Biochemical Alteration of Gravesoils between Season and Soil Type

By

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Abstract

Decomposition chemistry refers to the biochemical degradation processes which occur in soft tissue as decomposition proceeds. This study aims to investigate the relationship between the release of decomposition fluids into contrasting soil environments and their potential correlation with the presence of a decomposing carcass. Soil from two different carcass decomposition trials was utilized to determine if seasonal and soil variation altered the soils reaction to a carcass. The reaction was determined by investigating the soil available phosphorus, extractable lipid-phosphate, pH, moisture and fatty acid content. A significant increase in the relative concentration of extractable lipid-phosphate, soil available phosphorus, and fatty acid content was identified, confirming the flux in the microbial biomass in the soil. Contrary to these nutrients, there were no notable changes in the soil pH and moisture content. The findings of this study were able to highlight the future forensic potential of these techniques and demonstrate a need for further research.

Decomposition Gravesoil Postmortem Interval

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List of Abbreviations

ANOVAs	Analysis of variances
C	Carbon
CDI	Cadaver decomposition island
^{o}C	Degree Celsius
FAMEs	Fatty acid methyl esters
g	Grams
$K_2S_2O_8$	Potassium persulfate
L	Liter
lb	Pound
MeOH-KOH	Methanoic potassium hydroxide
M	Moles liter^{-1}
mL	Milliliter
mm	Millimeter
MS	Mass spectrometry
N	Normal
N	North
N	Nitrogen
NaOH	Sodium hydroxide
nm	Nanometer
P	Phosphorus
PMI	Post mortem interval
rpm	Revolutions per minute
Std	Standard
W	West
µL	Microliter
µg	Micrograms

Chapter 1

Introduction

1.0 Introduction

Forensic science is not defined by just one individual field of science, it is a field of study which incorporates many of the natural sciences, including chemistry. Just as forensic science includes many disciplines, forensic chemistry is also comprised of sub-disciplines including, toxicology, trace analysis, and fire analysis. Decomposition chemistry is a recent addition to the subsection of forensic chemistry and is gaining much attention due to the potential of newly developed methods for estimating time since death (Forbes et al., 2005, Vass et al., 1992). Decomposition chemistry specifically investigates the chemical degradation processes that occur in soft tissue as decomposition proceeds. Although the chemical pathways of decomposition have been studied for over a hundred years, the process is still not well understood (Dent et al., 2004). This gap in knowledge is a significant hindrance, as the accuracy and reliability of a forensic method determines admissibility in a court of law.

Post mortem interval (PMI) is defined as the period of time since death. Estimating the post mortem interval allows for the construction of a timeline prior to death. Presently there are a variety of methods that are used to approximate PMI including entomology (Mendonca et al., 2008), or using body temperature of the deceased (Henbge & Madea, 2004). These methods are not able to give a definitive PMI, thus they only provide an approximate time frame. There are few methods which use the surrounding environment to extract information about the time since death. Analysing gravesoils is a recent method explored by Vass et al. (1992), however not in great detail.

2.0 Carcass Decomposition

A number of stages are associated with the degradation of a carcass (Carter et al., 2007). As soft tissue decomposes, nutrients and bodily fluids are released into the surrounding environment, including the associated soil. Vass et al. (1992) observed that gravesoils receive a significant increase in volatile fatty acids and several nutrients (e.g. ammonium, sulphate), and the concentration ratio of these materials can be used to accurately estimate PMI. The same study by Vass et al. (1992) demonstrated that short chain volatile fatty acids are introduced to soil during cadaver decomposition and can be of forensic importance.

Cadaver decomposition is a complex process that is affected by internal and external factors. External factors include environmental conditions and the physicochemical environment where a body is placed (Carter and Tibbett 2008). Within soils, regulating factors include moisture content, pH, ion exchange capacity (Forbes et al., 2005) and texture (Carter et al., 2008). The internal factors affecting the rate of decomposition can be linked to the lipid and protein content in a body (Forbes et al, 2006). Although it is not possible to unify the rate of decomposition in all possible environments, there are certain characteristics through which the cadaver will likely progress (Dent et al., 2004).

The stages of decomposition, it must be noted, are subjective, and are only an attempt to better explain cadaver decomposition. The duration of these stages will vary depending upon the environmental factors surrounding the cadaver. However, the

current study will refer to five observed stages termed fresh, bloat, active decay, advanced decay, and skeletonization, including dry remains (Figure 1) (Payne, 1965).

Figure 1. Representation of the Stages of Decomposition (adapted from Payne, 1965).

2.1 Carcass Decomposition: Fresh Decomposition

Fresh stage decomposition begins immediately following death. This stage will continue until bloat is visible (Payne, 1965). The physical characteristics of the fresh stage include little to no odour. In Southern Ontario the fresh stage appears to last no longer than 3 days during both early and late summer (Benninger et al, 2008 and Payne,

1965). As fresh stage decomposition begins immediately following the cessation of the heart beating and the intake of oxygen it leds to the degradation of the cells by enzymatic self-digestion (Gill-King, 1997). The self-digestion process is referred to as autolysis and describes the hydrolytic splitting of the proteins, carbohydrates and fats within the body, with fats being degraded to a lesser degree (Dent et al., 2004). The hydrolytic splitting occurs through lysosomes that are present in the cytoplasmic granules in all cells, by releasing them into the cytoplasm (Clark et al., 1997). It has been predicted that autolysis is triggered by a decrease in the intracellular pH as a result of the lack of oxygen flow upon cessation (Clark et al., 1997). Autolysis allows for the proliferation of microorganisms, due to the disruption of the cell wall allowing the proteins, carbohydrates and fats to be released and used as nutrients (Clark et al., 1997). Although autolysis does not occur at a predictable rate, it is assumed that because lysosomes propagate the lysing, cells containing large amounts of lysosomes would be the first to go through autolysis (Clark et al., 1997). The initial changes that occur during autolysis are not visible to the naked eye, as they occur at the cellular level and can only be seen microscopically. After approximately 48 hours, the changes begin to be visible without magnification. Autolysis will proceed through all stages of decomposition.

2.2 Carcass Decomposition: Bloat

Bloat tends to begin shortly after the commencement of fresh decomposition (Benninger, 2007), but the time required for this stage is associated with temperature. This stage is characterized by the visible distension of the abdominal regions of the carcass, as well as any major extremities (Payne, 1965). Due to the distension, there will be a stretching and thus potential tearing. As well as being characterized by a visible

change, the characteristic odours associated with decomposition are very evident (Payne, 1965). The characteristic smell is coupled with the seepage of bodily fluids from the orifices, and effervescence of these bodily fluids, thus leaching into the soil and beginning to form the cadaver decomposition island (CDI). This island includes the soil beneath and surrounding the cadaver and often appears dark as a result of the leached bodily fluids (Amendt et al., 2004; Carter et al., 2007).

The biochemical changes occurring in the body during the bloat stage can be referred to as putrefaction. Putrefaction is defined as the biological alteration of the protein, lipid and carbohydrate components of the soft tissues, predominantly caused by enteric, anaerobic bacteria (Dent et al., 2004). These bacteria degrade the tissues and cells and release their products into the body cavity. These products include various gases such as hydrogen sulphide, and methane, by-products from anaerobic metabolism. The buildup of these gases causes bloating of the cadaver as the gases create pressure and expand the body.

2.3 Carcass Decomposition: Active Decay

Active decay takes place after bloat, and is signified by the deflation of the carcass, whilst liquidation of the carcass is occurring (Payne, 1965, Anderson and VanLaerhoven, 1996). Visually the carcass will appear to be very wet from the decomposition fluids, as well as the strong pungent odour (Anderson and VanLaerhoven, 1996). External signs of autolysis will now be visible, and the phenomenon of skin slippage may be apparent (Clark et al., 1997). Skin slippage is due to the release of the hydrolytic enzymes by cells at the dermal-epidermal junction of the skin, resulting in skin removal with movement of the carcass (Clark et al., 1997). In addition, intravascular

hemolysis causes marbling of the skin (Clark et al., 1997). This marbling is resultant of the blood vessels being outlined by deoxyhemoglobin, giving the signature blue colouring (Clark et al., 1997).

2.4 Carcass Decomposition: Advanced Decay

Advanced decay is the stage where the major sources of energy and nutrients remaining include soft tissues, hair, bone, and integument (Payne, 1965). At this stage any remaining soft tissue will have likely desiccated and become mummified. Due to the considerable loss of moisture and nutrients from the body, gross decomposition is usually minimal at this stage (Carter et al., 2007). The end result of the advanced decay process is the commencement of skeletonization of the remains, usually with the ligaments still intact (Dent et al., 2004).

2.5 Carcass Decomposition: Skeletonization/Dry Remains

During skeletonization any remaining soft tissue may be removed from the bones. This process can occur in two different forms; complete skeletonization where all the soft tissue has been removed and partial skeletonization where only portions of soft tissue are removed (Dent et al., 2004). The collagen will be lost via bacterial decomposition. The final product will be bone; however, the preservation of the bone is dependent upon the pH of the soil. If the soil is too acidic, its preservation will be less than if the soil is neutral or slightly alkaline (Dent et al., 2004), because the acidic environment will cause the breakdown of the organic parts of the body (Janaway, 1996).

3.0 Gravesoil Ecology

Soil is a complex matrix, as it is formed from the weathering of the rocks via biological, chemical and physical processes (Faulkner & Richardson, 1989). In forensic analysis, soil is primarily studied to determine physical changes to the soil to investigate footwear impressions, or as trace evidence; however soil has recently been studied to determine the biochemical changes associated with decomposition (Stokes et al., 2009). The ecology of gravesoils has become a viable resource for forensic scientists, as the decomposing remains will affect the soil-dwelling organisms (Parkinson, et al., 2009). The addition of the fluids from the remains has the ability to help or hinder the microbial species residing in the soil (Parkinson, et al., 2009). The fluids and carcass can be used as a substrate for these organisms, and can either help them flourish or repress their growth via the creation of unfavourable environmental conditions (Parkinson, et al., 2009). Particular unfavourable environmental conditions include that of alterations in pH of the soil and changes in the moisture content (Parkinson, et al., 2009).

3.1 Gravesoil Ecology: Microbial Biomass

As decomposition is heavily dependent upon microorganisms, from both the body itself and the environment surrounding it, the soil microbes become an important consideration (Parkinson, et al., 2009). The soil microbial biomass can be defined as the amount of microorganisms present in the soils, including bacteria, yeasts, fungi and protozoa (Zhang et al., 2005). This information is helpful in determining the amount of nutrients present in the soil, as the biomass provides insight into the size and structure of the soil microbial community (Jeannotte, et al., 2008).

Microbial populations are dynamic in soils, therefore, as the soil changes the microbial biomass can change with it. The microbial biomass size will change with the presence of additional nutrients, generally from the decomposition of a rich nutrient source (Ha et al., 2008). Similarly, the microbial biomass size will change if the nutrient levels lower, being an early indicator of potential changes of a nutrient source (Larkin, et al., 2006). Subsequently, some studies have shown that different soil types will lend themselves to different microbial biomass profiles (Larkin, et al., 2006). Particular soils, including ones with high silt and clay fractions, and those higher in total carbon content, pH and cation exchange capacity, tend to support a larger amount of microbial biomass (Larkin, et al., 2006).

As microbial biomass is made up of microorganisms, particular organisms will thrive in certain environments (Larkin, et al., 2006). Bacteria prefer neutral soil pH, whereas fungi prefer a more acid environment (Baath et al., 1992). Furthermore, bacteria have the ability to outcompete other organisms for nutrients present at a lower concentration, due to having high substrate affinities, surface to volume ratios, and metabolic rates (Bott & Kaplan, 1985). It has been suggested that during particular ogranic decomposition, for instance during the composting process, there is a dominance of bacteria during the initial stages, whereas during the latter stages, there is a dominance of fungi (Ha et al., 2008).

3.2 Gravesoil Ecology: Lipids

Lipids found in soils are considered to be biologically and chemically diverse, due to the variation in the original source of the lipids (Jeannotte et al., 2008). There are three major sources of the lipids in soil: plant, animal and microbial cells (Jeannotte et al.,

2008). Plant lipids are important not only to the plant, but to the animals that feed on those plants, as plant storage lipids are a major source of human and livestock nutrition (Murphy, 2008). The majority of the lipids found in plants are triacylglycerols, which contain acyl lipids (Murphy, 2008). The storage lipids that are found in plants are found throughout the entire plant itself, including the epidermis of the plant (Murphy, 2008). This is especially important as the epidermis of the plant is what comes into contact with the outside world including the soil and any neighbouring environments (Murphy, 2008).

Lipids are very important in animals as they provide metabolic insulation and storage. Lipids are composed of non-polar tails, and a polar head group, thus making them insoluble in water, and soluble in organic solvents. Lipids are in high abundance in mammals (60-85% of the remaining non-water components) as they are found principally in membrane bilayers, steroids, and as energy storage units (Dent et al., 2004). Thus, the decomposition of a cadaver will result in a significant influx of lipid into the soil. Due to the stability of these compounds in water, they will not degrade as readily as more soluble compounds under environmental conditions.

3.3 Gravesoil Ecology: Previous Studies

Previous studies have demonstrated that the soil and decomposition fluids will undergo sequential changes over time (Vass et al., 1992; Benninger et al., 2009; Stokes et al., 2009; Swann et al., In Press).

3.3.1 Fatty Acid Analysis

In a 1992 study, Vass et al. (1992) analysed short chain volatile fatty acids as well as cations and anions, in the soil solution. This particular study found 5 short chain

volatile fatty acids, propanoic acid (C3:0), iso-butyric acid (C4:0), *n*-butyric acid (C4:0), iso-valeric acid (C5:0), and *n*-valeric acid (C5:0), to be retained in the soil over a duration of approximately 150 days, as they are produced by the aerobic degradation of soft tissues (Vass et al., 1992). The abundance of these acids varied throughout the collection period however there were two notable areas of increased abundance that remain consistent within all of the fatty acids, occurring around accumulated degree days of 400 and 1050 (Vass et al., 1992). This same study found that there are seven ions (chloride, sulfate, sodium, ammonium, potassium, calcium, magnesium) that remain stable in the soil over the same trial cycle. Swann et al. (In Press 2009) found similar results to Vass and colleagues (1992), identifying propanoic acid (C3:0), iso-butyric acid (C4:0), *n*-butyric acid (C4:0), iso-valeric acid (C5:0), and *n*-valeric acid (C5:0 in the decomposition fluid released from pig carcasses in the absence of a soil matrix (Swann, et al., In Press). Long chain fatty acids including, myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) were also identified in the decomposition fluids (Swann, et al., In Press), which could be important to this study.

Structurally, fatty acids are carbon chains with a methyl group at one end, and a carboxyl group at the opposite end (McMurray, 1992). Fatty acids play a large role in the complex lipids, as they help with many biological functions: as a metabolic fuel, in the structure of all cell membranes, and as gene regulators (McMurray, 1992). All fatty acids will fall into two general categories: saturated, and unsaturated (McMurray, 1992). Unsaturated fatty acids can be further subdivided into two general categories; monounsaturated, having only one carbon-carbon double bond and polyunsaturated,

having more than one carbon-carbon double bond (McMurray, 1992). Fully saturated fatty acids are considered to be very stable, whereas unsaturated fatty acids have a greater susceptibility to oxidation (McMurray, 1992). Generally, the occurrence of oxidation increases as the number of double bonds increases (McMurray, 1992).

Fatty acids are of importance in the body, both when living and deceased. The majority of fatty acids are produced by the degradation of the adipose tissue. Adipose tissue is composed of approximately 60-85% lipids; where 90-99% of these lipids are triglycerides (Reynold and Cahill, 1965). Triglycerides are composed of three fatty acids attached to a glycerol backbone. The neutral fat of decomposing remains has the potential to undergo hydrolysis to produce fatty acids (Dent et al, 2004). These fatty acids will then undergo hydrogenation or oxidation (Dent et al, 2004) depending on the environmental conditions where decomposition is occurring. The fat can be hydrolyzed by intrinsic tissue lipases to produce saturated and unsaturated fatty acids (Dent et al, 2004). Hydrogenation and hydrolysis increases the amount of saturated fatty acids produced, whilst the amount of unsaturated fatty acids decreases (Dent et al, 2004). A summary of the processes involved in degradation can be seen in Figure 2. Saturated fatty acids have been detected in gravesoils more than fifty years after the bodies were deposited and are considered to be highly stable compounds in the environment (Forbes et al., 2002).

Figure 2. Flow chart of the adipose degradation processes (adapted from Dent et al, 2004)

Myristic acid (C14:0) is found in abundance in adipocere, the waxy tissue that can be formed during decomposition from the adipose tissue (Stuart et al., 2000; Vane & Trick, 2005) in the fluids collected from a decomposing carcass (Swann et al., In Press).

Palmitic acid (C16:0) is one of the most widely occurring saturated fatty acids, as it is present in fish oils (10 – 30%), in milk and body fats of land animals and in

vegetable fats (ranging from 5 – 50%) (Gunstone, 1996). Palmitic acid is known to be a component of adipocere that can be present in a gravesoil if the proper soil conditions prevail, including the moisture content and the oxygen exposure (Stuart et al., 2000; Vane & Trick, 2005). A previous study by Lorenzo et al. (2003), found the presence of palmitic acid upon analysis of the decomposition fluids released from the carcasses of pigs.

Stearic acid (C18:0) is less common biologically than palmitic acid, however it can be generated easily by the hydrogenation of oleic (C18:1) and linoleic (C18:2) acid (Gunstone, 1996). Stearic acid is also a major fatty acid in animals, fungi, and in very small portions in plants (McMurray, 1992). Stearic acid is a primary component of adipocere (Stuart et al., 2000; Vane & Trick, 2005).

Oleic acid (C18:1) is said to be produced in the largest abundance of all of the monoenoic fatty acids (Gunstone, 1996). This is a result of oleic acid being the precursor of other n-9 monoene acids, and of some polyene acids (Gunstone, 1996). As well as the biological abundance of this acid, it is also found naturally in large proportions in olive oil, almond oil, and a variety of other nut oils (Gunstone, 1996). Previous decomposition studies have found oleic acid to be abundant in the decomposition fluid from a pig carcass (Lorenzo, et al., 2003). This is consistent with oleic acid being a primary component of adipocere, resulting from the decomposition of adipose tissue (Vane & Trick, 2005)

3.3.2 Phosphate Analysis

Both Benninger et al. (2008) and Stokes et al. (2009) found an increase in the abundance of phosphates in the soil during carcass decay. Benninger et al. (2008), found

an increase in both soil available phosphorus, as well as extractable lipid phosphate for a trial completed in Southern Ontario. Stokes et al. (2009) found that the bicarbonate extractable phosphate, which utilizes a similar method to soil available phosphorus, abundance increased in varying soil types in Western Australia (Stokes et al., 2009). Thus there is a potential that phosphate is a useful decomposition product for forensic importance.

Phosphorus is not fixed from vegetation, or a lot of bacteria in the soil, however it is present from weathering of the rocks, and organic molecules released during decomposition. Phosphorus differs from other nutrients, as it is less water soluble the nitrogen and does not have a stable atmospheric gaseous phase (Filippelli, 2008). Thus, leaching through the soil takes a significant amount of time. Phosphorus is readily available in the biological world as mammalian bodies contain up to 1% of phosphours (Tortora and Grabowski, 2000), as it is required in the production of adenosine triphosphate (ATP), and in the bones to provide stability and strength (Filippelli, 2008).

Soil organic phosphorus is the most important form of phosphorus when considering the entire decomposition cycle, with the most active amount being biomass phosphorus (Stewart & Tiessen, 1987). Although the overall amount of soil organic phosphorus does not generally change in one year, seasonal variations have been reported (Stewart & Tiessen, 1987). Organic phosphorus is released by secretion, or cell lysis, into the soil, for uptake by soil organisms (Stewart & Tiessen, 1987). In order for plants to take up phosphorus, hydrolysis must occur to alter the organic phosphorus (Stewart & Tiessen, 1987). It can also remain in the soil via the interactions it has with the soil particles, and the side chains the organic phosphorus contains (Stewart & Tiessen, 1987).

Biomass phosphate can be considered the general name for many different types of phosphate, including but not limited to: nucleic acids, phosphate-esters and phospholipids (Stewart & Tiessen, 1987). The quantities of the metabolically active phosphate forms will vary between cells, as it is dependent upon the growth stage, and activity of the cells in particular (Stewart & Tiessen, 1987). Conversly, as phospholipids are concentrated in the cellular membranes, they will occur in relatively constant quantities (Stewart & Tiessen, 1987). Organic phosphate will vary as it is dependent upon the rate of uptake and release by the microrganisms present in the soil.

Phospholipids are created by the esterification of one of the alcohol groups in a glycerol, by a phosphoric acid molecule rather than a carboxylic acid. Phospholipids are the main component of cell membranes in the body, thus they are present in large quantities. Phospholipids have a unique structure, including a hydrophilic head, and a hydrophobic tail. Phospholipids also contain one saturated and one unsaturated chain leg of the hydrophobic tail. As other phospholipids degrade, an abundance of phosphates in the body are also released into the surrounding environment.

4.0 The Forensic Application of Gravesoil Ecology

The main purpose of this study was to utilize chemical analysis of soil, to investigate a potential correlation between the extent of decomposition fluids in a gravesoil and the postmortem interval. The nutrients being considered are the phosphate content, in particular the soil available phosphorus and the extractable lipid phosphate abundance, and the long chain fatty acid content. Previous studies have indicated that the phosphate content of the soil will remain elevated throughout the entirety of

decomposition, and does not return to baseline levels (Stokes et al., 2009). Further to this, Benninger et al. (2008), found a duel cycle of phosphate abundance, with the primary cycle nearing the end of the advanced decay stage, and the secondary cycle beginning approximately two weeks after the carcass had become skeletonized. We hypothesize that elevated phosphorus levels could be a viable option for estimating the postmortem interval. Thus, the phosphates must remain stable in the soil for an extended period of time.

