bold.

	Weighted		Unweighted	
	pseudo-F	*p*	*pseudo-F*	*p*
Treatment	**8.654**	**0.001**	**7.293**	**0.001**
Stage	**5.772**	**0.001**	**5.028**	**0.001**
Year	**12.37**	**0.001**	**7.368**	**0.001**
Season	**2.732**	**0.001**	**6.415**	**0.001**

Table 35. ANOSIM results determining significant difference between groups of samples based on treatment, decomposition stage, year and season for pooled samples the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 trials based on weighted or unweighted UniFrac distances. Significant effects ($p < 0.05$) are highlighted in bold.

	Weighted		Unweighted	
	R	***p***	***R***	***p***
Treatment	**0.104**	**0.001**	**0.041**	**0.001**
Stage	**0.2068**	**0.001**	**0.146**	**0.001**
Year	**0.1576**	**0.001**	**0.054**	**0.001**
Season	**0.0562**	**0.001**	**0.054**	**0.001**

Table 36. Weighted ANOSIM results determining significant differences between samples grouped according to treatment, trial, year and season per decomposition stage for pooled samples from the Spring 2011, Summer 2011, Spring 2012 and Summer 2012 experiments. Significant differences ($p < 0.05$) are highlighted in bold.

	Fresh		Bloat		Active		Advanced		Dry remains	
	R	***p***	***R***	***p***	***R***	***P***	***R***	***p***	***R***	***p***
Treatment	0.034	0.299	0.034	0.078	**0.078**	**0.007**	**0.123**	**0.001**	**0.191**	**0.001**
Trial	**0.492**	**0.001**	**0.425**	**0.001**	0.035	0.160	**0.092**	**0.003**	**0.339**	**0.001**
Season	0.164	0.118	0.038	0.138	-0.039	0.768	**0.045**	**0.005**	**0.123**	**0.003**
Year	**0.372**	**0.001**	**0.395**	**0.001**	**0.063**	**0.020**	**0.138**	**0.015**	**0.327**	**0.002**

Table 37. Summary of OTUs found to be significantly different between control and experimental samples collected during the fresh stages of all four experimental trials. Average total compositions of the OTUs are given for control and experimental samples.

OTU (Phylum; Class; Order)	*p*	Mean Control	Mean Experimental
Actinobacteria; Actinobacteria; Solirubrobacterales	0.004	7.63	1.12
Acidobacteria; Acidobacteria; Acidobacteriales	0.002	2.23	0.31
Chloroflexi; SOGA31;	0.000	1.57	0.18
Proteobacteria; Deltaproteobacteria; Myxococcales	0.001	1.56	0.4
Acidobacteria; Chloracidobacteria;	0.012	1.32	0.11
Actinobacteria; Actinobacteria; 0319-7L14	0.000	0.51	0.04
Proteobacteria; Betaproteobacteria; Rhodocyclales	0.017	0.50	0.09
Planctomycetes; Phycisphaerae;	0.013	0.31	0.08
Verrucomicrobia; Opitutae;	0.034	0.27	0.03
Actinobacteria; Actinobacteria	0.008	0.22	0.02
Actinobacteria; Actinobacter; Rubrobacterales	0.002	0.18	0.02
Chloroflexi; TK17;	0.045	0.17	0.01
Acidobacteria; Sva0725;	0.013	0.16	0.01
Chloroflexi; Anaerolineae; Caldilineales	0.000	0.09	0.00
Chloroflexi; Chloroflexi; Roseiflexales	0.001	0.09	0.00
Proteobacteria; Deltaproteobacteria; Desulfuromonadales	0.006	0.06	0.00
Proteobacteria; Deltaproteobacteria; Entotheonellales	0.010	0.04	0.00
Actinobacteria; Actinobacteria; Euzebiales	0.007	0.03	0.00
Proteobacteria; Deltaproteobacteria;	0.013	0.02	0.00
WS3; PRR-12;	0.015	0.02	0.00
Proteobacteria; Betaproteobacteria; Methylophilales	0.025	0.02	0.00

Table 38. Summary of OTUs found to be significantly different between control and experimental samples collected during the advanced decay stages of all four experimental trials. Average total compositions of the OTUs are given for control and experimental samples.

		Mean	
OTU (Phylum; Class; Order)	***p***	**Control**	**Experimental**
Actinobacteria; Actinobacteria; Solirubrobacterales	0.004	7.63	1.12
Acidobacteria; Acidobacteria; Acidobacteriales	0.002	2.23	0.31
Chloroflexi; SOGA31;	0.000	1.57	0.18
Proteobacteria; Deltaproteobacteria; Myxococcales	0.001	1.56	0.40
Acidobacteria; Chloracidobacteria;	0.012	1.32	0.11
Crenarchaeota; Thaumarchaeota; Nitrososphaerales	0.001	1.09	0.12
Actinobacteria; Actinobacteria; 0319-7L14	0.000	0.51	0.04
Proteobacteria; Betaproteobacteria; Rhodocyclales	0.017	0.50	0.09
Planctomycetes; Phycisphaerae;	0.013	0.31	0.08
Verrucomicrobia; Opitutae;	0.034	0.27	0.03
Actinobacteria; Actinobacteria;	0.008	0.22	0.02
Actinobacteria; Actinobacteria; Rubrobacterales	0.002	0.18	0.02
Chloroflexi; TK17;	0.045	0.17	0.01
Acidobacteria; Sva0725;	0.013	0.16	0.01
Chloroflexi; Chloroflexi; Roseiflexales	0.001	0.09	0.00
Chloroflexi; Anaerolineae; Caldilineales	0.000	0.09	0.00
Proteobacteria; Deltaproteobacteria; Desulfuromonadales	0.006	0.06	0.00
Proteobacteria; Deltaproteobacteria; Entotheonellales	0.010	0.04	0.00
Actinobacteria; Actinobacteria; Euzebiales	0.007	0.03	0.00
Proteobacteria; Deltaproteobacteria;	0.013	0.02	0.00
WS3; PRR-12;	0.015	0.02	0.00
Proteobacteria; Betaproteobacteria; Methylophilales	0.025	0.02	0.00

Table 39. Summary of OTUs found to be significantly different between control and experimental samples collected during the dry remains stages of all four experimental trials. Average total compositions of the OTUs are given for control and experimental samples.

		Mean	
OTU (Phylum)	***p***	**Control**	**Experimental**
Verrucomicrobia	0.001	4.05	0.95
Chloroflexi	0.000	3.37	0.64
Crenarchaeota	0.002	1.82	0.08
Planctomycetes	0.000	1.57	0.40
Gemmatimonadetes	0.000	1.04	0.22
Nitrospirae	0.000	0.14	0.01
Elusimicrobia	0.000	0.14	0.01
OP10	0.000	0.13	0.01
WS3	0.000	0.09	0.00
Chlorobi	0.001	0.07	0.00
CCM11b	0.000	0.03	0.00
Euryarchaeota	0.000	0.02	0.00
TM7	0.007	0.02	0.00
SC3	0.000	0.01	0.00
Chlamydiae	0.015	0.01	0.00

5.4 Discussion

Though fluctuations in soil pH were observed during each trial, soil pH was not found to have a significant effect on soil microbial community composition in any of the trials. The increase in soil pH which has been reported to occur with cadaver decomposition (Carter et al., 2010) was not observed during these experimental trials. Instead a drop in pH was typically observed during the bloat and early days of the active stage. This did not appear to produce a noteworthy shift within the microbial community.

With the exception of the slight acidification of soil at the beginning of each experiment soil pH of samples collected during each trial fluctuated only slightly within a range of soil pH which did not exceed 1 pH unit. Though studies using similar soil microbial community profiling methods suggest that community composition is closely defined by soil pH (Lauber et al., 2009; Rousk et al., 2010; Andrew et al., 2012), the correlation between community composition and soil pH is typically observed across multiple pH units.

Only a few OTUs were found to be correlated with soil pH during each individual trial. These included multiple Acidobacteria, Bacteroidetes, Planctomycetes, Chloroflexi and Proteobacteria. Acidobacteria were negatively correlated to soil pH which is to be expected as their presence in soil is known to be inversely proportional to soil pH (Lauber et al., 2009). Planctomycetes, which are often reported in acidic soils (Dedych and Kulichevskaya, 2013), were also negatively correlated to soil pH. The different Proteobacteria correlated both negatively and positively with soil pH. This phylum is extremely diverse and includes many different species each adapted to their own range of optimal pH.

Soil moisture was identified as a factor that had an overall effect on soil microbial activity and community FAME profiles in the previous chapters. Based on these observations it was expected that soil moisture would be associated with changes in microbial community composition detectable using NGS. Soil moisture was only found to have an effect on overall dissimilarities observed between samples collected during the Spring 2012 trial. During this trial a peak in soil moisture was associated with a shift in microbial community composition. Studies using next-generation sequencing to

characterize soil microbial communities have produced conflicting results concerning the potential effects of soil moisture. In their study of soil bacterial communities involved in decomposition process in the rainforest Leff et al. (2012) found that soil moisture had no effect on microbial community composition. Conversely various other ecological studies have reported a link between soil moisture and microbial community profiles (Shen et al., 2012; Lauber et al., 2013). The response of any soil microbial community is clearly complex requiring that a large number of variables, such as soil history (Evans and Wallenstein, 2012) and vegetation profiles (Mitchell et al., 2010) be taken into consideration. Soil moisture could potentially produce similar effects to decomposition requiring that precipitation data be taken into account when attempting to use soil microbial communities to establish timelines.

Though soil moisture was not a major driving force behind overall changes in soil microbial diversity a large number of OTUs were nonetheless found to be correlated with soil moisture within each experiment. Most of these bacterial groups were negatively correlated to soil moisture with their numbers decreasing when soil moisture peaked. Soil moisture was also negatively correlated to alpha-diversity during the Summer 2011 and Spring 2012 trials. Increases in soil moisture may have rendered the soil environment temporarily anaerobic thus favoring facultative anaerobes and anaerobes for a short period of time.

