ADAPTATION OF THE MICROBIAL DECOMPOSER COMMUNITY TO THE BURIAL OF SKELETAL MUSCLE TISSUE IN CONTRASTING SOILS

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This thesis is presented in partial fulfillment of the requirements for the
Master of Forensic Science

2008

Preface

I declare that the research presented in this 36 point thesis, as part of the 96 point Master degree in Forensic Science, at the University of Western Australia, is my own work. The results of the work have not been submitted for assessment, in full or part, within any other tertiary institute, except where due acknowledgement has been made in the text.

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Acknowledgements

Abstract

Microorganisms are known to be agents involved in the decomposition of organic matter. However, little is known about the participation of the microbial communities during the decomposition of mammalian skeletal muscle tissue. This study investigates the capacity of the soil microbial community to adapt to the decomposition of skeletal muscle tissue in differing soils. This has implications for the study of mass graves and sites of repeated burial.

A controlled laboratory experiment was designed to assess the adaptability of microbial communities present in three distinct soil types (sand, loamy sand and sandy clay loam) found near Perth, Western Australia. This experiment was split into two main stages. The initial decomposition stage involved the addition of porcine skeletal muscle tissue (SMT) (*Sus scrofa*) to each of the three soil types which were then left to decompose for a period of time. Controls were run in parallel, which had no porcine SMT present. The second decomposition stage involved a second addition of SMT to the soils obtained from the initial decomposition stage. Therefore, for each soil, SMT was either decomposed in the soil that had been pre-exposed to SMT or not.

The rate of decomposition, microbial activity (CO_2 respiration) and microbial biomass (substrate-induced respiration) were monitored during the second decomposition stage. The functional diversity of the microbial populations in the soil were assessed using Community-Level Physiological Profiling (CLPP). Across the three soil types, the re-introduction of SMT to the soil has led to its enhanced decomposition (measured by tissue mass loss and microbial activity) by the microbial communities. This microbial adaptation may have been facilitated by a functional change in the soil microbial communities.

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Figure 5.3 Microbial biomass (µg C_{mic} g^{-1} soil) estimated using substrate-induced respiration during the Second Decomposition Stage in sandy soil (i), loamy sand (ii) and sandy clay loam (iii) soils under the following conditions: no tissue added to the soil without pre-decomposition (control) (●), no tissue added to soil after pre-decomposition of skeletal muscle tissue (▲), tissue added to soil without pre-decomposition (O) and tissue added to soil after pre-decomposition of skeletal muscle tissue (△). The bars represent standard error where n=5.

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Figure 5.4 Represents the metabolic quotient of the microbial communities in the sandy soil (i), loamy sand (ii) and sandy clay loam (iii) soils under the following conditions: no tissue added to the soil without pre-decomposition (control) (●), no tissue added to soil after pre-decomposition of skeletal muscle tissue (▲), tissue added to soil without pre-decomposition (O) and tissue added to soil after pre-decomposition of skeletal muscle tissue (△). The bars represent standard error where n=5.

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Abbreviations

Cmic   Microbial biomass carbon

CO_2 Carbon dioxide

SMT   Skeletal muscle tissue

CLPP   Community – level physiological profiling

SIR   Substrate- induced respiration

qCO_2 Metabolic quotient

-/-   Control soil in phases 1 and 2

+/-   SMT added in phase 1 only

-/+   SMT added in phase 2 only

+/+   SMT added twice: phases 1 and 2

S   Sandy soil

LS   Loamy sand

SCL   Sandy clay loam

Chapter 1

Introduction

Taphonomy is the study of processes that affect organisms after the time of death (Calce and Rogers, 2007) and has considerable importance in the field of forensic science (Mann *et al.*1990. Pujol-Luz *et al.* 2006). Decomposition has been widely studied in disciplines such as entomology (Rodrigues and Bass, 1983, De Jong and Hoback, 2006) where the stages of decomposition (Johnson, 1975; Grassberger and Frank, 2004) and their relationship to assessing postmortem interval (Korshunov *et al.*, 2003. Manlove and Disney, 2008) have been documented. Further studies have been conducted on factors that have significant effects on taphonomic processes. These factors include climatic conditions, carcass size and microbiological factors (Adlam and Simmons, 2007).

From a forensic aspect, the potential contribution of soil microbiology has been recently recognized. Soil microbial DNA profiles have been investigated in an attempt to differentiate between differing soil samples (Lerner *et al.*, 2006). This can help link suspects to crime scenes using the soil evidence (Horswell *et al.*, 2002). The processes associated with cadaver decomposition and its effects on the surrounding soil ecology have also been explored in recent years (Carter *et al.*, 2007a; Hopkins, 2008; Brooks, 2008). These studies have shown the potential for the use of soil decomposition processes in forensic science. These processes

apparently can be used as both spatial evidence (to locate clandestine remains) (Carter and Tibbett 2003) and temporal evidence (to estimate time since death or time since burial) (Vass et al. 1992).

Soil microbial communities are diverse (Jackson *et al.* 2007) and are critical in maintaining soil ecosystem processes (Grayston *et al.* 2004). From methane emissions to decomposition, soil microbes are responsible for cycling nutrients (from the breakdown of plant and organic matter (Griffiths *et al.* 2003) and controlling trace gas concentrations in the atmosphere (Jackson *et al.* 2007). Soil microbial communities mediate the cycling of carbon and nitrogen, which is essential for maintaining ecosystem function (Balser and Firestone, 2005).

Environmental change or disturbance can stress microbial communities (Hazen and Stahl, 2006), therefore microbes can use physiological or compositional adaptation mechanisms to survive and remain active (Schimel *et al.* 2007). The physiological mechanisms are confined to the phenotypic diversity (e.g. Korona *et al.* 1994) of the species present while the compositional mechanisms involve a physical community change (e.g. Zak *et al.* 2003). The microbial community will change in response to environmental variation (Kelly *et al.* 1999) which may ultimately create shifts in the soil ecosystem processes (eg. nutrient flow and ecosystem-level C) (Schimel *et al.* 2007). Therefore, it is essential that soil microbial communities are dynamic

(Sonnleitner, 1998) and are able to rapidly adapt to a constantly changing environment (Hazen and Stahl, 2006).

Disturbance affects the activity and growth of the soil microbial communities (Peterson *et al*., 2002). For example, soil may be subject to changes in nutrient concentrations through leaching (Miller *et al.* 2005) or plant uptake (Schimel and Bennett, 2004), which would in turn affect the nutrient availability for the microbial community (Brandt *et al.,* 2004). The microbial community would have to be able to adapt to these new environmental conditions for optimal survival. Regulatory mechanisms are thought to be responsible for microbial adaptation (Brandt *et al.,* 2004) and have been suggested to occur in one of three ways. Firstly, certain enzymes may be altered in their expression levels (Sonnleitner, 1998) or specific enzymes may be induced to degrade the added resource (Macleod and Semple, 2002). Secondly, a genetic change may occur within microorganisms to increase these required metabolic capabilities. Thirdly, the number of organisms with the metabolic capabilities to metabolize the compound may increase (Leahy and Colwell, 1990; Macleod and Semple, 2002). These adaptation techniques either affect the phenotypic variation of the community (genetic changes or specific enzyme induction) or shape the microbial composition of the community. Such adaptations allow the cells to adapt to environmental changes accordingly (Sonnleitner, 1998).

Recent research has now made it possible to assess microbial community composition and determine their stability through time (Jackson *et al.* 2007) during the adaptation process. To extract living microorganisms from the soil to assess their adaptation would provide an unrepresentative result of the total microbial biomass as not all may be culturable (Degen, 1998). This removal may further result in either a stress response or change in the community structure which would impact any results obtained from the assessment (Rusk *et al.*, 2004). Therefore, molecular (e.g. DGGE, (Griffiths *et al.*, 2003)) and physiological (e.g. CLPP, Degens and Harris, 1997)) techniques have been developed to assess community level changes (Grayston *et al.*, 2004) directly within whole soil samples.

Soil microbial communities have been shown to adapt to a variety of added resources (Walker and Austin, 2003; Macleod and Semple, 2002; Rusk *et al.* 2004). For example, the addition of an organic material (such as compost) in the soil stimulated microbial activity which alternatively resulted in the enhanced degradation of a heavy mineral oil (Lee *et al.* 2008). Microbial communities have yielded a greater ability to utilize carbon sources upon the repeated application of farmyard manure over a 10 year period (Toyota and Kaninaga, 2006). Katayama *et al.* (1998) found that microbial communities in soils with repeated applications of farmyard manure possessed higher utilization abilities and were more stable. Soil microbial communities have also been shown to adapt to pesticides in the soil from either the repeated application of the same pesticide, or the application of another pesticide with a similar chemical structure (Arbeli and Fuentes, 2007).