Fatty acids associated with decomposition were first described the soil since 1992, when Vass and coworkers completed an initial study and found volatile short chain fatty acids to be retained in the soil approximately 100 days after the decomposition process was complete (Vass et al., 1992). Not only have short chain fatty acids been identified, but long chain fatty acids have also been found in gravesoils, as well as in the decomposition fluid without the soil matrix (Stuart et al., 2000; Swann et al., In Press). Due to long chain fatty acids being primary components of adipocere (Stuart et al, 2000), and being present in the decomposition fluids (Swann et al., In Press), we hypothesize that these fatty acids will be present in the soil even when there is no adipocere formation.

This study investigated decomposition fluids from pigs in contrasting environments, with one decomposition site located in a wooded area, and the other located in an open field. As indicated by the previous section there is a gap in the knowledge available with respect to the degradation processes which a carcass will proceed through. Therefore this study aims to provide further information regarding the decomposition processes in Southern Ontario. In addition, there will be a seasonal

variation between the two trials. We hypothesize that the seasonal variation will provide a difference with respect to the fatty acid composition and the extractable lipid phosphate, as the microbial community in the soil will have seasonal variation. We do not believe that the extractable lipid phosphorus will be affected by the change in season, and environment.

Chapter 2

Materials and Methods

2.0 Experimental Site

2.0.1 Summer 2007 Trial

The experiment consisted of two field trials. This first experiment was conducted in a wooded area in Oshawa, Ontario, Canada (43° 54' N 78° 52' W). The soil type at this location was an Alfisol with a loam texture. Soil physicochemical characteristics are presented in Table 1 (see Results section 3.0). The study area was located across a slight (5^{o}) gradient, with some drainage and depression areas. Dominant vegetation at the site included eastern white cedar (*Thuja occidentalis*), maples (*Acer* spp.) and trembling aspen (*Populus tremuloides*), therefore leaving the decomposition area heavily shaded (Figure 3).

Figure 3. Decomposition facility in Oshawa, Ontario, Canada during the summer of 2007

2.0.2 Summer/Fall 2008 Trial

The second and third experiments were conducted in an open field located in Oshawa, Ontario, Canada (Figure 4). The soil type at this location was an Alfisol with a sandy loam texture. The soil physiochemical characteristics are presented in Table 1 (see Results section 3.0).

Figure 4. Decomposition facility in Oshawa, Ontario, Canada during the fall of 2008

2.1 Experimental Design

2.1.1 Trials

Summer 2007

The first decomposition trial took place from 17th June to the 22nd October 2007, lasting 98 days. Sampling occurred every day for the first two weeks, alternating days for the next two weeks, and concluded by sampling once a month until the end of the trial. The sampling regime was more comprehensive compared to a previous trial completed in the same location (Benninger et al., 2008) due to the results from the previous trial showing that a greater number of sampling days could provide much needed insight to the two peak influxes of phosphate nutrients.

Summer 2008

The second decomposition trial commenced on the 17th of June, 2008. Extensive avian scavenging occurred because the carcasses were not sufficiently secured. The repercussions of the scavenging included multiple displacements of the carcasses, thus compromising the trial. The trial finished prematurely on the 31st of June, 2008. Due to the lack of completion within the trial, these samples will not be considered for this project.

Fall 2008

A third decomposition trial was started on the 28th August, 2008, and concluded on the 13th November, 2008, lasting 77 days, and represented the second complete trial for this study. Originally the trial was scheduled to end on the 3rd December, 2008, but

had to be completed earlier due to extensive snowfall. Sampling during this trial took place every day for the first two weeks, alternating days for the next two weeks, and concluded by sampling once a week until the completion of the trial.

2.1.2 Carcasses

Due to ethical restrictions involving the use of human cadavers for research purposes in Canada, pig carcasses were chosen as an acceptable model for decomposition (Schoenly et al., 2006) in this study. Pig carcasses are considered to be a suitable analog to human carcasses because of their similarity in fat distribution, internal organs, size of chest cavity, and the lack of heavy fur (Catts, 1992; Byrd & Castner, 2001). Likewise, humans and pigs share a comparable omnivorous diet, which suggests that they would contain similar gut flora (Anderson and VanLaerhoven, 1996). Five pig carcasses for the summer 2007 trial, each weighing approximately 55 lb, were euthanized by Phenobarbital by a veterinarian at a local pig farm prior to experiment. Five pig carcasses for the fall 2008 trial, each weighing approximately 100 lb, were euthanized via a captive head bolt at a local farm prior to experiment. All carcasses were wrapped in plastic and transferred to the experimental site approximately 1 hour after death.

Summer 2007

Pig carcasses were wrapped in mesh wire (3.81 cm hole diameter) and placed on the soil surface of the forest floor for 96 days as seen in Figure 5.

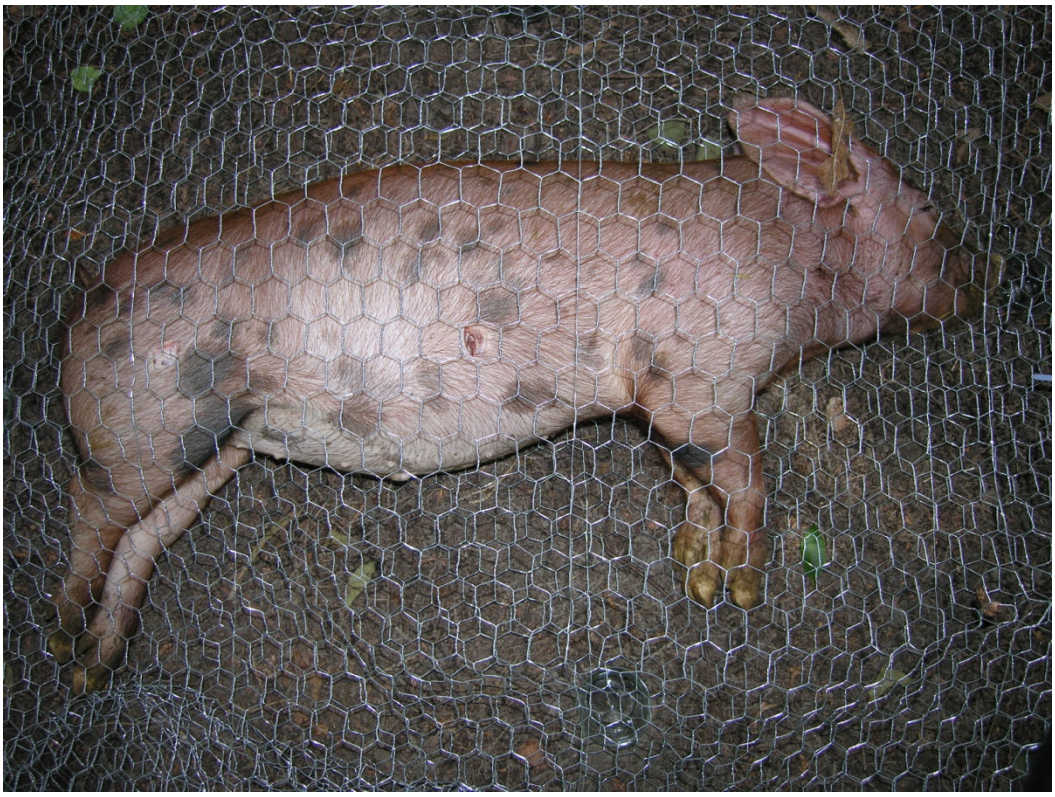

Figure 5. Experimental design of cage for scavenger protection in Oshawa, Ontario, Canada during the summer of 2007

The mesh wire was used to prevent scavenging by large carnivores and to assist with the lifting of the carcass for sampling without disturbing the surface site. Throughout the study period the weather was typical of an Ontario summer, with daily high temperatures averaging approximately 27.16^{o}C for the period when decomposition was most active. Rainfall during this time was minimal (see Figure 9 in Results Section).

The soil samples were collected from three regions for each pig carcass: the head, torso and rear region. During a similar study (Benninger et al., 2008) the experimental design included a control plot for each pig carcass. It was determined that there was no significant difference between each of the control plots. Thus only one communal control

plot was used for the summer 2007 and the fall 2008 trials. Samples were collected from three regions of the control plot, coinciding with the head, torso and rear regions of the decomposition sites. The samples were collected from a depth of approximately 0-2 cm from the surface.

Trowels were used to collect the soil samples in order to minimize the disruption of the carcass and the effect on the decomposition process. The trowels were disinfected with ethanol prior to each sample collection. During sample collection, approximately 50g of soil was obtained from each site and placed into sealable plastic bags. The soil samples were sieved using a 4 mm sieve, placed in sterile glass vials (Fischer Scientific, Canada) and stored in the freezer (-20 °C) until analysis. Due to the large number of samples generated, analysis was only completed on the torso samples, as it was determined that it would be the most representative sample by a previous study (Benninger, et al., 2008).

The samples collected at each site prior to the pigs being placed on the ground are referred to as "reference" samples. The samples that were collected from the communal control site are referred to as "control" samples, and the samples that were collected from the decomposition island of each specimen are referred to as the "gravesoil" samples.

Fall 2008

The experimental design used in the fall 2008 trial was identical to that used in the summer 2007 trial with the exception of the soil weight, and cage design. Approximately 20 g of soil was obtained from each site and placed into sealable plastic bags in the fall trial. The amount of sample taken was reduced in order to minimize the

disturbance of the grave environment. The cage used for scavenging protection differed in this trial, with a bottomless metal and wire cage placed over top of the carcass, thus the carcass was in direct contact with the soil (Figure 6). The carcass had to be shifted slightly in order to collect soil samples. The soil samples were then stored in the freezer (-20 °C) until analysis. As with the summer 2007 samples, analysis was only completed on the torso samples.

Figure 6. Experimental design of cage for scavenger protection in Oshawa, Ontario, Canada during the fall of 2008

Fall 2008 saw a larger abundance of precipitation in the form of rain and snow (see Figure 10 in the Results Section). During the time in which decomposition was the most active, an average daily temperature was seen to be 18.25^{o}C.

2.2 Soil Analysis

The majority of the methods used for soil characterization required that the soil be sub-sampled and dried in an oven at 105^{o}C for 24 hours. Soil samples were characterized for both the woodland and open field sites by electrical conductivity testing, moisture content, pH, total nitrogen and total carbon content.

2.2.1 Electrical conductivity

The electrical conductivity was analyzed on the reference samples only, for both 2007 and 2008 soils. These samples were sent to the University of Guelph Laboratory Services for analysis using an electrical conductivity meter, using a 2:1 water to soil ratio extraction, with the analysis being performed at room temperature on an Orion 4 Star meter (Greenberg et al., 1992). Each analysis was replicated three times.

2.2.2 Moisture content

Moisture content of the soil was measured by weighing 2 g of sieved field fresh soil into foil trays and drying at 105^{o}C for approximately 36 hours. The moisture content was measured for all of the analyzed samples using the following equation:

$$\% \text{ moisture} = \frac{\text{weight of wet soil} - \text{weight of dry soil}}{\text{weight of dry soil}} * 100$$

2.2.3 Soil pH

The pH of the reference soil was measured prior to the pig carcasses being placed on the soil. The summer 2007 trial utilized an in lab method for determining the pH of the soil, by using a 10:1 water to soil ratio and analyzing on a Mettler Toledo Seven Easy bench top pH meter. All soil from 2008 was analyzed for pH via a Kel Instruments (Wycokoff, USA) soil pH Meter. The pH meter allows for onsite analysis, as it is a portable pH probe.

2.2.4 Total carbon (C)

Total carbon was analyzed for all reference samples. These samples were sent to the University of Guelph Laboratory Services for analysis using an Elemental Analyzer (LECO SC444). The total carbon content was determined from the combustion and oxidation of the carbon to carbon dioxide. This is accomplished by burning the sample at

1350 ^{o}C while in a stream of pure O_2. The inorganic carbon was determined by ashing the sample at 475 ^{o}C for four hours prior to analysis (LECO, 1992). The organic carbon content was determined by the following formula:

Organic Carbon = Total Carbon – Inorganic Carbon

Each analysis was replicated three times.

2.2.5 Total nitrogen (N)

Total nitrogen was analyzed for all reference samples. These samples were sent to the University of Guelph Laboratory Services for analysis using an Elemental Analyzer (LECO FP-428) utilizing an adapted Dumas method (Dumas, 1831). Briefly the samples are combusted in a sealed system so that the released nitrogen compounds are reduced to the gaseous N_2, which is measured by a thermal conductivity cell (LECO, Operations). Each analysis was replicated three times.

2.2.6 Soil available phosphorous (P)

Soil available phosphorus refers to the phosphorus in the soil that is soluble for use as a nutrient. Approximately 1 g of oven dried soil was weighed into 15 mL centrifuge tubes. The extracting solution comprised ~0.03 M ammonium chloride (NH_4Cl) for the summer 2007 samples, but was altered to ~0.03 M ammonium fluoride (NH_4F) for the fall 2008 samples. Five (5) mL of concentrated hydrochloric acid (Sigma Aldrich, Canada) was added to the flask, and made to volume with deionized water (Reagent A). The colourimetric reagent was prepared using 0.006 M ascorbic acid, combined with 70 mL of Reagent A, in a 500 mL volumetric flask, and made to volume

with deionized water (Bray & Kurtz, 1945). Reagent A comprised 17.14 g ammonium molybdate A.R [$(NH_4)6Mo_7O_{24}.4H_20$] in 200 mL of warm deionized water; 0.392 g potassium antimonyl tartrate A.R. ($KSbO.C_4H_4O_6$) in 200 mL of water; and 200 mL of concentrated sulphuric acid made up to 2 L with deionized water. Seven (7) mL of the extracting solution was added to the dried samples and vortexed. The tubes were centrifuged at 4000 rpm for 10 minutes. Five hundred (500) uL of the supernatant was collected and placed into a 10 mL borosilicate culture tube. Two (2) mL of the colourimetric reagent was added to the supernatant. After the reagent and solution were mixed, the tubes were left to stand for 30 minutes.

The standards that were used in this study were diluted from a working standard that contained phosphorous at a concentration of 2.50 mg L^{-1}. Phosphorus was obtained from analytical grade potassium dihydrogen orthophosphate (EMD Chemicals, Canada). The total phosphorus concentration was determined by measuring the absorbance on a Genesys Spectrophotometer at a wavelength of 882 nm.

2.2.7 Extractable lipid phosphate analysis

The measurement of lipid-phosphate was part of the focus of this study because lipids are present in large concentrations within the body and as a consequence, are thought to be present in gravesoil due to decomposition. The method used in a similar study (Benninger et al., 2008) was effective, however lacked efficiency and consistency with respect to the analysis time. When determining the amount of phosphorus present in a sample, all forms of phosphate (inorganic and organic) must be converted to orthophosphate for detection. Primarily this is done using an oxidative digestion process,

which can be achieved via oxidizing acids (sulphuric, nitric or perchloric), or persulfate digestions (Frosegard et al., 1991). The primary method for digesting phosphates found in soil is a perchloric acid digestion. Perchloric acid is a highly regulated, dangerous chemical, and thus requires additional safety equipment which is not commonly found in laboratories, and is costly to purchase. Thus an alternative digestion process was created, which utilizes a mixture of sulphuric and nitric acid (5:1 ratio). This combination proved to be worthwhile, however it did lead to inconsistent digestion times causing reduced time efficiency. Thus, the focus of the method development was on the digestion phase, and improving the efficiency for bulk samples.

2.2.7.1 Original Method

Lipid Extraction

To extract and quantify phospholipids in the gravesoils the lipids must first be extracted from the soil (Kates, 1986). Approximately 2 g of soil was weighed into 15 mL centrifuge tubes. A 0.5 mL aliquot of deionized water was added and the sample was vortexed. Two milliliters of solvent I (methanol:chloroform 2:1) was added. The sample was vortexed and another 2 mL of solvent I added. The centrifuge tubes were capped, and allowed to stand at room temperature for two hours, with shaking every 15 minutes. After two hours, samples were vortexed and centrifuged at 2400 rpm for 15 minutes and the supernatant was transferred to a clean 15 mL centrifuge tube using a pasteur pipette. The pellet was then re-suspended in 4 mL of solvent II (methanol:chloroform:deionized water 2:1:0.8). The re-suspended pellets were vortexed

and then centrifuged at 2400 rpm for 15 minutes. The supernatant was combined with the previous extract (Figure 7).

Figure 7. Lipid extraction for the extractable lipid phosphate method (adapted from Kates, 1986)

Phase Separation

After the lipid extraction, the supernatants which contain the phosphorous, must be transferred to an organic solvent (Kates, 1986). Once the supernatants were combined, 2.5 mL of pure HPLC grade chloroform (EMD Chemicals, Canada) and 2.5 mL of 10mM ammonium sulfate (EMD Chemicals, Canada) was added. The glass centrifuge tube was

capped and vortexed to mix, and then centrifuged at 2500 rpm for five minutes. The organic layer of the centrifuged samples was transferred into a 10 mL glass culture tube using a pasteur pipette. The extract was subsequently washed with 2.5 mL of pure HPLC grade chloroform (Sigma Aldrich, Canada), and then centrifuged for five minutes at 2500 rpm. The organic layer was extracted and combined with the previous organic layer. The combined chloroform extracts was evaporated using a ThermoCorp Speed Vac overnight, to ensure complete evaporation of solvent.

Lipid Digestion/Phosphorus Conversion

To be able to quantify the amount of phosphorus present, the lipids must be digested to release the phosphorus, and the phosphorus converted to orthophosphate (Benninger, et al., 2008). Once the chloroform had evaporated, a glass bead was added into the centrifuge tube along with 1.2 mL concentrated sulphuric acid (Sigma Aldrich, Canada), and concentrated nitric acid (Sigma Aldrich, Canada) in a 5:1 ratio. The samples were then placed in a heated digestion block, at a temperature of 130^{o}C until samples turned colourless (12 hours). Once the samples became colourless, 2 mL of 6 M sodium hydroxide (NaOH) (EMD Chemicals, Canada) was added, and the tubes swirled to mix. One (1) mL of the acid-base mixture was added to a 15 mL glass culture tube, and one drop of phenolphthalein ($C_{20}H_{14}O_4$), a pH indicator, was added. The 6 M NaOH solution was added in 500 µL increments until the solution began to turn pink, indicating that it was approaching neutrality. The base was added drop wise until the pink colour remained. The neutral solutions were made to a 10 mL volume with deionized water.

Colourimetric Assay

To quantify the amount of phosphate present in the soil, a colourimetric assay was completed (Kates, 1986). A colourimetric reagent was prepared using 25 mL of 5N sulphuric acid (Sigma Aldrich, Canada), 2.5 mL of 4.11 x 10^{-3} M potassium antimonyl tartrate (EMD Chemicals, Canada), 7.5 mL of 3.24 x 10^{-2} M ammonium molybdate (EMD Chemicals, Canada), and 15 mL of 0.1 M ascorbic acid (EMD Chemicals, Canada). Approximately 1.6 mL of the colourimetric reagent was added to the centrifuge tubes containing the neutral pink solution, and allowed to develop for 10 minutes into a blue colour. The absorbance of the samples was measured using a Genesys 20 Spectrophotometer at a wavelength of 880 nm.

2.2.7.2 Alternative Lipid Digestion/Phosphorus Conversion Techniques

Acid Digestion

70% Nitric Acid

Rather than using a combination of oxidizing acids for determining the amount of phosphate present in the soil, it was thought that a stronger acid could provide a more consistent digestion time. Thus, 1.5 mL of 70% nitric acid was utilized, and heated until the acid became clear and colourless (12 hours). The 70% nitric acid gave inconsistent digestion times with not all solutions being clear and colourless after 12 hours of heating, similar to those found with the original method.

35% Nitric Acid

Since the concentrated nitric acid was not providing consistent digestion times, a diluted concentration was tried instead as it was thought that perhaps the acid was too strong. A 1.5 mL aliquot of 35% nitric acid was utilized, and added to 2 g of soil, and heated until the acid became clear and colourless (12 hours). This trial proved to provide a more consistent digestion time, with the bulk of the samples being completed at the 12 hour mark, and thus could have led to a possible alternative for the method.

Persulfate Digestion

Persulfate digestions are amongst the more common methodologies proposed in literature for the conversion of organic matter (Findlay et al., 1989). While acid digestions require high temperatures, persulfates utilize high temperatures and high pressure, therefore requiring the use of an autoclave. A conventional pressure cooker, traditionally used for cooking, was used to provide the same conditions as an autoclave. To determine that the pressure cooker was able to reach the desired temperature of 120^{o}C, autoclave tape was placed in various positions in the cooker to verify the colour change indicating that the desired temperature was reached.

Potassium Persulfate Trial 1

The initial trial tested 1 mL of a 5 g of potassium persulfate ($K_2S_2O_8$) (Fisher Scientific, Canada) in 100 mL of deionized water solution, which was added to 2 g of soil which had been previously analyzed with the initial method. After placing the tubes in the pressure cooker for an hour, a milliliter of this solution was added to a culture tube, and diluted with 9 mL of deionized water. Following this, the colourimetric assay

described previously was completed. This trial was not successful as there was no colour present following the assay. It was determined that the persulfate solution required neutralization prior to the assay.

