Nutrient dynamics of annual ryegrass as a tool in forensic investigation on burial sites

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Fresh	
	Commences immediately after death - autolysis, fluid filled blisters on the skin, skin slippage, drying of the nose, lips and fingers, skin marbling – livor mortis, prominent haemolysis.
Bloating	
	Putrefaction – discoloration of the head, green discolouration of the skin, formation of gas bullae, anaerobic fermentation, purging of gas and fluid, eventual maximal body swelling. - Heightened anaerobic bacteria activity (intestinal and stomach origin)
Destruction and Decay	
	Decomposition through volatile fatty acids and other products - Cease of bloating, broken skin, active autolysis, breakdown of internal organs, destruction of blood, putrefaction, collapse of abdominal cavity, early skeletonisation with ligaments, possible formation of adiopocere, mummification possible. - Predominant insect activity and possible carnivore activity
Dry	
	Diagenesis, absence of carrion fauna, bone exposure, small amounts of decaying tissue, leather like skin over bone,
Skeletonisation / Dry	
	Exposed bone >50% of the body – bone, teeth and resistant cartilage, chemical weathering of bone.
*Table adapted from Swann, Forbes, and Lewis (2010) and Stejskal (2012). General trend of decomposition in exposed environments. Largely dependent on temperature and environment.	

1. Introduction

The situation of a buried corpse presents a major problem to forensic investigator, post deposition estimation and gravesite location. Currently, there is no universal method that provides consistent and accurate post mortem interval (PMI) or gravesite location. The various disciplines within forensics have provided different perspectives in tackling this ever present problem (A. A. Vass, 2011). Studies within taphonomy carry great importance in PMI estimation by analysing the decomposition characteristics between the pre and postskeletonisation of a cadaver (Swann et al., 2010). Engaging with other factors, the onset of rigor mortis to livor mortis, or the progression of fresh decay to advanced decay can provide a valuable time frame. Yet a decomposing body attracts a variety of insect such as various families of flies and carrion beetles, of which the process and activity of certain species have demonstrated to be dependent and/or correlated with PMI (Byrd & Castner, 2009). Thus, entomology is another area of great importance with its methods of PMI estimation established and well documented for legal use (Byrd & Castner, 2009; Singh, Sharma, & Sharma, 2016). However, a myriad of factors influence the decomposition process, particularly in environments as complex as that of a buried cadaver.

The breakdown process fundamentally involves the reduction to primary constituents of carbohydrates, lipids and proteins until ultimately, dry matter is left (Cockle & Bell, 2015). The complex and multistage process of cadaver decomposition is greatly influenced by external factors such as temperature, humidity, exposure and the nature of terrestrial or aquatic environments (Sorg, Haglund, & Wren, 2012). The chemistry of grave soil can be analysed in order to detect decomposition by products, for example nitrogen level over extended periods of time (B. Anderson, Meyer, & Carter, 2013). The decomposition rate of experimental models also show variable differences for models left to decompose indoors as opposed to outdoors, as well as the differences between studies conducted in rural rather than suburban environments (G. S. Anderson, 2011). Furthermore, many studies have been conducted to

account for these factors in the task of deriving a PMI estimation method or to establish some form of decomposition rate framework. These will be discussed in further detail in the review.

Studies surrounding the interactions between decomposition products and flora show promising result in expanding the tools available in gravesite detection. Prevailing detection methods include ground probing, arial photography, remote sensing and search via cadaver dogs, methods vary by case and site and no one method carries a 100% success detection rate (France, 1992). Vegetation growth dynamics post-burial are useful as potential visual indicators of the age of gravesite, as well as indicators of a grave site itself (Caccianiga, Bottacin, & Cattaneo, 2012). Existing studies demonstrate the many steps forward to add to and improve on existing methods.

2. Human decomposition

In the study of cadaver decomposition there is major focus on the chemistry and physiological process that is of great interest in forensics.

Within the human body not all cells are the same, nor do they behave and function under the same conditions. The same can be said for cell death, e.g. cells containing high amounts of degradative enzymes such as proteases and lipases will decompose quicker than other cells (Stejskal, 2012).

Cellular processes function under ideal temperatures, nutrient supply, oxygen and pH levels. The changes to these components causes a change to cellular process at a systemic level (Stejskal, 2012). Following the death of an organism, these conditions will drastically change, and depend on the surrounding environment such as temperature and exposure. This is what ultimately varies the rates of decomposition and is the cause of so much difficulty surrounding estimation of time since death, or burial.

The physiochemical processes triggered after death follows a predictable set of processes and stages that continues over time until only dry matter remains. This is characterised by the preskeletonisation stage of autolysis to dry remains while skeletonisation is the absence of tissue and moisture and the predominant exposure of bone (Stokes, Forbes, & Tibbett, 2013; Swann et al., 2010). These processes commence with autolysis and the self-digestion of cells, soon followed by bloating which marks the beginning of putrefaction – the rotting of organic matter stage. Following putrefaction active decay occurs affected by insects, bacteria and fatty acids, this continues until moist skin and tissue are degraded, marking the start of skeletonisation (Swann et al., 2010). This process and the hallmarks of decomposition is summarised in Table 1.

Table 1: Hallmarks and details of decomposition	
Stage	Description
Pre-skeletisation – characteristic of the four stages of fresh, bloated, decay and dry	
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	Commences immediately after death - autolysis, fluid filled blisters on the skin, skin slippage, drying of the nose, lips and fingers, skin marbling – livor mortis, prominent haemolysis.
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	Putrefaction – discoloration of the head, green discolouration of the skin, formation of gas bullae, anaerobic fermentation, purging of gas and fluid, eventual maximal body swelling. - Heightened anaerobic bacteria activity (intestinal and stomach origin)
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As seen in Table 1 the body undergoes various stages and processes, however the distinction between the stages are not always clear and are ultimately dependent on external factors (Swann et al., 2010). An understanding of soft tissue breakdown is of great importance as varying physiological and environmental conditions bring about different results to grave site location and ultimately the post mortem interval.

a. Factors affecting decomposition

There is a great variation of results between the many studies that have looked at what affects decomposition and how. This is due to the myriad of ever-present external factors that influence the outcome of soft tissue breakdown. Temperature, water, soil, exposure, insects and microbiology all cooperate and play their part in this highly complex and variable process.

i. Environmental factors

Temperature is a major determinant of how the body will breakdown following death. It influences microbial activity, rates of decomposition, insect activity and soil conditions – temperature will almost always impact decomposition in one form or another (Cockle & Bell, 2015).

As a dominant driver of decomposition it can either accelerate chemical reactions that is associated with autolysis and bacterial activity, or slow it down (Sorg et al., 2012; Turner & Wiltshire, 1999). This can be anything from the presence of shade, vegetation, indoor or outdoor environments or specific locations such as lakes and valleys. Were a body left to decompose indoors the influence of temperature would be the access or barriers to direct sunlight, insect scavengers and room temperatures. Temperatures greater than 4°C generally allows for insects to reproduce and decomposition to proceed rather quickly without trouble (Sorg et al., 2012).

 Entomologically, the temperature of a room or given location will influence when flies will reach a corpse. Certain species such as blow flies, flesh flies, carrion and scarab beetle have

activity associated weather, season or geographical location (Singh et al., 2016; Sorg et al., 2012). Humidity coupled with temperature will also affect fly larvae development, and when they are able to access the body – most significantly that being the effects of large maggot mass (Magni, 2016; Weitzel, 2005). The activity within these masses can increase temperatures to high levels and would often further the progression of putrefaction or active decay, accounting for the general rapid speed of decomposition in an open environment (Magni, 2016; Weitzel, 2005).

In an Australian context, a study done by Archer (2004) in Victoria examined the effects of the four season on the rate of body mass loss. Newborn pigs were exposed to damp forest setting during the four seasons. There was clear indication that seasons with higher temperatures as well as rainfall saw greater loss of body mass – however this rate differed yearly due to the fluctuations in weather. Given that bodies related to homicides are not always discarded naked, as was conducted in this study, temperature plays an indirect if not a rather minimal role in influencing decomposition (Kelly, van der Linde, & Anderson, 2009).

Mummification refers to drying tissue due to the evaporation of liquid. This form of decomposition can occur in either extremely hot or cold climates, with its result being a rather tough leather like layer of skin covering the bones (Stejskal, 2012). This process is not often seen in clothed corpses.

The influence of changing conditions also go below the surface of the soil, influencing soil temperature and affecting microbial activity. Top soil (of 2.5cm or less) is subject to temperature fluctuations that can also allow odour to reach the surface attracting flies. The further one goes below the surface, there is less effect of fluctuations, leaving a stable and cooler environment (Stejskal, 2012).

Water, water environments, rainfall and moisture bring about different results to how decomposition proceeds, however temperature is rarely dissociated from it. The most recognisable product of decomposition - the adiopocere formation, is directly associated with

water level and moisture. The formation of adiopocere requires moisture, an aquatic environment, humidity, oxygen levels and the presence of bacteria (O'Brien & Kuehner, 2007). However, certain soils initiate the formation of this layer better than other types, permeable soils that retain water provide conditions that allow for the formation of the outer layer rather then decomposition, rather than high organic and low clay soils (Duraes et al., 2010). However as cited by O'Brien and Kuehner (2007) optimal formation conditions are not completely straightforward, described as the "Goldilocks Phenomenon" it takes a certain balance of moisture and bacteria and soil and other environmental elements at the 'Just right' condition to be able to completely form the layer.

As described by Stejskal (2012), decomposition does not always result in tissue breakdown by can also result in preservation through the adiopocere layer, this is achieved through saponification which is the hydrolysis of the body's fatty acids. The adiopocere layer is preserves so well that can inhibit post mortem changes (Duraes et al., 2010).

Compared to land, aquatic environments are extremely dynamic and unpredictable. According to Sorg et al. (2012) "…they tend to have shifting, three-dimensional dynamics that differ in chemistry, currents, and temperature according to region, season, depth, bottom type, and topography…". In this scenario adiopocere normally forms in aquatic environments with little current movement.