Research on the effect of soil type on decomposition of cadavers and their components have been limited with some suggestion that soil type has no effect on cadaver decomposition (Mant, 1987). This assumption has been brought into question by recent studies. Cayeula *et al.* (2008) have demonstrated the soils effect on the decomposition of meat and bone meals with varying mineralization rates in the differing soils. Decomposition rates have differed in diverse soils (Tibbett, 2008) with properties, such as contrasting pH, having a great effect (Haslam and Tibbett, 2009). Haslam and Tibbett (2009) found that the rate of decomposition in acidic soil (Podsol) was three times greater than the contrasting alkaline soil (Rendzina). The extent of microbial adaptation may also be influenced by soil texture (eg. Macleod and Semple, 2002) as soil microbial activity is shown to be greatly affected by the soil matrix (Torbert and Wood, 1992). Soils with greater amounts of clay are thought to lessen microbial decomposition as the clay particles bind to the organic matter and protect them from microbial attack (Beare *et al.* 1994; Watts *et al.* 2007).

The purpose of this study is to assess whether the microbial community could adapt to the decomposition of skeletal muscle tissue (SMT). It is hypothesized that the extent of adaptation would be affected by the soil environment. This current study examines how the microbial activity, biomass and rate of decomposition change as a result of the re-introduction of SMT. This study will determine whether the re-introduction of SMT increased microbial efficiency during the decomposition of this substrate, and whether catabolic diversity in community-level changes as a result. In an ecological context, this study illustrates a link between microbial adaptation and

subsequent functional changes after the addition of a carbon substrate. In a forensic context, this study illustrates the importance of burying bodies and/or their components in a soil with a well-known history, particularly when assessing post-mortem intervals.

Chapter 2

Review of Literature

2.1 Previous Taphonomic Research

There has been increased awareness of the importance of taphonomy in human forensic investigations (Pickering and Carlson, 2004) with recent studies focusing on the rate, extent and nature of cadaver decomposition in a number of differing burial situations (e.g. Forbes *et al.*, 2005a). Results of taphonomic studies can be applied to various forensic situations such as the investigation of clandestine graves (e.g. Wilson *et al.* 2007) or improving the estimation of time since death (e.g. Vass *et al.* 1992). There have been recent taphonomic studies that have used chemistry to assess certain decomposition processes. For example, infra-red spectroscopy, inductively-coupled plasma-mass spectrometry and gas chromatography-mass spectrometry have been implemented in studies investigating the formation of adipocere and how the surrounding burial environment affects this process (Forbes *et al.* 2005a; Forbes *et al.* 2005b). Burial factors were shown to both enhance (alkaline pH, warm temperatures and anaerobic conditions) and reduce (cold temperatures, aerobic conditions and the presence of lime) the rate of adipocere formation. This study gives an insight into the preservation of decomposing tissue in differing soil environments (Forbes *et al.* 2005a). Vass *et al.* 2002 looked at the chemistry associated with human decomposition to obtain a more accurate method of estimating postmortem interval. Biomarkers (such as amino acids, neurotransmitters

and decompositional by-products) in various organs (such as liver, heart and kidneys) produced distinct patterns which, in conjunction with cumulative degree hours (CDH), provide an accurate determination of postmortem interval. CDH is the average temperature of each 12 hour interval over the time period of cadaver decomposition. Obtaining this precise temperature data at the decomposition site is thought to be the main limiting factor of this method.

2.1.1 Microbial Research Relevant to Forensic Science

There have been recent advances in the study of processes associated with cadaver decomposition and its effects on the surrounding soil ecology (Carter *et al.*, 2007a). For example, various fungal species, which may be associated with the by-products of decomposing cadavers, may be used to identify, or potentially even date, gravesites (Carter and Tibbett, 2003; Tibbett and Carter, 2003; Sangara *et al.* 2008). Further examples include the recent study of soil microbial community DNA profiles (obtained using T-RFLP) to potentially connect soil evidence (from shoes or clothing) with suspects and/or crime scenes (Horswell *et al.* 2002). Incubation methods have also been developed to monitor microbial decomposition process (Tibbett *et al.,* 2004) and have been used to investigate the effects of temperature on microbial activity during the decomposition of skeletal muscle tissue (Carter and Tibbett, 2006). Slower microbial decomposition of skeletal muscle tissue was observed at lower temperatures as the peak rate of decomposition occurred during later time periods in the lower temperature incubations.

In a field setting, Hopkins *et al.* (2000) measured changes in soil characteristics, which included microbial biomass and microbial mediated processes obtained from the gravesite of three pigs. Measured CO_2 respiration, S reduction and N mineralization provided an insight into the microbial activity, while microbial biomass was monitored using substrate-induced respiration. The results from these measurements showed an increase in microbial activity and biomass with relation to the decomposition of the buried pigs. A recent study conducted by Wilson *et al.* (2007) involved the burial of pigs in three contrasting field sites to monitor the effects of specific microenvironments on decomposition. The buried cadavers modified the microenvironment by inducing an increased microbial load and soil pH changes. The differing soil conditions were also shown to have marked effects on cadaver decomposition with variations of cadaver decomposition occurring within a single site. This study highlights the importance of site-specific information and its implications on the rate, extent and nature of cadaver decomposition as it provides a greater understanding of the relationships between soil, soil microbial biomass and cadaver decomposition (Carter and Tibbett, 2008). This in turn will contribute to further developments in forensic fields, for example post-mortem interval determinations.

2.1.2 Relevant Research in Microbial Adaptation

Microbial adaptation has been shown to be imperative for organism survival and ecosystem function (Sonnleitner, 1998; Balser and Firestone, 2005). A significant amount of research has focused on enhanced degradation of various pesticides after their repeated applications. This enhanced degradation was shown to be due to microbial adaptation and was recognized soon after pesticide introduction to the market (Arbeli and Fuentes, 2007). Herbicides (Leasure,1964; Barriuso and Houot, 1996), insecticides (Harris *et al.* 1988; Singh *et al.*, 2003) and fungicides (Walker, 1987; Thom *et al.* 1997) are some pesticides whose enhanced degradation has been thoroughly researched. For example, there was a greater mineralization rate of the herbicide, atrazine, in the soils that had a continuous yearly application of this herbicide (Barriuso and Houot, 1996). Microbial adaptation to this herbicide is thought to be the reason behind the enhanced degradation of the herbicide in these soils (Barriuso and Houot, 1996). Thom *et al.* (1997) also demonstrated microbial adaptation to the fungicide difenoconazole, as the specific microorganisms able to degrade this herbicide in the soil, flourished.

Macleod and Semple (2002) demonstrated an enhanced biodegradation of the hydrophobic organic contaminant, pyrene, as a result of microbial adaptation. It was suggested that the biodegradation rates of this contaminant were affected by pyrene concentrations and length of the soil's prior exposure. The soil environment was also a factor that affected microbial adaptation as soil with a higher organic matter

content showed lower pyrene mineralization rates. The effects of differing soil environments were also observed in a study by Cayuela *et al.* (2008). The results from this study showed that the decomposition of meat and bone meal were greatly dependant on soil properties, specifically soil texture, as a higher sand content results in greater microbial mineralization rates of organic materials (Khalil *et al*., 2005).

2.2 Relevant Techniques

2.2.1 Carbon Dioxide Respiration

Soil microbial respiration may be quantified through the measurement of released carbon dioxide and has been positively correlated with the decomposition of soil organic matter and consequently microbial activity (Winding *et al.,* 2005). Measuring CO_2 respiration assesses the activity of the soil microbial community during the decomposition of various types of organic matter (Ajwa *et al.,* 1994) including muscle tissue (Carter and Tibbett, 2006).   The measurement of CO_2 respiration has proven to be an accurate representative of the activity of the microbial communities present within the soil environment (Putman, 1978).

2.2.2 Substrate-Induced Respiration (SIR)

The substrate-induced respiration method was developed by Anderson and Domsch in 1978 to provide an effective way to indirectly estimate the microbial biomass in

the soil. The soil microbial biomass consists of many organisms (e.g. bacteria, fungi, protozoa), each with specific roles (Brookes *et al.*1985). The soil microbial biomass may be considered as a single body and represents 2-5% of total soil carbon (Brookes *et al.*1985). SIR quantifies this initial respiration response of the microbial population to the added substrate in the soil which in turn gives an estimation of the carbon relating to the microbial biomass (Anderson and Domsch, 1978).

2.2.3 Metabolic Quotient ($q\mathrm{CO_2}$)

The microbial metabolic quotient has been used as an indicator of ecosystem development and disturbance (Wardle and Ghani, 1995). This quotient has been useful when assessing maturing ecosystems, as efficiency increases in relation to these changes (Anderson and Domsch, 1990). Even though $q\mathrm{CO_2}$ provides a useful measure of efficiency, its limitations fail to discriminate between the effects of disturbance and stress (Wardle and Ghani, 1995).