Potassium Persulfate Trial 2

This second trial used the 5 g of potassium persulfate ($K_2S_2O_8$) (Fisher Scientific, Canada) in 100 mL of deioinzed water solution for the digestion, which was added to 2 g of soil which had been previously analyzed with the initial method. Two (2) mL of 0.2 M sodium hydroxide (NaOH) (EMD Chemicals, Canada) was added to the sample, after it was heated in the pressure cooker for an hour. One (1) mL of this solution was added to a culture tube, and diluted with 9 mL of deionzied water. Following the dilution, the colourimetric reagent was added and the assay was completed. This trial was not successful as there was no colour change. It was determined that dilute acid needed to be added to the persulfate prior to digestion in order to have complete digestion (Findlay et al., 1989).

Potassium Persulfate Trial 3

In order to achieve the acid persulfate digestion, a solution of 0.45 g of potassium persulfate ($K_2S_2O_8$) (Fisher Scientific, Canada) was dissolved in 100 mL of 0.36 N sulphuric acid (Findlay et al., 1989). One (1) mL of this solution was added to the dried extract and placed in the pressure cooker for 1 hour. Next, 1 mL of 1.0 M sodium hydroxide (NaOH) (EMD Chemicals, Canada) was added, and the tubes swirled to mix. One (1) mL of solution was made to 10 mL with deionized water and transferred to a 15 mL empty glass culture tube. This trial was successful and the solution turned blue,

however it did not provide comparable absorbance readings to the original method. Thus, there was a lack of conversion of the phosphates, but the reason was unknown. There are two possibilities; the first being that the solution was not heated sufficiently. Potassium persulfate begins clear and colourless so there is no marker to know when the digestion is complete, contrasting the acid digestion method, which turns clear when the solution has been completely converted to orthophosphate. The second possibility is that the solution was not neutral.

2.2.7.3 Methodology

Although the third trial of the potassium persulfate digestion was workable, the decision was made to revert to the original method with several variations. The persulfate digestion did provide conversion of phosphate to orthophosphate, however, due to the lowered absorbance reading it was not deemed to be acceptable. Future work on the acidic persulfate digestion could prove to be viable, especially if a phosphate standard could be digested to determine recovery and allow for a better determination for altering the digestion time, and the pH of the solution.

Modifications

Modifications were made to three of the steps; the lipid extraction, the phase separation and the digestion to increase the speed of the assay. In the lipid extraction section, the modification added the use of 15 mL culture tubes rather than Pyrex centrifuge tubes. The samples were dried down for a maximum of 3 hours on the Speed Vac, as opposed to leaving the samples overnight from the previous method. This was changed to decrease the amount of time required to complete the assay. In order to

achieve a faster, and more consistent digestion (100% completion at 12 hours), the samples were digested with 1.5 mL of the mixed acid (Sulphuric:Nitric 5:1) rather than 1.2 mL. Also neutralization was completed with 6.5 M NaOH, rather than 6.0 M.

2.2.8 Fatty Acid Analysis

Fatty Acid Methyl Esters

Due to the lack of volatility of fatty acids, they require modification for analysis. The predominant method of analysis for fatty acids is gas chromatography (Drijber et al., 2000). In order for the fatty acids to be analyzed by gas chromatography, they must be converted to a highly volatile form. This occurs through a derivatization process where the fatty acids are converted to fatty acid methyl esters for analysis. This alternative form is more thermally stable, and is volatile, allowing for analysis to be completed via gas chromatography. As the fatty acids are hydrolyzed, they form a free fatty acid. Once these free fatty acids are reacted with a short chain hydrocarbon alcohol, such as methanol, an esterification reaction will occur resulting in the formation of a fatty acid methyl ester (Figure 8).

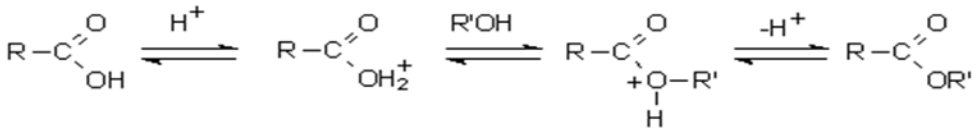

Figure 8. Acid catalyzed reaction for the derivatization of a fatty acid into a fatty acid methyl ester (Adapted from Christie, 1993)

One particular method for hydrolyzing, derivatizing and analyzing fatty acid methyl esters is through an adaptation of a soil extraction method (Drijber et al, 2000). This particular method utilizes a compound called methanolic potassium hydroxide to aid in the extraction of the fatty acids from the soil. This compound through its basicity, acts as a derivatizing agent to promote the esterification of the fatty acid. The fatty acid methyl esters formed are then protonated with acetic acid, which will cause the molecules to become hydrophobic. This allows the compounds to be removed from the soil via extraction into a hexane solution. The hydrophobic fatty acid methyl esters will migrate to the non polar hexane solution from the soil solution containing the polar water molecules, and thus allow for easy extraction. The hexane fatty acid methyl esters mixture is then injected into the gas chromatograph and analyzed. The hexane mixture is analyzed for short chain fatty acid methyl esters, phospholipid-linked fatty acid methyl esters, and ester-linked fatty acid methyl esters. Fatty acid methyl esters can be used to help identify many environmental components, including the relative size of microbial biomass, and rates of mammalian decay.

Fatty acid methyl esters (FAMEs) are used to approximate the health of the microbial community, and thus approximate the nutrient density (Haack et al., 1994). The method used was adapted from a previous method by Drijber et al., (2000). Two and half (2.5) grams of oven dried soil (for 36 hours) was weighed into a 15 mL Pyrex centrifuge tube. Five (5) mL of 0.2 M MeOH-KOH (Fischer Scientific) was added to the soils, vortexed, and placed in a 37 ^{o}C water bath for an hour, with intermittent shaking. After heating, 500 µL of 1 N acetic acid was added, and the solution checked for neutrality. Upon neutralization 2.5 mL of hexane (Fisher Scientific) was added, and the

sample vortexed and centrifuged at 6000 rpm for 10 minutes. The hexane layer was transferred to a borosilicate culture tube, and the residual was washed with another 2.5 mL of hexane by adding additional hexanes to the centrifuge tube, and re-extracting the organic layer. The combined hexane extracts were evaporated using a ThermoCorp Speed Vac for two hours, or until dryness was achieved. The dried samples were reconstituted in 1 mL of hexane (Fisher Scientific), and 10 µL of 0.4 g L^{-1} nonadecanoic acid internal standard was added. The re-dissolved samples were transferred to gas chromatography vials, using a pasteur pipette.

GC-MS Parameters

Separation of the FAMEs was completed by gas chromatography-mass spectrometry (Thermo-Finnigan Trace GC Ultra), with helium as a carrier gas (flow rate of 1.0 mL $minute^{-1}$) on a DB-5 (30 meters, 0.250 mm ID, 0.25µm film thickness) capillary column. Injection (1.0 µL) was completed in split mode with a ratio of 10:1, utilizing a Thermo Triplus autosampler. A pre-injection wash (9.0µL) with hexanes was completed prior to injection of three samples (1.0µL). The temperatures for the injector, transfer line and mass spectrometer were maintained at 240^{o}C, 240^{o}C and 200^{o}C respectively. The oven temperature was ramped from 80^{o}C to 150^{o}C at 12^{o}C per minute followed by an increase of 3^{o}C per minute until a final temperature of 240^{o}C, where the oven was held for 15 minutes. Fatty acid identification was performed by retention time, and confirmed with mass spectrometry using the NIST MS Search 2.0 library. A standard mix was used to determine the retention time, which included 21 fatty acid methyl esters.

2.2.9 Statistical Analysis

All statistical analyses were conducting using VSN International Ltd Genstat Ninth Edition. Analysis of variance between two means was determined by conducting one-way and two-way ANOVAs.

Chapter 3

Results

3.0 Soil and Environmental Conditions

The physicochemical characteristics of the soil were analyzed to determine if there was a different between the conditions of the soil. As indicated in Table 1, the two soils demonstrated similar conditions, when considering the pH, total carbon, total nitrogen, total phosphate and the electrical conductivity. Also there is only a slight variation in the texture of the soil, with the 2007 soil being loam and the 2008 soil being classified as sandy loam.

Table 1: Soil physicochemical characteristics obtained from the Summer 2007 and Fall 2008 Trial

		pH	Total C (%)	Total N (%)	Total P (µg/g)	Electrical Conductivity	Soil Texture
Summer 2007 Wooded Area	**Average**	7.70	5.25	0.27	5.67	1.33	Loam
	Std Error	0.09	0.79	0.01	1.52	0.07	
Fall 2008 Open Field	**Average**	6.2	3.4	0.13	25.83	1.21	Sandy Loam
	Std Error	0.00	0.31	0.02	11.01	0.14	

Throughout the 2007 study period the weather was typical of a Southern Ontario summer, with the temperatures averaging approximately 27.16^{o}C, with minimal rainfall (Figure 9). Throughout the 2008 study period there was a large abundance of precipitation in the form of rain and now (Figure 10). During the time in which decomposition was the most active, an average daily temperature was 18.25^{o}C.

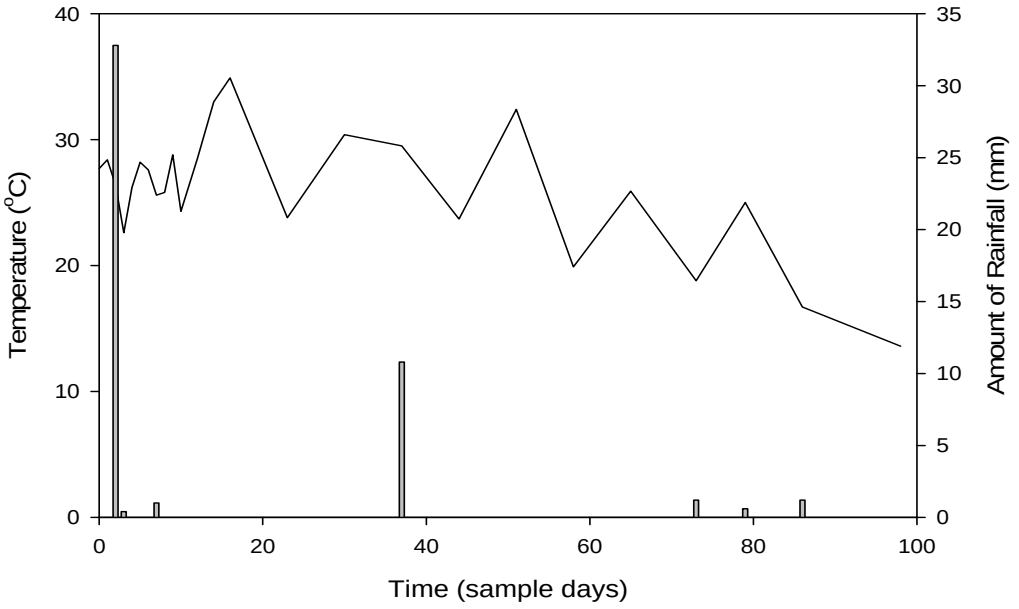

Figure 9. Average temperature and precipitation per sampling day taken from the Buttonville Airport in Oshawa, Ontario, Canada during the summer of 2007

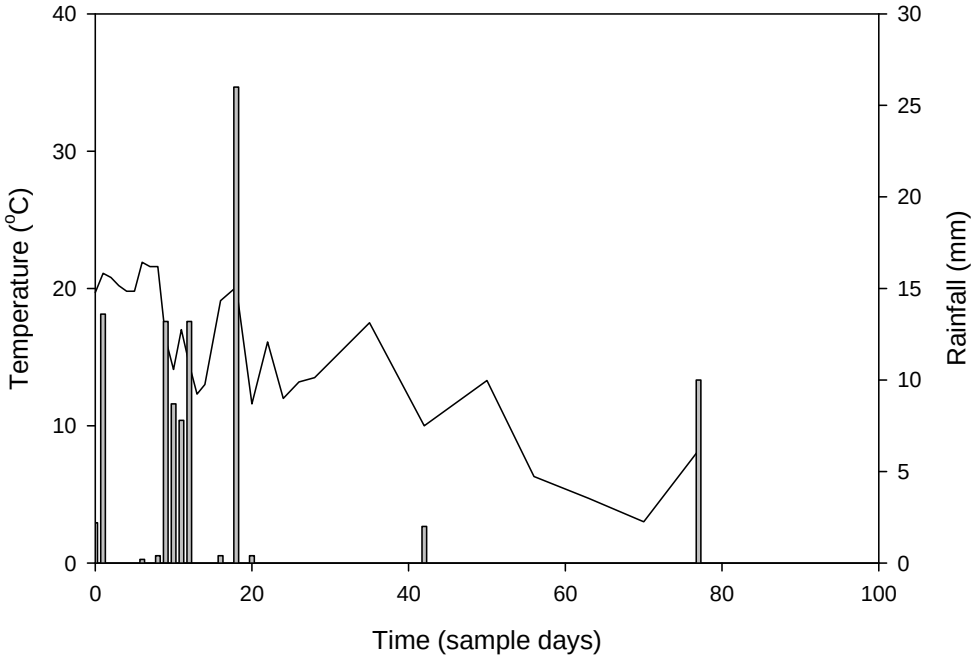

Figure 10. Average temperature and precipitation per sampling day taken from the Decomposition facility in Oshawa, Ontario, Canada during the fall of 2008

3.1 Decomposition Stages

3.1.1 Summer 2007 Trial

Figure 11. Pig (*Sus scrofa*) carcass during the a. fresh b. bloat c. active decay d. advanced decay and e. skeletonization/dry remains decompositions stages following placement on the soil surface in a woodland near Oshawa, Ontario, Canada in summer 2007. Arrows point to the CDI. Maggot masses which are referred to are located in the torso region of the body.

The fresh stage is defined as the initial time frame immediately following death and this stage was only present in the first day of the summer 2007 trial. Fresh stage is characterized by minimal observable changes on the carcass and a lack of macroscopic decomposition (Figure 11a).

Bloat was present from days 1 to 4 of the summer 2007 trial. This stage was characterized by observable discolouration and marbling of the skin (Figure 11b), significant bloating leading to tearing of the skin, and seepage of the decomposition fluids from the orifices.

Active decay was associated with an influx of decomposition fluids into the environment, thus creating a larger CDI (Figure 11c). The carcass had not fully deflated. However further discolouration was observed in comparison to the bloat stage, and there was an abundance of maggot activity.

Advanced decay was characterized by the mummification of tissues and skeletonization of the extremities (Figure 11d). There was little CDI observed, as there was little visible moisture left in the carcass.

Skeletonization occurred by day 14. Skeletonization was characterized by the majority of the bones being exposed and mummified tissue remaining (Figure 11e).

3.1.2 Fall 2008 Trial

Figure 12. Pig (*Sus scrofa*) carcass during the a. fresh b. bloat c. active decay d. advanced decay and e. skeletonization/dry remains decompositions stages following placement on the soil surface in an open field near Oshawa, Ontario, Canada in fall 2008. Arrows point to the CDI. Maggot masses which are referred to are located in the torso region of the body.

The fresh stage did not last longer than the second day of the fall 2008 trial. This stage was characterized by minimal observable changes on the carcass (Figure 12a).

Bloat was observed from days 2 to 5 of the fall 2008 trial. This stage was characterized by observable marbling of the skin (Figure 12b), significant bloating and distension of the abdomen, and seepage of the decomposition fluids from the orifices.

Active decay was associated with an influx of decomposition fluids into the environment, thus creating a larger CDI (Figure 12c). The carcass had deflated, and further discolouration was observed in comparison to the bloat stage. There was a vast amount of maggot activity on the carcass. It was also noted that advanced decay was beginning to occur in the region around the outer limbs.

Advanced decay was characterized by the mummification of tissues and skeletonization of the extremities (Figure 12d). There was still a large CDI observed, however there was little visible moisture left in the body, although it had rained extensively just prior to the photo being taken.

Skeletonization occurred by day 16 in the fall 2008 trial. The remains are visibly skeletal, with only mummified tissue remaining (Figure 12e). Any of the moisture present is due to increased rainfall during this time.

3.2 pH

3.2.1 Summer 2007 Trial

The pH values of the control soil and the gravesoils were analyzed to determine if there was a statistically significant change in soil pH during the decomposition of a pig

carcass. Gravesoil pH was significantly (p=0.030) higher than the control soil pH on days 16, 51 and 86 (Figure 13). However, visually the pH values remain relatively constant, with minimal fluctuations.

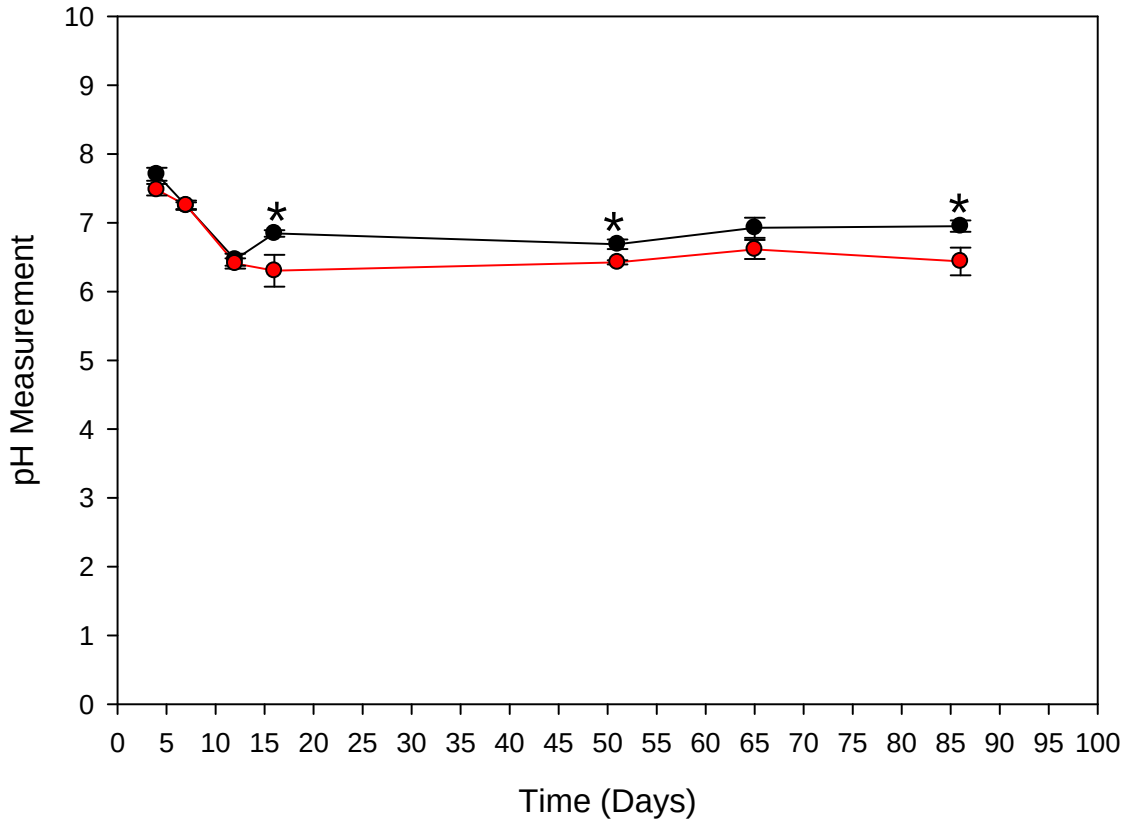

Figure 13. pH measurement of gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of a woodland near Oshawa, Ontario, Canada during summer 2007. *=least significant difference < 0.05 (unbalanced ANOVA, $F_{Type\ of\ Soil\ (1,\ 37)}$=5.09, p=0.030, $F_{Sample\ Day\ (1,\ 37)}$=13.55, p<0.001, $F_{int.\ (1,\ 37)}$=3.04, p=0.016)

3.2.2 Fall 2008 Trial

The fall 2008 trial demonstrated that there was only one individual point which reflected a difference as depicted in Figure 14. Day 77 not only exhibits significant difference from the gravesoil, but also from the control soils surrounding this sampling

day. Both gravesoils remained visually constant throughout the entire trial, excluding the aforementioned day.