The state of a body will ultimately depend on the location where it has either been buried or placed on soil. The properties of soil such as permeability, water retention, soft and loose or compact will impact how a body decomposes (Turner & Wiltshire, 1999). In a case of burial, the depth of the site of the grave will decide what temperatures a body will be exposed to. Top soil, sub soil and deep soil all experience different microclimates, top and sub soil are generally subject to temperature fluctuations, insect access and porosity (Stejskal, 2012). Deeper soil around 40-50cm are less affected by temperature changes, contain less oxygen and higher moisture levels. In all layers, microbes within the soil are present and also subject to the changes with anaerobic species in deeper soil layers (Tibbett, 2008).

ii. Physical factors

Many studies are done exploring insect colonisation and to explore the determinants of soft tissue decomposition. Yet a body is not always left naked, therefore wrapping and clothing carries its own variation. Wrapping may restrict access and potentially provide a microclimate for insect and bacterial activity. Restriction of insect access was dependent on how secure the clothing or wrapping is at covering exposed areas (Kelly et al., 2009). Clothing imposes varying affects in the advanced decay stage as closed environments retain moisture and keep decay going, thus slowing down overall mass loss. However, this will inevitably depend on if only the body is wrapped in only clothing, wrapped in plastic, blankets or even multiple layers.

3. Decomposition in buried environments

Decomposition changes rather significantly when there is no exposure to an open environment and all its nuances above ground. Buried environments pose a real problem in PMI estimation as it prevents a lot of typical external factors from acting on a body, as well as introducing new variables such as soil condition,

a. Effects on the body

A study by (Cockle & Bell, 2015) has affirmed that the rate of decomposition for buried and exposed subjects is a variable dependent on the stage of decomposition. Certain stages of the decomposition of buried cadavers were faster in buried environments, particularly for processes occurring immediately after death such as autolysis. The stage of putrefaction

proceeded much faster on the surface as expected while later stages of decomposition proceeded slightly faster in buried cases. In the case of exposed cadavers decomposition preceded faster due to access to carnivores, rainfall, insects and atmospheric temperate (Arpad A. Vass, 1992).

An experiment by (Turner & Wiltshire, 1999) revealed that cold anoxic conditions at the burial site of swine carcasses would result in the preservation of tissue, or rather the delayed on set of advanced decay. Soil provides a form of insulation barrier against solar radiation, creates a cool environment and slows down decomposition (Troutman, Moffatt, & Simmons, 2014). Carcasses had no signs of decay for at least several months, following the eventual onset of decay odour began attracting scavenger activity.

Rodriguez and Bass (1985) had come to conclude that an increase in temperature for buried human cadavers during decomposition was discovered to be directly proportional to the depth of the grave site. At deeper ground depth, this rate decreased. He found that cadavers at this level showed some signs of preservation, undergone minimal skeletonisation and had heavy adiopocere formation surrounding the body. The cadavers buried deep within soil, usually more than 1.2m below surface level, saw no carrion or insect activity.

Unexpectedly, in the same experiment, certain cadavers in soil heated up to a higher degree than the surrounding soil by up to a 10°C. It was stated that: "Shallow burial depths produced higher body temperatures during decomposition. Also with decreased burial depths the rise in body temperature occurred sooner and lasted longer". However, contention still exists between associated temperature changes, as data here is different to other studies (Weitzel, 2005; Wiltshire, 2008).

Similarly, decomposition proceeds differentially over time depending on how a carcass is positioned within the grave. Troutman et al. (2014) came to similar conclusions as Rodriguez and Bass (1985) in that carcasses at the centre mass or deeper within a grave did not breakdown as significantly as carcasses placed in shallow graves.

Due to the presence of anaerobic bacteria involved in the decomposition process, the bacterial activity produces heat in the tissue (Arpad A. Vass, 1992; Cockle & Bell, 2015; Weitzel, 2005). This increased production of heat is at its peak when the active decay stage is reached, thus potentially complicating further analysis for forensic purposes, especially that of the post mortem estimation.

b. Effects on the environment

Situations involving burials often refer to grave soil, which is the soil surrounding the decomposing carcass (Van Belle, Carter, & Forbes, 2009). This form of soil has a rich composition compared to rest of the soil body, due to decomposition products from the body leeching into the peripheral soil.

i. Plants

When there is evident soil disturbance it is more likely that plant matter in the immediate area is also altered, or completely disturbed. In experimental burial environments, depending on post burial interval, certain weed species can grow over graves with great biomass (C. J. Watson & Forbes, 2008).

ii. Insects

In burial sites, insect activity is not generally evident until scavengers begin accessing cadavers through digging, or the grave sites fall within 0.6 to 0.3m depth of soil (Rodriguez & Bass, 1985; Turner & Wiltshire, 1999). Certain insect species can be associated with burial such as larvae of *Morpholeria Kerteszi* burrowing down to reach carrion below the surface, or

Calliphoridae (blow fly) and Scrophagidae (flesh fly) at shallow grave (Turner & Wiltshire, 1999).

4. Post mortem interval estimation in buried corpses

Post mortem interval techniques can be from physical indicators or chemical indicators, each method is used case by case.

Certain decomposition products and stages can be loosely associated with a time since death (Stejskal, 2012). Algor mortis, livor mortis and rigor mortis generally occurring through to the 48 hour mark and is characterised by initial cooling of the body followed by bluish skin and the eventual stiffness of the muscles. However, it is heavily susceptible to temperature before death and possible pre-existing condition of the victim.

Michaud and Moreau (2011) studied that accumulated degree days (ADD) can be used as a baseline to establish and PMI estimate. Attempts have been made to develop a universal post-mortem interval formula for decomposition. A study by (A. A. Vass, 2011), comprised the important aspects of human decomposition such as temperature, moisture, oxygen pressure, and accumulated degree days, into two different mathematical formulas in an attempt at PMI (Arpad A. Vass, 1992). The effectiveness of this formula is limited to mid to eastern section of the US, and the corpse must be in a certain condition; pre-skeletonization, with tissue still soft and viable.

Oxalic acid after it has undergone a process of methylation is an important determinant affecting PMI decision. It is not normally targeted, but it is found to be easily detectable in tissue sampling (Arpad A. Vass et al., 2002). Liver tissue and brain tissue are also diagnostic

of early post mortem interval. Other promising means are biomarkers of amino acid and gamma amino butyric acid (GABA), proline and methionine. For a compound to be relevant in as a post mortem indicator, ratios compared to other biomarkers must be reproducible over time. Predominant breakdown products are normally from muscle and fat.

Volatile fatty acids are indicative of a post mortem interval, yet a lot of them are too variable to useful in a solid time since death framework estimation (Arpad A. Vass, 1992). Butyric, propionic and valeric acid are formed and released into soil solution in specific ratios, in temperature dependent setting can be used in a time since death determination. Potentially, under very strict circumstance, VFA can reveal perimortem weight, and possibly even racial affinity due to melatonin involvement. Yet the most accurate biochemical marker of PMI before putrefaction settles, is potassium content of the vitreous humor (Arpad A. Vass et al., 2002).

Ninhydrin reactive nitrogen levels can be measured from decomposing carcasses that were buried, they show great potential use for PMI estimation in the early post mortem period of decomposition, optimally the first 2 months (Van Belle et al., 2009).

Jaggers and Rogers (2009) underwent a study to document the morphological changes that occur to buried skeletal remains over periods of 60 and 150 days and to determine if it is possible to establish PMI. Result show that bone macroscopic characteristics did not change over the course of 150 days, regardless of the condition of the soil environment. After 5 months, very little morphological features were different. The results of this study conflict with others that skeletal remains in moist environments rapidly degrade after a few years.

Establishing such a valuable timeframe can provide critical information, particularly in matters of criminal investigation. A PMI can narrow down the time frame for missing person's cases,

assist in identification, account for or discredit alibis of persons of interest, as well as corroborate with other existing evidence that can influence the direction of investigation (Cockle & Bell, 2015; Moffatt, Simmons, & Lynch-Aird, 2016).

5. Identification of grave sites

Ideally the most reliable method of all is the witness account or a confession, however this is not an option that is always available. We must fall back into the many methods science offers in uncovering grave sites.

a. Cadaver dogs

Cadaver dogs are raised and trained to assist in the location of missing persons, or a deceased person remains. These dogs can detect volatile organic chemicals which is picked up by the scent cone during the act of sniffing, they are trained to pick up scents from the ground, a burial and even water (Lasseter, 2003). When considering previous studies cited so far, there is question is deeply situated graves would prove difficult for cadaver dog to detect as odour the dogs rely on would not be able to reach through the soil until much later in decomposition.

In the study by Lasseter (2003) cadaver dogs are rather proficient in finding graves while the buried corpse is well underway in the first stage of decomposition. However, in the trial involving skeletal remains, the dogs showed low detection rates and even the dog handlers were not could not pick up all the dog cues or allow them to thoroughly search. Cadaver dogs are a reliable method however much of the control is left to the handler's interpretation and discretion.

b. Forensic entomology

Blow flies, rove beetle, flesh flies and female flies depositing eggs on the surface of the top soil attempting to make their way down are entomological indicator of a possible shallow grave nearby (Byrd & Castner, 2009; Singh et al., 2016). This is especially prevalent following rainfall.

c. Geoscience methods

Ground penetrating radar (GPR) is a non-invasive archaeological search method that can be used in forensics to identify graves. It provides a real-time view below surfaces such as cement and asphalt and is an excellent method that does not compromise a crime scene or potential evidence (Schultz, Collins, & Falsetti, 2006).

The study by Buck (2003) aimed to use three types of geophysical equipment to test if its cost effective, and functional in detecting unmarked graves quicker than conventional archaeological methods. They found that in this case, it produced negative results as the GPR did not find target anomalies when tested out on a site suspected to have a buried body. In fact, it proved costlier in the long run as the GPR was meant to eliminate the need of extensive excavation as it was carried out in the end. The equipment itself also required experienced handling to understand some of the feedback the waves produced. The study by Schultz et al. (2006) showed that soil types involved in burials had an effect on whether something discernible can be detected. Sandy soil were ideal mediums for detecting bodies throughout all stages of decomposition until skeletonisation. Corpses buried in clay were difficult for the GPR to detect, this was suggested to be due to minerals and high clay content. In terms of disturbed graves, detection rates lower with time as soil becomes compact.

Physical disturbances refer to the visual indicator of possible graves during a search, this ranges from plant disturbance, growth, insect movement and soil characteristics.

