The Analysis Of Preserved And Degraded Human Skeletal Material: Understanding Relationships Between Bone And The Soil Environment.

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Abstract:

The relationship that soil interred human bone has with the burial environment has implications for the survival of the organic and inorganic components, including collagen and DNA. The study of both the Chapel House Farm Medieval cemetery, Poulton, Cheshire, UK, and contemporary skeletal remains from cemeteries from Liverpool, U.K. provides new data into the environmental conditions that human bone encounters in the burial environment that are either conducive to preservation or result in complete dissolution of both the organic and inorganic bone matrix.

Medieval bones from Chapel House Farm cemetery were analysed to establish the relationship between the organic and inorganic matrix of preserved human bone, and the interaction these had with their burial environment. The use of FTIR and XRF techniques proved to be effective mechanisms in assessing the relationship between the organic and inorganic molecules extracted from the different types of Medieval bone to assess their preservation. These analytical methods were able to establish the degree of soil component intrusion (movement) into the bone, the collagen content, as well as the condition of the mineral matrix of soil interred Medieval bone to be quantified using 2 mg of bone, reducing the need to destroy precious human bone samples.

The results from the study of both the Medieval preserved bones from Poulton and Modern contemporary dissolved human remains from Liverpool U.K., where the remains were known to have fully dissolved within 20-30 years post interment, found that the mobility of ions including: Ca, Fe, Mg, K, and P, out of the bone into the soil, does not happen at a constant or predictable rate, but is linked to the environmental soil conditions and burial dynamics. These include: hydrology, seasonal temperature, pH of the soil, burial depth as well as the initial health and age of the individual. Soil samples from two contemporary Liverpool cemeteries were taken from 14 single graves spanning seven time periods (2000- 1850AD) at four depths (0-110cm), along with control samples. They were subjected to X-ray Fluorescence semi-quantitative analysis. The results for the concentrations of five elements: Fe, Mg, Ca, P and K, were statistically tested for trends associated with interred dissolved human remains. The concentration of these ions remained at a relatively constant level in the top soil

(0-50 cm) through time. The results demonstrated a clear negative correlation between the levels of Ca and Mg with time, and depth, as well as a significant difference between these components and the control samples. The levels of Fe and K, demonstrated significant trends through two time periods at 50-110 cm, with peaks during initial decomposition and the war years (70-90 years). P demonstrated peaks at 50 and 150 years post interment at all depths.

There was a statistical difference in pH of the Liverpool cemetery soils in 50-110 cm depths, and a general increase in the pH from 5-7 in the cemetery soil with relation to time.

The presence of ions including: Ca, Fe, Mg, K, and P, derived from human remains from both the preserved Medieval and dissolved contemporary cemeteries persisted in the soil decades after the initial bone dissolution, making this a potentially new technique to assist in the detection of older forensic and historical soil interred human remains; and in addition provides information on the rate of release through time of chemical elements from decomposing human skeletal material.

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To all those that have gone before us: we thank you.
"Think not in life that you are no more, but thankful that you were."

This Thesis is dedicated to my children, thank you for your love, patience and encouragement xx

HAMLET *How long will a man lie i' the earth ere he rot?*

First Clown *I' faith, if he be not rotten before he die--as we have many pocky corpses now-a-days, that will scarce hold the laying in--he will last you some eight year or nine year: a tanner will last you nine year.*

HAMLET *Why he more than another?*

First Clown *Why, sir, his hide is so tanned with his trade, that he will keep out water a great while; and your water is a sore decayer of your whoreson dead body.*

William Shakespeare

Abbreviations:

FT-IR – Fourier Transform Infrared Spectroscopy

MFD – Microscopic Focal Destruction

PCR – Polymerase Chain Reaction

qPCR – Quantitative Polymerase Chain Reaction

SF/CI – Splitting Factor/Crystallinity Index

SK – Skeleton prefix, followed by a number.

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Chapter 1. Introduction to PhD Research and Aims

Bone and teeth remains found surviving in the soil environment have presented unique opportunities to study past human and animal populations. The DNA study of these ancient populations has grown in recent years as the knowledge and technology advanced to allow the polymorphic make-up of distinct genetic populations to be analysed. The first ancient DNA studies involved the extraction and amplification of the recently extinct quagga (Higuchi et al., 1984), before moving on to other animal studies and humans with some success (Schwarz et al., 2009; Poinar et al., 2008; Orlando, 2002; King et al., 2007; Chang et al., 2007; Capelli et al., 2003; Garcia-Bour et al., 2003; Weale et al., 2002; Falsetti et al., 1993). Currently the study of ancient DNA is well established as a technique but many obstacles continue to prevent the full understanding of degraded DNA from hard tissues; bones that appeared initially to be in excellent histological condition, proved to contain no useable DNA.

The human skeletons from Chapel House Farm, Medieval cemetery, Cheshire, U.K., were frequently excavated in an excellent state of preservation and initial DNA extraction and analysis of two juveniles (Figure 6) demonstrated a satisfactory DNA yield, but failed to amplify using standard polymerase chain reaction (PCR) protocols of decalcification with EDTA and a phenol chloroform iso amyl alcohol precipitation. The rationale for this PhD study stems from trying to understand the reasons for this, and it was considered at the start of the PhD that this could be due to the human DNA being the subject of contamination or degradation processes occurring within the soil.

PhD Aims:

The main aims of this PhD project were to investigate the processes of human bone degradation and soil component interaction with the burial environment to understand and explain the preservation, or not, of Medieval human bones. The thesis objectives have been divided into the following chapters:

- Chapter 1: Analysis of the DNA and cemetery soil component interaction of the Medieval, Chapel House Farm bones.
- Chapter 2: Study of the Medieval, Chapel House Farm bone preservation and their soil burial environment.
- Chapter 3: Study of the relationship between the organic and inorganic phases of the soil intruded bone from Medieval, Chapel House Farm.
- Chapter 4: A study of the degradation of bone, and the interaction of human remains with the burial environment using Modern late Nineteenth and early Twentieth cemeteries from Liverpool, Merseyside, U.K.