Temperature was found to influence weighted UniFrac distances for samples from the Summer 2011 trial indicating an effect on overall taxa abundance. Temperature was also correlated with community similarity during the Spring 2012 based on both weighted and unweighted distances. Lauber et al. (2013) also found that temperature was correlated

to community similarities and showed that the effects were dependent of soil land-use type. This is logical when taking into account that microbial growth is known to be influenced by temperature. Overall changes in temperature during the Summer 2011 and Spring 2012 trials did not differ from trends observed for the other corresponding trials according to season (see Chapter 3 – Figure 9). That temperature influenced microbial communities during two trials but did not have an effect during the other two may be indicative of a more complex effect combining factors such as precipitation, evaporation rates and vegetation growth.

OTU distributions and measures of alpha-diversity both indicated that decomposition produces a shift in the taxa which are present in experimental samples favoring a few bacteria associated with decomposition and reducing the abundance and diversity of native soil microbes. A similar displacement of the indigenous soil community by decomposition associated microbes was observed by Parkinson (2009). Across all four trials it was possible to observe a steady decrease in proportions of members of the Actinobacteria and Chloroflexi phyla within experimental samples. Alpha and Deltaproteobacteria were mostly negatively affected by decomposition. Archaea from the Crenarchaeota phylum (class Thaumarchaeota) were most abundant in soils prior to decomposition, decreasing gradually in abundance as decomposition progressed. This particular change was surprising as decomposition is known to release ammonia-rich fluids (Meyer et al., 2013) and Thaumarchaeota are ammonia-oxidizers (Treusch et al., 2005). Thaumarchaeota are only able to oxidize ammonia aerobically (Konneke et al., 2005) suggesting decomposition may have rendered the environment temporarily anaerobic.

Gammaproteobacteria were found in greater proportions in experimental samples and included Enterobacteriales, Pseudomonadales and Xanthomonadales. One group of Alphaproteobacteria, Caulobacterales, also increased in experimental soil during the Summer 2011 trial. Metcalf et al., (2013) also reported an increase in Gammaproteobacteria and Caulobacterales in soils associated with mouse carcass decomposition. Firmicutes were more abundant in experimental soils in many of the trials. Firmicutes which increased in experimental soils during the active and advanced stage were made-up of the orders Lactobacillales, Bacillales and Clostridiales. Lactobacilli and Bacilli which are common members of the human gut have been reported as bacteria involved decomposition process (Janaway et al., 2009). The surge in these Gram-positive obligate anaerobes and facultative anaerobes is consistent with reports that putrefactive processes are mostly anaerobic (Janaway et al., 2009). Both Lactobacilli and Clostridiae were shown to increase in proportions throughout decomposition in samples collected from swine carcasses (Pechal et al., 2013). The significant increase in Clostridiae, which originate mainly from the soil (Wells and Wilkins, 1996), may also indicate a change within the soil environment to more anaerobic conditions, possibly as a result of rain. It should be noted that an increase in Firmicutes was observed in control samples from the Spring 2011 trial and is believed to have been brought on by rainfall, confirming the potential of weather to influence microbial profiles and create false positives.

Overall, both 2011 trials suggested that there was no significant difference between control and experimental microbial profiles. The greater dissimilarity observed for both the control and experimental samples during these trials may have masked any

clear effects produced by decomposition. As both control samples and experimental samples exhibited changes over the course of the experiments it is believed that environmental variables may have had a strong influence on soil microbial community composition. Although the difference between control and experimental samples was not significant overall, groupings of samples according to decomposition stages were significantly different. This may have resulted from a combination of changes induced by both decomposition and changes in weather due to transitions between seasons over the course of the experiments. Soil moisture, soil pH and temperature were not found to have a significant effect on sample distances for these trials. Only accounting for variables such as soil moisture, soil pH and temperature individually may not offer a good explanation of soil community dissimilarities. The co-variability of such abiotic factors makes their effect on soil microbiology difficult to interpret. It appears that for both of these trials environmental factors may have masked the full effects of decomposition on soil microbial community composition. Changes in soil microbial community overtime may also be correlated to changes in vegetation which will inevitably be affected by weather conditions and seasons (Shanmugam et al., 2012, Jangid, et al., 2013).

Results from both 2012 trials indicated that sample profiles were significantly different overall according to treatment. During both of these trials there was a significant difference between treatments for the active decay, advanced decay and dry remains stages. Based on previous studies investigating gravesoil microbial communities, differentiation between control soils and gravesoils should be possible once the active stage is reached (Parkinson et al., 2009; Metcalf et al., 2013). It is unclear why the distinction between control and experimental samples during both 2012 trials would be

more apparent than what was observed during the 2011 trials. The degree of change observed within control soil microbial communities overtime appears to have bearing on the potential of discerning the changes brought on by decomposition in experimental samples.

The analysis of samples from all trials indicated that the majority of control samples collected across all trials shared a similar community composition. Though control samples grouped closely according to UniFrac distances it remained possible to distinguish between samples taken from different experimental trials. Samples grouped according to seasons or year were significantly different indicating that microbial community composition differed according to both factors. The variability of soil microbial communities according to season and year has already been reported (Lipson, 2007; DeBruyn et al., 2011) and is believed to differ according to soil type (Lauber et al., 2013). In Canada, overall differences in microbial community between years may be in part due to the freeze-thaw cycles observed during the year. The rate at which soils thaw in the spring can have damaging effects on microbial biomass (Schimel et al., 2007) which may alter community development in subsequent months.

Though overall trends for temperature and precipitations were similar for both spring and summer seasons across 2011 and 2012 each experiment was nonetheless characterized by short periods of either rain, drought, below average and above average temperature. These slight differences in weather conditions were shown to influence decomposition rates and appear to have affected microbial community composition over the course of each trial. The variations in weather conditions may also explain why each

trial was seen to produce distinct changes in community profiles when data is compared between trials.

Shade et al. (2013) documented the temporal changes in microbial community composition of different soils from Hawaii and Florida. They found that the patterns in microbial dynamics were often predictable. They emphasized how understanding these patterns are necessary to determine when a community is experiencing a disturbance and assessing how quickly it will be able to recover. Such information would also be essential in order to accurately interpret microbial timelines within forensic investigations. Both locations used in the study by Shade et al. fell within climatic areas where yearly average temperature ranges are quite small: 26°C to 32° C for Hawaii and 14°C to 27°C for Florida (Mayda, 2012). Climates of these sampling locations may be more suitable for recording changes in soil microbial community composition over periods of a few months. Based on data presented in this study achieving the same exploit in a region with distinct seasons and greater annual variations in temperatures may prove to be a challenging undertaking.

5.5. Conclusion

Results from all four experiments suggest that decomposition does cause microbial community composition to shift with the onset of active decay or early advanced decay. Distinguishing gravesoils from control soils based on microbial community profiles remains a difficult task due to the normal variability in microbial community composition observed in the absence of decomposition. The use of soil microbial profiles as PMI indicators in forensic investigations would require

environmental variables to be factored-in as well as a good knowledge of soil microbial dynamics for the area of interest.

The influence of environmental parameters on microbial communities differed between trials and specific effects remain unclear. It is believed that weather conditions may have masked or diminished the effects of decomposition on soil communities emphasizing the importance of considering environmental data when interpreting soil microbial dynamics. The shifts in microbial community composition which could be used as indicators in forensic taphonomy occurred at slightly different times during each experiment. This may also have been influenced by weather conditions. Rain and larval activity may both influence the rate at which microorganisms from a carcass or cadaver enter the soil environment thus changing the timeline which can be established from gravesoil microbial profiles.

The study presented here included a total of four trials conducted during two seasons and two years which produced varied results. Continuing similar experimental trials over a prolonged period of time at various time points during the year may help determine the true impact of seasonal changes on microbial communities as well as the decomposition process. As data presented here may only be valuable for forensic investigations undertaken within the same geographical region, obtaining microbial profiles from decomposition in different areas would be necessary for the widespread use of soil microbial profiles in forensic investigations.

Based on the changes observed within experimental soil profiles and similar published studies it appears that there may be a few groups of bacteria which could be

appropriate as indicators of decomposition. These included Gammaproteobacteria, Alphaproteobacteria and various Firmicutes. By accumulating further data from similar decomposition studies it will be possible to determine the potential of these indicator species at accurately estimating PMI.

CHAPTER 6
Conclusions and future considerations

6.1 Conclusions

In temperate climates such as in Southern Ontario, four seasons are typically observed over the course of a calendar year. Each season is characterized by a distinct range of temperatures and precipitation patterns which can alter microbial community composition. With global climate change these trends are liable to change over the course of the next few decades. Although environmental data from this study did indicate that temperatures gradually increased as seasons changed from spring to summer and gradually decreased as summer changed to fall, both years were also characterized by periods of extreme weather. The summer of 2011 was marked by a period of extreme heat during the month of July. Above average temperatures were also observed during the 2012 experimental period. Both years there were characterized by numerous thunderstorms while overall precipitations were sporadic.

Rates of decomposition differed between each experimental trial as a result of variable weather trends. Temperatures and precipitation had a clear impact on larval activity, microbial activity and the wetting and drying of tissues. The different rates of decomposition are believed to have an impact on the microbial responses between trials. Increased liquefaction rates or rainfall will facilitate the introduction of leachate into the soil while limited insect activity and periods of drought will slow down the interactions between decomposition products and the soil environment.

Different weather conditions can also alter microbial community composition both in the absence and presence of carrion. To truly consider the using timelines established from soil microbial community profiles within forensic investigations it is necessary to characterize the normal changes in microbial community composition in response to weather and seasonality. This task may become more difficult over the upcoming years as weather trends become more and more varied between years. Transitions between seasons are becoming blurred with above and below normal temperatures, unexpected precipitations and storms becoming more and more common. Changes in temperature and precipitation trends over the years will need to be taken into account in future studies as these may have significant effects on microbial community dynamics.