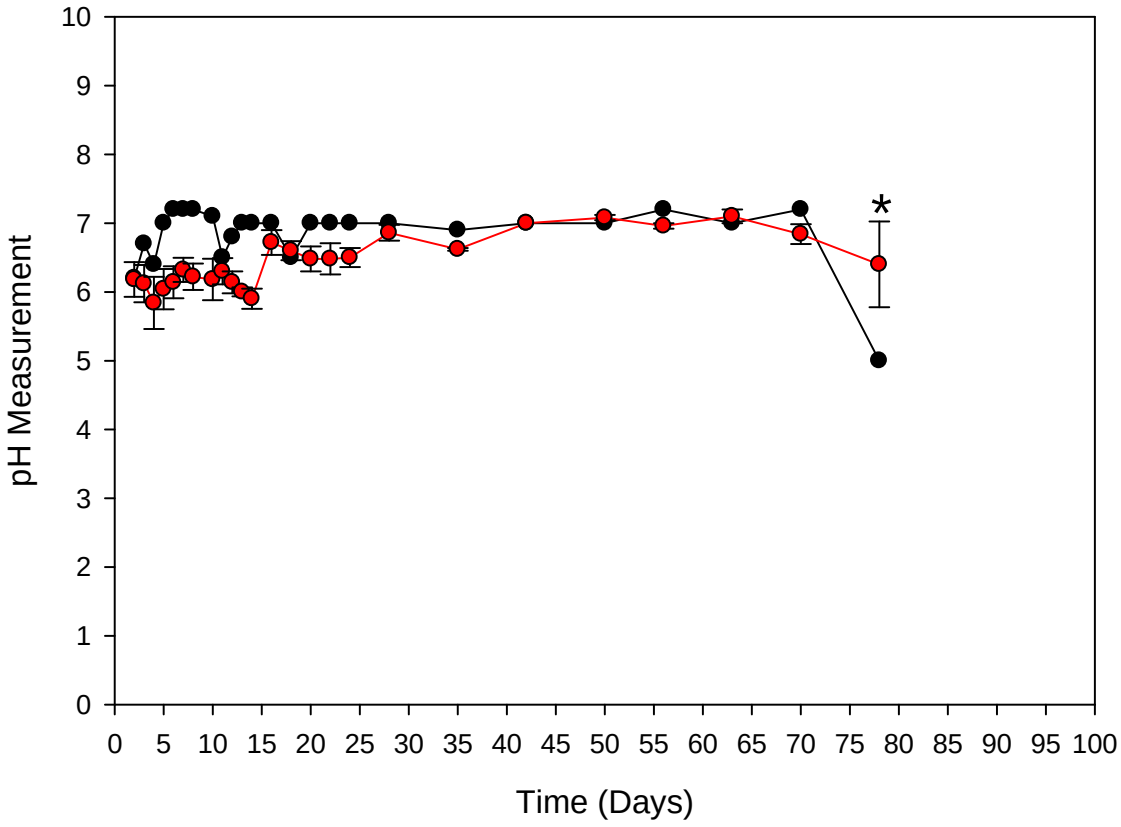

Figure 14. pH measurement of gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of an open field near Oshawa, Ontario, Canada during fall 2008. *=least significant difference < 0.05 (unbalanced ANOVA, $F_{Type\ of\ Soil\ (1,\ 77)}$=13.28 , p<0.001, $F_{Trial\ Day\ (1,\ 77)}$=2.68, p<0.001, $F_{int.\ (1,\ 77)}$=0.96, p=0.522)

3.3 Moisture Content

3.3.1 Summer 2007 Trial

The determination of moisture content was required for the lipid phosphate analysis and was therefore measured for all control and gravesoil samples as per the method described in the materials and methods section (Kates, 1986). As displayed in

Figure 15, days 0, 4 and 65 had significantly higher moisture content for the gravesoil in comparison to the control soil, with day 7 having a statistically (LSD<0.05) significant difference between the control soil and gravesoil. Day 37 showed a large increase in both the control soil and the gravesoil. With the exception of the aforementioned increase, the moisture content remained relatively constant for both the control and gravesoil throughout the study period on the days which were sampled.

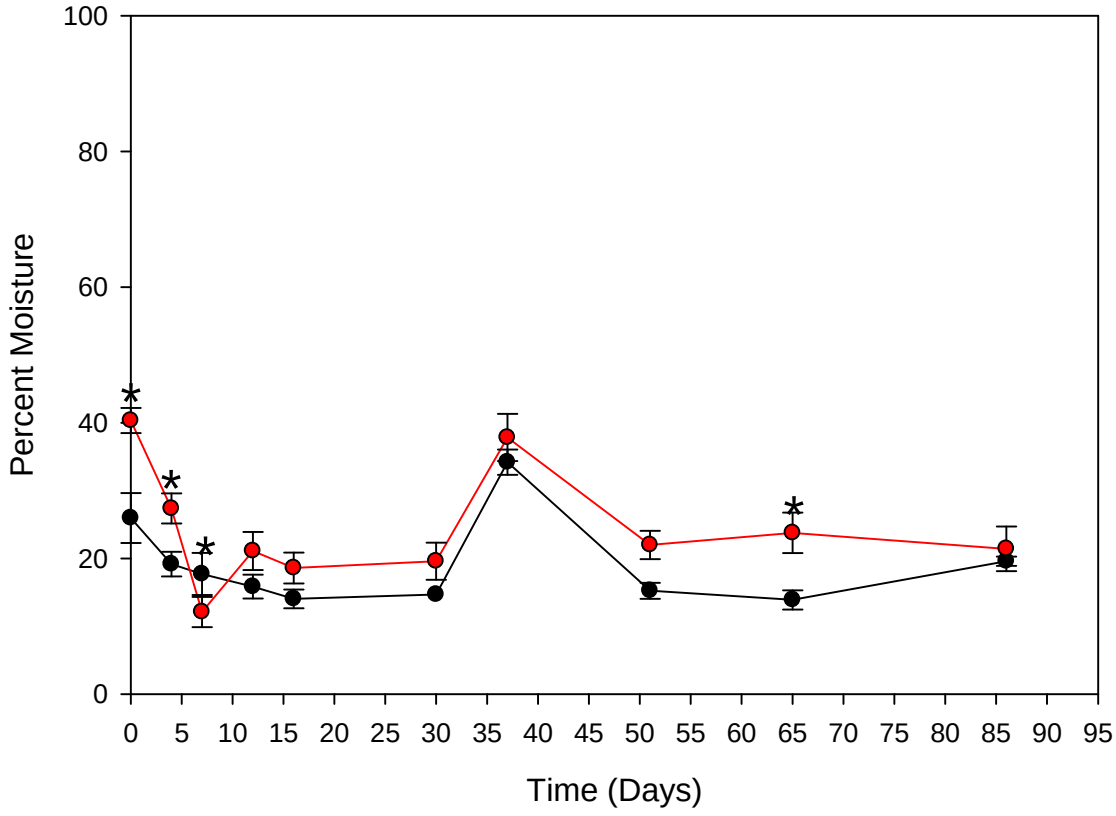

Figure 15. Moisture content of gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of a woodland near Oshawa, Ontario Canada during summer 2007. *=least significant difference < 0.05 (unbalanced ANOVA, $F_{Trial\ Day\ (1,\ 95)}$=8.24, p<0.001, $F_{Soil\ Type\ (1,\ 95)}$=28.63, p<0.001, $F_{int.\ (1,\ 95)}$=0.56, p=0.880)

3.3.2 Fall 2008 Trial

Fall 2008 sampling day 8 demonstrated significantly higher moisture content in the gravesoil than the respective control soil, with day 12 control soil being larger than the gravesoil (Figure 16). As demonstrated in Figure 16 both the gravesoil and the control soils moisture content remained relatively constant throughout the entire trial.

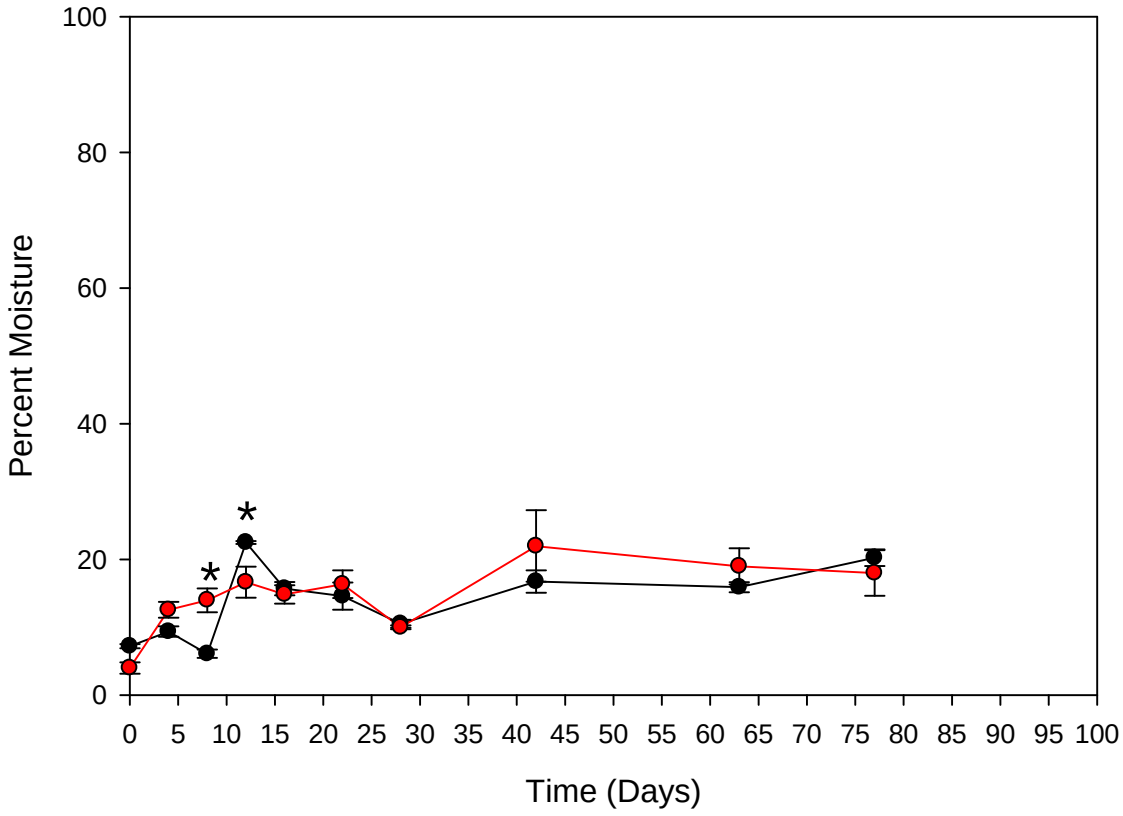

Figure 16. Moisture content of gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of an open field near Oshawa, Ontario, Canada during fall 2008. *=least significant difference < 0.05 (unbalanced ANOVA, $F_{\text{Trial Date (1,59)}}$ =8.16, p<0.001, $F_{\text{Soil Type (1, 59)}}$ =0.61, p<0.438, $F_{\text{int. (1, 59)}}$ =1.45, p=0.186)

3.4 Lipid Phosphate

3.4.1 Summer 2007 Trial

The extractable lipid phosphate content was measured as a secondary measure for estimating the microbial biomass in the soil, with FAMEs being the primary method. During summer 2007, days 7 and 86 were associated with a statistically (LSD<0.05) significantly higher concentration of lipid phosphate in the gravesoil compared to the control soil (Figure 17). Visually it can be seen that there is an increase in the abundance of extractable lipid phosphate content on days 32 and 52, however these are not statistically significant.

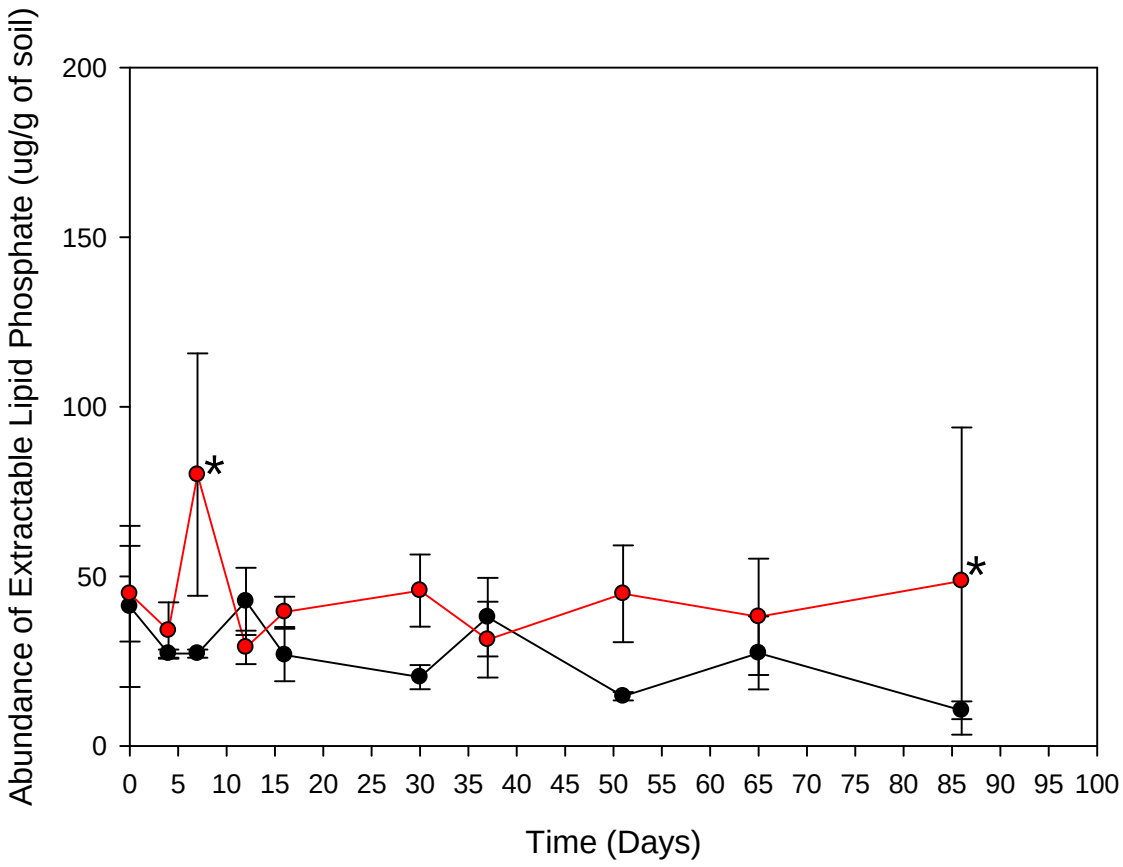

Figure 17. Lipid phosphate content of gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of a woodland near Oshawa, Ontario, Canada during summer 2007. *=least significant difference < 0.05 (unbalanced ANOVA, $F_{\text{Trial Day (1, 53)}}$=0.54, p=0.841, $F_{\text{Soil Type (1, 53)}}$=4.03, p=0.050, $F_{\text{int. (1, 53)}}$=0.68, p=0.726)

3.4.2 Fall 2008 Trial

The extractable lipid phosphate content for 2008 demonstrated a dual peak trend, with the first peak having two significant points (days 8 and 12), with the second peak at day 28 (Figure 18). The control soil values remained constant, with the exception of day 24. After day 40 the extractable lipid phosphate relative concentration returned to basal levels comparable to the control soil values.

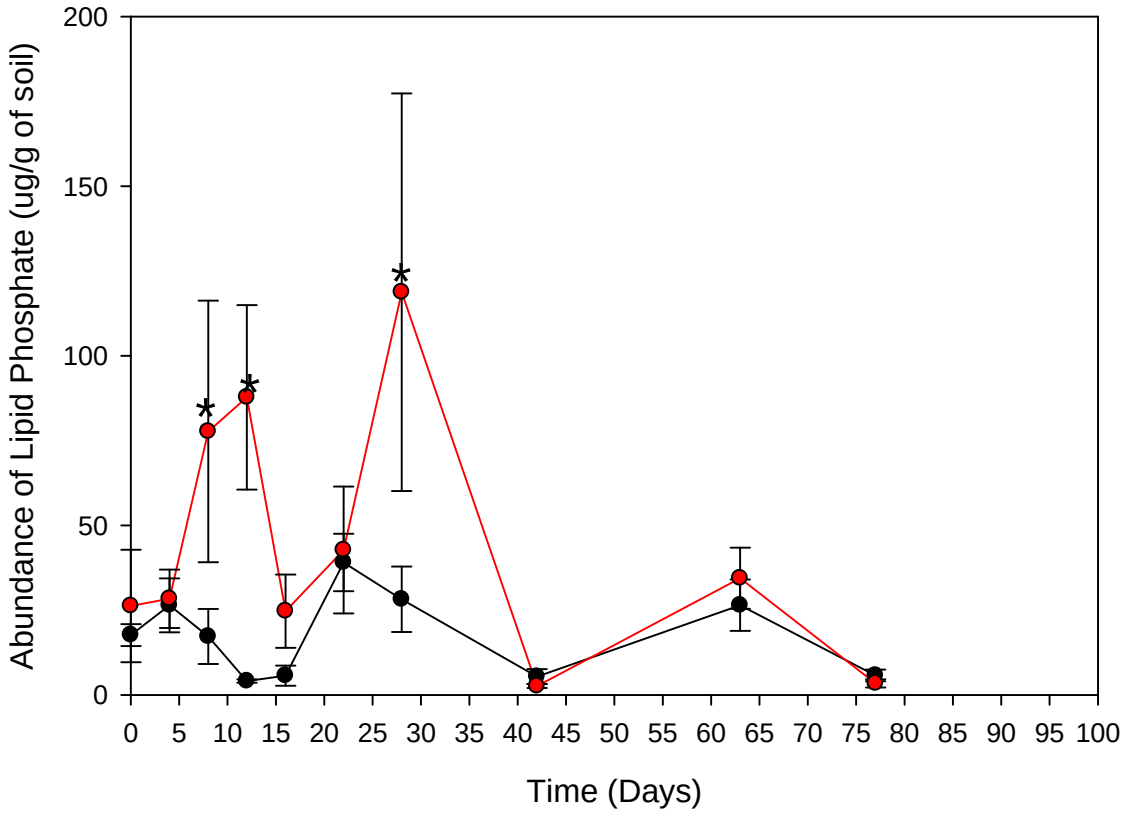

Figure 18. Lipid phosphate of gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of an open field near Oshawa, Ontario, Canada during fall 2008. *=least significant difference < 0.05 (unbalanced ANOVA, $F_{\text{Trial Day (1, 59)}}$ =2.57, p=0.014, $F_{\text{Soil Type (1, 59)}}$ =7.28, p<0.009, $F_{\text{int. (1, 59)}}$ =1.36, p=0.228)

3.4.3 Comparison of Inter-Year Soil Extractable Lipid-Phosphate

The extractable lipid phosphate concentration was compared between the two trials to determine if seasonal variation and soil variation would have an effect upon the results (Figure 19). When comparing the two seasons, the first peak in 2007, at approximately day 7, is similar to the beginning of a peak in the 2008 trial. Also, the second peak in 2008 trial, approximately day 30, when comparing to the 2007 trial, there

is another slight increase in 2007 around the same time. Although the 2008 trail does return to basal levels, the 2007 trial does not.

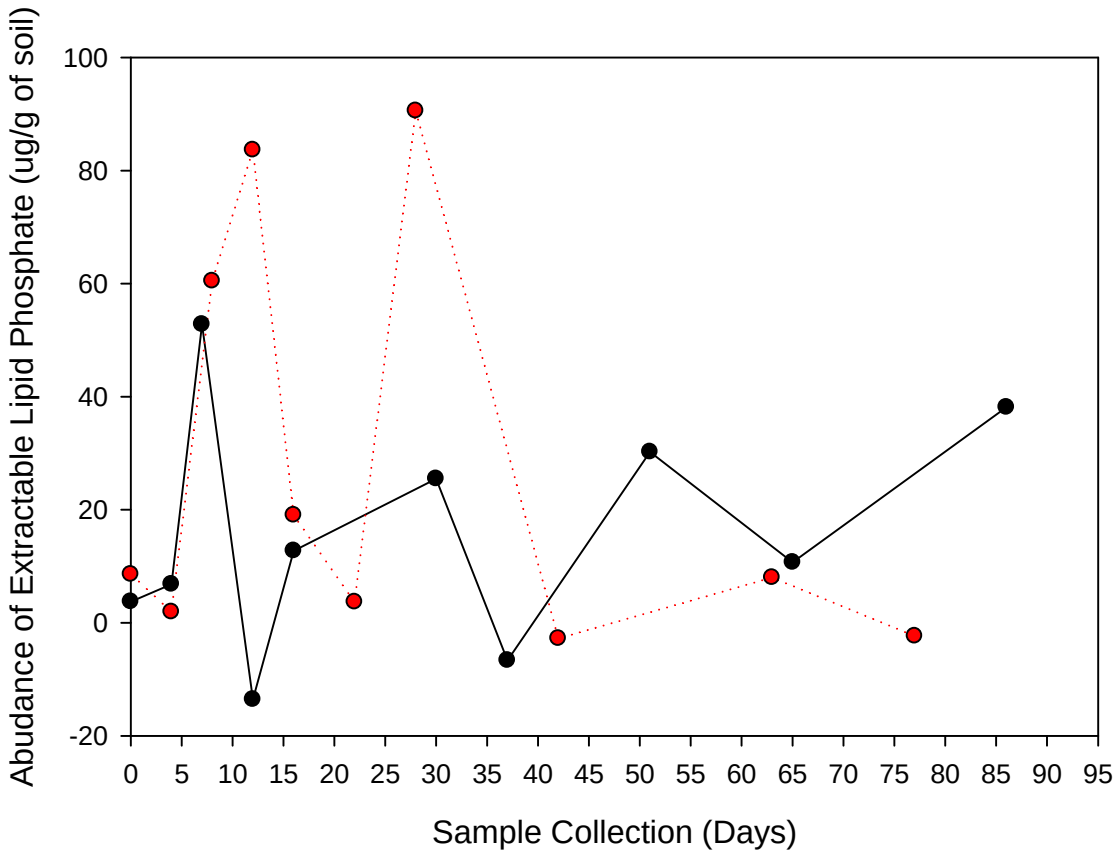

Figure 19. Background subtracted values of extractable lipid phosphate between the 2007 (●) and 2008 (●) gravesoil following the placement of a pig (*Sus scrofa*) carcass on the soil surface near Oshawa, Ontario, Canada

3.5 Soil Available Phosphorus

3.5.1 Summer 2007 Trial

Soil available phosphorus content was studied as it measures the capability of the soil as a nutrient source. Previous studies have shown that phosphorus content will increase with the presence of a decaying carcass (Stokes et al., 2008). The 2007 summer

trial exhibited two key peaks, with gravesoil phosphorus remaining elevated until the conclusion of the trial. As seen in Figure 20, there was a significantly ($p<0.001$) greater relative concentration of soil available phosphorus in gravesoil beginning at day 6 and continuing until day 86. The two large peaks were observed at days 12 and 56.