A common indicator of a grave is the disturbance in the top layer of the soil. During the disturbance of digging up soil, topsoil and deeper soil mix at the surface, producing a distinct discoloration of the different layers (Rodriguez & Bass, 1985). Between the first 6 to 12 months the discoloration does eventually lessen. Additionally, great undulations on the ground can be found in shallow graves, and secondary depressions within a primary one can be potentially indicative of a deeper grave. Secondary depressions come about in later decomposition within the body where the abdominal cavity collapses inwards.(Rodriguez & Bass, 1985; C. J. Watson & Forbes, 2008)

d. Botanical methods

The area of botany and palynology and mycology is a constantly growing area in regard to the search of graves.

Botany plays a very significant role in the detection of clandestine graves and is a widely recognised and credible field in forensic science. The surrounding vegetation can provide evidence of the changes to the environment because of a present grave site. The disturbance of soil alone impacts grass and vegetation regrowth more than any influence by putrefactions liquid (Caccianiga et al., 2012). Other studies have reported that at a certain burial depth, carcasses and the putrefaction liquid produced has minimal impact on plant growth (France, 1992; Van Belle et al., 2009). Beyond plants there is growing potential for fungi to be of forensic use and a reliable indicator in locating places where a corpse may be hidden (Hawksworth & Wiltshire, 2011). According to Hawksworth & Wiltshire, 2011, the morphological changes to fungi can be indicative a physical disturbance.

Overall there is never a guaranteed certainty of the location of a gravesite until the digging commences.

e. Time of plant and fungi growth

Alternatively, the study by Rodriguez and Bass (1985) concluded that plant regrowth occurred quickly when there was less disturbance to the initial topsoil even when there was time for plants to grow and establish on a burial site.

In fungi, species such as *Coprinnus comatus* and *Morchells* – once disturbed, won't spore for up to two years, this is characteristic of area with disturbed soil, logs or branches and fairly easy to recognise to the trained eye (Hawksworth & Wiltshire, 2011). It can also manifest physically on fungi, with the emergence of vertical stalks and horizontal caps.

ii. Succession of plants and fungi

Other types of vegetation have proven to be more robust to soil disruption. Redural and exotic plant species are quick to grow first such as crabgrass and witchgrass and these are suggested to be better indicators of grave sites rather than nutrient demanding plant species (C. J. Watson & Forbes, 2008). However, succeeding plant species would vary with different ecologies in and would and species would not be the same for any given area.

iii. Analysis of plant nutrients

Measuring nutrient content in grass is important as its indicative of assimilation, deficiencies and components present in soil. Analysis methods generally involve a form of dry ashing, spectrophotometry and wet acid digestion, these are dependent on the target nutrients namely nitrogen, sulphur, potassium, calcium and other micronutrients.

Wet digestion involves an acid actively digesting a form of sample in preparation for further treatment, generally oxidation processes. Up to three acids can be used to treat samples, and analysis involving these reagents target most elements such as potassium, phosphorous, calcium and magnesium (Levei, 2012). Further detail on wet digestion can be seen in Section 7c.

Dry ashing is a form of analysis that generally involves the use of plant material. Samples are oxidised at high temperatures before being ashed, this is soon followed by the addition of a dilute acid such as hydrochloric acid or nitric acid. There is greater range of target elements with the dry ashing method that includes micronutrients and trace elements such as boron and Molybdenum.

The Kjeldahl method is used primarily to estimate total protein content in samples. This method involves similar components of acid digestion with the use of sulphuric acid and a catalyst, this is then furthered by distillation (Anglov, 1999). Quantification is then used to determine the total nitrogen content in plant samples and also in animal feed (Marco. A., 2002).

6. Ryegrass in Australia

The Lolium genus, common in Australia is historically native to parts of Europe and North Africa but has since been naturalised in the Australian environment as a successful grass

species (Kloot, 1983). Annual ryegrass (*Lolium multiflorum*) is primarily a winter grass crop useful for pasturage and as experimental biomaterial (Li et al., 2014). In Australia there are many species present such as *L. perenne, L. rigidum, L. multiflorum, L. loliaceum*, including the presence of hybrid species (Kloot, 1983). In the United States the grass can be used as winter animal feed as its nutrition and growth rate make it palatable and economic for livestock (Venuto, Ward, & Twidwell, 2007). Other studies have taken full advantage of the capacity for growth, nutrient uptake and economic value that ryegrass offers (Abe, 1998; Brink, 2006). Such is the success of ryegrass that it has since become a weed of winter cereal crop, and its herbicide resistance is well documented, posing a major problem in the agricultural industry (Boutsalis, 2017).

a. Growing dynamics

The annual grass species is advantageous, robust, produces a large biomass and is extremely adaptable to various climates; being found in different soil and climatic conditions around Australia (Ferris, 2007). In the winter season, ryegrass generally is slow to establish in the soil, once the root system has settled it grows vigorously and densely (Pietola, 2005). The availability of nutrients, particularly nitrogen has noted effects on growth. Metabolic processes are stimulated, strong regrowth is promoted and leaf area can increase, internal components such as proteins and chlorophyll are also promoted to increase (Witkowska, 2008).

7. Nutrient analysis & Dumas combustion

a. Nutrients in soil & plants

An understanding of plant and soil interactions can give valuable insight into the changes caused by bioturbation. Through association of specific plant types to mechanical disturbances and regrowth; soil has been a heavy focus for many studies for a long time despite its difficult and dynamic nature (Wiltshire, 2008). However, A combination of plant nutrient analysis in a forensic application is not an area that is extensively studied, particularly methods measuring plant nutrients for locating graves and estimating PMI's.

A form of forensic plant chemistry is being used in South Africa to stop theft and illegal trade of the nation's cycads plants. This is done using stable isotope analysis of plant tissue to track a plants movement throughout its life: "In nature, the relative abundances of a chemical element's isotopes — which differ in the number of neutrons they have in their nuclei — vary from place to place. As organisms grow, they take building blocks from their environment, making these isotope signatures part of their bodies." When comparing the isotopes of relocated plants to native ones, it is possible to tell the difference between the two with confidence (Nordling, 2014).

The follow up study saw various target elements (N, C, O and more) as indicative of different environmental changes e.g. presence of fertilizer, air quality, aerosols, and water availability. As a result, the study was successful in using isotopes in detecting a difference between relocated plants and native (Retief, West, & Pfab, 2014). This is a novel approach of using plant chemistry which if appropriated can be applied to gravesite location and possible post mortem interval.

b. The role of nitrogen

Interspecific facilitation involves one plant species positively altering the immediate environment of another (Høgh-Jensen, 2000). Nitrogen transfer is the underlying process of

considerable interest. The purpose of the study by (Høgh-Jensen, 2000) was to quantify the nitrogen transfer between ryegrass and clover and relied on the assumption of equal distribution and absorption and constant enrichment over time. Results show that active transfer occurred until it plateaued. When a swine carcass decomposes, nitrogen is released into the surrounding grave soil (Van Belle et al., 2009; Arpad A. Vass et al., 2002). Soil levels of NRN can therefore be detected and used as grave soil underneath a carcass shows significant NRN concentration up to 6 months after decomposition.

Nitrogen levels in soil can remain persistent. It's eventual return to basal levels is speculated to be due to soil to soil ammonifiers and nitrifies that convert organic and ammonium nitrogen to nitrite and nitrate through nitrification reactions Van Belle et al. (2009). The products of this process assist plants in acquiring nutrients from nearby carcasses. Plants can continue to uptake significant levels of nitrogen from the carcass, and if analysed, can be distinguished from grass growing on a grave and grass from another area (Arpad A. Vass, 1992; Danell, 2002; Melis et al., 2007).

c. Methods of analysis

Analysing nitrogen levels in plant content includes commonly used methods such as Kjeldahl analysis, acid digestion and nuclear magnetic resonance (NMR).

NMR in the study of plants is a versatile method that has been used in nutrient analysis and nitrogen and metabolite assessment (Mesnard, 2004). NMR targets magnetic

moments within nitrogen and is advantageous as these levels can be observed directly. NMR is a flexible technique and useful in a variety of other cell based application, however is not as sensitive as other techniques such as mass spectrometry (Chatham, 2001).

Acid digestion is used extensively in the analysis of nitrogen in plant matter. The sulphuric – hydrogen peroxide acids digests plant samples, however the procedure itself can be time consuming (Lowther, 2008). Combined with microwave assistance this method has good product recovery, quick digestion times and can function with low sample and reagent usage (Levei, 2012).

The Kjeldahl method is an internationally recognised method used for a wide range of applications including nitrogen determination, it is predominantly the method of choice in agriculture (Jung, 2003). The common process is the use of acids and other reagents in the presence of a catalyst. A common issue with this method is its use of potentially hazardous reagents and the possibility that it does not convert all forms of nitrogen (Michael, 2002). More recently, the Kjeldahl method is being replaced by the more automated method of Dumas combustion.

i. Dumas method of combustion

The nitrogen method of combustion (Dumas) is a well-established procedure of quantifying nitrogen content in a variety of materials. It measures all forms of nitrogen including nitrite and nitrate, the combustion method also extends to the determination of carbon, nitrogen, sulphur and hydrogen elements (Marco. A., 2002). The basis of this method a dry oxidation process where samples are subjected to combustion under

high temperatures up to 1000°C, reduction to nitrogen gas and measurement with a thermal conductivity detector (Jung, 2003).

The method of combustion provides some advantages. The process is quick and eliminates the use of toxic reagents and hazardous procedures. No additional treatments or procedures are required, minimising loss of total nitrogen (Schindler, 2008). Furthermore, the Dumas method has been shown to provide a higher result then its competing method, Kjedahl (Schindler, 2008). This has been speculated to be attributed to non-protein forms of nitrogen being detected in the Dumas process. One critical evaluation about the Dumas method is the possibility of overstating or understating nitrogen content that can occur when characterising deficiency and sufficiency of plant matter (E. H. Simonne, Harris, & Mills, 1998).

1. Applications

This Dumas method of nitrogen analysis is widely used in agriculture and the food industry. Its application is predominantly for products such as fishmeal and animal feed as trading prices and market value is normally determined by crude protein value (Marco. A., 2002; Miller et al., 2007). The applications of this method can even extend in the assessment of quality control of food products such as soybeans, cereals and dairy products. These products are normally required to meet a standard of nutrition requirements of which this method has been tried and tested for its accuracy and appropriateness in application (Michael, 2002; A. H. Simonne, Eitenmiller, R.R., Mills, H.A., Cresman, C.P., 1997).