One objective of this study was to document changes in soil moisture and soil pH throughout the decomposition process and the influence these factors may have on soil microbial communities. Soil moisture influenced both microbial activity and community composition. Precipitation and evaporation may both affect soil moisture reiterating the importance of accounting for climatic conditions when studying soil microbes during the decomposition process. The effect of soil pH on microbial activity and community profiles was not as evident as that of soil moisture. This may reflect the ability of microbial communities at this location to adapt to various changes in soil pH throughout the year following precipitations and vegetation life cycles. It has often been reported that decomposition causes soil pH to increase following the purging of decomposition fluids (Carter et al., 2008) yet this was not observed during the current study. The initial alkaline pH and buffering capacity of soil at the decomposition facility is believed to play

a role in the pH variability observed during decomposition and the effects these changes may have on soil microbiology. The effect of soil moisture and soil pH on microbial communities are likely to be specific to soil type and decomposition studies should be carried out in a variety of soils in different geographical areas to fully assess how these variables impact soil microbial dynamics during decomposition.

Both FAME and metagenomic profiles were able to show changes within soil microbial communities as a result of decomposition processes. The onset of active decay or advanced decay was typically associated with the first noticeable shifts in soil microbial profiles. Experimental samples collected during both these stages demonstrated the greatest degree of separation from control samples. Once the carcasses reached the dry remains stage experimental samples often showed minimal variation to control samples suggesting the effects of decomposition were attenuated overtime.

FAME profiles may prove useful as a preliminary analysis to confirm the presence of a transit grave or decomposition site. Marker fatty acids such as 3OH 12:0, 12:0, 16:0 and 18:0 were repeatedly identified in experimental samples and may prove valuable in establishing PMIs based on their arrival and persistence within gravesoils. Metagenomic profiles were able to provide specific information concerning the microorganisms associated with the shifts in soil microbial community composition resulting from decomposition. Bacteria commonly associated with putrefactive processes were frequently identified in experimental samples indicating these microbes may prove useful as gravesoil indicators. The dynamics between the microbial population originating from carcasses and native soil microorganisms were similar across all

experiments. With continued research it may be possible to define typical changes in soil microbiology overtime and correlate these to post-mortem intervals.

6.2 Future considerations

One of the major issues identified from this study was the influence that climatic conditions and can have on microbial community activity and composition. Thus there is a need for a better understanding of these changes before microbial profiling can be used in forensic investigations. Vegetation may also prove to be an important factor in determining soil microbial profiles within the first soil horizon. Microbial communities present in sub-surface horizons will not be directly influenced by changes in aboveground weather and vegetation. Microbial diversity has also been shown to decrease considerably as depth increases (Eilers et al., 2012). Changes in microbial communities during belowground decomposition may prove easier to monitor than on the soil surface. Profiling microbial communities of cadavers and gravesoils during interment will confirm the potential of using microbial profiles to establish PMI under burial conditions.

Experiments conducted during this study utilized pig carcasses as human cadaver analogues. Pig carcasses provide an adequate replacement to humans and allow for replicates to be easily obtained. The use of human cadavers in similar studies would nonetheless prove beneficial to provide data more comparable to death scenes observed during forensic investigations. Different body compositions, causes of death and state of health at time of death may all affect the decomposition microbiology and should be studied to allow for microbial data to be correctly interpreted.

Clothing or coverings were not placed on carrion during the experiments presented here yet homicide victims are often found clothed, wrapped or covered with various debris. The presence of body coverings is known to affect rates of decomposition (Campobasso et al., 2001) which can influence the rate at which leachate from cadavers enters the surrounding soil environment. Attempts at concealing cadavers using plant litter, branches or vegetation may also facilitate the colonization of carrion by environmental bacteria and fungi. As these different conditions can influence microbial profiles obtained from cadavers or gravesoils they should investigated in future studies.

Larval colonization of carrion had a significant effect on observed rates of decomposition for each experimental trial. The presence of maggots on carcasses was also believed to influence the microbial content of the carcasses. It has been shown that the exclusion of insects will considerably slow down decomposition (Simmons et al., 2010a). Forensic investigations from across Canada have also indicated that insects may rarely play a role in cadaver decomposition across the country (Cockle, 2012). Obtaining microbial profiles from carrion and gravesoils in studies where insects are excluded from the bodies may provide novel information more relevant to certain death scenes.

Finally, experiments conducted as part of this study were only carried out using one soil type within one region. Findings from this research may only be applicable to similar soil types and climates. Similar experiments to those conducted in this study should be carried out across multiple soil profiles and geographic regions. This will provide forensic scientists with a catalogue of typical decomposition rates and microbial responses for various locations allowing for the increased use of microbial analyses within forensic taphonomy.

CHAPTER 7
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CHAPTER 8
Appendices

APPENDIX A
Supplementary Tables - Microcosm studies

Table 40. Statistical summary table of Student's t-tests or Mann-Whitney rank sum test (*) on soil microbial activity measures for control microcosms and experimental microcosms at 5°C and 20°C. Significant differences ($p < 0.05$) are highlighted in bold.

	5°C		20°C	
	t	***p***	***t***	***p***
Week 1	1.362	0.245	8.00*	4.00
Week 2	-1.738	0.157	-1.570	0.191
Week 3	**-4.714**	**0.009**	-1.118	0.326
Week 4	**-4.320**	**0.012**	**-11.094**	**< 0.001**
Week 5	**4.047**	**0.016**	**.4-064**	**0.015**
Week 6	-2.467	0.069	**-9.313**	**< 0.001**
Week 7	**-5.942**	**0.004**	**-3.930**	**0.017**
Week 8	-1.090	0.337	6.00*	0.100

Table 41. Summary of t-tests or Mann-Whitney rank sum tests (*) on microbial activity measures for control microcosms and experimental microcosms at 20%, 40%, 60% and 80% WHC. Significant differences ($p < 0.05$) are highlighted in bold.

	20%		40%		60%		80%	
	t	***p***	***t***	***p***	***t***	***p***	***T***	***p***
Week 1	**-2.981**	**0.041**	**5.869**	**0.004**	2.245	0.088	**4.942**	**0.008**
Week 2	**4.083**	**0.015**	**-4.674**	**0.009**	1.201	0.296	**3.150**	**0.034**
Week 3	-1.243	0.282	**5.390**	**0.006**	0.307	0.774	-2.607	0.059
Week 4	-1.167	0.308	-2.525	0.065	-0.155	0.884	2.553	0.063
Week 5	**6.925**	**0.002**	13.0*	0.400	0.676	0.536	**3.213**	**0.033**
Week 6	**5.936**	**0.004**	1.412	0.231	2.015	0.114	-0.737	0.502
Week 7	**7.168**	**0.002**	2.281	0.0847	**3.255**	**0.031**	1.212	0.292
Week 8	-1.828	0.142	-1.645	0.175	0.994	0.377	2.209	0.092

Table 42. Summary of t-tests on microbial activity measures for experimental controls presenting substantial fungal growths versus none to little fungal growths for both microcosms experiments. Significant differences ($p < 0.05$) were not observed.

	t	***p***
5°C	-1.948	0.123
20°C	1.025	0.332
20% WHC	2.981	0.206
40% WHC	0.906	0.391
60% WHC	-0.921	0.292
80% WHC	1.390	0.202

APPENDIX B
Supplementary Tables and Figures - FAME community profiles

PCA PLOTS FOR INDIVIDUAL DAYS OF THE ADVANCED DECAY STAGE-SPRING 2011

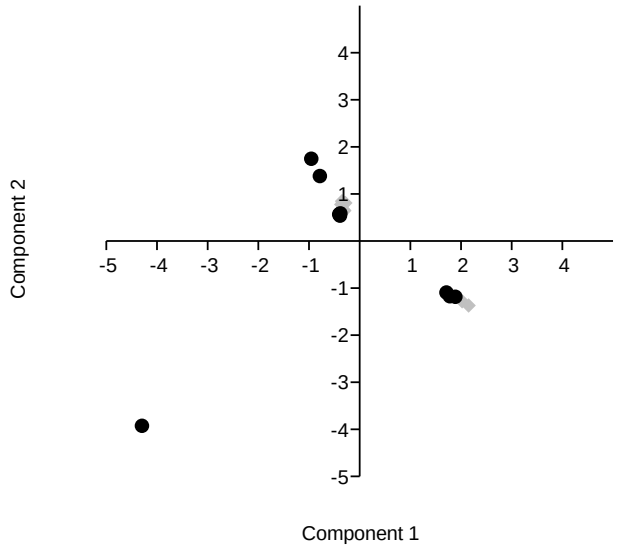

Figure 39. PCA of control (♦) and experimental (●) samples based on FAM E profiles for day 11 of the Spring 2011 trial. PC1 explains 40% of the variation; PC2 33% of the variation.

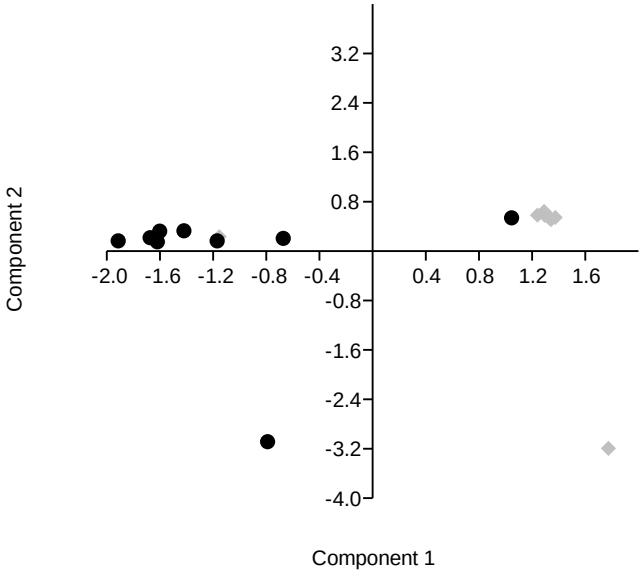

Figure 38. PCA of control (♦) and experimental (●) samples based on FAM E profiles for day 14 of the Spring 2011 trial. PC1 explains 48% of the variation; PC2 explains 32% of the variation.