2.2.4 Community-Level Physiological Profiling (CLPP)

Certain microbial species within the general soil microbial community may respire at differing rates when it comes to utilizing different substrates (Campbell *et al.*, 2003). This CLPP method tests the ability of the microbial communities to utilize sole-carbon substrates that have been added to the soil samples (Bloem *et al.*, 2003) where each individual soil sample is provided with a different carbon source (Yao *et*

al., 2000).   The amount of carbon dioxide measured after incubation, is an indication of the community's utilization of that corresponding substrate (Bloem *et al.*, 2003). This allows for the construction of catabolic fingerprints (Campbell *et al.*, 2003) of the active microorganisms within the community (Degens *et al,.* 2000) which ultimately provide a measure of the functional diversity of the microbial community within the soil. Therefore this technique is highly advantageous with respect to this study, as it monitors the changes within the microbial community that are functionally relevant, at specific time periods. There is an alternative method developed by Campbell et al. (2003) that measures the carbon dioxide evolution of microbial communities from whole soil samples using sole-carbon sources on microtiter plates which is thought to be equally effective (Leckie, 2005).

Chapter 3

Soil Characterisation

Soil is a complex and heterogeneous environment with a range of physical, chemical and biological characteristics (Tibbett, 2008) which, when combined, make each soil environment unique. Contributing factors include; finely divided crystalline and amorphous minerals, inert and decomposing organic matter, living and metabolizing microbiota as well as animals, plants and pollens. All of which make the soil a highly complex matrix (Horswell et al., 2002).

The soils used in this study were collected from areas within the south-western part of Australia. This area has a Mediterranean - type climate (Lamont *et al.*1999) with averaging temperatures of this area ranges from 30° C in January (hottest month) to 9° C in August (coldest month). The average rainfall of this area ranges between 600mm – 800mm annually (Australian Government: Bureau of Meteorology).

Three different soil types were used for this project. The relative proportions of sand, silt and clay were determined for each soil to establish respective textural classes. Hand-texturing was carried out by Georgina Holbeche (from Earth and Geographical Sciences, University of Western Australia). The three soil types were classified as: sand, loamy sand and sandy clay loam. pH (H_2O) of the soils were

measured by Kathryn Stokes (from Centre for Forensic Science) using a standard method (Rayment and Higginson, 1992).

3.1 Soil Type and Location

Bulk soil samples were collected during winter after a period of sustained rain. The fresh soil was therefore relatively moist. Fresh bulk soil samples were sieved (6mm) and moisture content was adjusted to 50%.
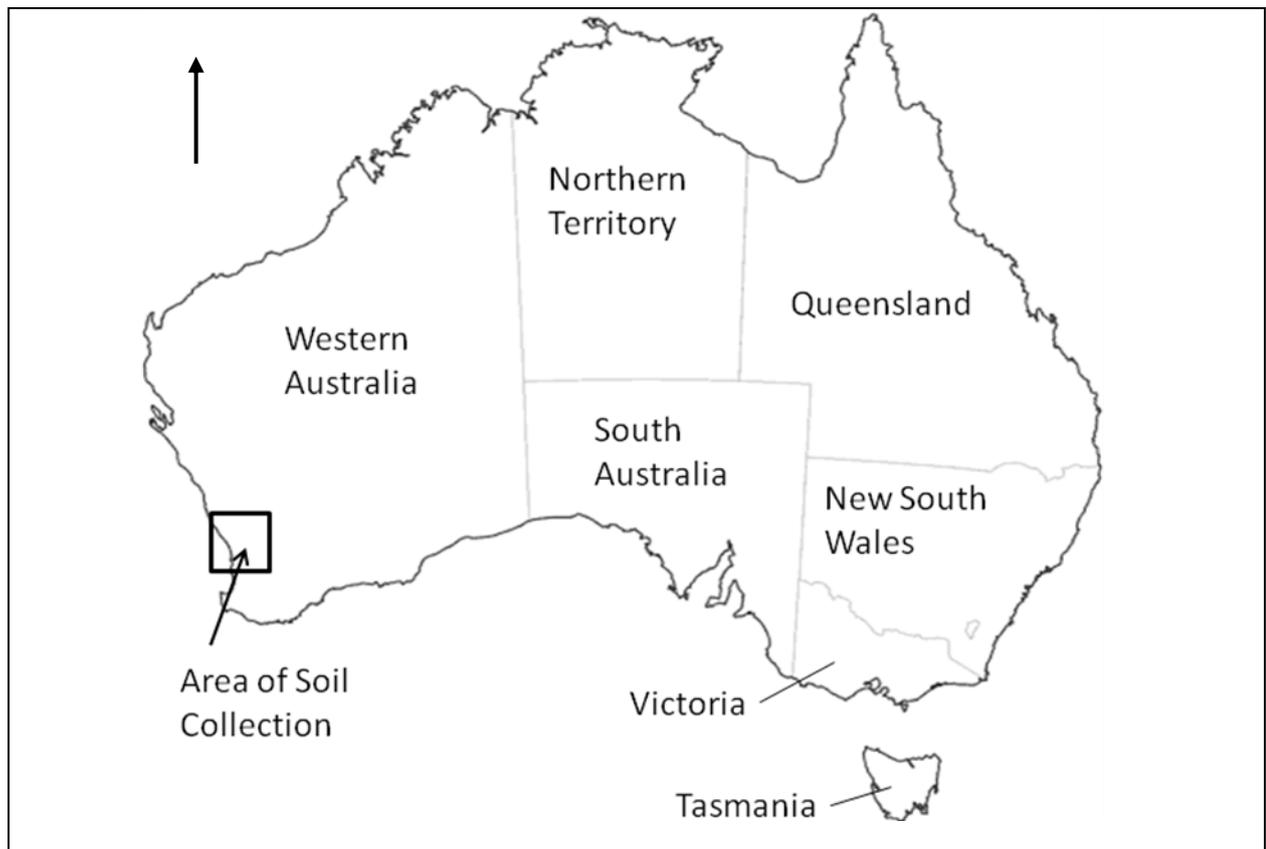
Figure 3.1 Map of Australia Showing Soil Collection Area

(i)

(ii)

(iii)

Figure 3.2 (i) Sample of grey sand (5Y5/1) with pH 5.5-6 collected from HWMR; (ii) Sample of reddish yellow (7.5YR6/8) Spearwood sand (loamy sand) with pH 8.5; (iii) Sample of the sandy clay loam (pH 6) which is dark yellowish brown in colour (10YR 3/6) when dry

Table 3.1 A Summery Soil Characteristics

Soil type	Location	pH	Munsell Colour	Vegetation	Other notes
Sand	Harry Waring Marsupial Reserve (115.8 °E 32.2 °S)	5.5 - 6	grey sand (5Y5/1)	*Banksia* woodlands	This is a Bassendean sand: consists of coarse (0.5 – 1 mm) and medium (0.18 - 0.5 mm) sands (Salama *et al*. 2005). Low nutrient content
Loamy Sand	The outskirts of WA Limestone minig area (32 °17"43.98'S, 150°48"57.0'E)	8.5	reddish yellow (7.5YR6/8)	Natural Tuart Forest	This is a Spearwood sand: has a higher proportion of fine sand (0.063 - 0.18mm) and lower percentage of coarse sand (0.5 - 1.0mm) than Bassendean
Sandy Clay Loam	Darling Scarp in Gooseberry Hill (31°57's, 116°03'E)	6	dark yellowish brown (10YR 3/6)	*Carymbra calophylla - E wandoo* woodland with thickets of *Hakea* and *Dryandra*	This is a shallow epidolerite-derived soil (Lamont *et al*.1999)

Chapter 4

Materials and Methods

4.1   Decomposition Stages and Coding

This research study was completed in two stages and the experimental design was based on Tibbett *et al.* (2004). The initial decomposition phase used large masses of each soil type, which were amended with organic porcine skeletal muscle tissue (obtained from the loin of *Sus scrofa*) and left to fully decompose for a period of time (Section 4.1.1). Controls were also set up which had no porcine muscle tissue added. The second decomposition phase used soil from the initial decomposition phase in smaller scale incubations with further skeletal muscle tissue (SMT) amendments according to a pre-defined treatment structure (Section 4.1.2). For each soil type, all possible combinations of SMT amendments (+) and controls (−) were included for the decomposition phases 1 and 2. These were −/− (control soil in phases 1 and 2) ,+/− (SMT added in phase 1 only) ,−/+ (SMT added in phase 2 only) and +/+ (SMT added twice: phases 1 and 2).

4.1.1 Initial Decomposition Phase

Three different soils, sand, loamy sand and sandy clay loam, were used in this study, each of which, were collected from their respective locations (details in Chapter 3) and sieved through a 6mm grid. Subsequently, 8kg of each soil were weighed into their respective 30L containers. There were two of these containers for each soil type and each was labeled accordingly (see Table 4.1).