The use of this assessment on plant matter, stems and soil is still an emerging area. The study by E. H. Simonne et al. (1998) has tested Dumas' capacity on vegetable leaves with promising results of improved accuracy due to a greater nitrogen percentage being detected in comparison to other methods. Similarly, Dumas based instruments have proven superior in total nitrogen determination of agricultural materials which include plant materials (M. E. Watson & Galliher, 2001).

8. Aims

The use of plant and fungi have largely been attributed to gravesite indicators. Limited studies have attempted to explore the role of grass nutrients in gravesite location and estimation of deposition. The following manuscript aims to provide a proof of concept of whether nitrogen combustion (Dumas) can be utilised in the assessment of nutrient level of ryegrass, growing on decomposing pork.

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Part 2

Nutrient dynamics of annual ryegrass as a tool in forensic investigation on burial sites

Manuscript

Contents

Name	Depth	Colour	Gravel	Texture	Ammonium Nitrogen	Nitrate Nitrogen	Phosphorus Colwell	Potassium Colwell	Sulphur	Organic Carbon	Conductivity	pH Level (CaCl2)	pH Level (H2O)	DTPA Copper	DTPA Iron	DTPA Manganese	DTPA Zinc	Exc. Aluminium	Exc. Calcium	Exc. Magnesium	Exc. Potassium	Exc. Sodium	Boron Hot CaCl2	PBI
			%		mg/Kg	mg/Kg	mg/Kg	mg/Kg	mg/Kg	%	dS/m	pH	pH	mg/Kg	mg/Kg	mg/Kg	mg/Kg	meq/100g	meq/100g	meq/100g	meq/100g	meq/100g	mg/Kg	
QS1	0-10	WH	5	1.0	<1	<1	<2	<15	0.7	0.11	<0.010	5.3	6.1	0.14	3.47	0.45	0.11	0.015	0.03	0.02	0.02	0.03	0.13	2.7
QS2	0-10	WH	5	1.0	<1	<1	<2	<15	0.7	0.05	<0.010	5.4	6.2	0.18	3.18	0.30	0.20	0.013	0.05	0.02	0.04	0.03	0.11	2.0
QS3	0-10	WH	5	1.0	<1	1	<2	<15	0.6	< 0.05	<0.010	5.2	6.1	0.14	3.69	0.34	0.11	0.010	0.05	0.02	0.02	0.02	< 0.10	1.7
RIVER SAND1	0-10	LTBR	5	1.0	<1	1	<2	<15	1.0	< 0.05	<0.010	5.6	6.3	0.18	3.29	0.39	0.18	0.106	0.07	0.05	0.02	0.01	0.11	6.3
RIVER SAND2	0-10	LTBR	5	1.0	<1	1	<2	<15	1.1	< 0.05	<0.010	5.7	6.3	0.16	3.46	0.41	0.27	0.098	0.06	0.04	0.02	0.01	< 0.10	3.8
RIVER SAND3	0-10	LTBR	5	1.0	<1	<1	<2	<15	1.4	< 0.05	<0.010	5.9	6.4	0.29	2.77	0.40	0.24	0.071	0.07	0.04	0.02	0.01	< 0.10	6.7
SOIL SAMPLE	-	GRBR	10-15	2.0	3	12	54	36	7.9	1.77	0.065	4.8	5.9	2.98	36.62	1.95	1.62	0.484	2.67	0.44	0.07	0.30	0.34	130.8

Table 1. Soil analysis results table, washed river sand and native soil.

Data summary

Trt	Soil	Applied	DM (t/ha) (H1)	DM (t/ha) (H2)	DM (t/ha) total
1	Soil	Control	1.62	0.73	2.35
2	Soil	Treatment (No fertiliser)	1.52	0.48	2.01
3	Soil	Treatment (Pork)	0.12	0.16	0.28
4	Sand	Control	0.25	0.04	0.29
5	Sand	Treatment (No fertiliser)	0.08	0.08	0.16
6	Sand	Treatment (pork)	0.03	0.05	0.08
		Prob	<0.001	<0.001	<0.001
		LSD	0.3029	0.165	0.3388

Table 2. Data summary of averaged dry plant weights in tonnes/hectare, values of P<0.05 were considered significant.

SOIL (HARVEST)	Group (Pots)	Boron	Calcium	Chloride	Copper	Iron	Magnesium	Manganese	Molybdenum	Nitrate	Phosphorus	Potassium	Sodium	Sulfur	Total Nitrogen	Zinc
		mg/Kg	%	%	mg/Kg	mg/Kg	%	mg/Kg	µg/Kg	mg/Kg	%	%	%	%	%	mg/Kg
SAND (H1)	CONTROL (P1, 2, 3)	37.71	0.58	2.49	15.60	211.00	0.34	24.28	2225.33	41.38	0.46	3.76	0.53	0.30	2.31	38.84
SAND (H1)	TREATMENT PORK (P1, 2, 3)														3.98	
SAND (H1)	TREATMENT NO FERTILISER (P1, 2)														1.81	
SAND (H2)	CONTROL (P1, 2, 3)	31.50	0.5	3.08	16.38	69.62	0.29	37.26	1704.47	70.80	0.55	4.76	0.41	0.27	2.50	64.96
SAND (H2)	TREATMENT PORK (P1, 2, 3)														5.01	
SAND (H2)	TREATMENT NO FERTILISER (P1, 2)														2.76	
SOIL (H1)	CONTROL (P1, 2, 3)	17.93	0.54	2.63	11.13	81.32	0.31	53.62	1521.40	1873.80	0.27	3.54	1.04	0.26	3.58	31.31
SOIL (H1)	TREATMENT PORK (P1, 2, 3)	23.33	0.38		14.93	124.46	0.22	44.22			0.23	3.90	0.60	0.45	4.59	29.91
SOIL (H1)	TREATMENT NO FERTILISER (P1, 2)														2.84	
SOIL (H2)	CONTROL (P1, 2, 3)	16.98	0.46	3.79	11.80	105.29	0.33	87.59	2009.86	232.61	0.48	3.52	1.69	0.30	3.22	39.97
SOIL (H2)	TREATMENT PORK (P1, 2, 3)	17.39	0.38		17.45	100.20	0.25	71.35			0.37	4.50	0.86	0.47	5.38	39.34
SOIL (H2)	TREATMENT NO FERTILISER (P1, 2)	20.16	0.49		11.73	89.54	0.30	91.12			0.49	3.29	1.41	0.31	2.71	35.37

Table 3. Nutrient analysis of all samples according to soil type and treatment. Not all samples underwent the same analysis due to weight restriction.

Type	Species	Boron	Calcium	Chloride	Copper	Iron	Magnesium	Manganese	Molybdenum	Nitrate	Phosphorus	Potassium	Sodium	Sulfur	Total Nitrogen	Zinc
		mg/Kg	%	%	mg/Kg	mg/Kg	%	mg/Kg	mg/Kg	mg/Kg	%	%	%	%	%	mg/Kg
Young Tissue	Perennial Ryegrass	3.0 - 5.0	0.15 - 0.20	-	4.0 - 6.0	40 - 60	0.15 - 0.20	15.0 - 20.0	0.11 - 0.13	-	0.20 - 0.28	1.4 - 1.9	-	0.18 - 0.22	3 - 3.5	10.0 - 15.0

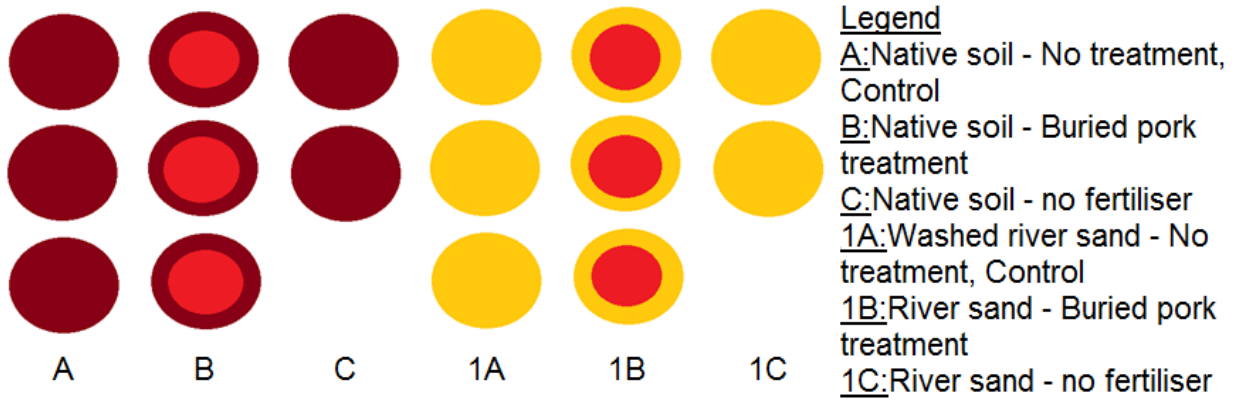Table 4. Average nutrient levels, adapted from *Plant Analysis Manual: An interpretation, 1997.*