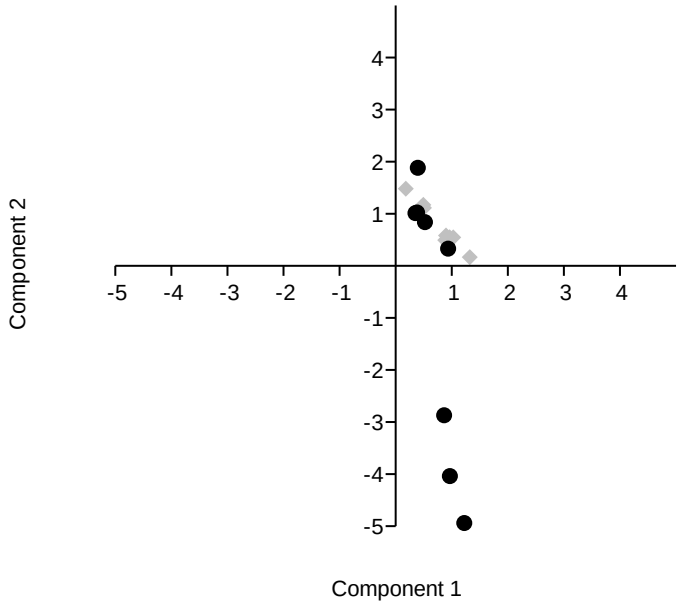

Figure 41. PCA of control (♦) and experimental (●) samples based on FAM E profiles for day 17 of the Spring 2011 trial. PC1 explains 51% of the variation; PC2 explains 19% of the variation.

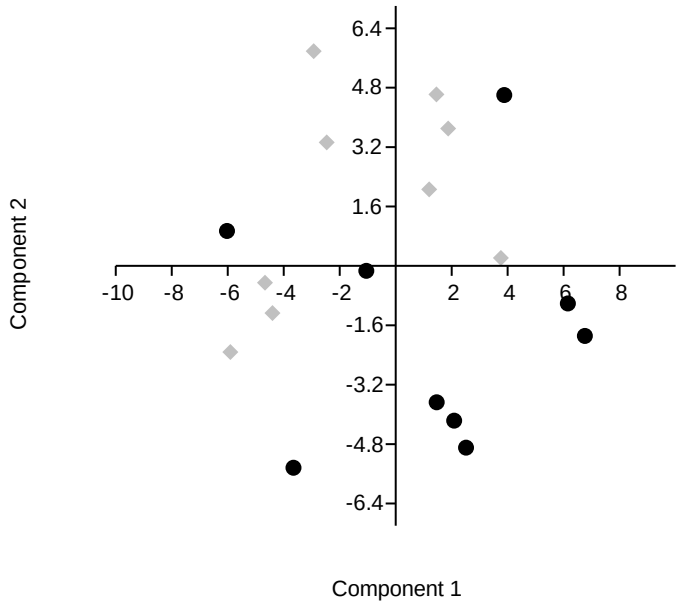

Figure 40. PCA of control (♦) and experimental (●) samples based on FAME profiles for day 20 of the Spring 2011 trial. PC1 explains 51% of the variation; PC2 explains 26% of the variation.

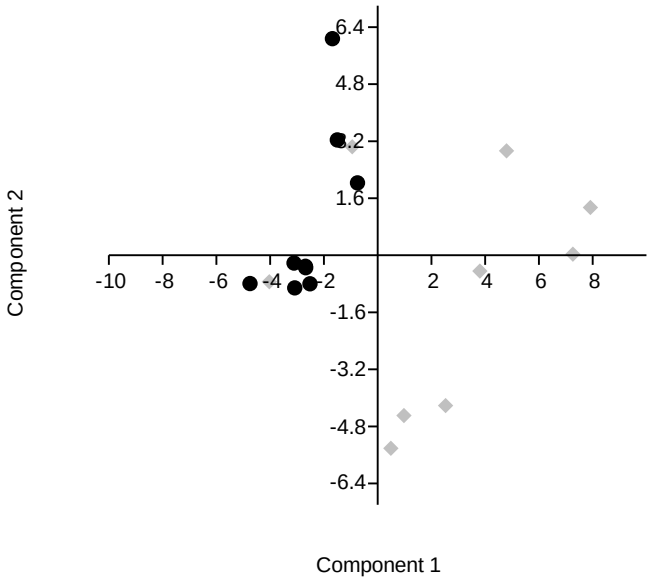

Figure 42. PCA of control (♦) and experimental (●) samples based on FAME profiles for day 27 of the Spring 2011 trial. PC1 explains 53% of the variation; PC2 explains 19% of the variation.

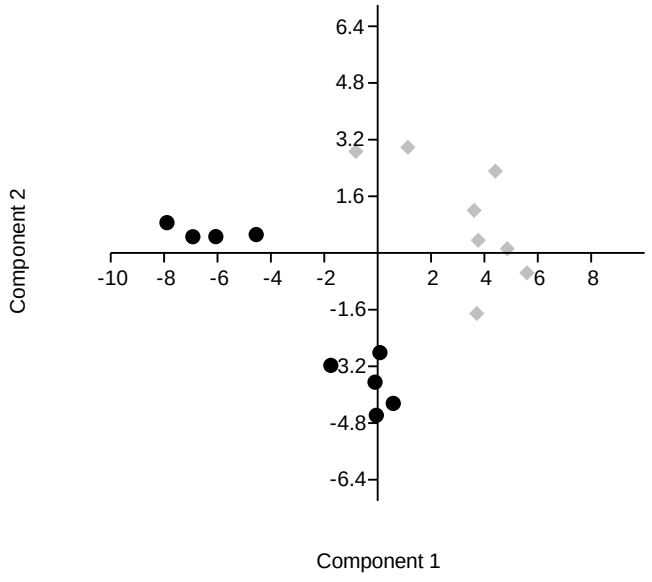

Figure 43. PCA of control (♦) and experimental (●) samples based on FAME profiles for day 41 of the Spring 2011 trial. PC1 explains 35% of the variation; PC2 explains 20% of the variation.

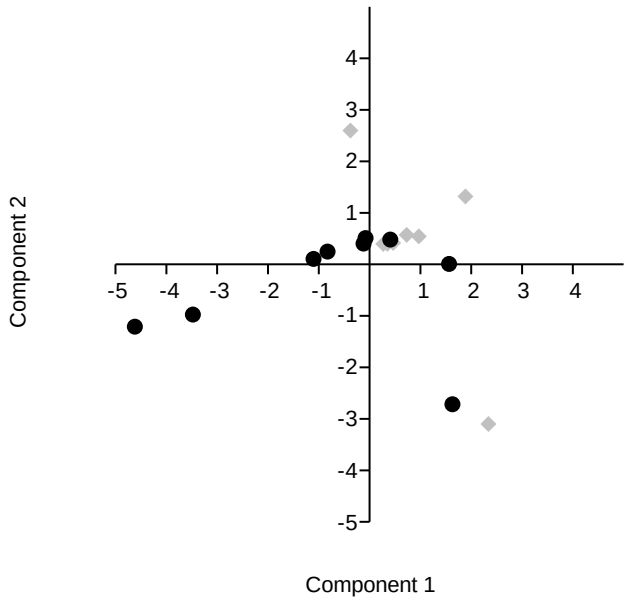

Figure 44. PCA of control (♦) and experimental (●) samples based on FAME profiles for day 34 of the Spring 2011 trial. PC1 explains 46% of the variation; PC2 explains 21% of the variation.

PCA PLOTS FOR INDIVIDUAL DAYS OF THE ADVANCED DECAY STAGE - SUMMER 2011

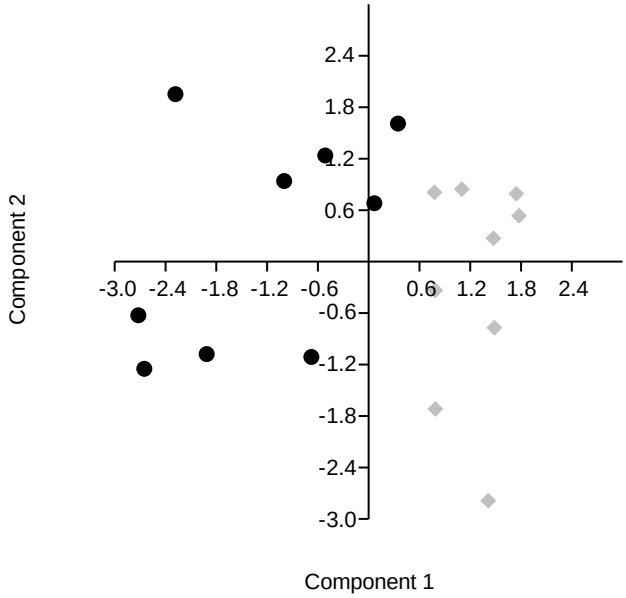

Figure 46. PCA of control (♦) and experimental (●) samples based on FAME profiles for day 20 of the Summer 2011 trial. PC1 explains 32% of the variation; PC2 explains 22% of the variation.