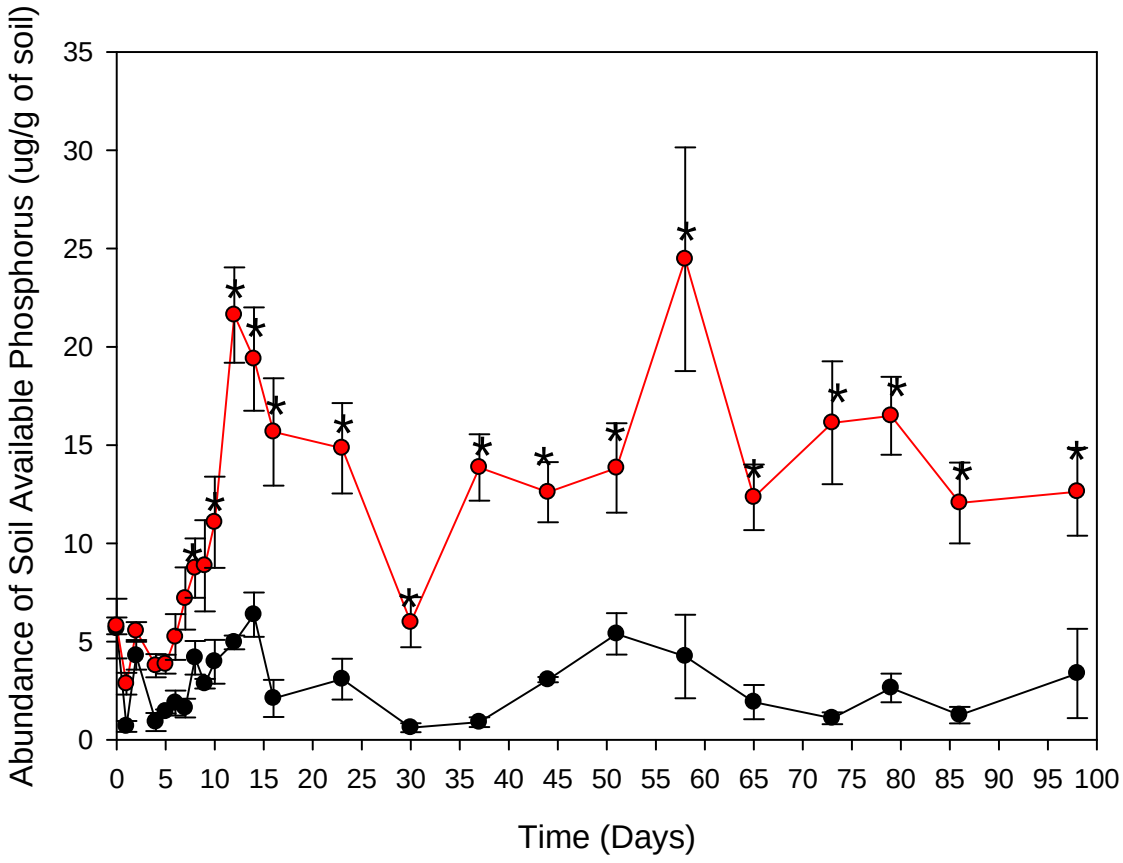

Figure 20. Soil available phosphorus content of gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of a woodland near Oshawa, Ontario, Canada during summer 2007. *=least significant difference < 0.05 (unbalanced ANOVA, $F_{\text{Trial Date }(1,\,322)} = 9.76$, $p<0.001$, $F_{\text{Soil Type }(1,\,322)} = 119.61$, $p<0.001$, $F_{\text{int. }(1,\,322)} = 1.93$, $p=0.007$)

3.5.2 Fall 2008 Trial

The soil available phosphorous levels in the control soils did not remain constant throughout the trial (Figure 21), thus the trend of the gravesoil is not clear. However, there are numerous days which demonstrated significantly ($p=0.024$) larger

concentrations of soil available phosphorous in the gravesoil than the control soil. There were 14 of the 29 days which had individual significance when compared to the control soils; days 0, 6, 8, 9, 11, 12, 13, 14, 16, 20, 24, 26, 42 and 56.

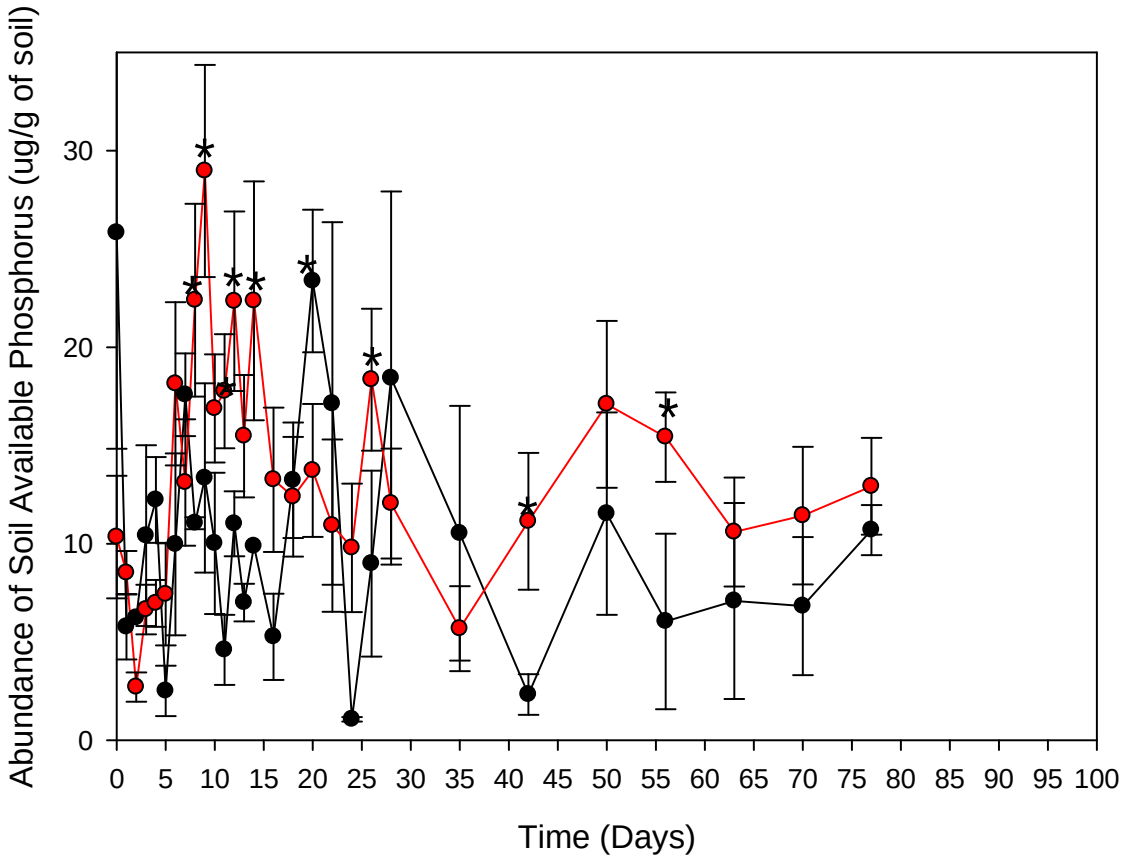

Figure 21. Soil available phosphorus of gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of an open field near Oshawa, Ontario, Canada during fall 2008. *=least significant difference < 0.05 (unbalanced ANOVA, $F_{\text{Soil Type (1, 332)}} = 5.12$, $p = 0.024$, $F_{\text{Trial Day (1, 332)}} = 2.74$, $p < 0.001$, $F_{\text{int. (1, 346)}} = 1.09$, $p = 0.346$)

3.4.3 Comparison of Inter-Year Soil Available Phosphorus Variation

When comparing the overall seasonal effect on the soil available phosphorus content, a difference between the summer 2007 and the fall 2008 season was observed (Figure 22). The summer 2007 trial had less variability throughout the duration of the sampling days, in comparison to the fall 2008. Although the comparison is not explicitly

clear, there is a similar increase in both trials at days 15, around day 35 and at day 55. Importantly, both sample sets exhibited increases in the concentration of soil available phosphorus with the addition of a carcass.

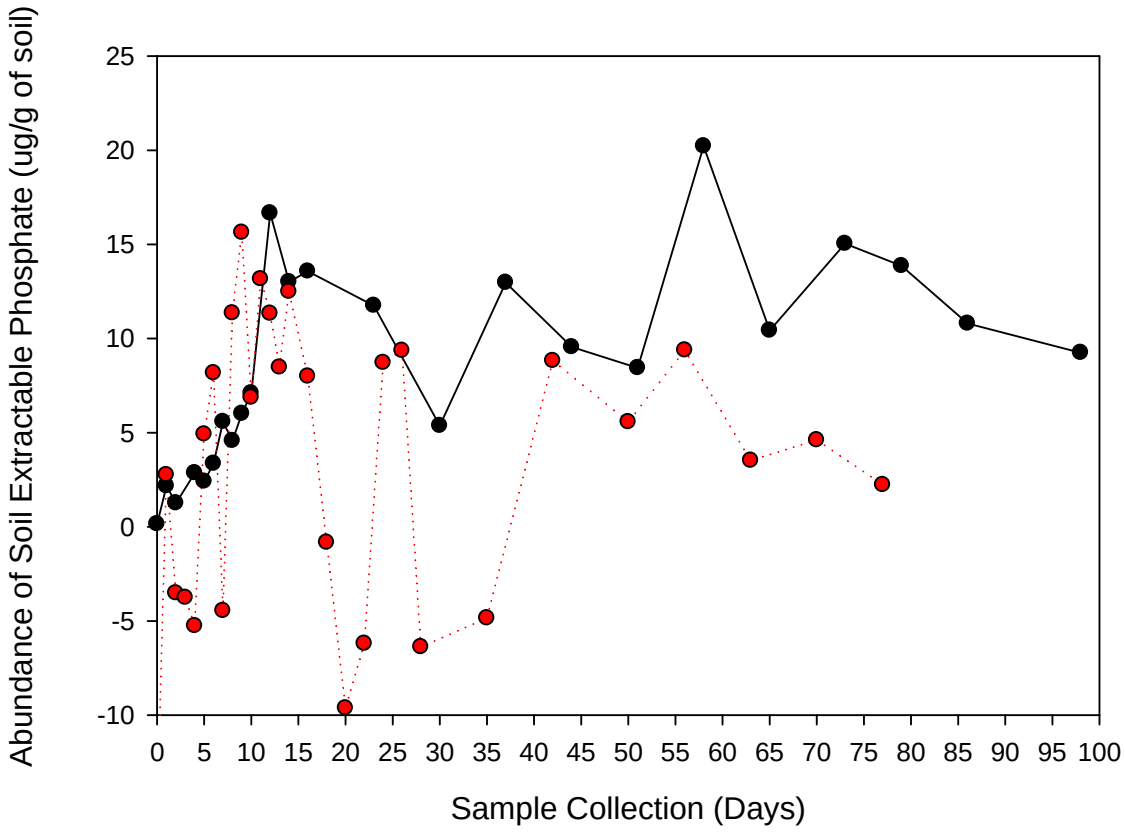

Figure 22. Background subtracted values of extractable lipid phosphate between the 2007 (●) and 2008 (●) gravesoil following the placement of a pig (*Sus scrofa*) carcass on the soil surface near Oshawa, Ontario, Canada

3.6 Fatty Acids

The analysis of the soil samples involved identifying 17 fatty acids, ranging from C8:0 – C22:0, however only 5 were considered of interest to this study because they have demonstrated significance in prior research (Forbes et al., 2004), and tend to be stable for longer periods of time. The five fatty acids were myristic acid (C14:0), palmitic acid

(C16:0), heptadecanoic acid (C17:0), stearic acid (C18:0), and oleic acid (C18:1) as seen in the sample gas chromatograph septrum (Figure 23). Similar fatty acids were found in the gravesoils as demonstrated by Figure 24.

3.5.1 Sample Gas Chromatography Spectra

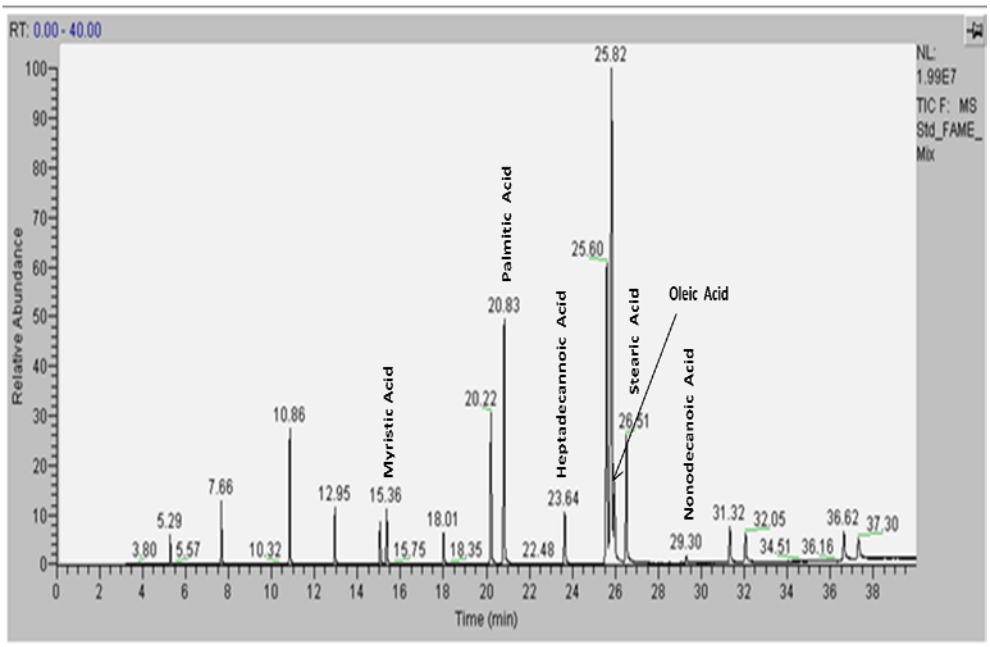

Figure 23. Total ion count spectrum obtained from the gas chromatographer for the standard FAME mix indicating the presence of myristic acid methyl ester, palmitic acid methyl ester, heptadecanoic acid methyl ester, oleic acid methyl ester, stearic acid methyl ester, and nonadecanoic acid methyl ester

Figure 24. Total ion count spectra obtained from the gas chromatographer for a representative sample indicating the presence of myristic acid methyl ester, palmitic acid methyl ester, heptadecanoic acid methyl ester, oleic acid methyl ester, stearic acid methyl ester, and nonadecanoic acid methyl ester

3.6.2 Myristic Acid (C14:0)

Summer 2007 Trial

Myristic acid was analyzed in the form of myristic acid methyl ester, as the derivatized form has greater volatility. Both the control soils and the gravesoil were analyzed using GC-MS. There was a greater relative concentration of myristic acid noted in individual sample days; 1, 9, 10, 12, 14, 37 and 79. A small dual peak was observed, with the first peak starting at day 14, and the second peak starting at day 37 (Figure 25).

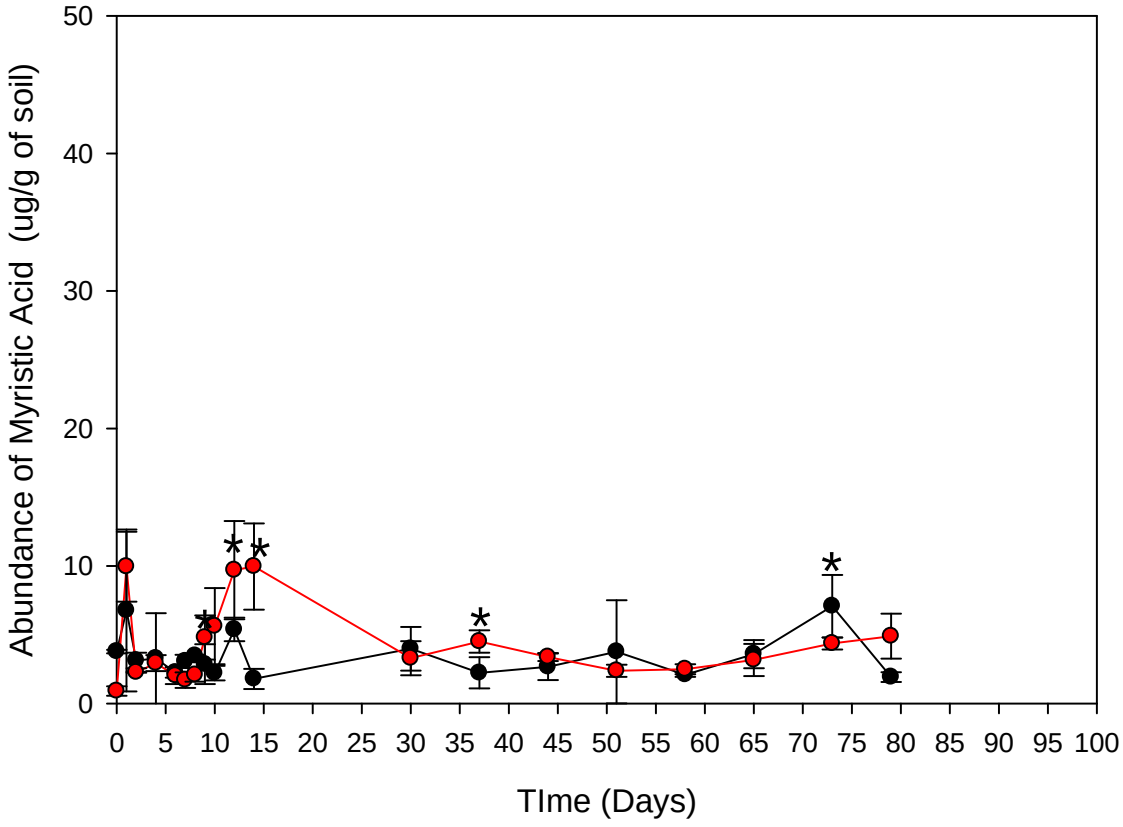

Figure 25. Myristic acid content in gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of a woodland near Oshawa, Ontario, Canada during summer 2007. *=least significant difference < 0.05 (unbalanced ANOVA, $F_{Trial\ Day\ (19,\ 109)}$=2.40, p=0.002, $F_{Soil\ Type\ (1,\ 109)}$=1.42, p=0.235, $F_{int.\ (18,\ 109)}$=0.98, p=0.492)

Fall 2008 Trial

The fall 2008 trial showed a very visible dual peak trend, with the first peak starting at day 35, with two prior days (24 and 28) also being significantly different to the control (Figure 26). The second peak is present at day 70. Day 56 has a significantly (LSD<0.05) larger control mean than the gravesoil mean however it is also significantly larger than the adjacent control means. While control peaks 16 and 18 are not significantly larger than that of the gravesoil values, they are associated with large error and are also not consistent with the surrounding control values.

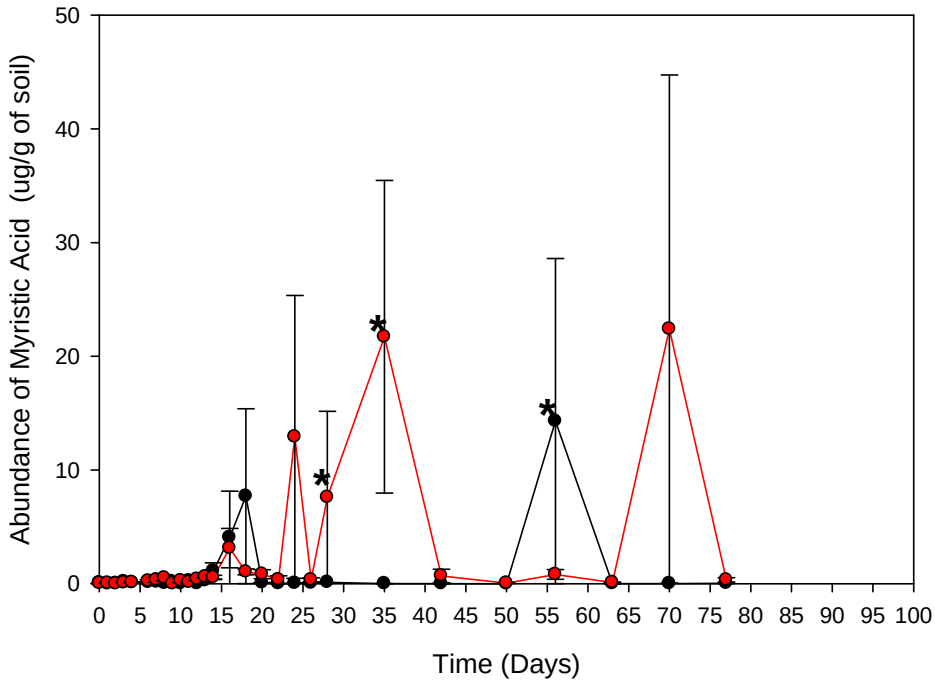

Figure 26. Myristic acid content in gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of an open field near Oshawa, Ontario, Canada during fall 2008. *=least significant difference < 0.05 (unbalanced ANOVA $F_{trial\ day(28,\ 128)}$=0.83, p=0.715, $F_{Soil\ Type\ (1,\ 128)}$=1.32, p=0.253, $F_{int.\ (26,\ 128)}$=0.58, p=0.947)

Comparison of Inter-Year Soil Myristic Acid Variation

As the two trial locations contained varying soil types, and also took place during different seasons, identifying potential differences between the two trials is an essential part of this study. Myristic acid did not show any trends between the two seasons when the control values were subtracted from the gravesoil (Figure 27).