Figure 1. Two growing mediums, three repetitions per group. Two repetitions per non-fertiliser group. A) Native soil+fertiliser+ryegrass, B) Native soil+fertiliser+ryegrass+pork, C) Native soil+ryegrass, 1A) washed river sand+ryegrass+fertiliser, 1B) river sand+ryegrass+fertiliser+pork, 1C) river sand+ryegrass
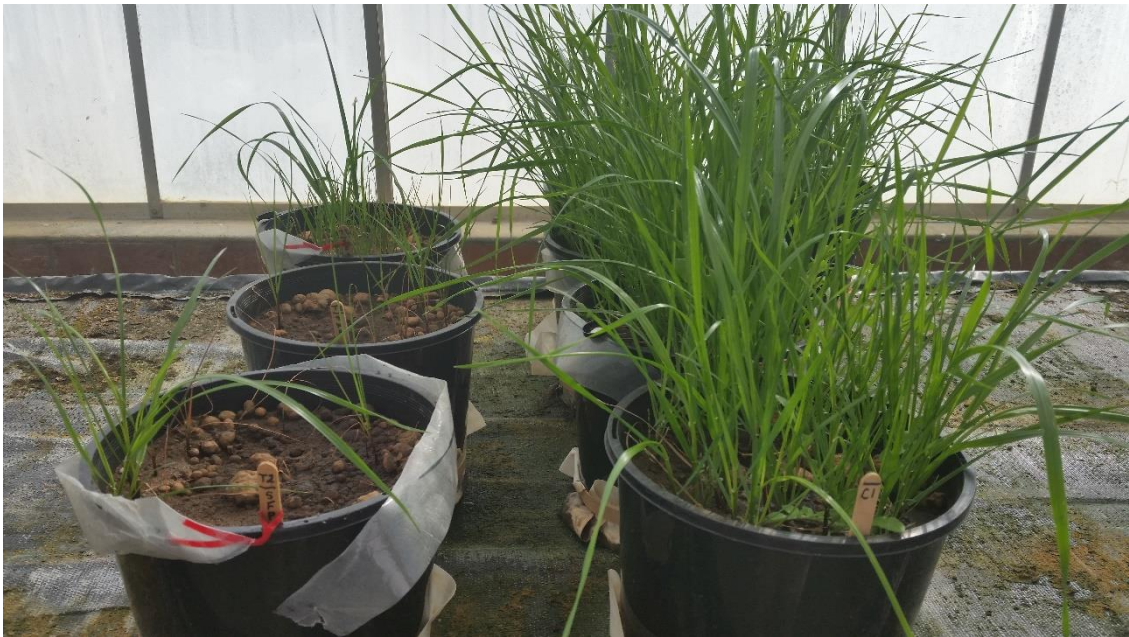

Figure 2. Grass biomass difference on the 5th week, native soil control (right), buried pork (left) and non fertiliser (right, rear two) groups prior to first harvest.
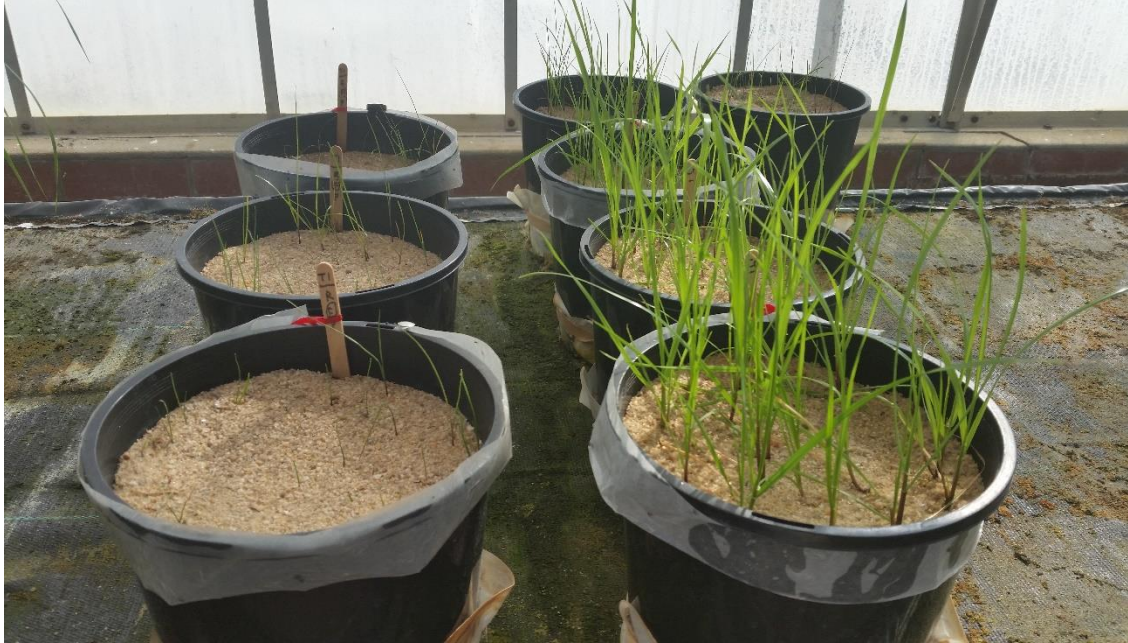
Figure 3. Grass growth of washed river sand group prior to the first harvest. Control group (right), pork (left) and non- fertiliser (right, rear two).


Figure 4. Ryegrass regrowth one week after the first harvest. Comparison between two growing medium groups

Figure 5. Holes on the surface of Pork Treatment pots from the washed river sand group.

Figure 6. Averaged samples, graphed in tonnes per hectare at 95% confidence, $P<0.001$. A) Native soil – control, B) Native soil – non fertiliser, C) Native soil – pork treatment, 1A) Washed river sand – control, 1B) River sand – non fertiliser, 1C) River sand – pork treatment
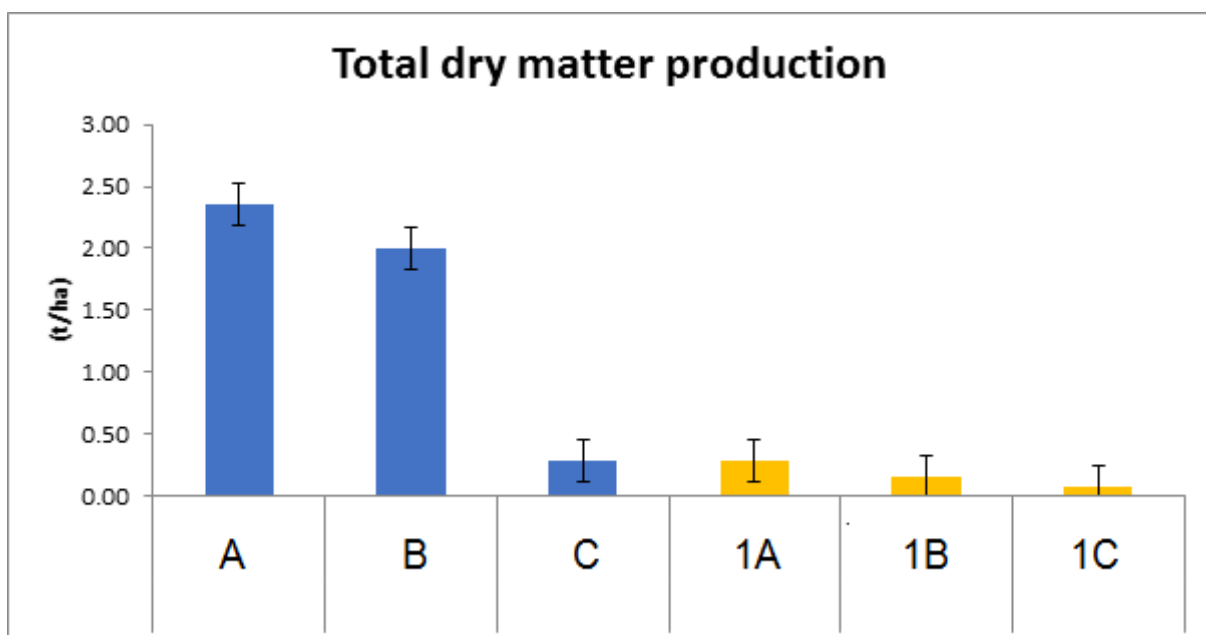
Nutrient dynamics of annual ryegrass as a tool in forensic investigation on burial sitess

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Abstract

The burial of a corpse presents forensic investigators with the problems of gravesite location and post deposition estimation. Botany has a predominant role in the assessment of graves, its potential for providing temporal and gravesite indication is continually being researched. This research aimed to provide a proof of concept of the use of Dumas combustion on ryegrass growing on decomposing pork, and provide temporal and grave indication. The experimental design, involved growing tetraploid ryegrass seeds over two different growing mediums; washed river sand and ground soil. Grass was left to grow within the growing mediums which consisted of repetitions of buried pork, non-fertiliser treated and non-pork treated groups. Grass was harvested, dried and underwent nitrogen and ICP analysis. The dry matter yield of grass that resulted, show some level of growth difference between grass grow on buried pork and non-treated control groups. Nitrogen levels between these groups was also very different with increased levels seen between all groups on the second harvest, non-fertiliser treated groups were an exception. Further research is warranted to explore the full use of Dumas and validate the observations noted.

1. Introduction

The situation of a corpse that has been buried presents forensic science with two major problems, a time since death or deposition estimation, and location of the grave site itself. The understanding of decomposition is crucial in tackling these two problems, and many studies have been dedicated to explaining the major difference of this process between the surface and below soil (Rodriguez & Bass, 1985; Sorg, Haglund, & Wren, 2012). Standard processes following death is the catabolism of major molecules such as lipids, nucleic acids, proteins, carbohydrates, which in turn release degradative by-products including volatile organic compounds; in swine carcasses these also include oxygenated compounds and nitrogen compounds in a general decomposition process (Dekeirsschieter et al., 2009). A body that has been buried is effectively shielded from many of the immediate effects of temperature, moisture, entomological and scavenger activity, temperature alone is different at certain depths with deeper burials showing significant delay in onset of late stage decomposition (Troutman, Moffatt, & Simmons, 2014; A. A. Vass, 2011). Burial even delays the arrival of insects - a valuable tool for the estimation of time since death (or minimum Post Mortem Interval, minPMI), from reaching the body (Singh, Sharma, & Sharma, 2016). Therefore the min PMI evaluation of a buried corpse is difficult as many of the morphological features accompanying the hallmarks of decomposition are no longer accurate, or unavailable for analysis. Grave site location comprises of a completely

different affair with many of the existing search techniques revolving around cadaver dogs, ground penetrating radars (GPR), probes and vegetation dynamics of the local region (Buck, 2003; France, 1992; Lasseter, 2003). Much of the direction of searches would rely on witness testimony, criminal confessions or last know whereabout, no search method is 100% effective and must be adapted to the scenario. Given the high variability surrounding the search and estimation of a buried corpse, there is an increasing interest in botany as it offers the possibility of considering both situations.