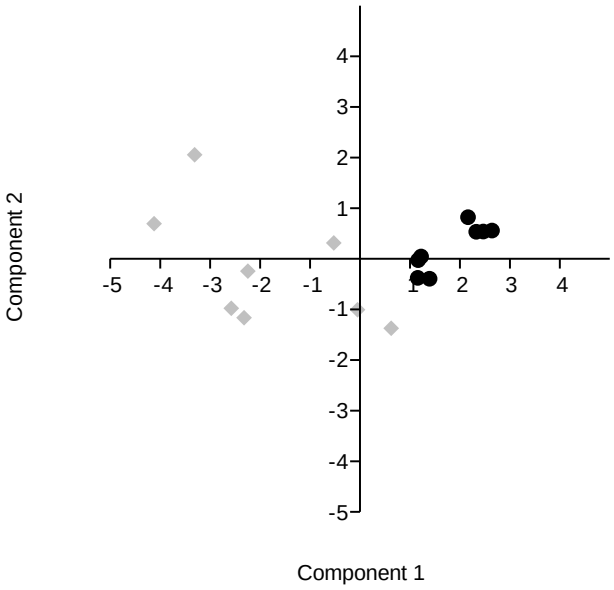

Figure 45. PCA of control (♦) and experimental (●) samples based on FAME profiles for day 27 of the Summer 2011 trial. PC1 explains 64% of the variation; PC2 explains 10% of the variation.

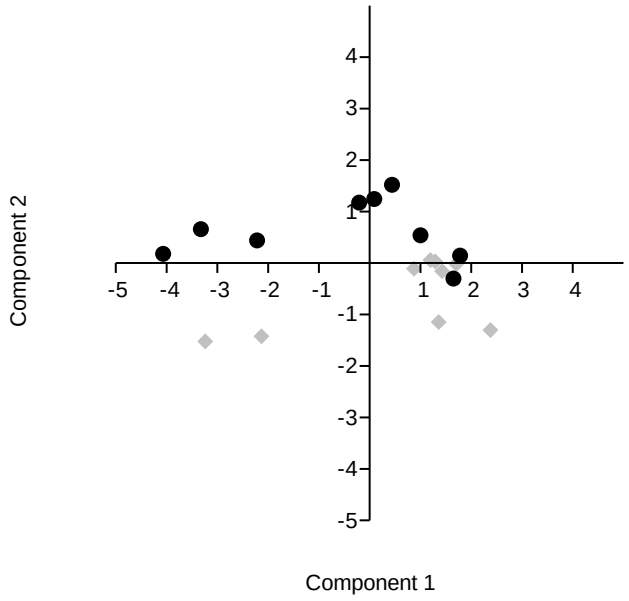

Figure 47. PCA of control (♦) and experimental (●) samples based on FAME profiles for day 34 of the Summer 2011 trial. PC1 explains 60% of the variation; PC2 explains 12% of the variation.

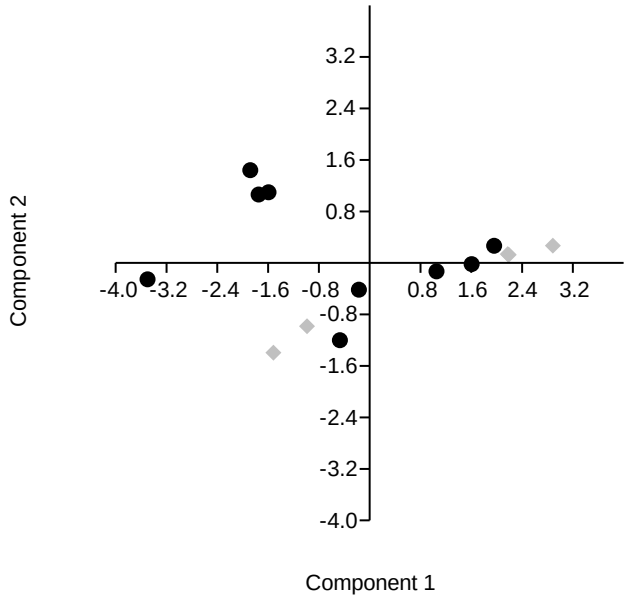

Figure 48. PCA of control (♦) and experimental (●) samples based on FAME profiles for day 41 of the Summer 2011 trial. PC1 explains 62% of the variation; PC2 explains 11% of the variation.

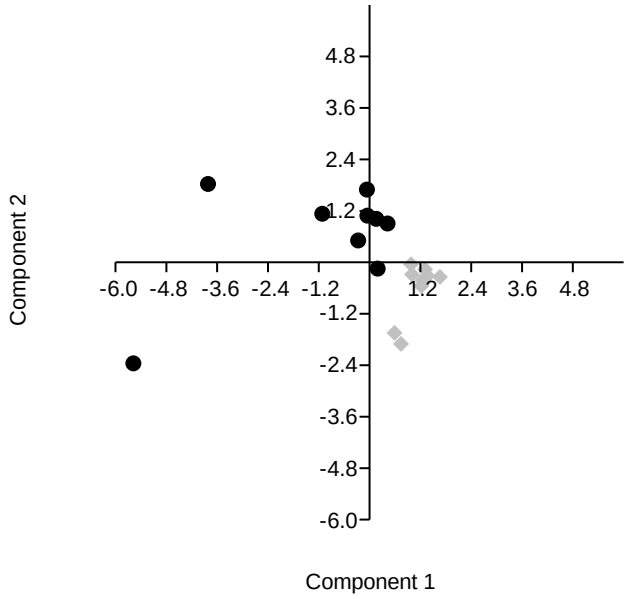

Figure 49. PCA of control (♦) and experimental samples based on FAME profiles for day 48 of the Summer 2011 trial. PC1 explains 58% of the variation, PC2 explains 23% of the variation.

PCA PLOTS FOR INDIVIDUAL DAYS OF THE DRY REMAINS STAGE OF - SPRING 2012

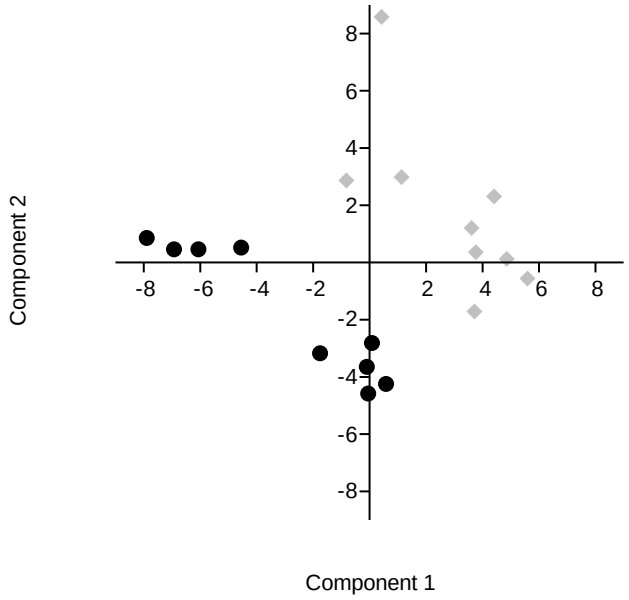

Figure 50. PCA of control (♦) and experimental (●) samples based on FAME profiles for day 34 of the Spring 2012 trial. PC1 explains 31% of the variation, PC2 explains 18% of the variation.

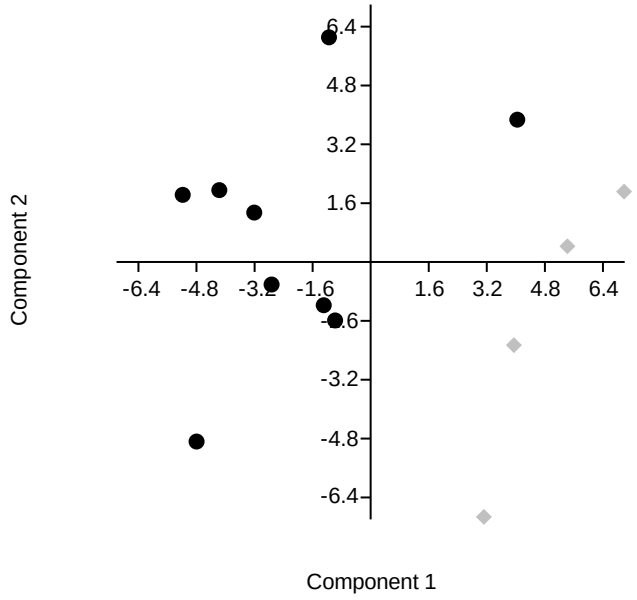

Figure 51. PCA of control (♦) and experimental (●) samples based on FAME profiles for day 41 of the Spring 2012 trial. PC1 explains 36% of the variation, PC2 explains 25% of the variation.

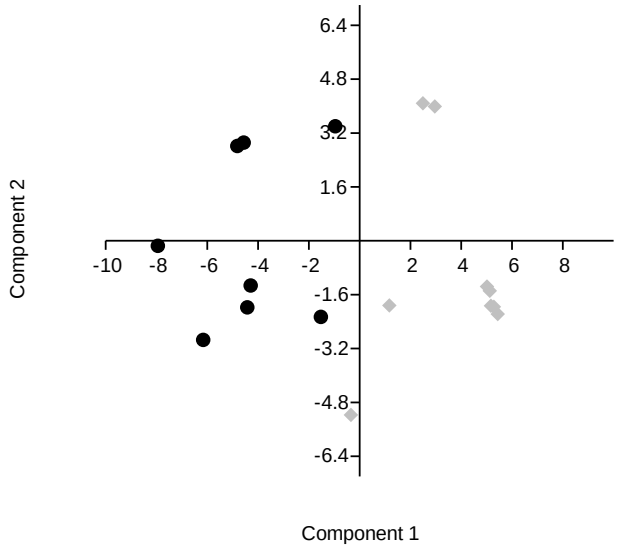

Figure 52. PCA of control (♦) and experimental (●) samples based on FAME profiles for day 48 of the Spring 2012 trial. PC1 explains 38% of the variation, PC2 explains 20% of the variation.