Chapel House Farm, Medieval cemetery is located at Poulton, Cheshire, U.K. (OS ref. SJ 3959) in-between the Duke of Westminster's estate and the Welsh border. Figure 1 shows the general location of the site in relation to Poulton and the surrounding area. The site has a long history of human habitation, mostly of an agrarian life-style with little industrialisation. The site has evidence of human occupation since before Mesolithic times with a strong religious emphasis; this has been demonstrated by evidence of a wooden henge, Roman altars, and later successive Christian chapels. The present derelict Cistercian chapel was established in the 12th century, before being leased to the Manley family, then becoming abandoned following the death of the last heirless Manley in 1580. Following this the land was sold to the Grosvenor Estate, which in turn was sold on to a farming family (Emery et al., 1995).

The area around the cemetery has been used for ritual death rites since before Roman occupation of the site whilst the fort at Deva (Chester) was in use, with copious cremated remains being found in the soil. The cemetery occupies a plot measuring approximately 27 m x 35 m (Figure 4) and was originally enclosed by a boundary ditch. The human skeletal remains were found in a variety of states of preservation ranging from remarkably good to very poor. One cemetery interment has been radiocarbon dated (J Ohman: pers. Com.) to 1280-1320 AD (Beta Analytic, Miami), in line with the recorded dates of activity of the monastery, making at least some of the burials over 700 years old. Bones of the hands and feet, and the undeveloped bones of sub-adults such as the long bones of the axial skeleton, suffered more degradation than the developed robust bones of the adults.

The site has generally been dedicated to farming with the land being used mainly for poor quality grazing, including the chapel site as it fell into disrepair and the stone robbed, with only the sandstone foundations remaining. By the time initial archaeological excavation work started in 1995 the cemetery site had been abandoned for several centuries, covered by overgrown pasture and afforded no protection from encroachment by grazing farm animals. By examining the chapel site, it is evident that some of the present day exposed foundations, and recovered scattered blocks, at least in part, consisted of reclaimed Roman worked stone. At present the chapel and cemetery site does not have a fully recognised boundary, and continues to be explored during on-going archaeological excavation. There is some evidence for it having been enclosed with the typical Medieval boundary ditch, as the area slopes downwards, to a hedge-line, with a drainage ditch behind. Although the chapel site was long abandoned, its location was recognised on historical maps (Figure 5), and limited amateur excavations have been conducted in the past. The land adjacent to the cemetery formed part of RAF Poulton during WWII, and the disused runway along with its associated, derelict features remain in place today (Figures 2&3).

Figure 1: Map of the UK showing the location of the Medieval, Chapel House Farm cemetery, just outside Chester on the Welsh border (Map: © itravel).