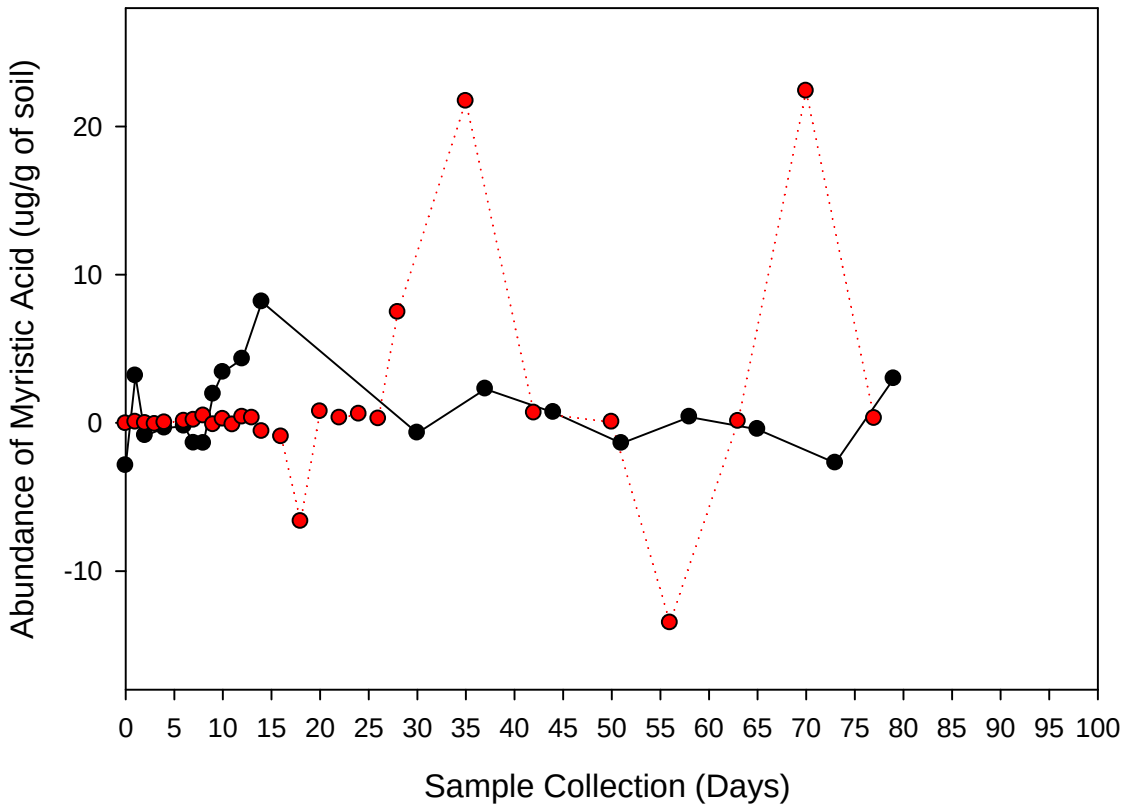

Figure 27. Background subtracted values of myristic acid between the 2007 (●) and 2008 (●) gravesoil following the placement of a pig (Sus scrofa) carcass on the soil surface near Oshawa, Ontario, Canada

3.6.3 Palmitic Acid (C16:0)

Summer 2007 Trial

In Figure 28, it can be seen that there are two peaks of increased concentration in the palmitic acid concentration, one occurred at the beginning of the trial, and the second occurred toward the end of the trial. The individual days which were significant from their respective control values include days 1, 4, 6, 10, 44, 51, and 58. In addition, days 0 and 30 had a larger control value than gravesoil value, and they were significantly (p=0.012) larger from that of the control values in the surrounding sample days.

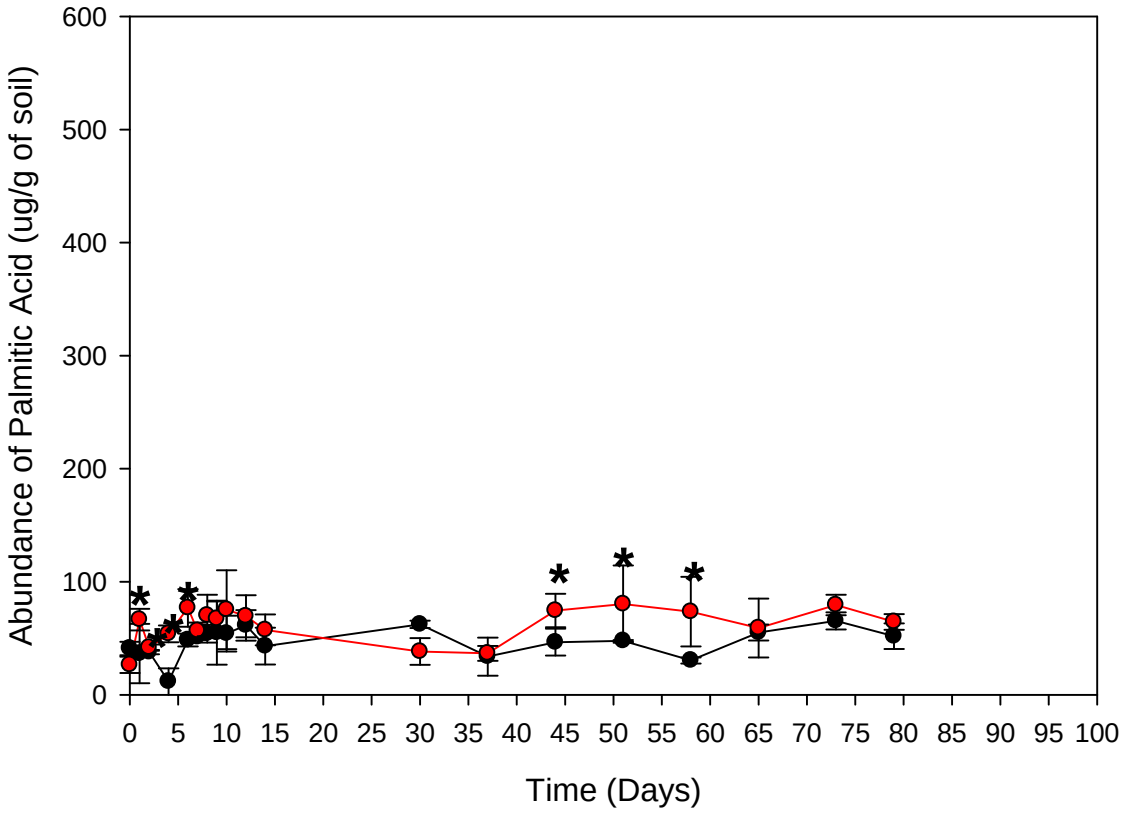

Figure 28. Palmitic acid content of gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of a woodland near Oshawa, Ontario, Canada during summer 2007 *=least significant difference < 0.05 (unbalanced ANOVA, $F_{\text{Trial Day (19, 109)}}$ =1.03 p=0.431, $F_{\text{Soil Type (1, 109)}}$ =6.48, p=0.012, $F_{\text{int. (18, 109)}}$ =0.51, p=0949)

Fall 2008 Trial

The fall 2008 trial had two prominent peaks (Figure 29), with the two apexes at days 35 and 70, respectively. The apex at 35 was preceded by one other significant day (LSD<0.05) (28) with these concentrations being higher than the respective control days.

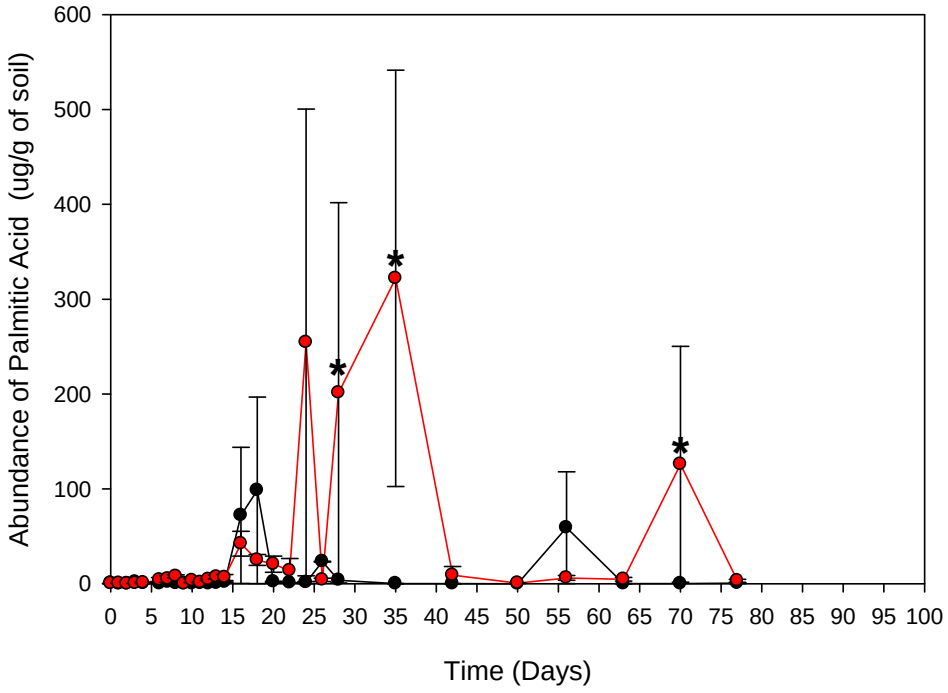

Figure 29. Palmitic acid content in gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of an open field near Oshawa, Ontario, Canada during fall 2008. *=least significant difference < 0.05 (unbalanced ANOVA $F_{\text{trial day (28, 128)}}$ =0.98, p=0.508, $F_{\text{Soil Type (1, 128)}}$ =1.81, p=0.181, $F_{\text{int. (26, 128)}}$ =0.58, p=0.944)

Comparison of Inter-Year Soil Palmitic Acid Variation

A comparison of the inter-year data (Figure 30) demonstrates that there are no similarities in the peaks seen in each individual year. However, the soils do responded to the addition of a carcass to the soil surface. Each season demonstrated similar increases in relative concentration on particular sampling days.

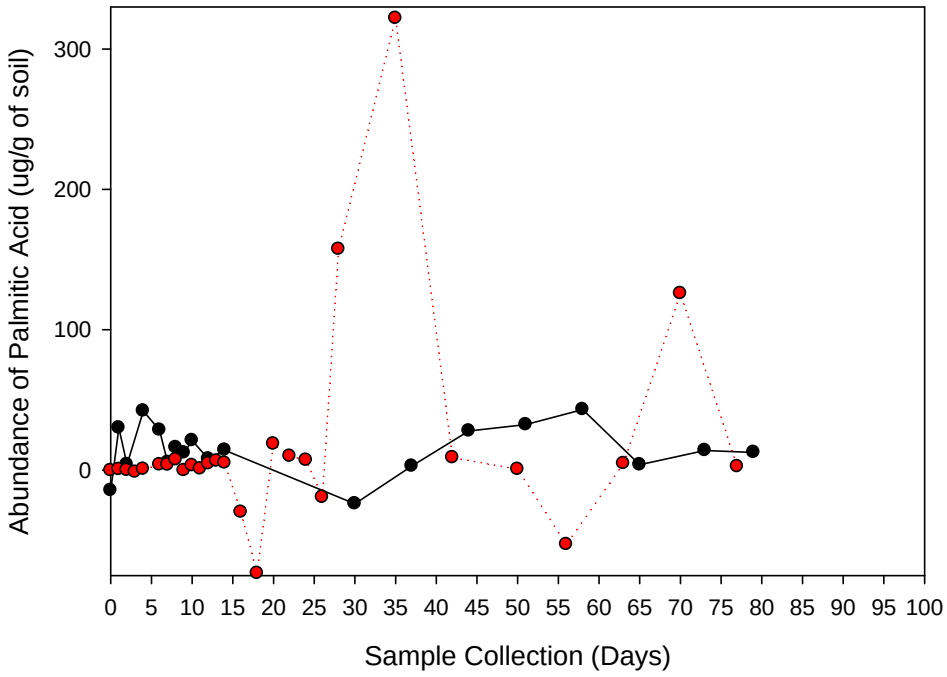

Figure 30. Background subtracted values of palmitic acid between the 2007 (●) and 2008 (●) gravesoil following the placement of a pig (*Sus scrofa*) carcass on the soil surface near Oshawa, Ontario, Canada

3.6.4 Heptadecanoic Acid (C17:0)

Summer 2007 Trial

The concentration of heptadecanoic acid in gravesoil (Figure 31) was greater after day 35 and did not return to basal levels by the end of the study. The control soils and gravesoil remained relatively constant during the early to active stages of decomposition.

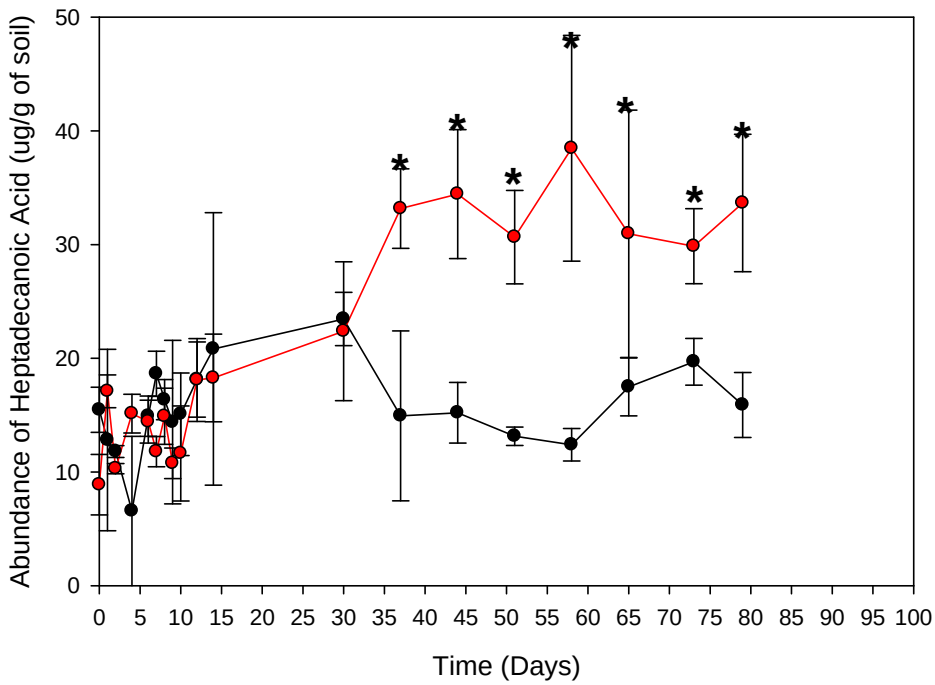

Figure 31. Heptadecanoic acid content of gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of a woodland near Oshawa, Ontario, Canada during summer 2007. *=least significant difference < 0.05 (unbalanced ANOVA, $F_{Trial\ Day\ (19,\ 109)}$=3.70 , p<0.001, $F_{Soil\ Type\ (1,\ 109)}$=13.63, p<0.001, $F_{int.\ (18,\ 109)}$=2.45, p=0.002)

Fall 2008 Trial

Fall 2008 displayed the dual peak trend with some gravesoil values being higher than that of the control values: day 35 and day 70 had a significantly greater concentration (LSD<0.05) of heptadecanoic acid. As demonstrated by Figure 32, two days (16 and 18) do have significantly larger control means compared to the gravesoil means. They also possess significantly larger control means than their adjacent control means.

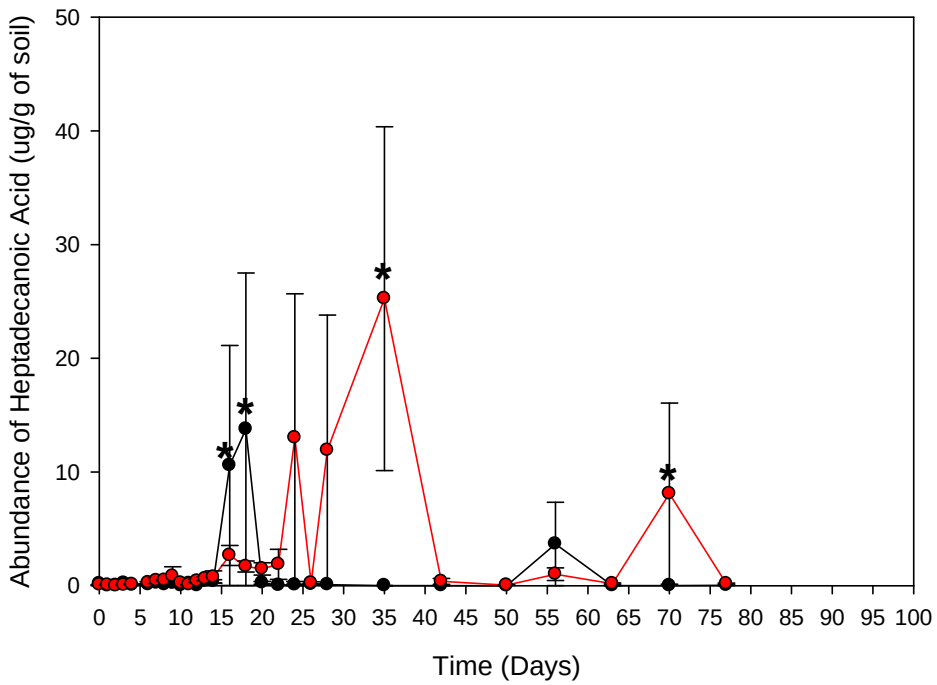

Figure 32. Heptadecanoic acid content in gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of an open field near Oshawa, Ontario, Canada during fall 2008. *= least significant difference < 0.05 (unbalanced ANOVA $F_{trial\ day(28,\ 128)}$=1.02, p=0.463, $F_{Soil\ Type\ (1,\ 128)}$=1.01, p=0.297, $F_{int.\ (26,\ 128)}$=0.72, p=0.835)

Comparison of Inter-Year Soil Heptadecanoic Acid Variation

When comparing both trials heptadecanoic acid content, it can be seen that there is a difference in the trends exhibited (Figure 33). The two years did not show a similar trend, and only showed a few similar peaks.

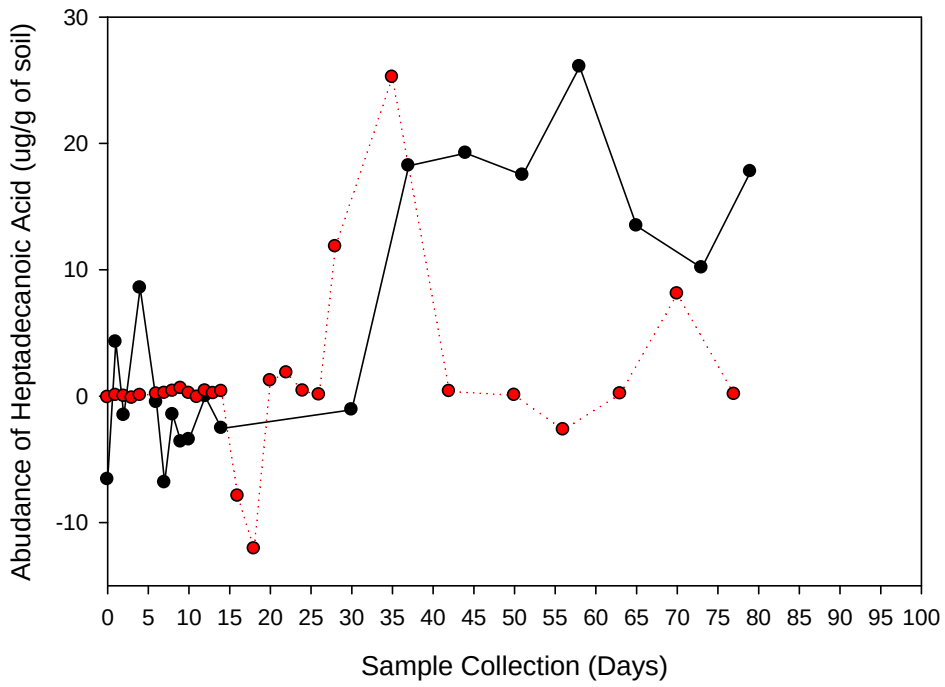

Figure 33. Background subtracted values of heptadecanoic acid between the 2007 (●) and 2008 (●) gravesoil following the placement of a pig (*Sus scrofa*) carcass on the soil surface near Oshawa, Ontario, Canada

3.6.5 Oleic Acid (C18:1)

Summer 2007 Trial

The oleic acid relative concentration in the summer 2007 trial was associated with three peaks, the first peak apex occurred at day 12, the second peak occurred at day 44, and the third major peak occurred at day 58 (Figure 34). Although day 79 does represent a significant (LSD<0.05) relative concentration when comparing the gravesoil to the control soil, the soils appear to return to basal levels.

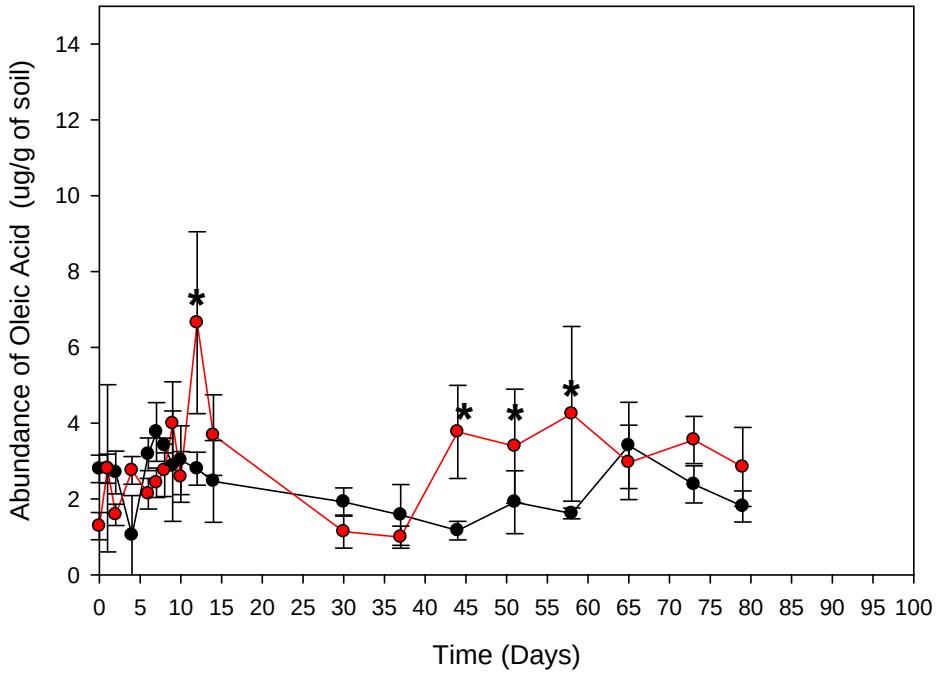

Figure 34. Oleic acid content of gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of a woodland near Oshawa, Ontario, Canada during summer 2007. *= least significant difference < 0.05 (unbalanced ANOVA, $F_{Trial\ Day\ (19,\ 109)}$=1.45 , p=0.120, $F_{Soil\ Type\ (1,\ 109)}$=1.76, p=0.187, $F_{int.\ (18,\ 109)}$=1.11, p=0.356)

Fall 2008 Trial

The oleic acid concentration for the fall 2008 trial also had the triple peak seen during the summer 2007 trial (Figure 35). The initial cycle began at day 24, a secondary peak occurring at day 35. The third peak occurred at day 70. The control samples demonstrated some fluctuations throughout the trial.