Table 4.1 Experimental coding for soil types and treatments

Soil Type	Treatment	Experimental Label
Sandy soil	Tissue added	S+
	No tissue added	S-
Loamy sand	Tissue added	LS+
	No tissue added	LS-
Sandy clay loam	Tissue added	SCL+
	No Tissue added	SCL-

The water holding capacity of each soil type was calculated and subsequently adjusted to 50% water holding capacity. This percentage is generally accepted to be the optimum for greatest microbial activity (Linn and Doran, 1984). Once the water holding capacity was adjusted, the soil was allowed to equilibrate for 7 days. Following this equilibration time, the porcine skeletal muscle tissue (SMT) (*Sus scrofa*) was added to the soil. One container for each soil type was used as a control where no SMT was added. A total of 240g of porcine SMT was added to each

respective container. This SMT was cut into 2g portions before being added to the soil. The contents of each container (including the controls) were then mixed thoroughly. Loosely fitting plastic lids were placed on each container which allowed for some aeration throughout the experiment. These containers were stored in the dark at approximately 25°C (in a temperature-controlled room) for a period of 70 days. During this incubation time, the soil was turned every 24 hours to maintain an aerobic environment. The water holding capacity percentage was maintained throughout the experiment by spraying the surface of the soil with deionized water every 3-4 days.

After the 70 day incubation, soil samples were removed from each container. Community-level physiological profiling (CLPP) was completed on these soil samples (see Section 4.3).

4.1.2 Second Decomposition Phase

This second stage decomposition was much more specific and closely monitored. One hundred grams of soil from each of the containers (S+, S-, LS+, LS-, SCL+ and SCL-) were weighed and placed into their respective jars (40 jars for each container). The soil from each container underwent two tissue treatments. The first treatment involved the addition of 1.5g (this weight is based on Tibbett *et al.*2004) of porcine skeletal muscle tissue (*Sus scrofa*) (the exact weight of which was recorded for the measurement of tissue mass loss) to 20 of the jars. Each tissue sample was buried in

the middle of the soil sample and was not disturbed until its respective harvest. The remaining 20 jars underwent the second treatment of no skeletal muscle tissue addition. The jars were incubated in the dark at 25°C (in a temperature-controlled room) for the duration of the experiment (28 days) (see Table 4.2)

Table 4.2 Second decomposition coding indicating soil type and treatments of samples

Initial decomposition experimental label	Second Decomposition Treatment	Second Decomposition Experimental Label
S+	Tissue added (+)	S+/+
	No tissue added (-)	S+/-
S-	Tissue added (+)	S-/+
	No tissue added (-)	S-/-
LS+	Tissue added (+)	LS+/+
	No tissue added (-)	LS+/-
LS-	Tissue added (+)	LS+/-
	No tissue added (-)	LS-/-
SCL+	Tissue added (+)	SCL+/+
	No tissue added (-)	SCL+/-
SCL-	Tissue added (+)	SCL-/+
	No tissue added (-)	SCL-/-

Each tissue treatment was replicated five times and a total of four sequential harvests were set up for each tissue treatment. Figure 4.1 illustrates the second decomposition phase for each container.

Figure 4.1 A diagram showing the experimental set-up of the second decomposition

A 30ml vial containing NaOH was placed within each jar from the fourth sequential harvest so that carbon dioxide (CO_2) respiration could subsequently be measured throughout the experiment (see Section 4.2). These vials were exchanged every 24 hours for the first two weeks and then every 48 hours thereafter. During the exchange time, all of the jars were opened and fanned to allow an exchange of head-space gases.

Destructive harvests were completed at 7 day intervals where the SMT was removed, placed in its respective plastic bag, sealed and labeled. These SMT samples were stored for tissue mass loss analysis. During the harvests, the soil surrounding the

tissue was extracted using a 50ml container and stored. From the control samples, the soil from the centre of the jar was removed. The soil from each harvest was stored and labeled in its respective sealed plastic bag. These samples (soil and tissue) were then stored at -20°C. Table 4.3 summarizes the methods used in this study.

4.2   Carbon Dioxide Respiration

This method establishes the respiration rate (activity) of the microbial biomass within each of the soil samples. This method (see Rowell, 1994) was used during the second decomposition and was carried out each day for the first 14 days of the experiment and then every second day until the completion of the second decomposition.

Ten millimeters of 0.3M sodium hydroxide (NaOH) was pipetted into 30ml vials (these were known as NaOH traps). These vials were then placed into their respective jars (fourth harvest jars) and each jar was sealed tightly. The jars were then returned to the incubator and left overnight. After 24-hours, the NaOH traps were removed from the jars and sealed. New NaOH traps were placed in the jars which were then sealed and returned to the incubator. This was done continuously throughout the second decomposition. The NaOH from each vial was transferred into its respective 250ml conical flask where 10ml of 0.5M barium chloride ($BaCl_2$) was then added. Five drops of phenolphthalein indicator was added to the flask

which turned the solution pink. Hydrochloric acid (HCl)(0.1M) was back titrated into the flask until the colour changed from pink to colourless. This was performed for each trap. The volume of HCl used for each titration was recorded.

NaOH was used for the traps because of its affinity for CO_2. $BaCl_2$ was then added so that when the HCl was titrated, it would only react with the residual NaOH as the $BaCl_2$ is responsible for precipitating out the resulting Na_2CO_3 formed when the NaOH reacted with the CO_2 (Rowell, 1994).

4.3   Tissue Mass Loss

Tissue mass loss is a simple and direct method of measuring the rate of decomposition (Tibbett *et al.* 2004). This method was carried out during the second decomposition phase on the pre-weighed SMT. Four harvests were completed throughout the second decomposition and these harvests were done at 7 day intervals. Each tissue sample removed from each harvest was placed in a labeled plastic bag and frozen at -20°C. Each SMT sample was then washed in deionized water to remove any soil present on the tissue. The SMT samples were then re-weighed.

4.4   Community-Level Physiological Profiling (CLPP)

Microbial species within the soil community may respire at differing rates when utilizing different substrates (Campbell *et al*., 2003). CLPP is a measure of the short-term respiration responses to a range of simple organic substrates added to each soil sample.

The following CLPP procedure was carried out based on the Degens and Harris (1997) method, which was modified by Lalor *et al.* (2007). This procedure was performed on soil samples from both the initial and second decomposition phases. For the initial decomposition a total of four replicates were assessed from each of the samples: S+, S-, LS+, LS-, SCL+ and SCL-. It took a total of 4 days to complete the CLPP procedure on the initial decomposition. For the second decomposition, the CLPP procedure was carried out on the first three replicates of each of the samples.

20 organic substrates were used in this procedure. These substrates were classed as amino acids (10mM), amines (10mM), carbohydrates (75mM) and carboxylic acids (100mM). The pH of each of the substrate solutions were adjusted to 5.5 – 6.0 using concentrated HCl or NaOH (see Table 4.4).

Table 4.4 The substrates used in the CLPP analysis of the soil types and their respective treatments

Substrate	Class	Concentration
L-arginine		
L-asparagine		
L-glutamic acid		
L-histidine	Amino Acids	10mM
L-lysine		
L-serine		
D- glucosamine	Amines	10mM
L-glutamine		
L-ascorbic acid		
Citric acid		
Fumaric acid		
α-ketoglutaric		
DL-malic acid		
Malonic acid	Carboxylic Acids	100mM
Pantothenic acid		
Quinic acid		
Succinic acid		
L-tartaric acid		
Uric acid		
D-glucose	Carbohydrates	75mM

4.4.1 Procedure

One gram (dry weight equivalent) of soil from each sample was weighed out into their respective McCartney bottles. A total of 23 McCartney bottles were needed for each soil sample. Each substrate solution (2ml) was added to its respective bottle with a time interval of 30 seconds per substrate addition. Three blank bottles were sealed for each soil sample. One of these had 2 ml of de-ionised water added so that the basal microbial activity could be monitored. Each McCartney bottle was sealed tightly with a suba-seal, swirled and then incubated at 25°C for a period of 4 hours in

the dark. After 2 hours of incubation, each bottle was swirled again and returned to the incubator. The resulting concentration of CO_2 within each bottle was measured using an ADC series 225 Infra-Red Gas Analyser (IRGA).

Before the measurement of the samples, several 0.3ml, 0.2ml and 0.1ml samples of the 4.90% CO_2 standard were injected into the IRGA using a polythene syringe, which the samples were adjusted against. One milliliter of the head space gas was drawn from each sample and injected into the IRGA. This was carried out twice for each sample to ensure the level of accuracy was maintained.

4.5   Substrate - Induced Respiration (SIR)

The substrate - induced respiration method was developed by Anderson and Domsch (1978) in order to provide an effective and inexpensive way to indirectly measure microbial biomass carbon. SIR quantifies the initial respiration response of the microbial population to the added substrate in the soil which in turn gives an estimation of the carbon relating to the microbial biomass (Anderson and Domsch, 1978).

The following SIR method (Anderson and Domsch, 1978) used was modified by Carter (2006). It was performed on all the soil samples from the second decomposition, two hours after each sequential harvest. Five grams (dry weight equivalent) soil samples were weighed out into their respective McCartney bottles.

To each sample, a glucose solution (6mg g^{-1} soil) was added so that a total water holding capacity of the sample was calibrated to 95%. The glucose solution was added to the respective samples at 30 second intervals. A blank bottle was sealed for each treatment which had the equivalent amount of deionised water added in place of the glucose solution. These bottles were then sealed with suba-seals, shaken, and placed in 25°C incubator for 4 hours in the dark. After 2 hours of incubation, the bottles were shaken for a second time and immediately returned to the incubator.