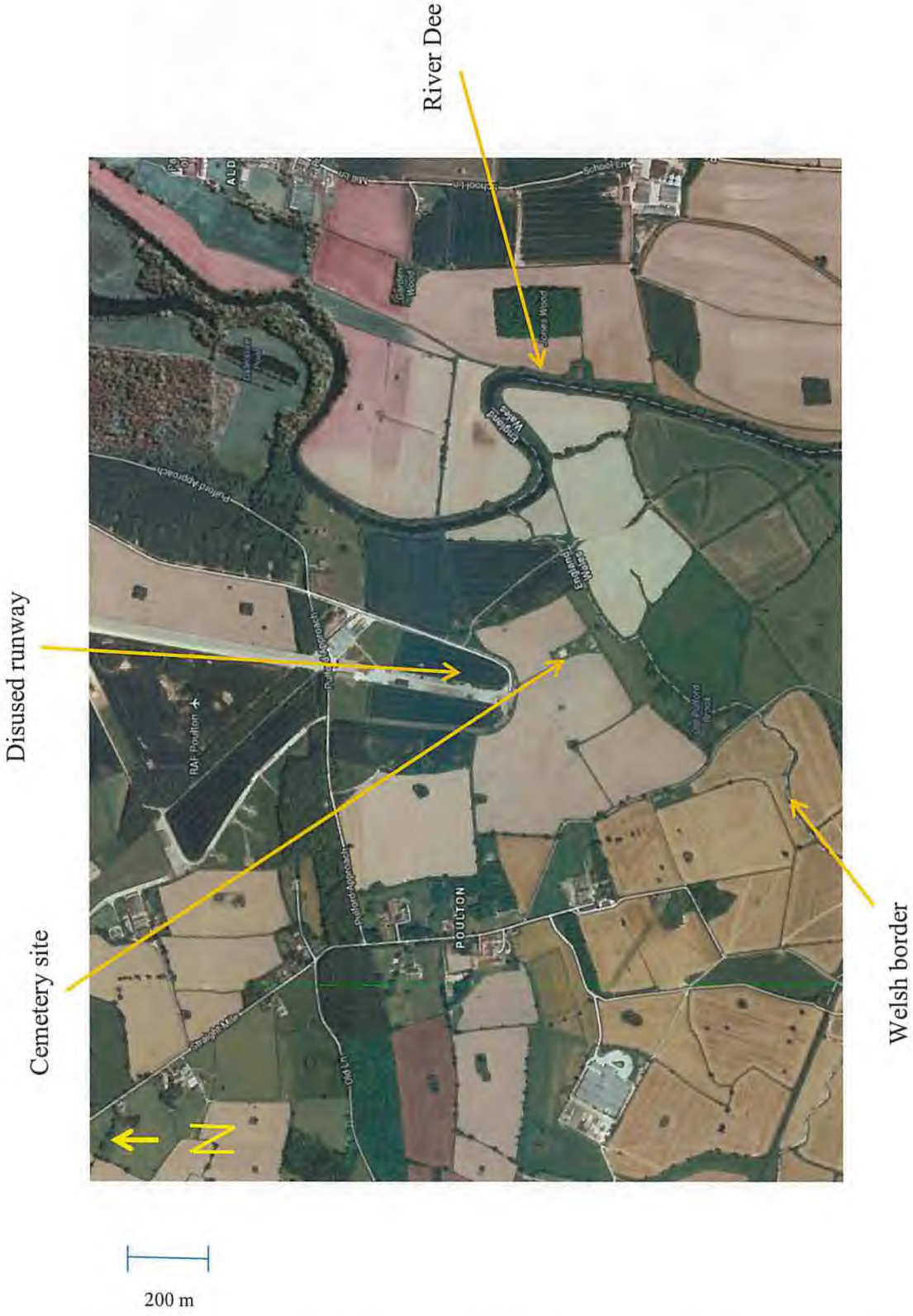


Figure 2: Location map of Medieval, Chapel House farm cemetery, Poulton, Cheshire (data © Google map 2014).

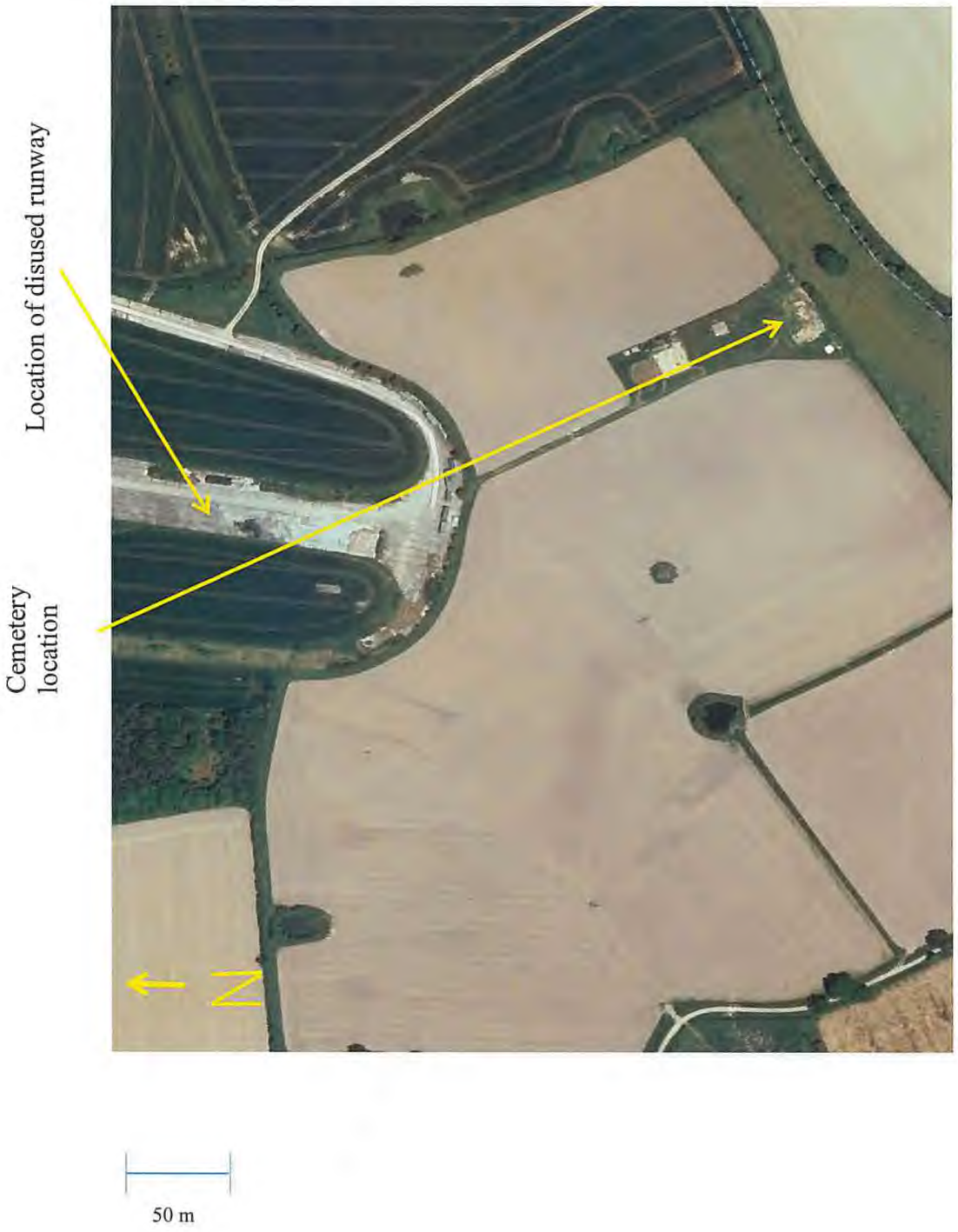


Figure 3: Location map of Medieval, Chapel House Farm cemetery and WWII runway (data © Google map 2014).