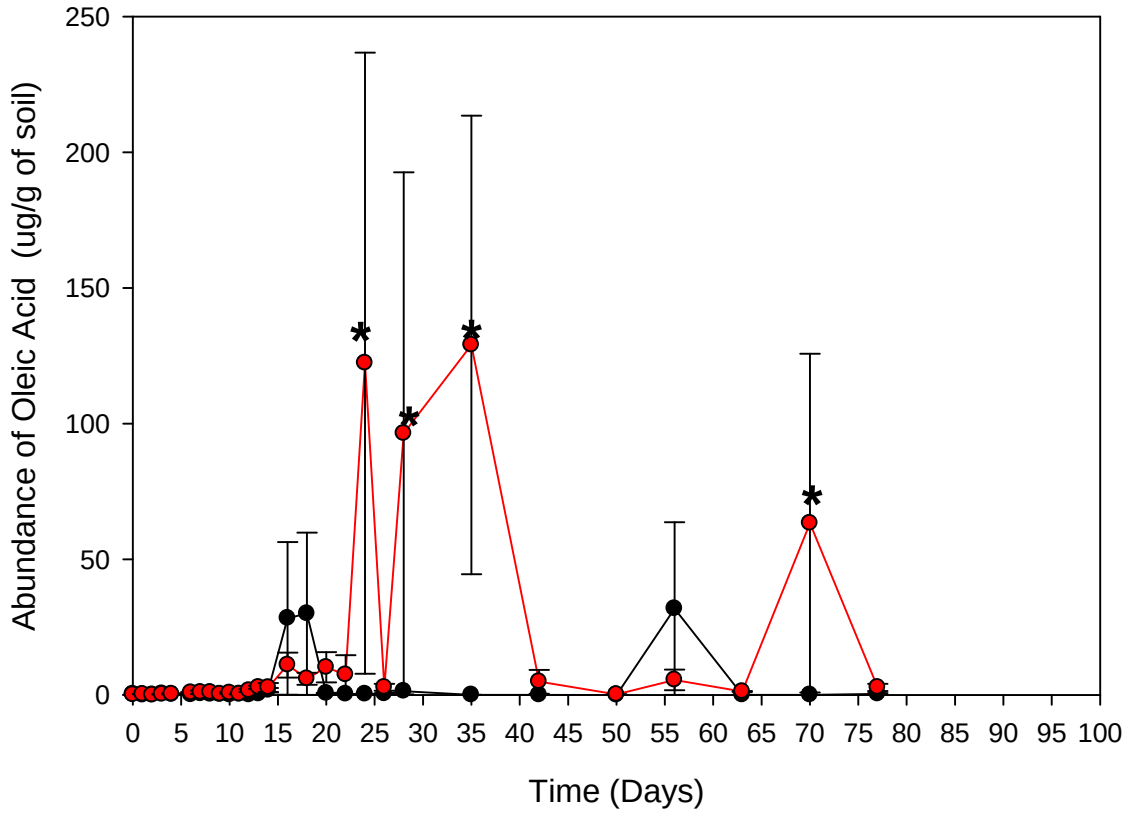

Figure 35. Oleic acid content in gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of an open field near Oshawa, Ontario, Canada during fall 2008. *= least significant difference < 0.05 Not to scale with previous figure (unbalanced ANOVA $F_{trial\ day(28, 128)}$=0.85, p=0.682, $F_{Soil\ Type\ (1, 128)}$=1.63, p=0.205, $F_{int.\ (26, 128)}$=0.46, p=0.989)

Comparison of Inter-Year Soil Oleic Acid Variation

There was a significant difference between the two trial years (Figure 36). There was a variation in the major peak locations, as well as an additional peak present in summer 2007.

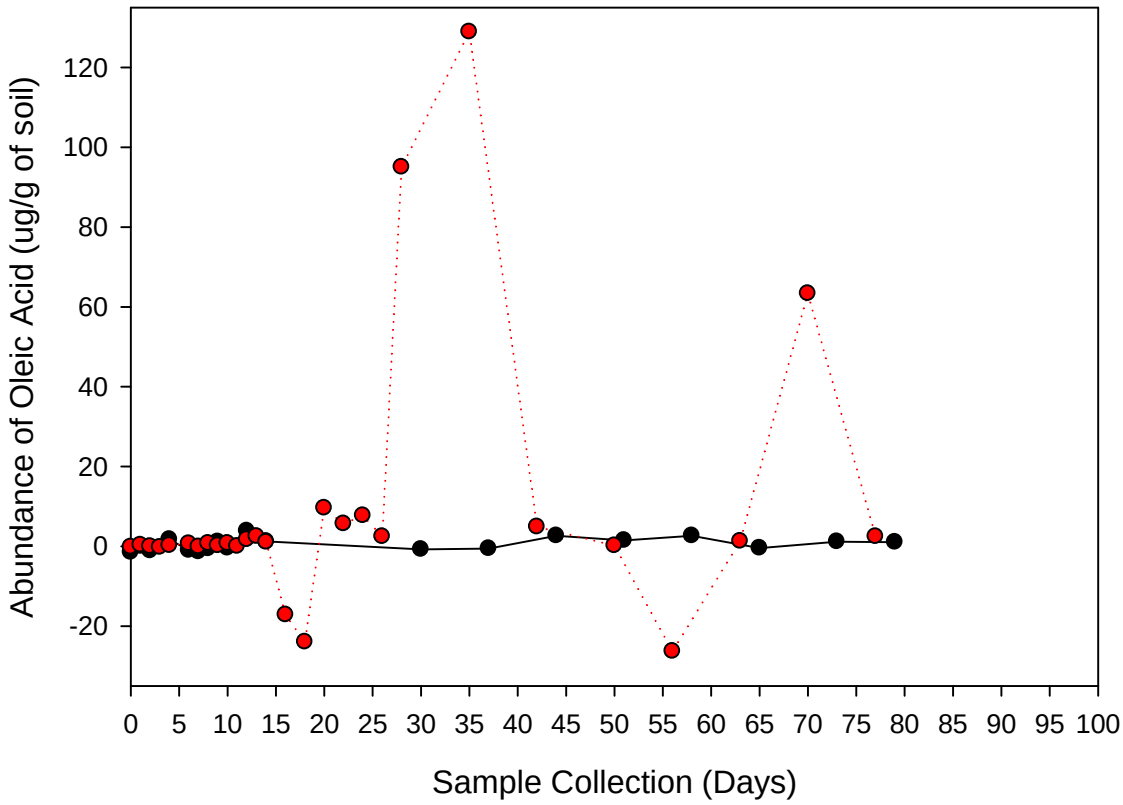

Figure 36. Background subtracted values of oleic acid between the 2007 (●) and 2008 (●) gravesoil following the placement of a pig (*Sus scrofa*) carcass on the soil surface near Oshawa, Ontario, Canada

3.6.6 Stearic Acid (C18:0)

Summer 2007 Trial

The summer 2007 trial demonstrated that stearic acid had a two peak trend during the course of the decomposition study (Figure 37). The first peak began at day 7, with a minor peak at day 8, and the major peak occurred at day 12. The second major peak began at day 37, and the apex occurred at day 44. The gravesoil means only returned to

control levels for a brief period at approximately day 65, and then continued to significantly increase for the duration of the study.

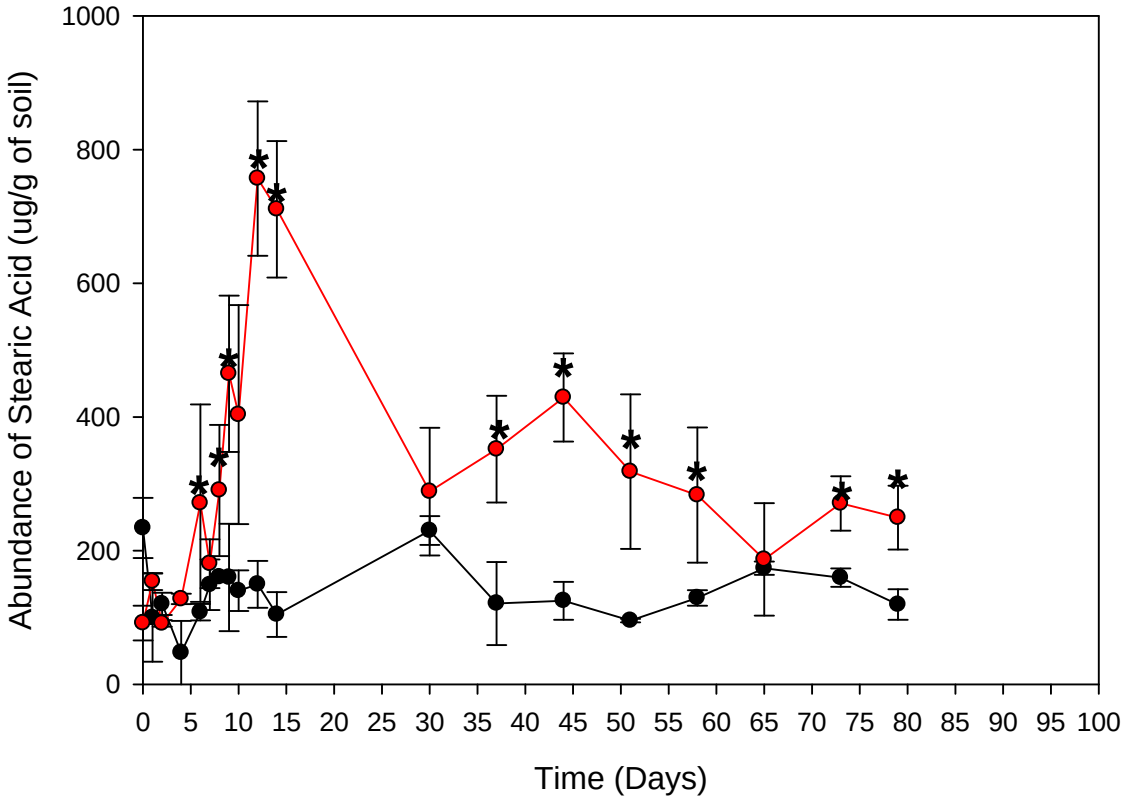

Figure 37. Stearic acid content of gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of a woodland near Oshawa, Ontario, Canada during summer 2007. *= least significant difference < 0.05 (unbalanced ANOVA, $F_{Trial\ Day\ (19,\ 109)}=4.09$, $p<0.001$, $F_{Soil\ Type\ (1,\ 109)}=36.34$, $p<0.001$, $F_{int.\ (18,\ 109)}=2.34$, $p=0.004$)

Fall 2008 Trial

A similar two peak trend was observed for stearic acid concentrations in the fall 2008 trial (Figure 38). The first significant (LSD<0.05) cycle commenced at day 28, with the apex at day 35, and the second cycle occurred at day 70. Although these are the only two significant peaks, it is apparent that there was an increase in both the control soil and the gravesoil around days 12 and 14. This increase was seen in all five fatty acids.

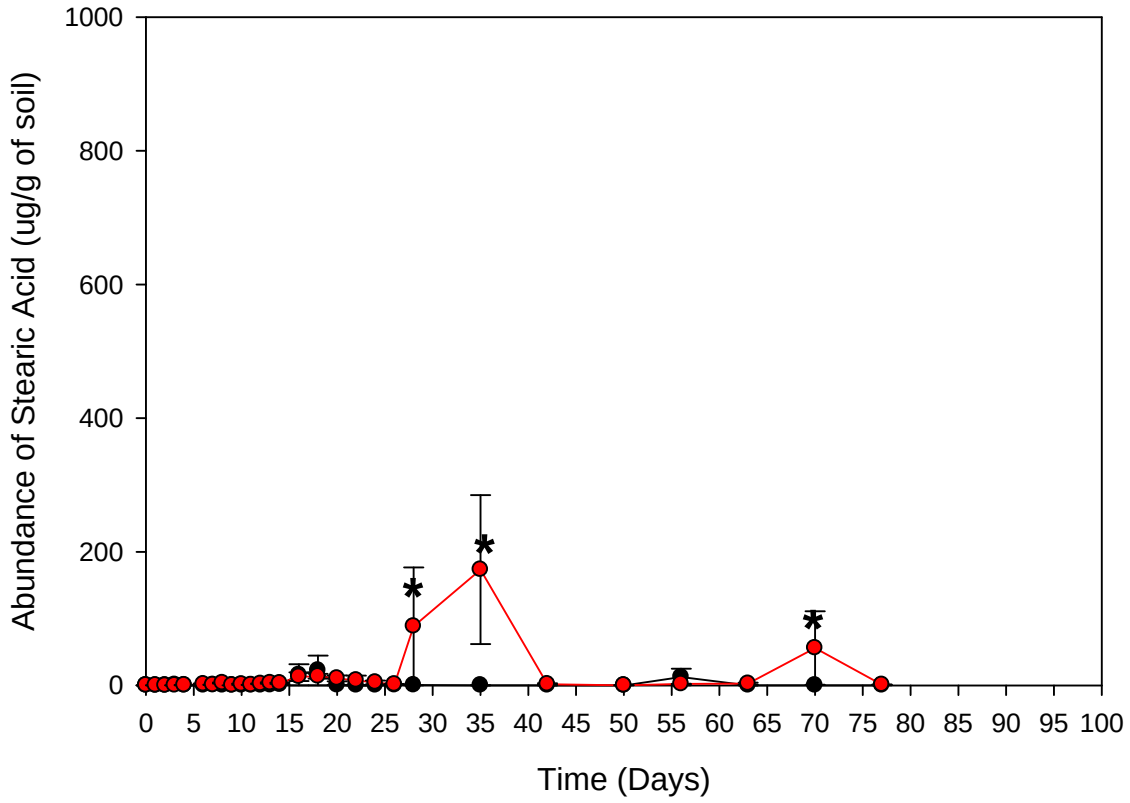

Figure 38. Stearic acid of gravesoil (●) and control soil (●) following the placement of a pig (*Sus scrofa*) carcass on the soil surface of an open field near Oshawa, Ontario, Canada during fall 2008. *= least significant difference < 0.05 ($F_{trial\ day(28,\ 128)}$=0.97, p=0.514, $F_{Soil\ Type\ (1,\ 128)}$=2.36, p=0.127, $F_{int.\ (26,\ 128)}$=0.54, p=0.965)

Comparison of Inter-Year Soil Stearic Acid Variation

The comparison between the overall means of each trial demonstrated a significant difference between the two trials, with the 2007 summer trial having larger values (Figure 39). When comparing visually it was seen that there is no similarity between the peaks in each year, with the 2007 peaks being observed earlier, and the 2008 trial only demonstrating one peak overall.

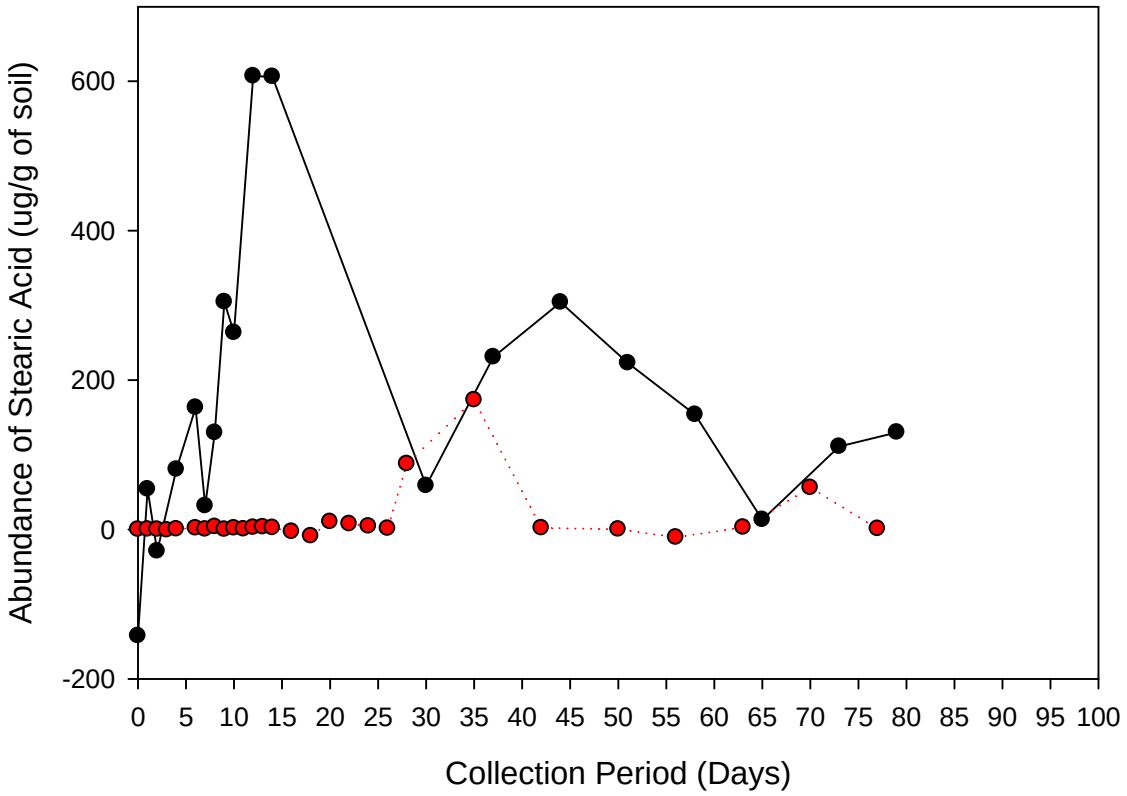

Figure 39. Background subtracted values of stearic acid between the 2007 (●) and 2008 (●) gravesoil following the placement of a pig (*Sus scrofa*) carcass on the soil surface near Oshawa, Ontario, Canada

3.6.7 Fatty Acid Comparison

Comparison of all five fatty acids in 2007

The vast abundance of stearic acid has demonstrated that there is no similar peaks which occur in the same abundance (Figure 40).

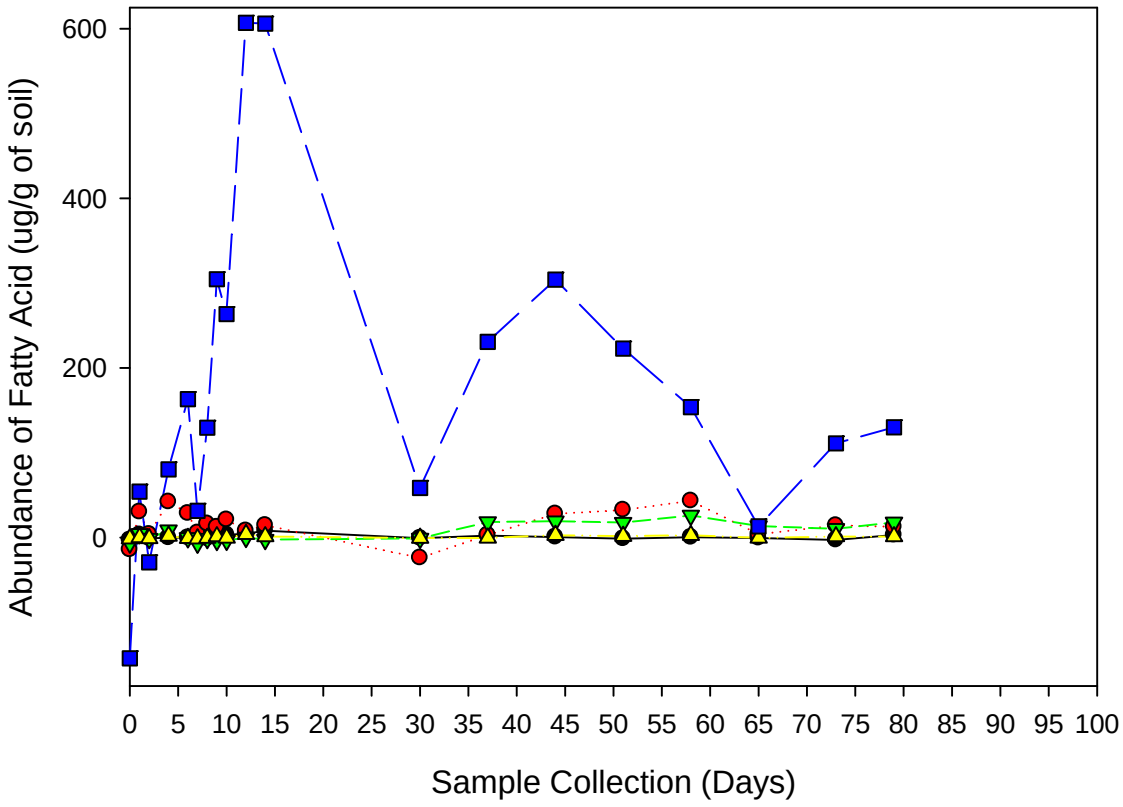

Figure 40. Background subtracted values of fatty acids between the myristic acid (●), palmitic acid (●), heptadecanoic acid (▼), oleic acid (▲) and stearic acid (■) gravesoil following the placement of a pig (*Sus scrofa*) carcass on the soil surface in 2007

In order to observe any noticeable similarities between the fatty acids in the 2007 season stearic acid was removed as it inhibited the ability to see the trends. Figure 41 demonstrates that there is a visual increase in the palmitic and heptadecanoic acid content in the early decomposition days, and continuing in to the later decomposition days (approximately day 55). As well there is a similar increase in abundance of myristic acid and palmitic acid around day 12.