Botanical indicators are a valuable tool in the search for clandestine graves as vegetation response is visually detectable and can have attributed post burial interval characteristics. Visual manifestations of gravesite disruptions are indicative by present fungi species up to 2 years following disturbance (Hawksworth & Wiltshire, 2011). Fungi colonisation as a method of post deposition estimation has already been successfully applied in criminal investigation. Post deposition estimation in years is also possible through the analysis of a cross section of plant stem showing periods of growth and dormancy (Willey, 1987). Depending on local ecology, weed species are likely to be early successors of disturbed terrain, the difference between disturbed and undisturbed soils can be distinguished through present weed species and resultant biomass of grass over the grave; whether it be in abundance or complete absence would be dependent on the time since burial (Caccianiga, Bottacin, & Cattaneo, 2012; Watson & Forbes, 2008). In Australia, the *Lolium Rigidum* (annual ryegrass) species is an example of a highly successful and robust grass weed species that produces a high amount of biomass quickly and is adaptable to various climates and habitats (Ferris, 2007). Such is its success, that in the past three decades the grass species has since become one of the most herbicide resistant weeds in the world, posing a

major problem in agriculture (Ferris, 2007; Kloot, 1983). The characteristics in ryegrass can thus add to existing search and post burial estimation tools in forensic science because of its extensive use in studies relating to nutrient and mineral uptake from a nutrient source, including nitrogen and phosphorous compounds (Abe & Ozaki, 1998; H. F. Høgh-Jensen, Vibeke ; Schjoerring, Jan K., 2001; H. S. Høgh-Jensen, Jan, 2000; Li et al., 2014; Venuto, Ward, & Twidwell, 2007). Ryegrass nutrient dynamics can potentially be used as an gravesite indicator and post deposition method given the enrichment of soil environment from a decomposing corpse (Benninger, Carter, & Forbes, 2008).

During decomposition, it is described that the surrounding soil receives a 'localised pulse of nutrients', these products present in soil can be transferred to flora like ryegrass and detected in dry matter product using nitrogen combustion (Benninger et al., 2008). All manner of minerals and nutrients are released from a decomposing corpse, soil sciences have found amino acids, volatile fatty acids (VFA), putrescine and cadaverine to be indicative of a corpse (Stokes, Forbes, & Tibbett, 2013; Arpad A. Vass et al., 2002). There is great interest in nitrogen and nitrogen based compounds such as ammonium and nitrate, these have been demonstrated to increase with extended post mortem intervals, additionally, has far reaching effects on pH and processes such as subsequent nitrification which have been demonstrated to be detectable in soil up to 1 year following deposition (Anderson, Meyer, & Carter, 2013). Domestic pig (*Sus scrofa domesticus*, Erxleben) makes a suitable analogue in decomposition studies as its products are relatively similar, with the addition of other compounds such as potassium, phosphorous and sodium that can be potentially traced (Tomovic et al., 2015). Nitrogen combustion (Dumas method) offers a quick

and accurate analysis of samples and alleviates the need for toxic reagents (Marco. A., 2002; Miller et al., 2007). This method of combustion is used to quantify total nitrogen content and is normally used in studies involving quality and assessment of nitrogen and proteins of animal feed, cereals and plants (Miller et al., 2007; Simonne, 1997). Dumas combustion also has the capacity to be a determinant for carbon, nitrogen and sulphur in various compounds (Hansen, 1989; Marco. A., 2002). At the time of this research no study had been encountered in relation to the combined use of Dumas combustion and effects of soil-to-ryegrass nutrient intake in the forensic context. Fewer still are studies that have explored the nutrients absorbed by weed grass species in the forensic context.

With limited studies exploring the role of grass nutrients in gravesites, this research aims to provide a new tool for burial site location and attempt to provide temporal information regarding burial time. With the use of *Sus Scrofa* (will be referred to as pork) this study aims to provide a proof of concept of whether nitrogen combustion can be used to assess nutrient dynamics of ryegrass growing on decomposing pork.

2. Materials & methods

The research was conducted at the Department of Agriculture and Food, Western Australia (DAFWA) located in South Perth between March and May, 2017.

A total of 16 round pots measuring 18cm in height and 298.65 cm^2 were filled with two different soils resulting in 3 control pots and 5 treatment pots per soil type. Controls and treatment groups were marked and then placed in a glasshouse located at

DAFWA. Temperatures within the facility were kept at a constant 21°C from start to end of the research.

2.1 Soil

For this experiment, two types of soil were used:

a) "Native soil": ground soil collected at Manjimup (WA).

b) "Washed river sand" soil: soil containing minimal nutrients, provided by DAFWA which.

Both soil types filled 8 pots each up to 7cm below the top of the pots (Refer to Figure 1). Following the burial of the pork cuts (see 2.2), fertiliser by Macro Pro Extra© was spread over the surface of all 12 pots (control and treatment), two pots from each group did not receive this treatment, these are referred to as 'no fertiliser' group. Fertiliser for the washed river sand group was spread over the surface at a rate of 125kg/ha (0.37g), fertiliser for the native soil group was added at a rate of 44.6kg/ha (0.13g).

2.2 Pork

Pork loin on the bone was purchased direct from wholesaler butcher Goodchild Meats in Perth and was delivered frozen. Two pieces 1cm thick and weighing an average of 251g +/- 5g in total was buried in each of the three treatment pots per group. Pork was buried 7cm from the soil surface, leaving a 1cm gap between the from the edge of the pot.

The Buzz© Fly paper glue trap, was lined on four pots of each soil group and kept on the pots until the end of the experiment and sent off for analysis by an entomologist.

2.3 Seeds

Seeds of *L. multiflorum (*Angus ryegrass) were sourced from Landmark Operations Ltd. and lightly sown without prior germination beneath the surface of all pots in all groups. Seeds were buried at a rate of 20 seeds evenly spread along the surface.
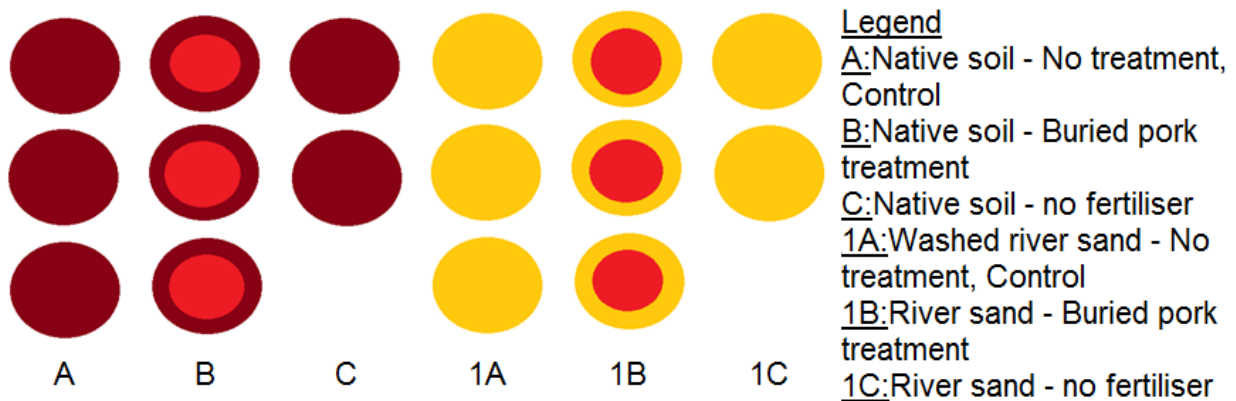

Figure 1. Two growing mediums, three repetitions per group. Two repetitions per non-fertiliser group. A) Native soil+fertiliser+ryegrass, B) Native soil+fertiliser+ryegrass+pork, C) Native soil+ryegrass, 1A) washed river sand+ryegrass+fertiliser, 1B) river sand+ryegrass+fertiliser+pork, 1C) river sand+ryegrass

2.4 Watering

To aid seed growth, the misting of the surface was required daily until the seedlings sprouted. After 10 days, watering switched to 213ml daily per pot in accordance to total annual rainfall, this was administered by the watering mats in the glasshouse until the end of the experiment (Maddern, 2016).

2.5 Harvesting

All pots were harvested 1cm from the base of the grass twice during the experiment; on the sixth and eight weeks after seeding. These were placed into individual paper bags for drying.

Plant cuttings from all the pork treatment groups were placed in a conventional fan forced oven for 30 minutes at 5°C. Plant cuttings from all the control and non-fertiliser groups were placed in the oven at 5°C for 2.5 hours.

2.6 Nutrient Analysis

Prior to use in the experiment, samples of each soil type were tested for pre-existing nutrient levels. These samples were sent to Wesfarmers Chemicals, Energy & Fertilisers (CSBP Ltd.) located at Bibra Lake.

Following the harvest and drying of the plant cuttings, samples were weighed and refrigerated before analysis at CSBP, Bibra Lake. Combining of single samples was required due to the very light weights of the samples not allowing for the original anticipated comprehensive test (involving Dumas) on all grass cuttings. Comprehensive, nitrogen and inductively coupled plasma (ICP) and trace nitrogen tests were conducted on the dried samples that were too light in weight.

2.7 Statistical Analysis

Statistical analysis for dry weights of the grass samples were conducted on GenStat® edition 18. A one-way analysis of variance (ANOVA), followed by post-hoc was used to determine any statistical difference between the group.

3. Results

3.1 Soil Analysis

One set of analysis was conducted on the tested soil groups prior to planting. The washed river sand was not completely void of nutrients, however traces of nitrogen based compounds were below levels of 1mg/Kg (Refer to Table 1). Pre-existing nutrient levels within the native soil group were higher in comparison, particularly between nitrate, nitrogen and phosphorous (12mg/kg), diethylenetriaminepentaacetic acid (DPTA) Iron and magnesium. Ammonium nitrogen levels between both soil types were relatively even with washed river sand at <1mg/kg and native soil at 3mg/kg.

3.2 Growth Observation

An attempt was made to germinate the ryegrass seeds prior to sowing, they were incubated for approximately a week and had overdeveloped. The decision was made to sow ungerminated seeds directly into the soil.

Throughout the experiment, growth in all native soil groups was generally the strongest. The native soil group saw a distinguishably greater amount of grass biomass produced per pot as opposed to the river sand group. Native soil controls grew densely compared to its treatment group and river sand control group. Overall, pots containing no fertiliser grew denser than treatment pots in both groups.

Figure 2. Grass biomass difference on the 5th week, native soil control (right), buried pork (left) and non fertiliser (right, rear two) groups prior to first harvest.