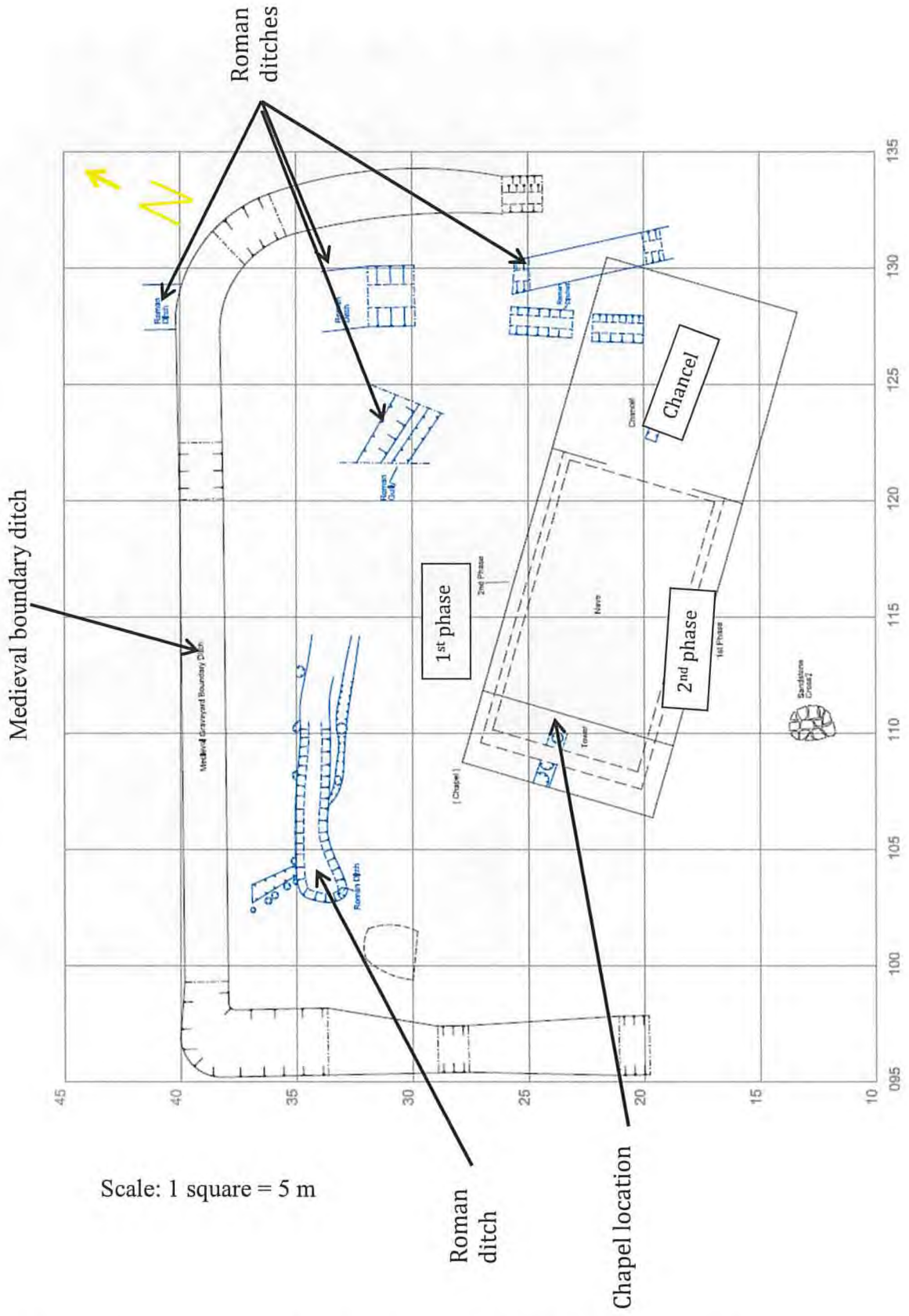


Figure 4: Chapel House Farm, Medieval cemetery (black) with Roman (blue) archaeological features (adapted from map by R. Carpenter and M Emery 2012).

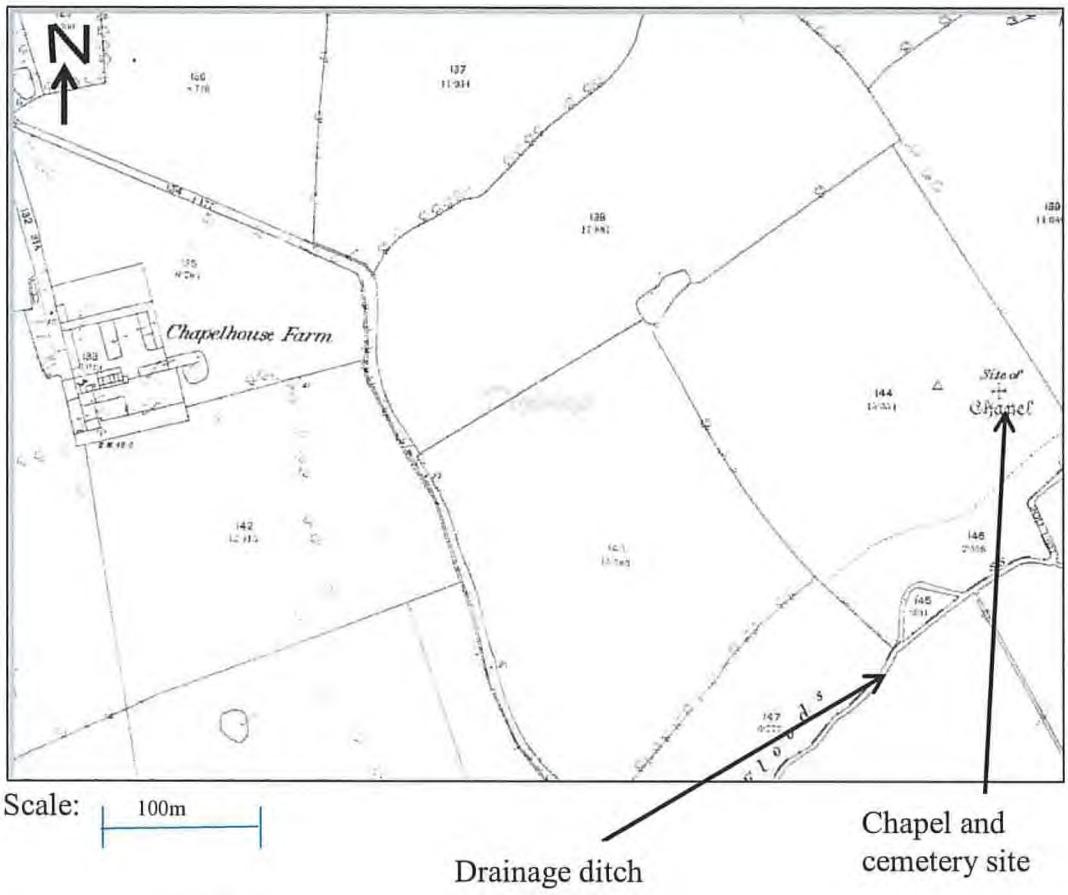


Figure 5: 1870 Ordnance Survey map, showing the location of the Medieval cemetery and Chapel at Poulton.