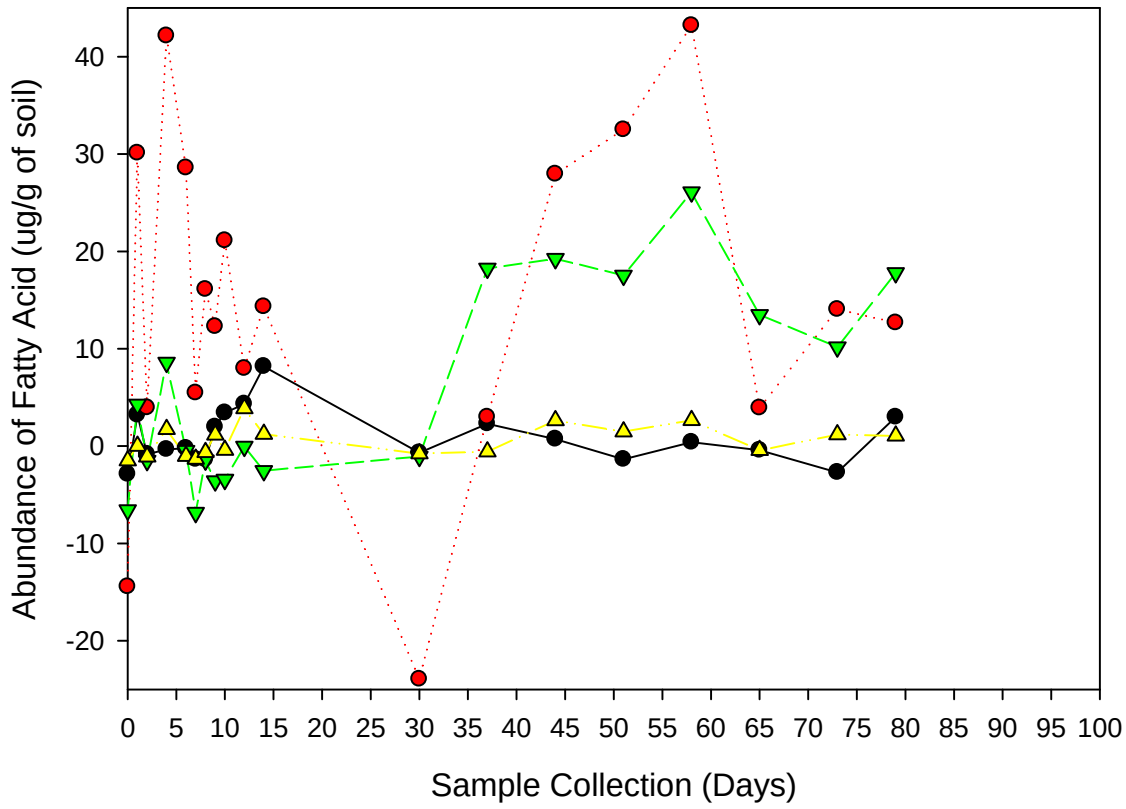

Figure 41. Background subtracted values of fatty acids between the myristic acid (●), palmitic acid (●), heptadecanoic acid (▼) and oleic acid (▲) gravesoil following the placement of a pig (*Sus scrofa*) carcass on the soil surface near Oshawa, Ontario minus Stearic Acid

Comparison of all five fatty acids in 2008

In 2008 all five fatty acids show a larger increase at day 35 and again at approximately day 72 (Figure 42). These peaks are present in all samples, with palmitic acid being the most abundant, and myristic acid being the least abundant. There is a visual decrease in the abundance of all of the fatty acids at approximately day 18, and again at approximately day 56. There is a secondary peak being seen in all 5 fatty acids at approximately day 72.

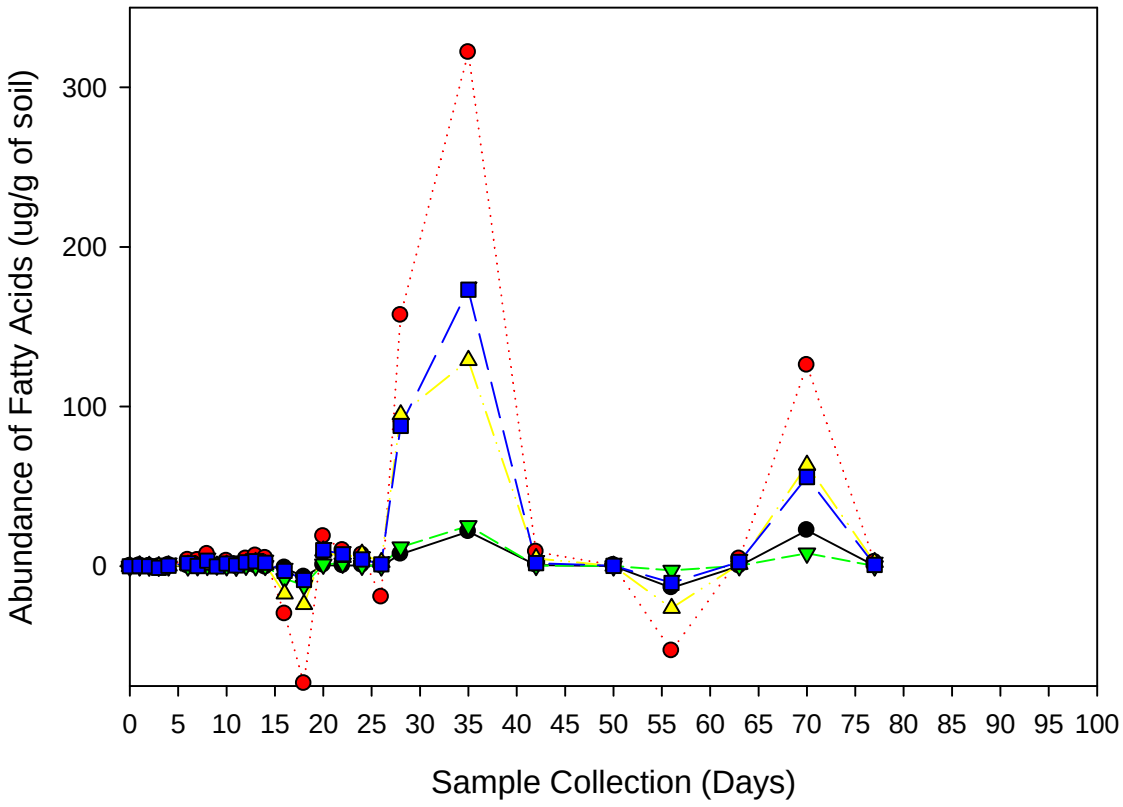

Figure 42. Background subtracted values of fatty acids between the myristic acid (●), palmitic acid (●), heptadecanoic acid (▼) and oleic acid (▲) gravesoil following the placement of a pig (*Sus scrofa*) carcass on the soil surface near Oshawa, Ontario in 2008.

Chapter 4

Discussion/ Conclusion

4.0 Discussion/Conclusion

The results found in this study demonstrate that carcass decomposition does alter the soil environment. The decomposition of a carcass had a significant effect on the chemical measurements analyzed: extractable lipid phosphate, soil available phosphorus, and fatty acid composition, specifically myristic acid, palmitic acid, heptadecanoic acid, stearic acid, and oleic acid. These effects were modified by altering the trial location and the seasonal variation. The alteration of the trial location included an additional soil texture, and the seasonal variation including changes in precipitation and temperature. In contrast, there was little effect on the soil pH values and the moisture content due to the decomposition of the carcass. These findings are similar to the decomposition of other organic matter, such as the addition of pea residue matter to soil (Ha et al., 2008). Therefore we conclude that soil pH is not a reliable indicator of gravesoils in certain soil types; some fatty acids can be of use to estimate the presence of a decomposition carcass, in particular stearic acid and heptadecanoic acid; phosphates found in soil can show the presence of additional nutrients potentially derived from the carcass; seasonal variation, including precipitation, did not alter the trends seen, although it did alter the relative concentrations of the fatty acids, and phosphoric nutrients; and finally variations in soil type did not have any observable effect on the rate of decomposition, or the trends present in the chemical analysis of fatty acids, and phosphorus.

The soil pH was not greatly affected by the decomposition processes, thus suggesting it is not an accurate estimate of the presence of a carcass in all soil types. In both the 2007 summer trial and the fall 2008 trial the gravesoil pH decreased in comparison to that of the control soil. This is consistent with the findings found in a

similar study completed by Benninger and colleagues (Benninger et al., 2008). As with the previous trial in southern Ontario soils, certain soils used as part of a study in Western Australia (Stokes et al., 2009) showed a similar decrease. Other studies completed have showed there to be a larger basification of the soil while the carcass proceeds through decomposition, for example Vass et al. (1992). Hopkins et al. (2000) has also observed this increase in basification trend and has attributed it to the accumulation of ammonium. The decrease in pH, thus the acidification, could result from the accumulation of nitrates in the soil, as nitrate follows ammonium in the nitrogen cycle. It is becoming an accepted notion that those references to an increase in pH of the soil upon decomposition only holds true for soils which are initially acidic (Stokes et al., 2009), based upon multiple studies exhibiting the similar trends with soils which contain a more basic initial pH, demonstrating an acidification of the soil rather than basification. This is further comfirmed by a study completed in the United Kingdom where an increase in pH was seen, only in an acidic environment, while the other site tested showed a decrease in pH, where the initial soil pH would be considered basic (Wilson et al., 2007). While a change in the soil pH, either to become more basic, or more acidic, is seen with regards to the presence of a decomposing carcass, it is not an accurate method to determine the presence due to the fact that change is minimal, and not always significant. As described by the materials and methods section, two different methods were used to analyze the pH, thus a direct comparison between the two trials (seasonal variation and soil type) cannot be made because the number of variations between the seasons is too large. Future studies need to be conducted on both of the soil types to verify that each individual method is consistent, and that they are comparable to one another. Another limitation to

the pH measurements taken for the summer 2007 trial is that the pH was not measured in the field, and instead used a bench top method, thus changing the error associated with the readings which were obtained.

A change in relative concentration of fatty acids was seen in the gravesoils compared to the control soils, for both seasons. In particular, heptadecanoic acid (C17:0) and stearic acid (C18:0) have the potential to be the most reliable indicators of the presence of gravesoils due to the increase seen in the gravesoil, compared to the control soils. There was an unusual trend that was noticed across all fatty acids analyzed in the 2008 fall trial, that days 16, 18 and 56 consistently had significantly larger control values than treatment values. The only way to determine if this was scientist error, or representative of the soil chemistry, would be to analyze more samples. Due to these large irregularities, it could have altered the significance of the gravesoil. These irregularities can be lower by increasing the sample size and analyzing all soil collected.

As myristic acid (C14:0) has been found previously as a component of adipocere formation (Vane & Trick, 2005), it was not unexpected to find it in the soil beneath a decaying carcass. A study completed in Western Australia found a dual peak trend in the abundance of myristic acid when the decomposition fluids were studied without soil, with the first peak being seen around days 12 and the second by day 25 (Swann et al., In Press), indicating a similarity to the increase in the myristic acid found in gravesoils. The 2008 fall trial found myristic acid has three peak influxes with the first having its apex at day 28, the second being at day 35, and the third being at day 70. In comparison to the 2007 summer trial these peak influxes are late, and occurring after the intense decomposition period has ceased. The 2007 trial indicated a large abundance of myristic

acid present in the first 20 days of the trial. Although myristic acid is present in adipocere, and in the decomposition fluids of pig carcasses, it does not appear to be the most reliable marker for the presence of gravesoils. As demonstrated by Figure 25 and 26, there does not appear to be a reliable trend that extends past sampling day 20 for determining the presence of gravesoils (Figure 27).

Palmitic acid (C16:0), although it demonstrated some increases in the relative concentration between the gravesoils and the control soils, also does not provide great potential for being an indicator of presence of gravesoil, due to the lack of consistent trends between trials. In the first of the two trials (Figure 28), there was no evidence of any reliable trends; in contrast the 2008 fall trial (Figure 29) did show the potential for a dual peak trend, however the lack of consistency between the two trials suggests soil and environmental variations affect the retention of the palmitic acid. Conversely the study completed in Western Australia found that palmitic acid, followed the two cycling pattern with the first being around day 14, and the second at approximately day 25 (Swann et al., In Press). This study did not consider the interaction of the decomposition fluids with the soil, as it studied the fluids alone thus the soil interaction could have led to the change in trend, as was in seen in this experiment. Palmitic acid is one of the primary fatty acids found in adipocere, the decomposition product of adipose tissue (Stuart et al., 2000). Although palmitic acid has been seen previously in decomposition studies, it is formed from the hydrogenation of palmitoleic acid (Dent et al., 2004), which was not found in abundance in this trial. Thus it is not unexpected that we did not find a reliable trend.

Heptadecanoic acid (C17:0) is the one of the few fatty acids, which demonstrated a larger relative increase after day 30 in the 2007 summer trial (Figure 31). A similar

trend was seen in the 2008 trial (Figure 32), where there is a large increase in relative concentration around day 30 and a secondary increase around day 70. The presence of heptadecanoic acid was unexpected, as it is not generally associated with decomposition, as it is not predominately studied (Vane and Trick, 2005). Although unexpected, it does have a forensic potential, as the increase appears later in the decomposition period, thus providing us with the ability to determine the presence of gravesoil over a longer period of time, allowing for extended use. Oleic acid (C18:1) demonstrated a few key peaks in both of the trials (Figure 34, and 35), however there was no relationship between the two sets of data, the 2007 trial and 2008 trial. These peaks do not correspond to one another, and do not remain elevated for a sufficient period of time to determine if there is any potential for forensic importance. It is not uncommon to find an increased abundance of oleic acid present in the soil, as it is present during adipocere formation (Vane & Trick, 2005), and is also found in the decomposition fluids from a decaying pig carcass (Swann et al., In Press).

The final fatty acid quantified, stearic acid (C18:0), demonstrates the largest forensic potential from this study. The 2007 summer trial showed a dual peak cycle (Figure 37), which has also been seen previously in the decomposition fluids of a decaying carcass, without the influence of the soil matrix (Swann et al., In Press). Not only is there a dual peak cycle seen in the 2007 trial, the relative concentration of the stearic acid remains elevated when compared to the control soils throughout the entire study dates. Thus it has the potential to be found in the soil long after the carcass stops releasing decomposition fluids. The 2008 trial (Figure 38) does not demonstrate as prominent a cycle when compared to the 2007 trial; however there are very distinctive

increases in the relative concentration of stearic acid. Due to these distinctive increases, stearic acid has the potential to be a reliable marker for the presence of a gravesoil.

The limitation to the FAME results is that the pre-existing research in this field, with respect to forensics, is limiting, thus there are very little data that can be compared. Additional replicates from the trial should be analyzed to ensure the results that were obtained are representative; only the torso soils from all 5 pig sites were analyzed, as well as all locations for the control. The head and rear samples should be analyzed for consistency. In addition to studying these five fatty acids in greater details, other long chain fatty acids including linoleic acid, and palmitoleic acid, should also be analyzed as they are natural precursors to oleic acid and palmitic acid (Dent et al., 2004).

Both soil available phosphorus, and extractable lipid phosphate show forensic potential, as their relative gravesoil concentrations increased in comparison to the control soil with time, however soil available phosphorus does demonstrate a greater potential. Benninger et al. (2008) found similar results in a previous study in the same location while Stokes et al. (2009) observed increased concentrations of bicarbonate extractable phosphorous in gravesoils in Western Australia. A limitation to utilizing phosphate as a biomarker for the presence of a gravesoil, is that other decaying organic matter will provide the phosphates as well as a decomposing carcass (Ha et al., 2008). Although there could be some variation within the concentration of the additional phosphate in the soil, it could be used as a presumptive test to determine if there is a potential for a body to have been present. Studies have also shown that an increase in pH will decrease the phosphorus absorption (Jiao et al., 2007), and since the summer 2007 soils had a larger pH value, the decrease in soil available phosphorus abundance could be expected. A

limitation to this assay is the inability to develop a standard through the digestion process. The standard is an inorganic phosphate, whereas the assay tests for organic phosphates.

Both of the trials completed in this study were completed over the course of two different seasons. Originally it was hypothesized that the seasonal changes would primarily affect the two assays, which focus on the microbial biomass; extractable lipid phosphate and the fatty acid content. As expected the seasonal change did not have an effect on the rate of decomposition as the average mean temperature varied between both trials, as well as the amount of precipitation. This is not uncommon, as the amount of precipitation might not affect the rate of decomposition (Vass et al., 1992). Although the vast amount of precipitation did not have an effect on the rate of decomposition, or the presence of the chemicals in the soil, it may have altered the concentrations of the chemicals, by diluting the amount of decomposition fluid seeping into the soil. Thus the seasonal variation only affected the relative abundance, not the mechanism in which the soil reacted to the additional organic matter. Not only did the seasonal variation show little to no change between the two studies, the variation in soil type also did not have a large impact on the decomposition trials.

The rates of carcass decomposition were consistent between both studies, even with the variation in the soil type. However, when compared to other studies completed in Canada (Gill, 2005, Anderson and VanLaerHoven, 1996), the rates of decomposition were only consistent with two other studies, one which was also located in Southern Ontario (Benninger et al., 2008), and another completed in rural Manitoba (Gill, 2005). Gill saw a delay in decomposition when in a shaded environment (Gill, 2005). This was

not seen in our experiment; however we did see seasonal variation playing a role with a slight delay in the decomposition, which occurred in direct sunlight. Another study located in Southern British Columbia in 1992, did not show the same rate of decay, regardless of the similarities in the temperature and the seasons (Anderson and VanLaerhoven, 1996). Anderson and VanLaerhoven's study exhibited slower decay rates beginning with bloat and continuing throughout the processes until skeletonization. Soil type was not influential in the reaction of the chemicals to the soil matrix. All of the assays saw very similar trends and responses to the decomposing carcass regardless of the change in soil type. Although it was not seen in this study as the two soil types were similar, soil type may have an effect on the rate of decomposition, as seen in burial trials (Wilson et al., 2007). Unfortunately, these studies do contain numerous variables (change in location, season, and sun exposure) making it difficult to determine whether the altered variables are causing the observed differences in decomposition.

As mentioned previously, a limitation to this study is that all soil samples collected were not analyzed due to time constraints. If all soil had been analyzed, as well as all days, a more accurate representation of the chemical processes in the soil would have been obtained. Due to the first trial in 2008 (Summer 2008) having to be ended abruptly, it allowed for a seasonal variation between 2007 and 2008. Initially the only variation was going to be soil type. Having two variables does not allow for a positive identification of the cause of the peak shifts, and for differences in the relative concentrations. Since the correlation between soil chemistry and decomposition stages in an attempt to find a post mortem interval is a relatively new field of study, the trials need to be repeated for validation purposes. As Canada goes through all four seasons, it would

be valuable to conduct trials lasting through all of the major seasons to determine if the peak shifts are seasonal. Testing a variety of soil types would be another future recommendation to eliminate the limitations of this trial. As storing the soils at -20^{o}C could have a negative impact on some of the assays; soil samples should be split upon collection, with one sample being stored at -20^{o}C, and the other being stored at room temperature to determine the effect of freezing on soil chemistry.

The sampling methodology utilized in the 2008 study had been changed to avoid disrupting the microbial community by collecting less soil; however sampling from different soil strata was not taken into consideration. Particularly in sandy sub surfaces, there is an alteration in the soil microbiology as the depth of the soil increases (Zhou et al, 2004). In future studies it would be beneficial to grid under the carcass, to ensure that only soil strata was collected for analysis (Parkinson et al., 2009), or to loosen and homogenize the soil prior to carcass placement to avoid multiple strata sampling (Parkinson et al., 2009). Additionally, the method by which the soils were ground could have added variation to the samples. Grinding of soils has previously demonstrated an increased abundance in eukaryotic phospholipids fatty acids, which can either over estimate or underestimate the structure of the community (Allison and Miller, 2005). Thus, it is important that all samples, both control and gravesoil, are treated to the exact same grinding processes to eliminate as much variation as possible.

The purpose of this study was to examine the soil chemistry during the process of decomposition in two separate sites, during two different years and seasons, and determine if the chemical parameters of the soil will react similarly regardless of the variation in parameters. The study has proved that the chemical parameters of the soil

will react in a similar form, regardless of the variation in the season, soil type, and direct sunlight. In particular the phosphate content of the soil has forensic potential, as it remains elevated for a long duration after the carcass has skeletonized regardless of soil type and seasonal variation. Similarly heptadecanoic acid (C17:0) could be useful as a tool for estimating postmortem interval after day 30, and into the later stages of decomposition. Stearic acid (C18:0) also remained elevated throughout the decomposition period, and into the later stages of decomposition, with a two key peak formation. While direct comparison between the two trials cannot be made due to the variation in parameters, many of these assays provide potential for forensic use, as they reside in the soil long after decomposition has ceased. Presently, there is no method for estimating postmortem interval in the extended postmortem period, and further study of these chemical compounds in soil may provide a direct correlation with the time since death of decomposed remains.

Chapter 5

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5.0 References

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