The washed river sand group grew sparingly across all pots, with control groups producing a greater amount of biomass. The treatment group initially grew at a steady rate, approximately two weeks after planting there was visible evidence of withering; grass tips drying, rigid stalks dropping. Complete death of the treatment group occurred three weeks after planting, reseeding was then required.
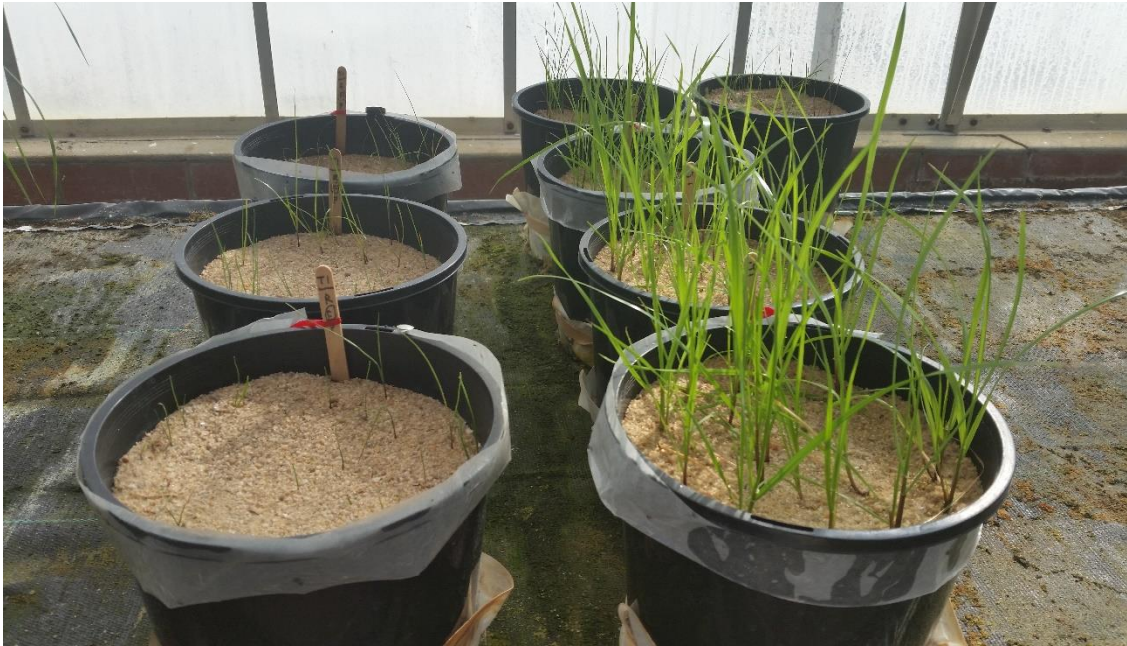
Figure 3. Grass growth of washed river sand group prior to the first harvest. Control group (right), pork (left) and non- fertiliser (right, rear two).

During observation, it is noted that in all treatment pots, initial sprouting was seen all over the treatment pots, further into growth stalks situated around the centre of pots began to darken in brown colour and lose rigidity two weeks after planting. Strong growth was generally seen around the edge of the pot further into the growth period. Reseeding was required for all sand treatment pots due to complete death of grass three weeks after planting, 40 seeds were sown to maximise growth before harvest.

Figure 4. Ryegrass regrowth one week after the first harvest. Comparison between two growing medium groups

Following the first harvest after six weeks since planting, grass regrowth occurred one week following collection.

3.3 Insect Activity

An established ant colony was present in one pot from the native soil treatment group within the first week of seeding. The colony was terminated using RichGro© ant killer according to the recommended instructions. Ant activity left small holes over the

surface of the pot.Other insect activity was detected five weeks after planting with flies suspected to be a species of corpse related fly. Small holes were noted on the surface of treatment pots from both native soil and river sand groups at the time these flies were detected. Notable change to the surface of control groups could not be detected as clearly with a dense biomass of ryegrass. Following the conclusion of the experiment flies captured on the tape were confirmed to be Diptera: Phoridae species, commonly known as phorid flies.

Figure 5. Holes on the surface of Pork Treatment pots from the washed river sand group.

3.4 Dry matter yield

Total dry matter (DM) produced by all groups according to the data is considered statistically significant, with the soil groups producing the biggest total difference (Table 2.) compared to the washed river sand group. Pork treatment from the sand group produced the least dry matter in comparison to the soil counterpart, while control samples from both groups generally saw more DM produced. Overall, total mass

produced significantly dropped between the first and second harvest, with the first harvest seeing the greatest DM produced.

|			Data summary		
Trt	Soil	Applied	DM (t/ha) (H1)	DM (t/ha) (H2)	DM (t/ha) total
1	Soil	Control	1.62	0.73	2.35
2	Soil	Treatment (No fertiliser)	1.52	0.48	2.01
3	Soil	Treatment (Pork)	0.12	0.16	0.28
4	Sand	Control	0.25	0.04	0.29
5	Sand	Treatment (No fertiliser)	0.08	0.08	0.16
6	Sand	Treatment (pork)	0.03	0.05	0.08
		Prob	<0.001	<0.001	<0.001
		LSD	0.3029	0.165	0.3388

Table 2. Data summary of averaged dry plant weights in tonnes/hectare, values of P<0.05 were considered significant.

These differences are seen in Figure 6. where the dry matter yield between the two soil types are contrasted. In the native soil group, the effect of the added fertiliser was not as contrasting as the effect added pork had on the treatment group, in comparison to the control there is a highly significant difference that is visually and statistically detected.

This dynamic between treatment, non-fertiliser and control groups is mimicked in the DM yield in the river sand group on a much smaller level. However, with only three replicates per treatment the results seen are only preliminary.

3.5 Nutrient Analysis

Figure 6. Averaged samples, graphed in tonnes per hectare at 95% confidence, P<0.001. A) Native soil – control, B) Native soil – non fertiliser, C) Native soil – pork treatment, 1A) Washed river sand – control, 1B) River sand – non fertiliser, 1C) River sand – pork treatment
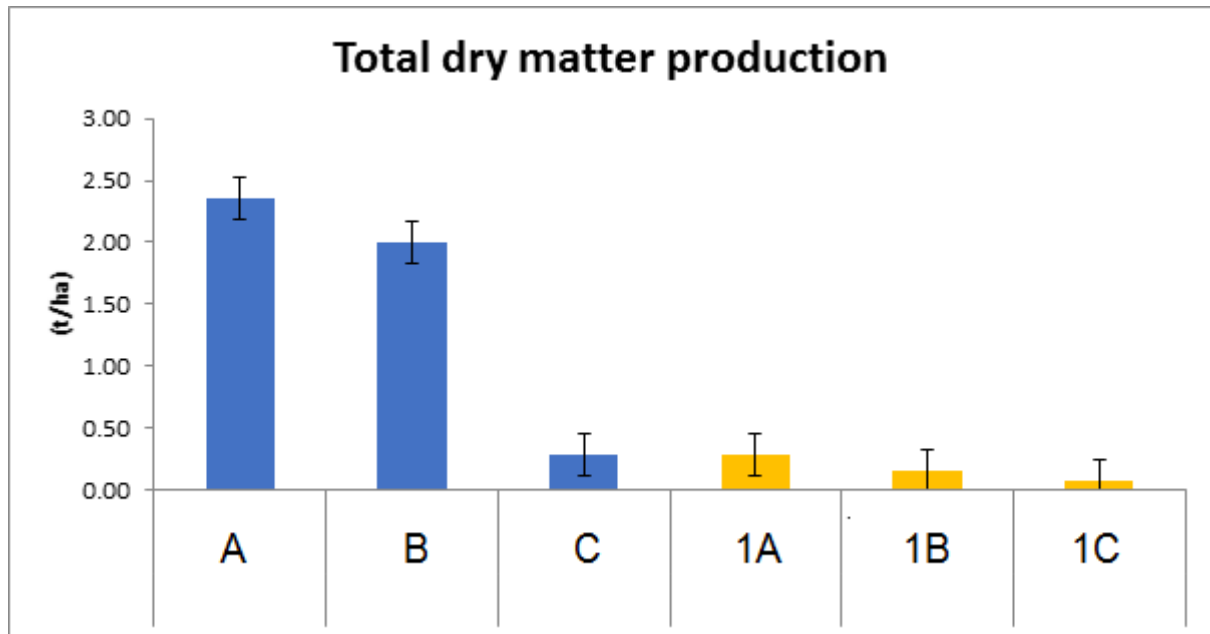
The following results are in comparison to Table 4. and only exhibit the guideline for nutrient deficiency in ryegrass at the stage of growth producing young tissue (leaf blades harvested during active growth).

Comprehensive tests involving Dumas combustion could not be carried out on all samples due to the light weight of dry matter yield. All three samples from each control and treatment group were therefore combined for testing. Comprehensive tests were carried out on all controls from all groups in both first and second harvests, trace nitrogen testing was carried out on soil treatments with no fertilizer (first harvest) and no fertiliser in sand groups (second harvest). ICP plus nitrogen was conducted on remaining groups that reached minimum combined weight for analysis. As seen in Table 3. not all samples were able to successfully undergo this analysis.

SOIL (HARVEST)	Group (Pots)	Boron	Calcium	Chloride	Copper	Iron	Magnesium	Manganese	Molybdenum	Nitrate	Phosphorus	Potassium	Sodium	Sulfur	Total Nitrogen	Zinc
		mg/Kg	%	%	mg/Kg	mg/Kg	%	mg/Kg	µg/Kg	mg/Kg	%	%	%	%	%	mg/Kg
SAND (H1)	CONTROL (P1, 2, 3)	37.71	0.58	2.49	15.60	211.00	0.34	24.28	2225.33	41.38	0.46	3.76	0.53	0.30	2.31	38.84
SAND (H1)	TREATMENT PORK (P1, 2, 3)														3.98	
SAND (H1)	TREATMENT NO FERTILISER (P1, 2)														1.81	
SAND (H2)	CONTROL (P1, 2, 3)	31.50	0.5	3.08	16.38	69.62	0.29	37.26	1704.47	70.80	0.55	4.76	0.41	0.27	2.50	64.96
SAND (H2)	TREATMENT PORK (P1, 2, 3)														5.01	
SAND (H2)	TREATMENT NO FERTILISER (P1, 2)														2.76	
SOIL (H1)	CONTROL (P1, 2, 3)	17.93	0.54	2.63	11.13	81.32	0.31	53.62	1521.40	1873.80	0.27	3.54	1.04	0.26	3.58	31.31
SOIL (H1)	TREATMENT PORK (P1, 2, 3)	23.33	0.38		14.93	124.46	0.22	44.22			0.23	3.90	0.60	0.45	4.59	29.91
SOIL (H1)	TREATMENT NO FERTILISER (P1, 2)														2.84	
SOIL (H2)	CONTROL (P1, 2, 3)	16.98	0.46	3.79	11.80	105.29	0.33	87.59	2009.86	232.61	0.48	3.52	1.69	0.30	3.22	39.97
SOIL (H2)	TREATMENT PORK (P1, 2, 3)	17.39	0.38		17.45	100.20	0.25	71.35			0.37	4.50	0.86	0.47	5.38	39.34
SOIL (H2)	TREATMENT NO FERTILISER (P1, 2)	20.16	0.49		11.73	89.54	0.30	91.12			0.49	3.29	1.41	0.31	2.71	35.37

Table 3. Nutrient analysis of all samples according to soil type and treatment. Not all samples underwent the same analysis due to weight restriction.