Finds such as flint arrow heads, fragments of Roman and Medieval pottery, fishing weights, building material, jewellery, as well as large quantities of human and animal bone had been reported for many years. In this area some of the shallower burials from the cemetery produced bones with considerable plough damage from being brought to the surface.

Archaeological excavations began in 1995 and developed into The Poulton Research Project (a charitable trust). Large quantities of animal bone continue to be excavated along with human skeletal material. The majority of the excavated human skeletal material consisted of shallow, (<40cm deep) mainly Christian East-West orientated burials, (along with the rare West- East orientation) with a large amount of complete as well as disarticulated human remains. These human remains consist of mixed sex and age, indicating a civilian population. The skeletal remains vary in their

preservation and condition, ranging from complete individuals, fragmented disarticulated single bones, to almost completely decomposed powdered remains. To date (September 2014) in excess of 600 complete sets of human remains have been excavated along with a large quantity of disarticulated material, animal teeth and bones, as follows:

141 adult females

108 adult males

92 unsexed adults

251 sub adults (<18 years)

Cremated bone was commonly found in the grave soil, and was thought to be Roman in origin, as open pyre cremation was a common practice until Christianity became the dominant religion and grave interment became the norm. There was little evidence to indicate the type of burial but the occasional shroud pin or coffin nail has been found; but it is thought that the burials were mostly shrouded, not coffined. Routine soil sifting was not routinely done at this site, due to the heavy clay soil.

Figure 6: Poulton cemetery: An example of disturbed older burials to make way for a new child double interment (SK 183 and 184).

Chapter 2. Analysis of the DNA from Chapel House Farm, Poulton, Cheshire, UK: Exploring DNA Contamination and Degradation from Human Bone.

Objectives

The objectives of this study were to extract ancient DNA and to establish the condition of the DNA from a selection of four adult bones of different types from the Medieval, Chapel House Farm, (Poulton) collection, housed at Liverpool John Moores, University:

- To analyse the Poulton bone DNA using different extraction techniques and primers, to remove or reduce soil product contamination and improve the DNA yield.
- To assess the inhibition of the Polymerase Chain Reaction (PCR) using a soil spiking test, and also assess the degree of degradation of the DNA.
- To devise protocols that may improve the PCR and DNA amplification outcome of the different types of Medieval bones.

2.1. Introduction

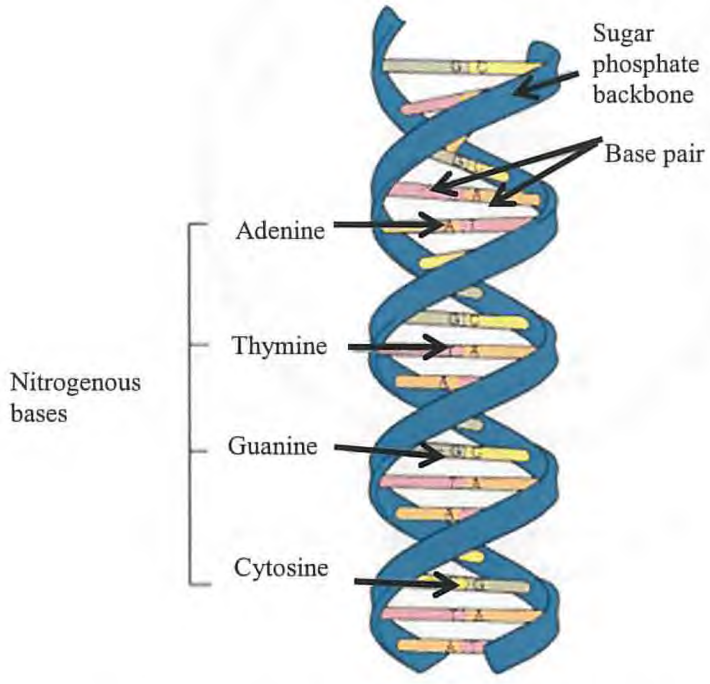


Figure 1: The structure and composition of nucleic DNA (Image adapted from: The National Genome Research Institute, ©2013: www.genome.gov).

The double helix structure of DNA was first described comprehensively by Watson and Crick (1953) and consisted of four nitrogenous bases of adenine, thymine (purines), guanine and cytosine (pyrimidines), supported by a sugar-phosphate backbone (Figure 1). These bases were usually paired in a specific order: adenine with thymine and guanine with cytosine. Variations from this are referred to as polymorphisms, these determine individuality, familial relationships as well as global groups and some disease susceptibility. The research and technology progressed rapidly over the decades to using thermally stable enzymes and template DNA to develop the polymerase chain reaction (PCR) which enabled multiple copies (amplification) of DNA strands to be analysed from a single cell, and eventually to sequencing of the whole human genome (Mullis and Faloona, 1987; Sanger et al., 1977; Lander and Consortium, 2001; Venter and Consortium, 2001).