As with the CLPP method, the IRGA was used here to measure the resulting CO_2 concentration in the head space gas of each sample. The process used, with respect to the use of the IRGA, was carried out in the same fashion as the CLPP method described previously (Section 4.4). The microbial biomass C was then calculated using the Anderson and Domsch (1978) equation: $x = 40.4y + 0.37$.

4.6   Metabolic Efficiency

The metabolic quotient ($q$$CO_2$) was calculated to assess microbial metabolic efficiency during their utilization of the SMT substrate. The $q$$CO_2$ was calculated by dividing the basal respiration (µg CO_2-C g-1soil h-1) by the microbial biomass C (µg $C_{mic}$$g^{-1}$ soil) and was expressed as µg CO_2-C$µg^{-1}$ $C_{mic}$$h^{-1}$ (Dilly and Munch,1998).

4.7   Statistical Analyses

The statistical package SAS was used to analyse the data received from CO_2 respiration, tissue mass loss and SIR. Analysis of variance (ANOVA) was used to examine the tissue mass loss and SIR results. Repeated measures ANOVA was used to assess the CO_2 respiration data. This modified version of ANOVA was required to take into account the differing time periods throughout the CO_2 respiration analysis. Analysis of the CO_2 responses from the CLPP procedure was completed using the Primer 6 statistical package. Non-metric multidimensional scaling (MDS) (Clarke, 1993) and an analysis of similarity (ANOSIM) were applied to this CLPP data. This resemblance matrix was based on Euclidean distance and the data was first standardized using square-root transformations before analyzed.

Chapter 5

Results

5.1   Tissue Mass Loss

The pretreatments of SMT and the differing soil types both showed significant effects at each harvest time (P<0.001). Generally, all pretreated samples with SMT (+/+) had a greater rate of tissue mass loss than the samples that were not pre-exposed to tissue (-/+). All tissue within the pretreated samples were fully decomposed at the end of the 28 day incubation, leaving no SMT present. In the -/+ samples, the sandy loam and sandy clay loam soils reached a maximum tissue mass loss of 66% and 69% respectively. In the equivalent sandy soil samples a maximum tissue mass loss of approximately 95% was recorded at the fourth harvest.

(i)

(ii)

(iii)
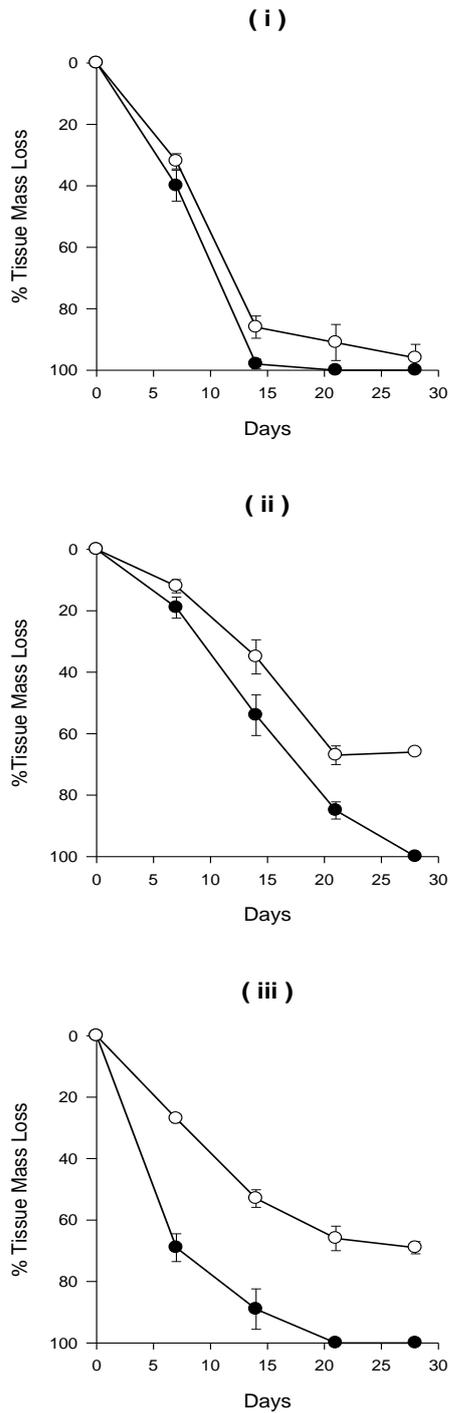
Figure 5.1 Percentage tissue mass loss during the second decomposition stage. 1.5g of porcine (*Sus scrofa)* muscle tissue was buried in 100g of (i) sandy soil, (ii) loamy sand and (iii) sandy clay loam, obtained from the initial decomposition stage. The treatments are as follows: Tissue added after pre-decomposition (●) and tissue added without pre-decomposition (O), Bars represent standard errors where n=5.

5.2   CO$_2$ Respiration

Both soil type and SMT treatments have significant effects on microbial activity (P<0.001). In both the sand and sandy clay loam soils, the +/+ samples show less activity than the -/+ samples. In contrast, the loamy sand shows little difference in microbial activity between the +/+ and +/- samples.
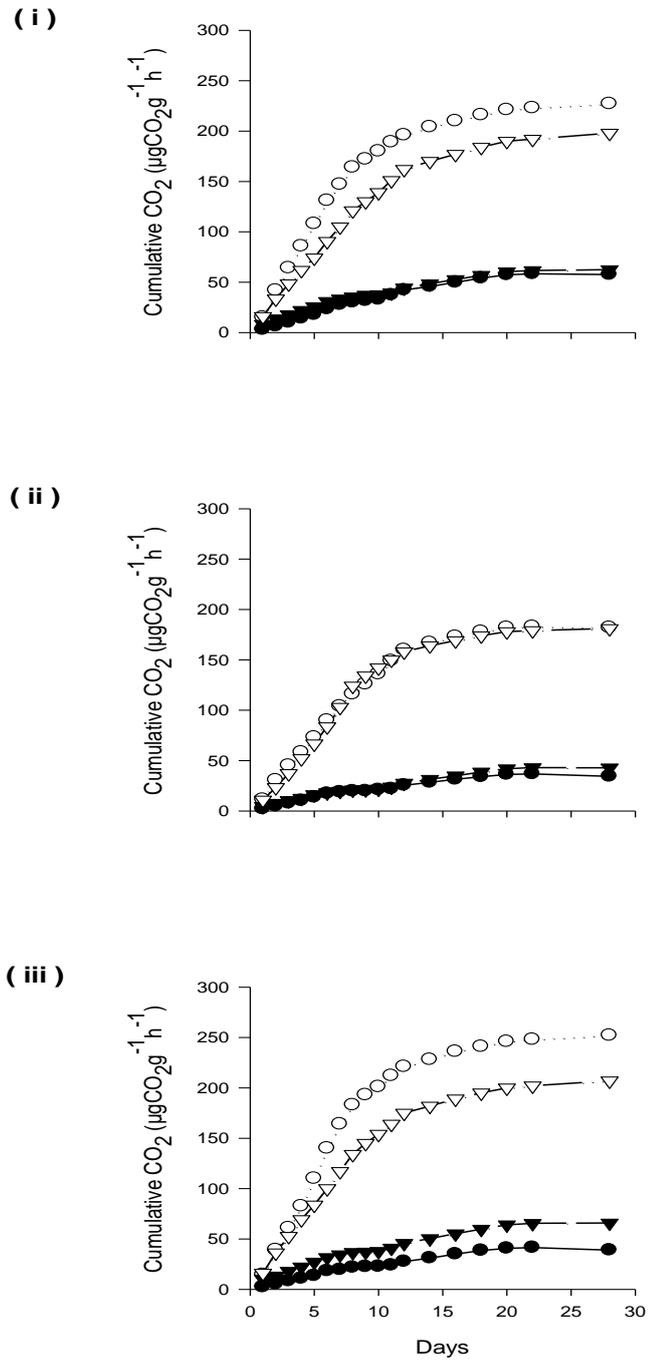
Figure 5.2 Cumulative CO_2-C during the second stage decomposition following the burial of 1.5g porcine muscle tissue (*Sus scrofa*) in 100g soil samples retrieved from the initial decomposition stage. The soils used are (i) sandy soil, (ii) loamy sand and (iii) sandy clay loam found in areas round Perth, Western Australia. The treatments are as follows: tissue added without pre-decomposition (O), no tissue added without pre-decomposition (control) (●), tissue added after pre-decomposition (△) and no tissue added after pre-decomposition (▼). (n=5)

5.3   Substrate - Induced Respiration

Microbial biomass within all the soil types generally decreased over the duration of the second incubation across the treatment combinations.   A number of complex three-way interactions were observed where tissue treatments, soil and time had a combined effect (see appendix).   In the sand and sandy clay loam soils, the +/+ samples had a lower microbial biomass than the -/+ samples.   In contrast, the loamy sand generally showed little difference in microbial biomass between the +/+ and -/+ samples expect for day 14, where +/+ had the highest microbial biomass.