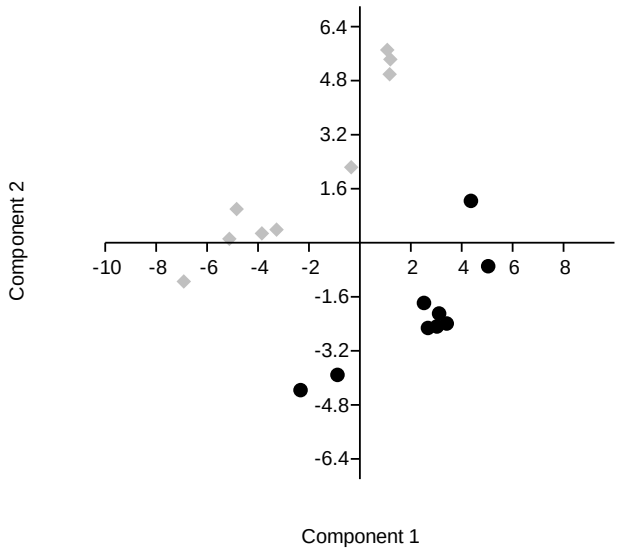

Figure 53. PCA of control (♦) and experimental (●) samples based on FAME profiles for day 62 of the Spring 2012 trial. PC1 explains 31% of the variation, PC2 explains 22% of the variation.

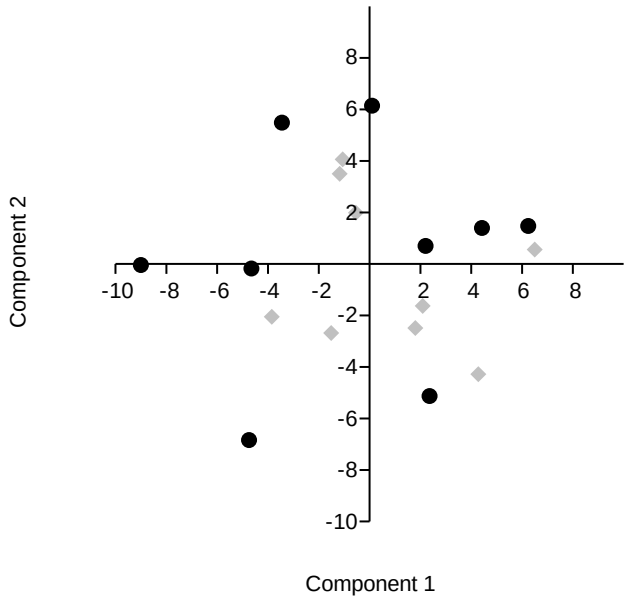

Figure 54. PCA of control (♦) and experimental (●) samples based on FAME profiles for day 41 of the Spring 2012 trial. PC1 explains 25% of the variation, PC2 explains 18% of the variation.

Table 43. ANOVA results for the effects of season and year on FAMEs common to all four experiment trials during the fresh stage of decomposition. Significant differences ($p < 0.05$) are highlighted in bold.

	Season		**Year**	
	F	***p***	***F***	***p***
16:1ω11c	0.485	0.524	0.498	0.519
***α*15:0**	1.104	0.353	**7.941**	**0.048**
***i*15:0**	**119.1**	**<0.001**	**9.121**	**0.039**
***i*16:0**	**152.4**	**<0.001**	7.066	0.057
3OH 12:0	**54.19**	**0.002**	48.89	0.002
3OH 14:0	**22.1**	**0.009**	0.266	0.633
10:0	**20.25**	**0.011**	**8.845**	**0.041**
12:0	**12.61**	**0.024**	6.821	0.059
18:2ω6	**37.83**	**0.004**	0.906	0.395
17:0	**10.62**	**0.031**	0.634	0.470
14:0	**249.3**	**<0.001**	**28.68**	**0.006**
18:1ω7c	**28.77**	**0.006**	0.299	0.6137
18:1ω9t	**97.41**	**0.001**	**137.9**	**<0.001**
16:0	0.704	0.449	4.127	0.112
16:1ω9c	**76.53**	**0.001**	4.274	0.108
15:0	**49.3**	**0.002**	0.546	0.501
18:0	**8.493**	**0.043**	4.056	0.114

Table 44. ANOVA results for the effects of season and year on FAMEs common to all four experiment trials during the bloat stage of decomposition. Significant differences ($p < 0.05$) are highlighted in bold.

	Season		Year	
	F	***p***	***F***	***p***
16:1ω11c	**169.0**	**<0.001**	**181.3**	**<0.001**
***α*15:0**	**12.66**	**0.024**	**14.95**	**0.018**
***i*15:0**	**59.99**	**0.001**	**58.14**	**0.002**
***i*16:0**	**47.02**	**0.002**	**44.90**	**0.003**
3OH 12:0	**9.513**	**0.037**	**9.305**	**0.038**
3OH 14:0	0.841	0.411	1.147	0.345
10:0	**2.2E4**	**<0.001**	**2.1E4**	**<0.001**
12:0	**4.1E4**	**<0.001**	**3.9E4**	**<0.001**
18:2ω6	6.47	0.063	6.47	0.063
17:0	**42.10**	**0.003**	**41.75**	**0.003**
14:0	**33.01**	**0.005**	**20.24**	**0.011**
18:1ω7c	**351.3**	**<0.001**	**344.3**	**<0.001**
18:1ω9t	**249.2**	**<0.001**	**754.9**	**<0.001**
16:0	**61.72**	**0.001**	**61.56**	**0.001**
16:1ω9c	**77.79**	**0.001**	**74.31**	**0.001**
15:0	**6107**	**<0.001**	**5785**	**<0.001**
18:0	3.661	0.128	3.654	0.129

Table 45. ANOVA results for the effects of season and year on FAMEs common to all four experiment trials during the active decay stage of decomposition. Significant differences ($p < 0.05$) are highlighted in bold.

	Season		Year	
	F	***p***	***F***	***p***
16:1ω11c	**10.19**	**0.033**	1.532	0.284
***α*15:0**	5.708	0.075	5.969	0.071
***i*15:0**	0.006	0.942	0.006	0.942
***i*16:0**	0.024	0.884	1.727	0.259
3OH 12:0	6.183	0.067	6.183	0.067
3OH 14:0	0.748	0.436	1.256	0.325
10:0	**8.065**	**0.047**	**8.065**	**0.047**
12:0	3.535	0.141	3.353	0.141
18:2ω6	5.056	0.088	5.056	0.088
17:0	3.052	0.156	3.052	0.156
14:0	**16.34**	**0.016**	**16.34**	**0.016**
18:1ω7c	**26.44**	**0.007**	**26.44**	**0.007**
18:1ω9t	**39.76**	**0.003**	**41.94**	**0.003**
16:0	5.414	0.081	2.233	0.209
16:1ω9c	0.002	0.966	0.002	0.966
15:0	0.806	0.420	0.806	0.420
18:0	0.441	0.543	0.441	0.543

Table 46. ANOVA results for the effects of season and year on FAMEs common to all four experiment trials during the advanced decay stage of decomposition. Significant differences ($p < 0.05$) are highlighted in bold.

	Season		Year	
	F	***p***	***F***	***p***
16:1ω11c	**8.210**	**0.046**	**8.307**	**0.045**
***α*15:0**	**948.1**	**<0.001**	**959.2**	**<0.001**
***i*15:0**	**1302**	**<0.001**	**1311**	**<0.001**
***i*16:0**	**46.10**	**0.002**	**46.71**	**0.002**
3OH 12:0	3.186	0.149	3.191	0.149
3OH 14:0	**1201**	**<0.001**	**1295**	**<0.001**
10:0	**30.25**	**0.005**	**29.85**	**0.005**
12:0	**9.512**	**0.037**	**9.559**	**0.037**
18:2ω6	5.123	0.086	4.938	0.090
17:0	**9.728**	**0.036**	**12.67**	**0.024**
14:0	**50.94**	**0.002**	**49.21**	**0.002**
18:1ω7c	**1100**	**<0.001**	**1206**	**<0.001**
18:1ω9t	4.312	0.106	4.601	0.099
16:0	**39.82**	**0.003**	**39.20**	**0.003**
16:1ω9c	**51.08**	**0.002**	**52.09**	**0.002**
15:0	**38.47**	**0.003**	**37.88**	**0.004**
18:0	**66.12**	**0.001**	**64.09**	**0.001**

Table 47. ANOVA results for the effects of season and year on FAMEs common to all four experiment trials during the dry remains stage of decomposition. Significant differences ($p < 0.05$) are highlighted in bold.