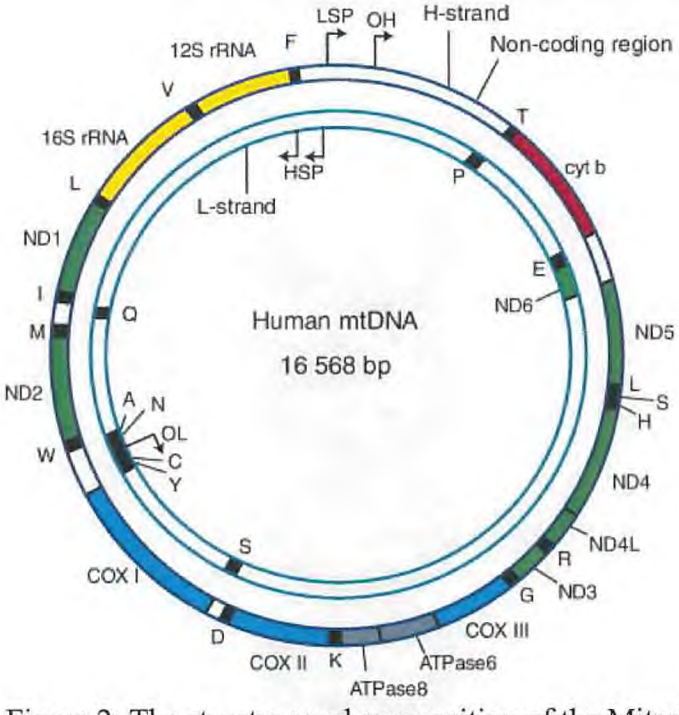


Figure 2: The structure and composition of the Mitochondrial DNA loop (Wanrooij and Falkenberg, 2010).

Soft tissues provide a rich source of cells (50-500 ng/mg), blood (20,000-40,000 ng/ml) with the hard tissues containing much less (3-10ng/mg) (Butler, 2005). There are two sources of DNA in eukaryotes: nucleic which is found in the cell nucleus and mitochondrial of which there are numerous copies to be found in the cell cytoplasm

(Anderson et al., 1999; Anderson et al., 1981). There are about 3 billion base pairs in nucleic DNA, but only 16,569 base pairs in the circular loop structure of mitochondrial DNA (Figure 2). Mitochondrial DNA is only inherited maternally (to both sons and daughters) in contrast to nuclear DNA which is inherited equally both paternally and maternally (Manfredi et al., 1997). As there are numerous mitochondria found in the cell, this DNA can be used for those samples where the nucleic DNA has been unsuccessfully amplified in both forensic and historic case work (Chaitanya et al., 2014; Hofreiter et al., 2001; Just et al., 2011). Mitochondrial DNA has also assisted in the development of maternal line population maps, and in conjunction with male Y chromosome lineages has made great strides in understanding the unique make-up and mobility of human populations across the globe (Falsetti and Sokal, 1993; Capelli et al., 2003; Garcia-Bour et al., 2003; Helgason et al., 2000; Immel et al., 2006; King et al., 2007; Melchior et al., 2008; Schurr et al., 1999; Weale et al., 2002; Wilson et al., 2001).

Variations commonly occur when comparing different population groups (haplogroups) to the human genome, these polymorphisms are usually single nucleotides (SNPs) and what may be common in one group (haplotypes) may be rare in another (Brookes, 1999; Consortium, 2007; Kline et al., 2005; Ramensky, 2002). Polymorphisms are usually benign but can be associated with an increased risk of developing a disorder (Ashley-Koch et al., 2000; Myerowitz and Costigan, 1988). It is now possible to determine the sex of an individual using the X and Y amelogenin alleles, (Davis et al., 2013; Faerman et al., 1995; Stone et al., 1996) and the colour of eye and hair using melanocytic genes (Branicki et al., 2008; Duffy et al., 2007; Fox et al., 1997; Schioth et al., 1999; Sturm and Frudakis, 2004; Sturm et al., 2001; Tully, 2007; Valverde et al., 1995; Walsh et al., 2013). The advancements in DNA technology have been used to solve identification of degraded skeletal remains using familial relationships, through the use of mitochondrial and nucleic short tandem repeats (STRs) or single nucleotide polymorphisms (SNPs) and this technology continues to develop further (Coble et al., 2009; Freire-Aradas et al., 2012; Gill et al., 1994; Green et al., 2013; Hawass et al., 2010; Just et al., 2011; King et al., 2014; Romero et al., 2012; Zhou et al., 2014). The presence of disease DNA has been isolated from ancient human remains, which has important implications for disease

antiquity and demography (Baron et al., 1996; Donoghue et al., 1998; Guhl et al., 1999).