Type	Species	Boron	Calcium	Chloride	Copper	Iron	Magnesium	Manganese	Molybdenum	Nitrate	Phosphorus	Potassium	Sodium	Sulfur	Total Nitrogen	Zinc
		mg/Kg	%	%	mg/Kg	mg/Kg	%	mg/Kg	mg/Kg	mg/Kg	%	%	%	%	%	mg/Kg
Young Tissue	Perennial Ryegrass	3.0 - 5.0	0.15 - 0.20	-	4.0 - 6.0	40 - 60	0.15 - 0.20	15.0 - 20.0	0.11 - 0.13	-	0.20 - 0.28	1.4 - 1.9	-	0.18 - 0.22	3 - 3.5	10.0 - 15.0

Table 4. Average nutrient levels, adapted from *Plant Analysis Manual: An interpretation, 1997*.

Overall, nutrient levels appear to differ between the first and second harvest for both soil groups. In the sand groups, there is a generally an increase in nutrient levels in control groups for second harvest, notably in zinc, nitrogen, nitrate, potassium and manganese. Total nitrogen content increased in the second harvest in both pork and no fertiliser treatment groups in comparison to the controls. Due to light weights of these groups no other nutrient levels could be successfully analysed.

The native soil group followed similar harvest trends for total nitrogen levels. Pork treatments saw an increase while controls and no fertiliser treatments slightly

decreased in nitrogen levels. Pork treatment saw an overall increase across all tested nutrients except for boron, calcium and iron. Further comparison of the no fertiliser group can't be made with the absence of data from the first harvest.

Between both groups, nitrogen levels of pork treatments saw the greatest difference compared to controls and fertilisers; sand at +1.03% and native soil at +0.79% increase. For additional compounds of interest in pork such as potassium, phosphorous and sodium, increases were seen between harvests for treatments involving pork, this increase was also seen for control groups. Further comparison of the no fertiliser group can't be made with the absence of data from the first harvest.

In reference to Table 3. possible nitrogen deficiencies were seen in no fertiliser samples across all groups with total nitrogen below the 3.0 – 3.5% range, this also includes sand control groups from both harvests. High levels were seen in pork treatments involving all groups with the highest being 5.38% in native soil. Phosphorous levels across all groups were within and beyond the range of 0.20 – 0.28%, total percentage increased across all groups.

4. Discussion

Preliminary results show that Dumas method of nitrogen combustion can be used in the forensic context for gravesite location and post mortem estimation with further investigation needed to validate noted observations. This research has uncovered some trends and occurrences that are of interest; ryegrass growth in the presence of pork, resulting dry matter yield and present nutrients.

Prior to the first day of planting an attempt was made to germinate seeds, after incubation it was deemed the seeds had overdeveloped and not suitable for planting.

Instead, seeds were planted directly into pot soil – eliminating the possibility of false negatives.

4.1 Growth Observation

A major observation noted is that in all treatment pots, seeds initially growing at the centre of the pots were quick to die within two to three weeks of planting despite the addition of fertiliser on the surface. Normal growth was seen around the edge of the pot, appearing to border the edges of the buried pork, which was not an expected result. This phenomenon of death of vegetation can be associated with advanced decay stages, where death can be triggered by nitrogen toxicity, soil pH, volatile fatty acids (VFAs) in soil or cadaver smothering (Carter, Yellowlees, & Tibbett, 2007; Arpad A. Vass et al., 2002).

The lack of data on soil pH and VFAs leaves nitrogen and pork presence a possibility in growth hindrance. Pork treatment groups saw the highest percentage of total nitrogen in dry matter yield when compared to the average range in Table 3, this group had difficulty growing centrally in the pot right where the pork is present, and collected plant tissue was predominantly from the pot edges. Nitrogen toxicity is possible given the understanding that nitrogen levels in soil follows a steep gradient with greater distance from a cadaver, total nitrogen released directly at the centre of the pot would likely be higher than that seen in Table 2. (Danell, 2002). It cannot be stated conclusively that nitrogen toxicity can occur in ryegrass growth because of the lack of studies exploring this. The effect of the pork presence itself is also another possibility, acting as a physical barrier, making it difficult for young roots to establish themselves in the soil, particularly in shallow burial situations (Hunter, 1996). The type of soil itself

is another variable with looser, less compact grainy soil types making it difficult for plants to establish themselves securely (Jaggers & Rogers, 2009).

Some studies indicate the presence of a corpse itself may not cause the observed presence and growth of vegetation but rather the mechanical disturbance and subsequent aeration of the soil itself (Caccianiga et al., 2012; Watson & Forbes, 2008). This can be attributed to bioturbation on a large scale, the effects of bioturbation on corpses at the soil level is to date undocumented however the major shifting in soils sets plants succession into motion (France, 1992; Meysman, Middelburg, & Heip, 2006). Dominant plants become disturbed and pioneer species, generally weed and opportunistic plants, flourish (Watson & Forbes, 2008).

4.2 Nutrient Dynamics

The bulking of the individual samples has prevented meaningful statistical analysis of the differences between the groups, furthered by the necessary use of the ICP test brings uncertainty to the result. However, the nutrients present provide some insight in ryegrass behaviour. The high total nitrogen content of up to 5.38% seen in the treatment pots is likely not greatly influenced by added fertiliser or existing soil nutrient levels. These conditions were replicated in the control groups with similar fertiliser and soil content; nitrogen levels still differed. The added pork is likely affecting growing conditions and peripheral ryegrass is responding to the nitrification process of organic nitrogen to its inorganic (ammonium and nitrate) form that the roots are able to absorb, with high concentrations being present in soil surrounding the pork ("Soil and Plant Nutrition," 2012; Stokes et al., 2013; Van Belle, Carter, & Forbes, 2009). It can be suggested that the levels released exceed that present in Macro Pro Extra© fertiliser

initially added. As an improvement, more data would be required to explore and validate these possibilities.

In context of wider studies, the results of this research contradicts a few existing taphonomy results where revegetation of the area had occurred, and in some cases, to almost indistinguishable levels in comparison to undisturbed graves with exception of varying vegetation (Danell, 2002; France, 1992; Rodriguez & Bass, 1985). Many of these studies have attributed this to influx of nutrients, primarily, nitrogen, phosphorous, potassium and calcium compounds to the strong response normally seen (Melis et al., 2007). Initial expectations in this research were abundant growth after the establishment of grass, this was not the case as noted in Figure 3. and followed up by Figure 4, grass was not able to be re-established by the conclusion of the experiment. However, it should also be clarified that this research was conducted over a very short period in comparison to the long term studies mentioned, which is often the case when exploring post deposition possibilities. Another existing variation can lie within this research exclusive use of pork loin which consists of muscle, where whole cadavers often used, eventually involve the break down of offal (organs and entrails) which has an average higher nutrient content (Tomovic et al., 2015). The strong growth response seen on other wider studies could have experienced strong growth due to the release of offal nutrients in early decomposition.

4.3 Insect Activity

The arrival of insects prompted inspection of the glasshouse which was not completely sealed from the outside environment, it is a possibility the insects arrived through the opening and closing of the door, a small hole from broken glass, and the air vents. The phorid fly species is normally associated with buried or concealed cadavers (Martin-

Vega, Gomez-Gomez, & Baz, 2011). The coffin fly has been observed moving and hovering over the surface of the treatment pots containing pork and it is unclear if the flies have successfully reached the pork 7cm deep. With the presence of small holes over the surface on the treatment pots of both soil and sand, it can be suggested that the flies have attempted to burrow down to the decomposing pork meat. This is entirely possible as the coffin fly larvae have been found as deep as 60cm below the surface within two weeks of burial, similar timing seen in this research (Pastula & Merritt, 2013).

4.4 Recommendations

A few limitations became apparent as the research progressed. Time and budget constraints were a major factor on the limiting data available to analyse. Ryegrass could only be harvested twice in the four month growth period and samples could not be analysed individually due to limited growth and weight restrictions for combustion analysis. The targeted analysis of Dumas combustion could not be conducted on all samples as intended, and grass nutrient data could not undergo further analysis to be deemed statistically significant.

A few recommendations can be made to improve on the research attempted. Increasing growth period up to a minimum of 12 months will allow the ryegrass to grow sufficiently from the second harvest onwards. More than two harvests must be made to establish and statistically verify observations noted in the results and explore any nutrient dynamics associated with increased post deposition time. The lack of grass growth on pork treatment pots could be improved using larger growing areas to observe the stated nitrogen gradient, providing opportunity to observe growth around

the edges and possible nutrient intake from leeching decomposition products, and fully realise the potential of Dumas combustion.

5. Conclusion

Nutrient dynamics of ryegrass using dumas to locate and estimate a time of deposition of a piece of pork, is a concept that has not been investigated before. The resulting death of grass was an unexpected event that could be linked to bioturbation effects or the physical presence of pork. The presence of pork has affected dry matter yield, and the resulting conditions have increased nitrogen presence in grass tested using Dumas combustion and ICP. Further studies in this area will be able to realise the full potential of dumas combustion in gravesite location and post deposition estimation.

Disclaimer

No declared conflict of interest

Reference

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