	Season		Year	
	F	***p***	***F***	***p***
16:1ω11c	0.136	0.731	0.153	0.716
***α*15:0**	**508.6**	**<0.001**	**474.5**	**<0.001**
***i*15:0**	**259.0**	**<0.001**	**223.3**	**<0.001**
***i*16:0**	**14.88**	**0.018**	**9.60**	**0.036**
3OH 12:0	2.14	0.217	1.025	0.369
3OH 14:0	5.87	0.073	4.902	0.091
10:0	**13.93**	**0.020**	3.347	0.141
12:0	4.82	0.093	3.838	0.122
18:2ω6	**8.587**	**0.043**	**2.578**	**0.184**
17:0	0.001	0.979	**31.91**	**0.005**
14:0	**657.4**	**<0.001**	**22.81**	**0.009**
18:1ω7c	**20.05**	**0.011**	**174.4**	**<0.001**
18:1ω9t	1.205	0.334	3.17	0.149
16:0	5.276	0.083	**27.08**	**0.006**
16:1ω9c	3.008	0.158	4.686	0.096
15:0	**331.6**	**<0.001**	**18.53**	**0.013**
18:0	0.566	0.494	1.085	0.356

APPENDIX C
Supplementary Figures - Soil metagenomes

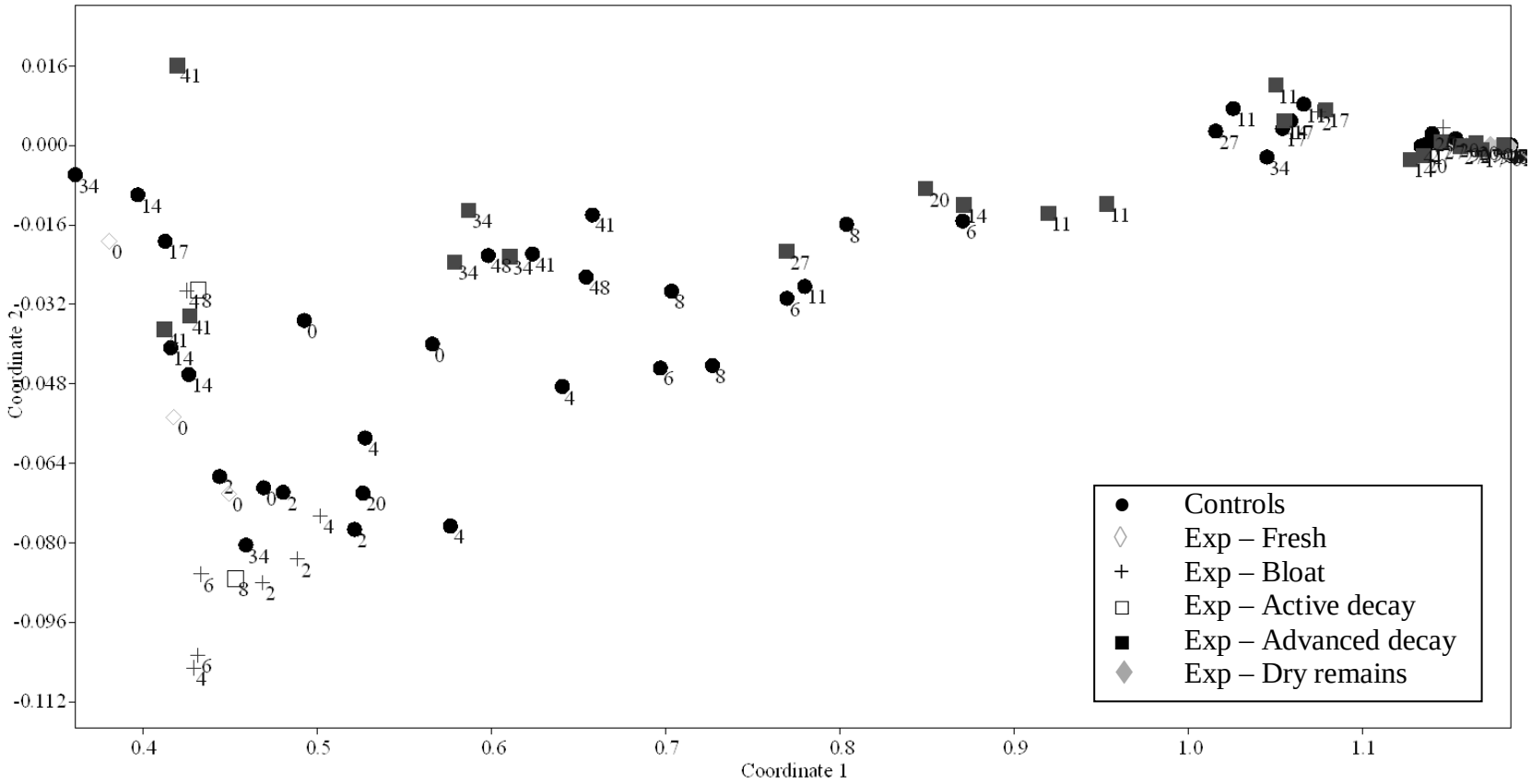

Figure 55. Unweighted UniFrac distances of control samples and experimental samples per decomposition stage in Spring 2011 using principal coordinate analysis

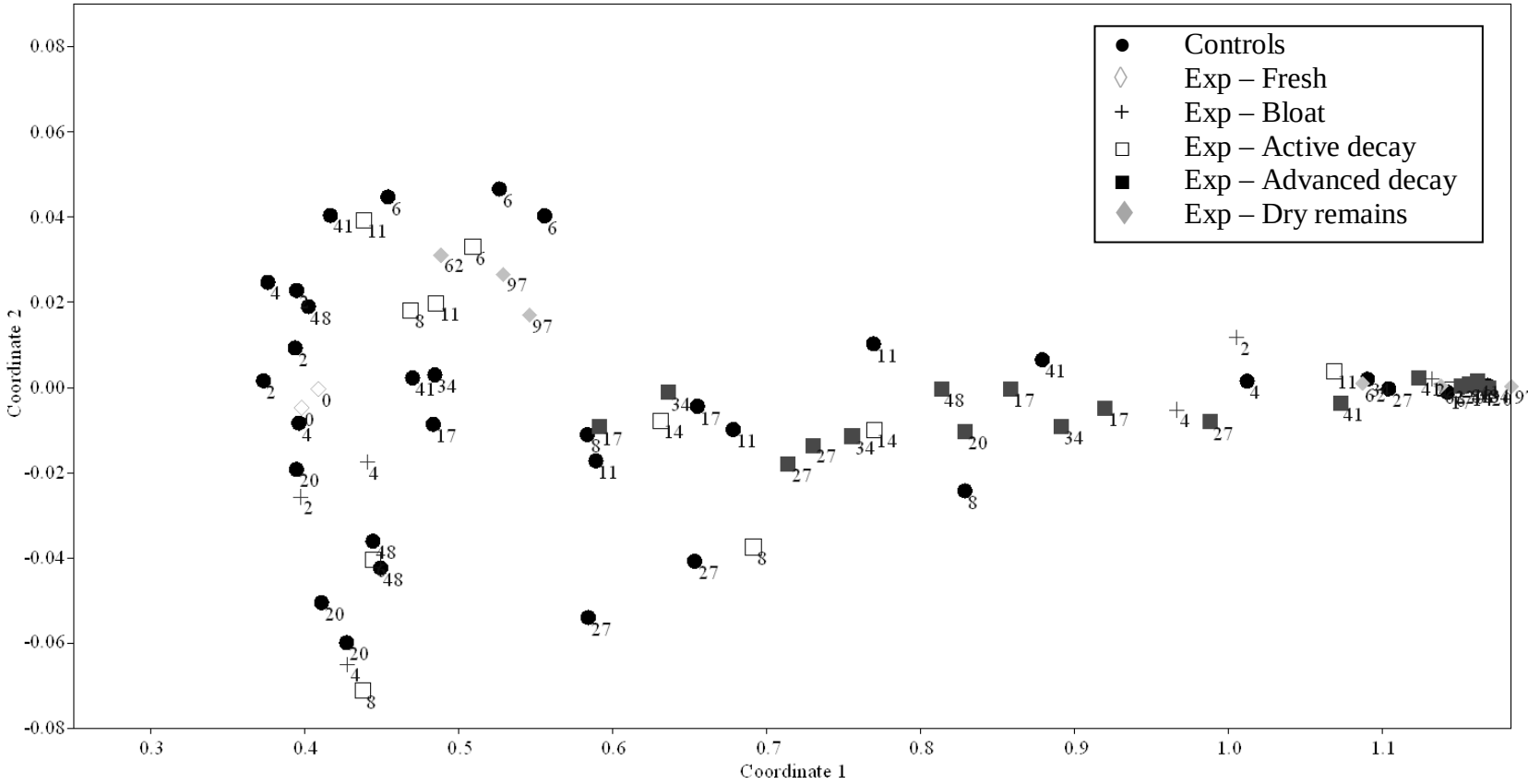

Figure 56. Unweighted UniFrac distances of control samples and experimental samples per decomposition stage in Summer 2011 using principal coordinate analysis