2.1.1. The Polymerase Chain Reaction (PCR)

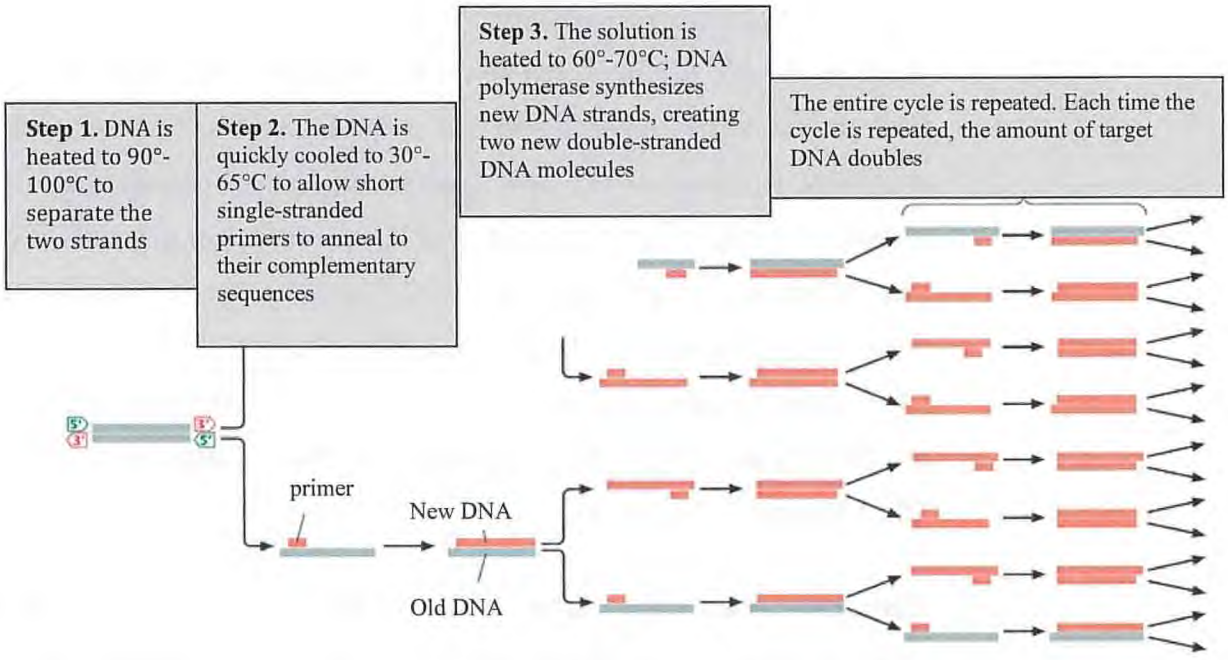


Figure 3: Steps used during amplification of strands of DNA using the polymerase chain reaction (image adapted from Nature © 2005 W.H. Freeman and company, all rights reserved).

The polymerase chain reaction is a technique that was developed to amplify target DNA strands in order that they can be analysed for medical, scientific or forensic purposes (Saiki et al., 1988; Mullis and Faloona, 1987). The process of DNA amplification involves the exponential increase of target molecules facilitated by thermally stable enzymes (polymerases), by repeated cycles of heating and cooling (Figure 3). At the end of a set number of cycles the DNA product bands could be sequenced or viewed in Ethidium Bromide (EtBr) stained agarose gel under UV light. This technology allowed previously low copy DNA (LCN) samples to be successfully amplified even from a single cell. The development of new DNA extraction and purification techniques and enzymes opened up the opportunities to analyse samples that were not thought suitable due to contamination of the DNA with enzyme inhibiting components, including blood and soil products (Kermekchiev et al., 2008; Abbaszadegan et al., 1993; Sutlovic et al., 2007).

Quantitative PCR (qPCR) enables the DNA to be quantified at the end of a set number of cycles. The detection of amplification is indicated where the fluorescence of the DNA product is greater than the background effect, referred to as the cycle threshold (CT), this is followed by an exponential then linear product growth, and finally a plateau stage (Figure 4).

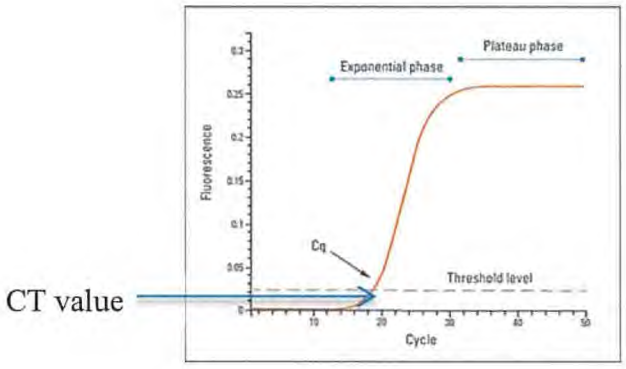


Figure 4: Distinct stages of qPCR, the CT (Cq) value being 19 (image from Thermoscientificbio).

The technology for degraded qPCR DNA work usually uses fluorescent dyes such as SYBR Green, with the intensity increasing with DNA amplification. This allows quantification of the end product and calculation of cycle efficiency, the amplified DNA bands can still be viewed on an EtBr stained agarose gel under UV light.

2.1.2. PCR Efficiency

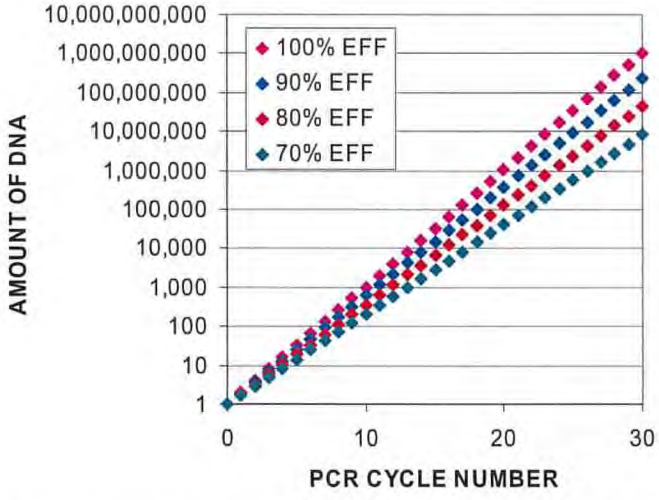


Figure 5: Comparisons of the effects different efficiencies on the final amount of DNA (Saiki et al 1988).

As each PCR cycle results in a doubling of the DNA this results in an efficiency of 2 (100%). The efficiency may be reduced if there is a problem with the reaction due to contamination, degradation, or the reaction components. As the amplification relies on exponential increase this could result in even small reductions in the efficiency of the PCR having very much greater effects on the quantity of the final DNA products (Figure 5). The lower number of cycles (CT value) the higher the loading of useable DNA, especially in degraded DNA work; it can be useful to conduct a series dilution test to assess this (Higuchi et al., 1993).