(i)
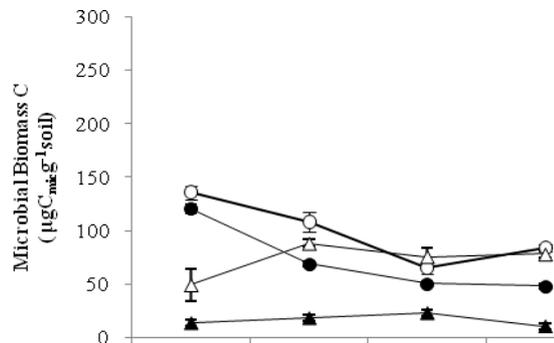
(ii)
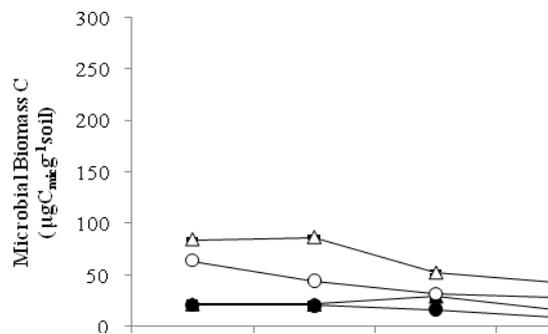
(iii)
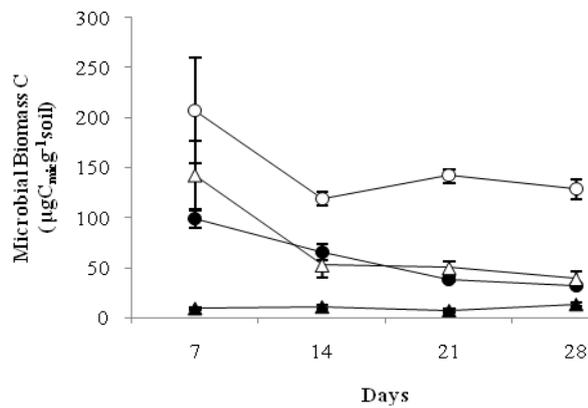
Figure 5.3 Microbial biomass (µg C_{mic} g^{-1} soil) estimated using substrate-induced respiration during the Second Decomposition Stage in sandy soil (i), loamy sand (ii) and sandy clay loam (iii) soils under the following conditions: no tissue added to the soil without pre-decomposition (control) (●), no tissue added to soil after pre-decomposition of skeletal muscle tissue (▲), tissue added to soil without pre-decomposition (O) and tissue added to soil after pre-decomposition of skeletal muscle tissue (△). The bars represent standard error where n=5.

5.4   Metabolic Quotient

Generally, the -/+ and +/+ samples showed an increase in microbial efficiency during the 28 day incubation. Significant differences between the treatments +/+ and +/- were generally observed throughout the incubation across all the soils ($P>0.001$).   In the sand and loamy sand, significant differences between -/+ and +/+ treatments were only noted during the first half of the incubation, however in the sandy clay loam, significant differences were generally observed throughout the incubation ($P<0.001$). In the sand, +/+ generally had the greatest significant differences in the first and last harvests when compared to the rest of the treatments. The treatment +/+ had the greatest significant differences in comparison to the rest of the treatments during the first half of the incubation in the loamy sand and for the duration of the incubation in the sandy clay loam.

(i)
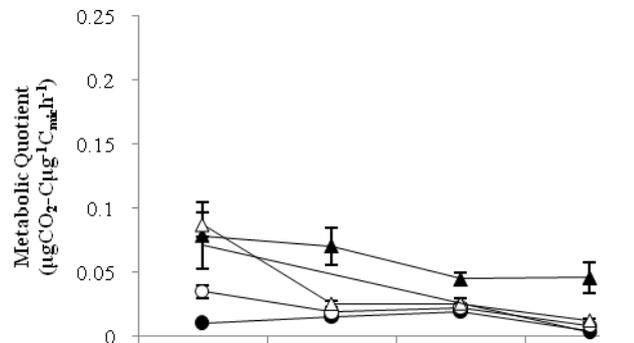
(ii)
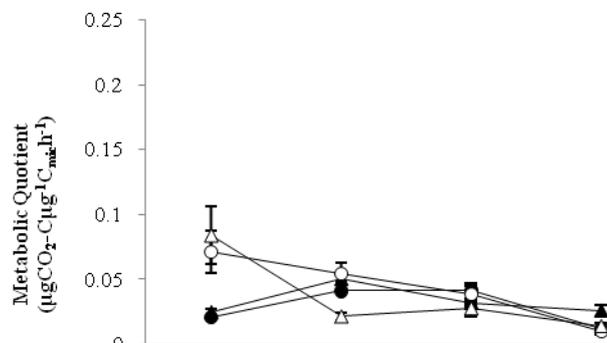
(iii)
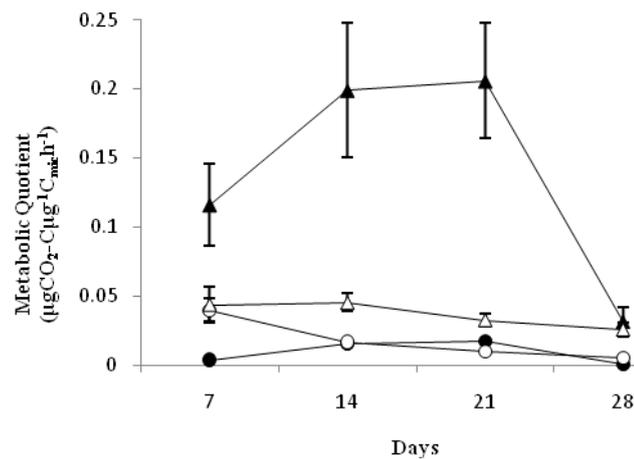
Figure 5.4 Represents the metabolic quotient of the microbial communities in the sandy soil (i), loamy sand (ii) and sandy clay loam (iii) soils under the following conditions: no tissue added to the soil without pre-decomposition (control) (●), no tissue added to soil after pre-decomposition of skeletal muscle tissue (▲), tissue added to soil without pre-decomposition (O) and tissue added to soil after pre-decomposition of skeletal muscle tissue (△). The bars represent standard error where n=5.

5.5   Community – Level Physiological Profiling

5.5.1   CLPP after Initial Decomposition

From the non-metric multidimensional scaling plot (MDS plot) of the samples, clear groupings can be seen between the control soils and the soils with added SMT (Figure 5.5). A two-way ANOSIM, applied to all the CLPP data, showed a significant difference ($P<0.001$) between the treatments (Global R:0.84). Pair-wise comparisons between the soils showed no significant differences between the sand and sandy clay loam ($P<0.4$). However there were differences between the sandy clay loam and loamy sand ($P<0.029$) as well as the loamy sand and sand ($P<0.014$).
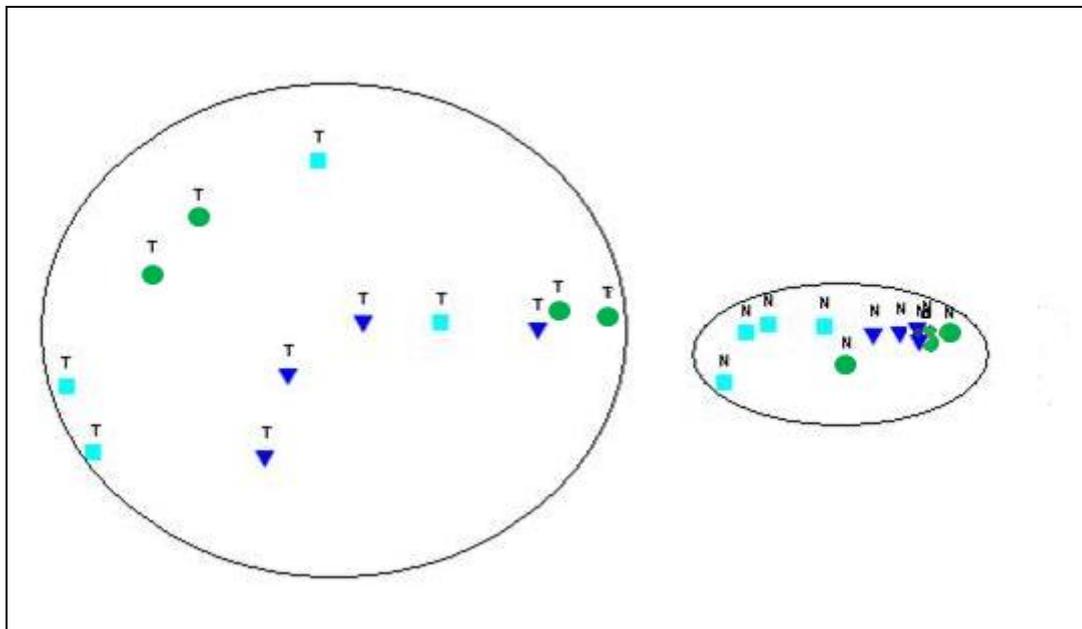
Figure 5.5 A 2D multidimensional scaling ordination of CLPP data after the initial incubation (70 days) from sandy soil (●), loamy sand (▼) and sandy clay loam(■) soils, on their controls (N) and samples with added tissue (T) (2D stress: 0.03). (n=4)