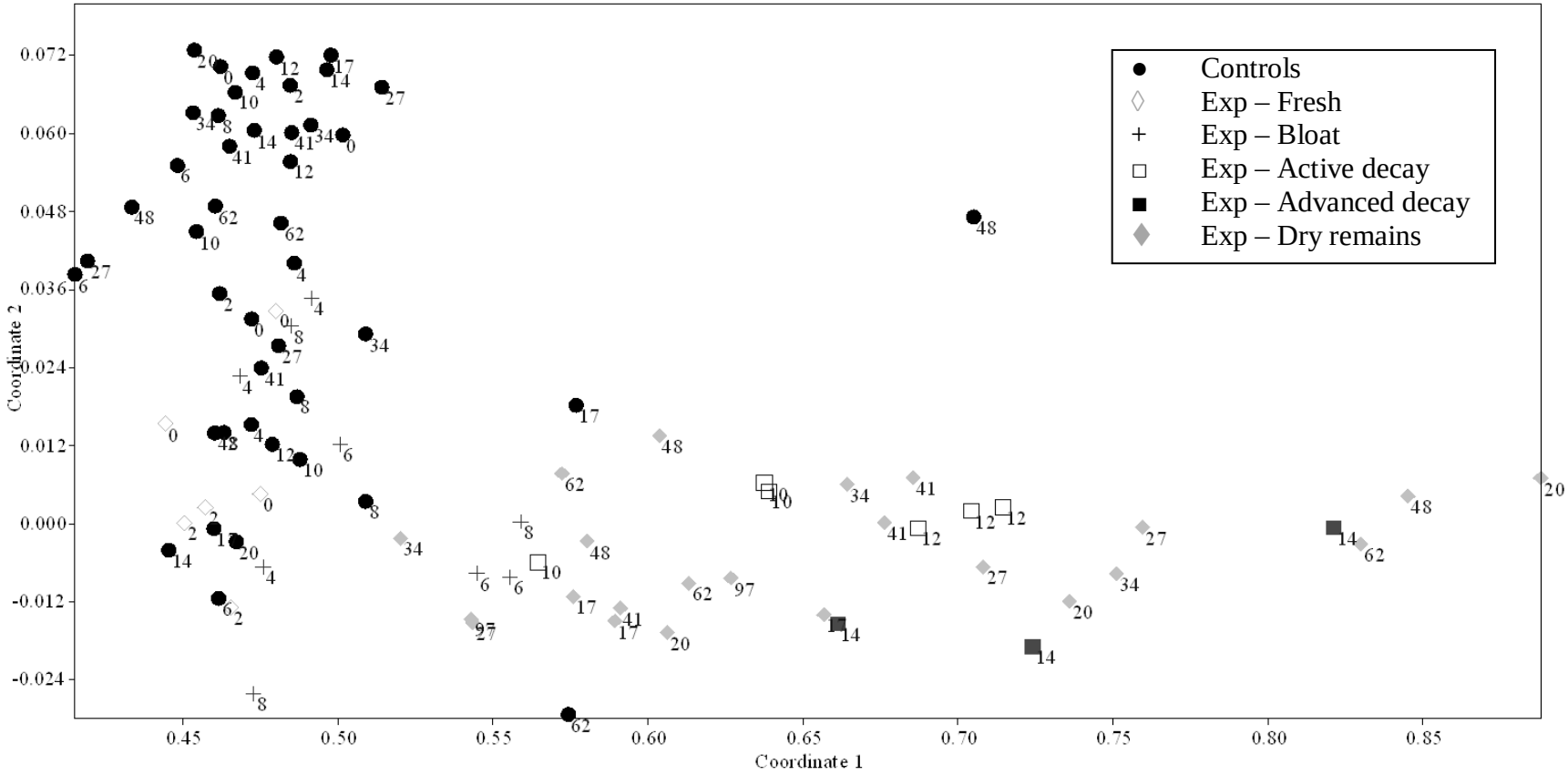

Figure 57. Unweighted UniFrac distances of control samples and experimental samples per decomposition stage in Spring 2012 using principal coordinate analysis

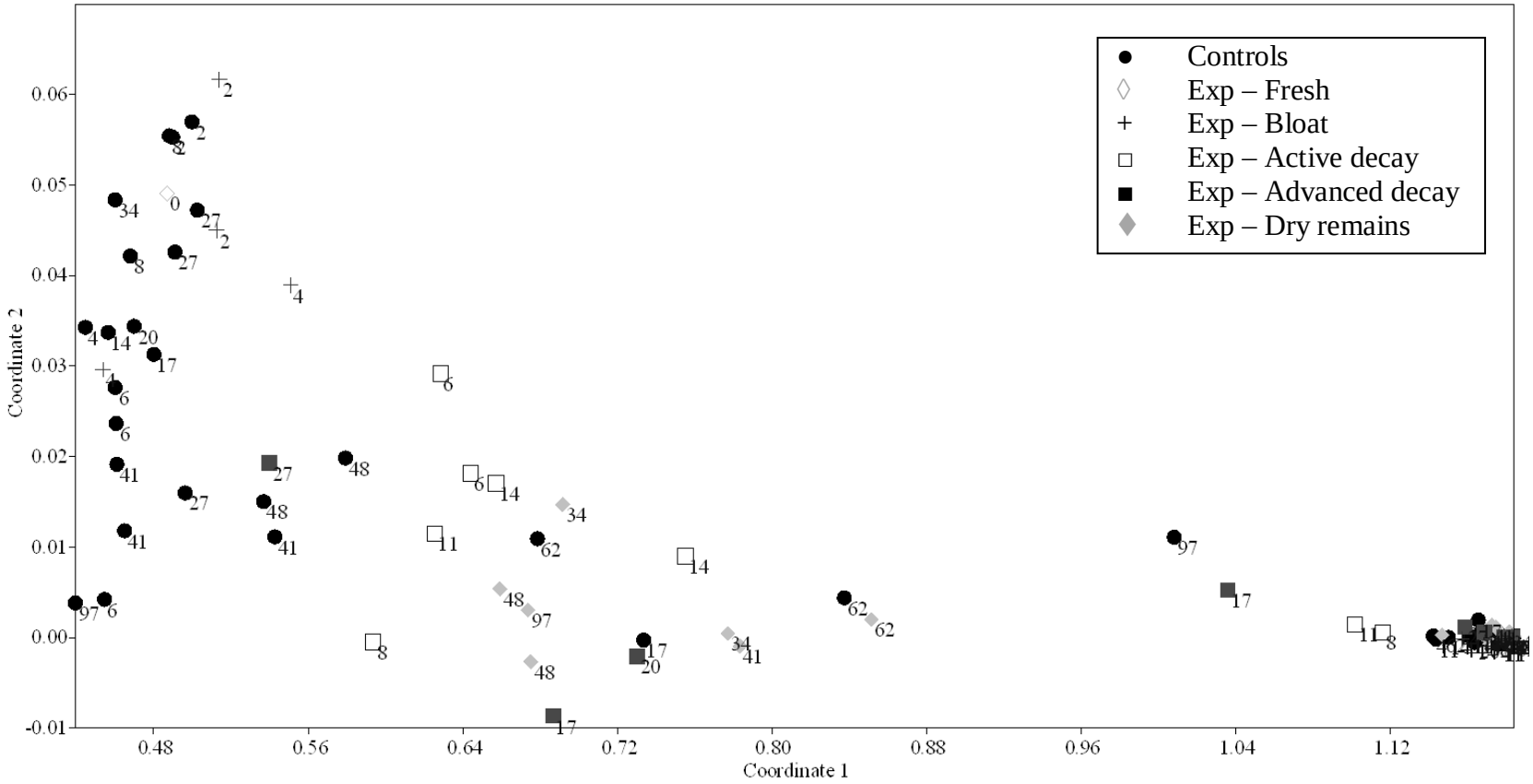

Figure 58. Unweighted UniFrac distances of control samples and experimental samples per decomposition stage in Summer 2012 using principal coordinate analysis

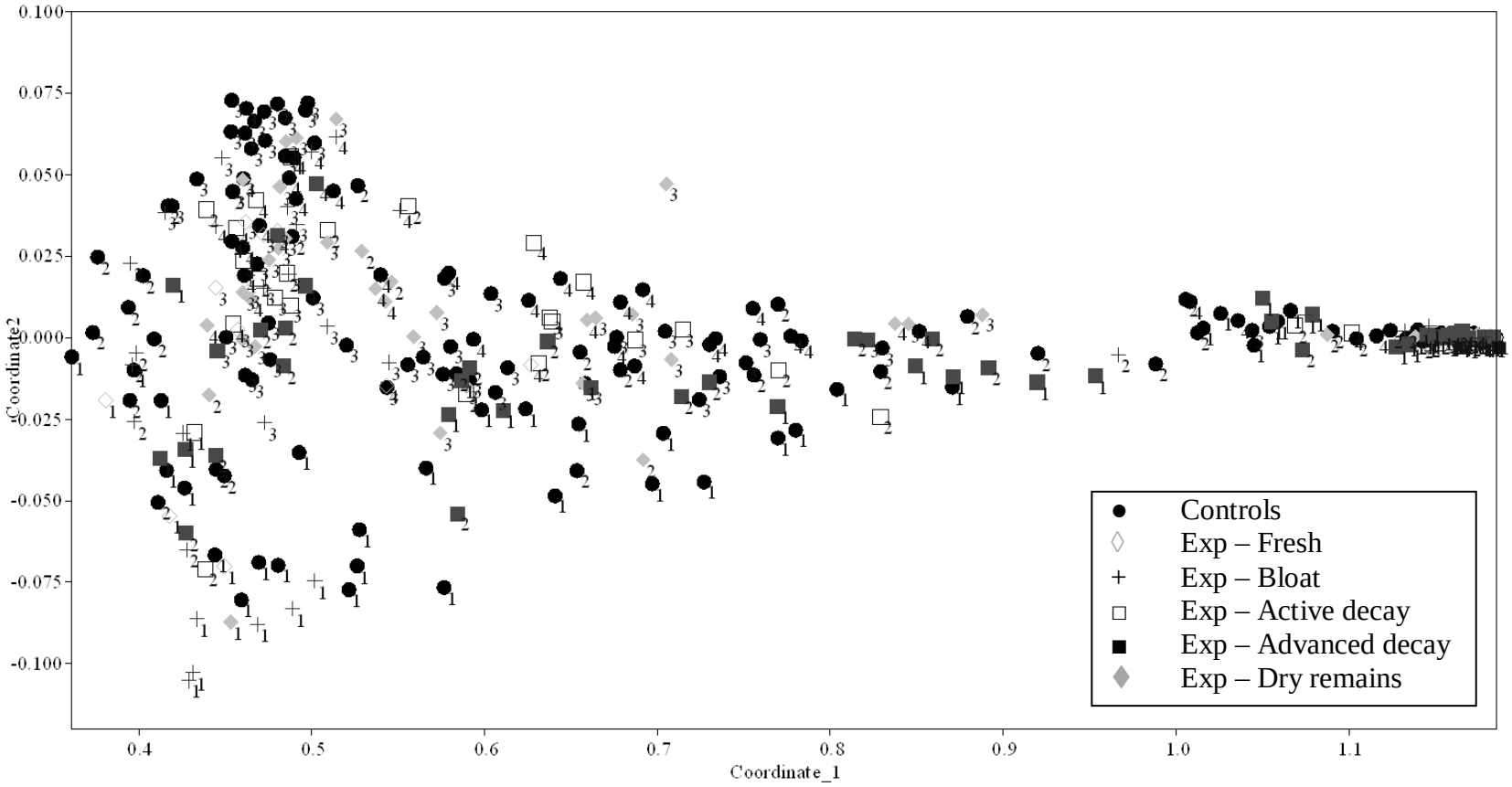

Figure 59. Unweighted UniFrac distances of control samples and experimental samples per decomposition stage using principal coordinate analysis for the Spring 2011 (1), Summer 2011 (2), Spring 2012 (3) and Summer 2012 (4) trials. Samples are labelled according to trial.