One–way ANOSIMs were applied to the CLPP data for each treatment type. The control data shows significance differences between the soil types (P<0.004) (Global R: 0.5). Pair-wise comparisons between the soil types of this control data showed significant differences between both the sand and loamy sand (P<0.029) as well as the sandy clay loam and sandy loam soils (P<0.029). There was, however, no significant difference observed between sandy clay loam and sandy soils (P<0.49). The one-way ANOSIM of the soil samples treated with tissue showed no significant difference between the soil types (P<0.41) (Global R: 0.005) with pair-wise comparisons supporting this (sand and sandy clay loam (P<0.29); sand and loamy sand (P<0.43); sandy clay loam and loamy sand (P<0.4))

5.5.2 CLPP after Second Decomposition

The MDS plots for each soil type show differing grouping patterns between the treatment combinations (Figure 5.7).
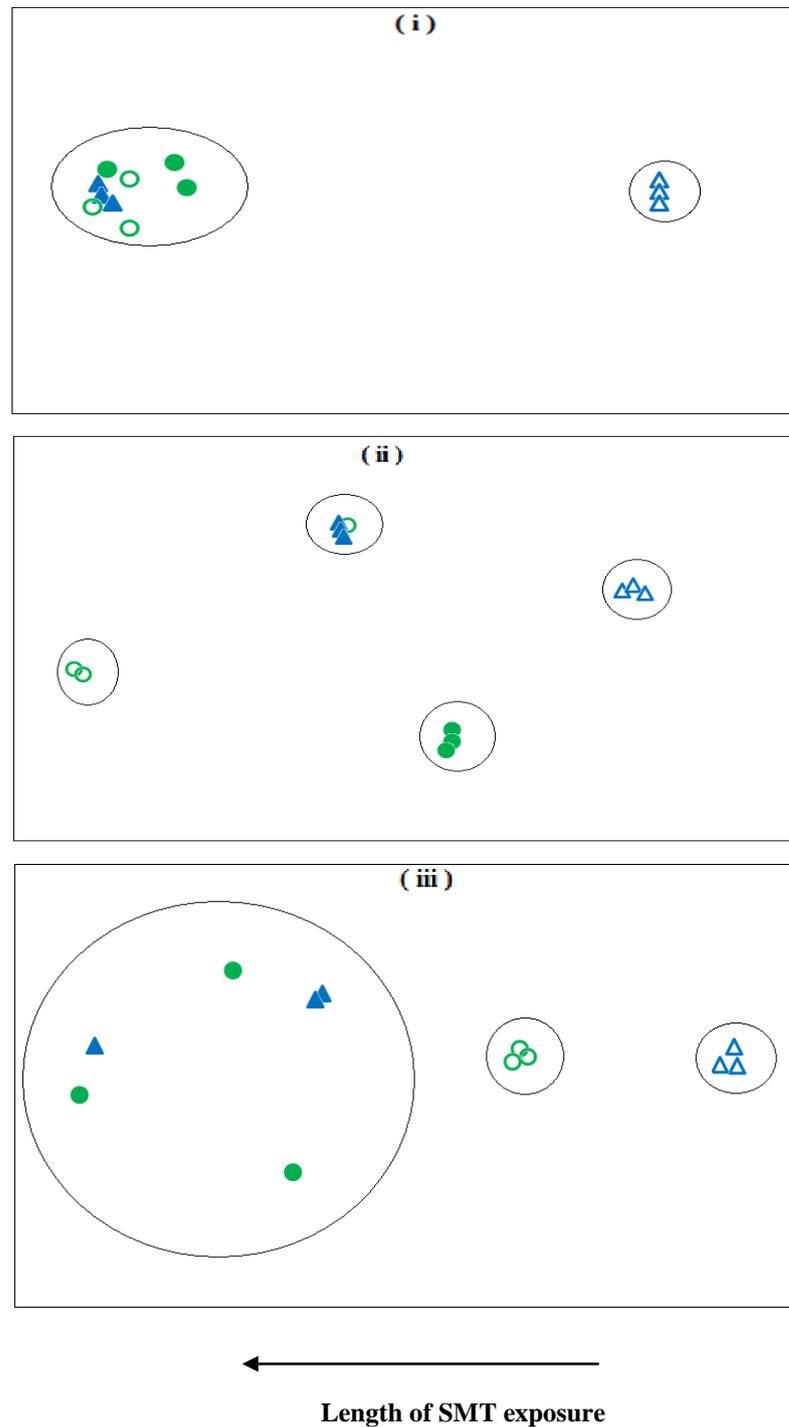
Length of SMT exposure

Figure 5.6 2D multidimensional scaling ordinations of the community level physiological profiling data from the (i) sandy soil (2D stress: 0.01), (ii) loamy sand (2D stress: 0.01) and (iii) sandy clay loam soil (2D stress: 0) following the second decomposition: Tissue added after pre-decomposition (●), no tissue added after pre-decomposition(▲), tissue added without pre-decomposition(○), no tissue added without pre-decomposition(△). (n=3)

Functional changes were first noticed after the initial incubation due to the SMT treatments. These changes were defined by the soil after re-treatment with SMT as the functional patterns of the microbial communities differed between the three soil types only after the second incubation

In the sandy soils (Figure 5.7i), similar functional changes occurred within microbial communities, regardless of when the SMT was added as the controls samples (-/-) are clearly grouped away from the rest of the samples (+/-, -/+, +/+). Within the loamy sand soils, each different combination of SMT treatment had a unique effect on the functional response of microbial communities producing different functional profiles according to treatment combination (Figure 5.7ii). In the sandy clay loam soils, there are 3 clear groupings (Figure 5.7iii), the controls, the -/+ samples and then both the +/- and +/+ samples. These changes in the functional capacity of the microbial communities seemed to be affected by the length of time the soils were exposed to SMT. The longer the exposure, the similar the functional capacity of the microbial communities became.

Chapter 6

Discussion

Across the three soil types, the ability of the microbial communities to adapt to the burial of SMT was illustrated by the increased efficiency and rate of SMT decomposition after being pre-exposed to the SMT.   The specific process of adaptation may not be clear, however, the change observed in the catabolic diversity of the microbial communities after the initial SMT addition, might suggest a functional change facilitated this adaptation.   The extent of adaptation was influenced by soil texture with rates of decomposition of SMT decreasing in the order of sand, sandy clay loam and loamy sand.   This result is consistent with other research which showed mineralization rates increased in soils with the highest sand content (Cayuela, 2008; Côté *et al.*2000).   This result is also consistent with research by Haslam and Tibbett (2009), where greater rates of decomposition were noted in soils of a lower initial pH (sand had a pH of 5.5, sandy clay loam had a pH of 6 and loamy sand had a pH of 8.5).   In this current study, the present data indicated that the maximum rate of decomposition always occurred in the first half of the second incubation.   There may be two reasons for this. Firstly, most of the moisture from SMT may be lost during the early stages of incubation. Secondly, the mass and quality of the resource (SMT) may have significantly lessened throughout the duration of the incubation (see Carter and Tibbett, 2006).

6.1. Evidence of Microbial Adaptation

Following the re-introduction of SMT, a more rapid rate of decomposition was most likely due to changes in the functionality (microbial activity, catabolic diversity) of the microbial communities, resulting in the adaptation of this decomposer community (possibly through rapid proliferation of zymogenous microbes). The adaptation of microbial communities to the introduction of organic resources has been observed in previous studies. Recently, Carter and Tibbett, (2008) demonstrated enhanced microbial decomposition to the repeated introduction of ovine (*Ovis aries*) skeletal muscle tissue without a related increase in microbial biomass, thus indicating microbial adaptation. Macleod and Semple (2002) were responsible for showing microbial adaptation to the presence of hydrophobic organic contaminants, such as pyrene, in the soil. The rate of this adaptation to pyrene was also shown to be dependent on soil environment, paralleling similar observations to our current study. Further examples of microbial adaptation include the repeated application of herbicides which resulted in an enhanced rate of decomposition (Jayachandran, 1997; Walker and Austin, 2003). However, some of these studies showed an increase in microbial biomass and C mineralisation (Hendry et al, 1988; Moorman, 1990; Dzantor and Felsot 1989). These responses were not evident in the present study. Another aspect that may have some effect on the microbial responses is the carryover of nutrients between incubations. Nutrient carryover is inevitable and to counter this would cause disturbances which may compromise the integrity of the experiment.

The decreased microbial biomass, activity and metabolic quotient associated with the +/+ samples are likely consequences of microbial adaptation. Microbial adaptation can lower the resilience of the community to other stressors (Rusk, 2004) thereby reducing their ability to cope with changes in their immediate environment. The decrease in soil microbial biomass may also be related to the maintenance of the microcosm. In a recent study, soil chemistry has been shown to change with time in similar incubations, even in the control soil, suggesting their systems are not in equilibrium (Stokes *et al.* 2009). The relative inefficiency (metabolic quotient) observed in the +/- samples may be due to microbial stress in response to resource depletion (lack of SMT) of the recently adapted microbial community. These findings, in conjunction with changes in catabolic diversity, may infer that the microbial communities changed functionally to utilize the SMT in a more efficient way.

6.2. The Influence of Soil Texture

Soil texture is commonly known to have an effect on microbial function (Fang *et al.*, 2007; Fang *et al.*, 2005; Bossio *et al.* 1998; Buyer *et al.*, 1999). In the current study, soil texture was observed to influence microbial adaptation where the efficiency and rate of decomposition by the microbial communities in the loamy sand were less than the sand and sandy clay loam soils. In the sandy loam soil, changes in the rate of decomposition of SMT and the catabolic diversity of the microbial communities were observed between the SMT treatments. However these results were not

reflected in the microbial biomass, activity or metabolic quotient. This suggests apparent adaptation was a result of functional change rather than changes in microbial biomass or magnitude of activity. These differences observed between the soils might be due to the clay and organic matter content of the soils. Clays are well known to adsorb organic compounds (e.g. enzymes and organic matter) (Carter *et al.*2007b; Marx *et al.* 2005), which can result in the sequestration of the substrate in the soil aggregates and therefore protection from microbial decomposition (Khalil *et al.*, 2005). This may have occurred in the sandy loam soil. Although its clay content is less than the sandy clay loam, its organic matter content may be higher. This high organic matter content, in conjunction with its clay content, may limit decomposition in this soil. Macleod and Semple (2002) observed lower pyrene mineralization in the soil with a higher organic matter content. Other research has shown that the sorption of organic compounds increases with higher organic carbon contents in the soil (Means et al., 1980; Weissenfels et al., 1992). Fang *et al.* 2007 found that soil texture was a significant factor during the decomposition of corn residues and showed that a silty clay soil had increased C-mineralization when compared to the sandy loam soil.

6.3.   The Comparison of Microbial Decomposition Patterns

The general decomposition patterns (mass loss, C mineralization) in the current study were similar to those observed in Carter and Tibbett (2006). Both studies

showed similar exponential decay curves, with the degree of decomposition being the only differing factor between soils from either study. These similar exponential patterns were also observed in a recent study by Cayuela *et al.* (2008), where differing meat and bone meal compositions were amended to soils. The rates of decomposition differed between soil types and meal compositions, however the general exponential pattern of decomposition remained similar. These similarities might be a significant finding with respect to forensic taphonomy. The use of soils in forensic taphonomy would benefit greatly from the identification of universal processes and patterns applicable to gravesoils regardless of location or physicochemical characteristics.

6.4.   The Relevance of these Findings to Forensic Science

Ultimately, forensic evidence can be grouped into two major categories: spatial evidence and temporal evidence. This study has important research and case work implications for both these types of evidence. From the current study, it has been shown that SMT decomposes differently in soils that have been pre-exposed to this substrate. Therefore, to obtain accurate results during taphonomic studies, it is important to bury bodies and/or their components in soils that have not been pre-exposed to these remains or at least know the history of the soils in this regard. This may be particularly relevant to the study of post-mortem interval, as rates of decomposition are shown to vary according to pre-exposure. Vass *et al.* (1992)

found that specific volatile fatty acids (VFAs) and various cations and anions were released in the soil from decomposing human cadavers in predictable patterns which could enhance the accuracy of postmortem interval determinations. The current study suggests that the accuracy of VFAs as an estimation of post-mortem interval would be dependent on the soil, as those results may differ given a proximity to previous cadaver decomposition(s). Therefore it is recommended that future research should be conducted in uncontaminated soils where ever possible. With regards to case work, the current findings might have important implications on serial dump sites or mass burial situations.

Soil is most commonly used as spatial evidence in forensic science (Fitzpatrick, 2008). Soil collected from clothing and objects are routinely compared to soils collected from crime scenes. Comparisons can be based upon physicochemical characteristics (Junger, 1996; Pye *et al.,* 2006; Thanasoulias *et al.,*2002; Cox *et al.*, 2000) and biological characteristics (Horswell, *et al.* 2002; Lerner *et al.* 2006; Heath *et al.*, 2006; Horrocks *et al.* 1999). The current research addresses the biological aspect of spatial evidence. Previous research showed soil microbial community DNA profiling could potentially link a suspect to a crime scene (Horswell, *et al.* 2002). The functional profiles of the microbial communities obtained in our research may prove complimentary to microbial DNA profiling and enhance the specificity of soil microbial characterization.

6.5   Conclusions and Further Research

In conclusion, the hypotheses put forward in this study have been accepted. The re-introduction of SMT to the soil has led to its enhanced decomposition (measured by tissue mass loss and microbial activity), which may have been facilitated by a functional change in the soil microbial communities. The extent of this SMT decomposition has varied according to soil type. Across the soils, differences in catabolic diversity, tissue mass loss, microbial activity and biomass support this finding.

These encouraging outcomes of the present research should be extended with further related research. Now that the adaptation of microbial communities to the presence of SMT substrates has been confirmed, cadavers may be used in place of SMT to assess how microbial adaptation may change to the addition of a more complex substrate. This will help establish the applicability of this approach to real life scenarios. Walker and Austin (2003) showed that repeated herbicide amendment of soils led to faster rates degradation of the herbicide. A corresponding effect from repeated SMT burials on soil microbial communities might be anticipated. A variation in time between repeated burials would also help assess the long term effects of the changes that occurred within the microbial community as a result of the SMT additions.   Further work in this field may incorporate the use of techniques such as 16S-based denaturing gradient gel electrophoresis (DGGE) and phospholipid fatty acid analysis (PLFAs). DGGE is a molecular technique that would have

assessed the genetic diversity present within the soils' microbial community structure and would have provided further indications of the changes that may have occurred within the microbial community (Griffiths *et al.*, 2003). PLFAs would have provided a biochemical fingerprint of the soil microbial community (Drenovsky *et al.* 2004) and would have given an indication of any structural changes that may have occurred throughout the incubation (Bloem *et al.* 2003). Along with CLPP, these techniques would have provided a greater understanding of microbial community changes that occur during the adaptation process.

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Appendix

Substrate-Induced Respiration Statistics

Source	DF	Type I SS	Mean Square	F Value	Pr > F
treat1	1	249.8725949	249.8725949	139.66	<.0001
treat2	1	748.7826959	748.7826959	418.52	<.0001
treat1*treat2	1	36.8478144	36.8478144	20.60	<.0001
Soil	2	185.2750946	92.6375473	51.78	<.0001
treat1*soil	2	326.1767536	163.0883768	91.16	<.0001
treat2*soil	2	55.5869563	27.7934782	15.53	<.0001
treat1*treat2*soil	2	15.4379590	7.7189795	4.31	0.0147
Harvest	3	153.7147489	51.2382496	28.64	<.0001
treat1*harvest	3	38.0544904	12.6848301	7.09	0.0002
treat2*harvest	3	7.4506157	2.4835386	1.39	0.2477
treat1*treat2*harves	3	14.1577875	4.7192625	2.64	0.0509
soil*harvest	6	37.2043519	6.2007253	3.47	0.0028
treat1*soil*harvest	6	38.8965255	6.4827543	3.62	0.0020
treat2*soil*harvest	6	41.5029425	6.9171571	3.87	0.0011
trea*trea*soil*harve	6	22.3758663	3.7293111	2.08	0.0568

Source	DF	Type III SS	Mean Square	F Value	Pr > F
treat1	1	249.8725949	249.8725949	139.66	<.0001
treat2	1	748.7826959	748.7826959	418.52	<.0001
treat1*treat2	1	36.8478144	36.8478144	20.60	<.0001
Soil	2	185.2750946	92.6375473	51.78	<.0001
treat1*soil	2	326.1767536	163.0883768	91.16	<.0001
treat2*soil	2	55.5869563	27.7934782	15.53	<.0001
treat1*treat2*soil	2	15.4379590	7.7189795	4.31	0.0147
Harvest	3	153.7147489	51.2382496	28.64	<.0001
treat1*harvest	3	38.0544904	12.6848301	7.09	0.0002
treat2*harvest	3	7.4506157	2.4835386	1.39	0.2477
treat1*treat2*harves	3	14.1577875	4.7192625	2.64	0.0509
soil*harvest	6	37.2043519	6.2007253	3.47	0.0028
treat1*soil*harvest	6	38.8965255	6.4827543	3.62	0.0020
treat2*soil*harvest	6	41.5029425	6.9171571	3.87	0.0011
trea*trea*soil*harve	6	22.3758663	3.7293111	2.08	0.0568