It is common practice to assess the efficiency of the PCR by using either a PCR where a target component series dilution test is used and by observing the intensity of fluorescence in the bands of EtBr gels (Higuchi et al., 1993); or a target component series dilution qPCR and mathematical calculations (Pfaffl, 2001).

2.1.3. Spiking Tests

"Spiking" involves the deliberate inoculation of a substance with another of known quantity and quality under controlled conditions, and has been beneficial in the assessment of the working of inhibitors during the PCR. "Spiking" has been used as a positive confirmatory test for the presence of inhibitors in ancient DNA studies involving fossil remains (Yoder et al., 1999) but only for the direct effect of inhibitors of PCR and not the effect of the contaminants on the DNA. It is known that spiking contemporary genomic DNA with ancient extractions can cause problems with the template DNA amplification (Pusch and Bachmann, 2004).

2.1.4. DNA Primers

DNA primers are short lengths of template DNA that are used to bind to target DNA. As the long DNA strand degrades it breaks up into increasingly smaller strands, the more degraded the target DNA is, the shorter the primer would need to be. For degraded DNA work there are four kinds of primers used: STR (short tandem repeats) miniSTR, Y-STR (paternal lineages), which all use nucleic DNA, and mitochondrial DNA (maternal lineages). These short primers use repeat sequences that are unique to the individual and their close biological family. They can be used to establish species and sex, as well identifying the individual and close familial relationships in both forensic and historic work (Alonso et al., 2005; Butler et al., 2003; Chang et al., 2007;

Coble and Butler, 2005; Gill et al., 2006; Grubwieser et al., 2005; Poinar et al., 2003; Schwarz et al., 2009; Vanek et al., 2009; Wiegand and Kleiber, 2001; Wurmb-Schwark et al., 2009). The amplicon (DNA product) length can vary between <100bp to >300bp. In the case of single nucleotide polymorphisms (SNPs) sequencing the target area(s) of the genome would assist in identifying the haplotype (Brookes, 1999; Consortium, 2007; Freire-Aradas et al., 2012; Ramensky, 2002; Romanini et al., 2012).

2.1.5. Degraded Bone DNA

Unless the environmental conditions are conducive to the preservation of the soft tissue, bone is often the only remaining tissue that can be retrieved after an extended length of time. DNA is most abundant in the living organic tissues, and quickly begins to degrade once the organism dies. The taphonomy of bone is poorly understood, mainly due to the vast range of environmental conditions that affect its preservation (Nielsen-Marsh et al., 2006).

The amplification of soil interred ancient DNA from bone has often been dogged with problems of failure, with many resorting to the use of coprolite (Poinar et al., 2003), cave or crypt interment analysis (Fletcher et al., 2003; Orlando et al., 2002; Reich et al., 2010), remains that have been preserved in the permafrost or by mummification (Schwarz et al., 2009; Willerslev et al., 2004; Guhl et al., 1999; Hawass et al., 2010). Each sample studied comes with its own unique characteristics, and approach in the techniques that need to be employed when processing the remains for further study; and a successful outcome with one particular sample does not necessarily mean success with another (Hummell, 2003). Even bones in an apparently excellent histological condition may not have useable DNA, especially if they have been stored at room temperature for several years or so. Most problems arise from the effects of DNA degradation, contamination and inhibition of the PCR from soil components or combinations of these, and assessing which of these is a problem in any given sample, can be a case of personal experience along with trial and error.

DNA preservation is very sensitive to fluctuations in temperature and water content in warm environments. Most successful ancient DNA studies have been from "thermally young" samples: cool, thermally stable environments, with low available water

content (Smith et al., 2001). DNA degradation primarily occurs via depurination and destabilization followed by fragmentation, involving hydrolytic and oxidative decomposition; however the DNA can be provided with limited protection through adsorption to the hydroxyapatite matrix of bone (Guarino et al., 1999; Lindahl, 1993), or the formation of glycosidic cross-links with Maillard products (O'Rourke et al., 2000; Poinar et al., 2003).

Screening bone samples for preservation of the DNA has its merits with freshly excavated material but has set-backs especially if using old stored remains, and many of the technologies require expertise and specialised equipment that may not be routinely available (Haynes and Searle, 2002; Poinar and Stankiewicz, 1999; Smith et al., 2003; Trueman and Martill, 2002).

2.1.6. DNA Contamination

It has become increasingly important to recognise the ever present problem of contamination of ancient excavated material with modern DNA, which can be air-borne, transferred from the laboratory operator or from manufacturing of consumables (Hummel, 2003; Marota et al., 2002; Willerslev and Cooper, 2005). It has been shown that modern DNA spiked with ancient DNA can cause non-authentic sequences to be generated (Pusch and Bachmann, 2004). Bacteria can originate from both ancient and modern sources, and may dominate the DNA extraction particularly from geological samples, but there is good reason to expect the same phenomenon could occur with soil intruded bone samples (Hebsgaard et al., 2005; Poinar et al., 2006). Cross-contamination from other samples can happen where multiple samples are being processed at the same time, or the samples are co-mingled (Willerslev and Cooper, 2005). Primer contamination has also been identified as a source of contamination of PCR products where all precautions had been taken to minimise the risk (Deguilloux et al., 2011). Strategies to limit the contamination of samples from exogenous DNA have been incorporated into laboratories that routinely process degraded DNA samples (Hummel, 2003; Melchior et al., 2008; Yang and Watt, 2005). It is important to limit the risk of contamination as much as possible and acceptable standards and accreditation are available, especially where work is hoped to be scientifically published or for police evidence.

2.1.7. Humic Acids and DNA

Humic acids can be seen as a brown coloured solution that often results during the preparation stage of decalcification by the application of buffer solutions to soil interred powdered bone for DNA study. This brown colouration from these humic acids originates from the biological break-down of plant, animal, and microorganisms and is the one of the primary sources of the colour of soil (Kumada, 1965). The most common contaminants that can affect the amplification of DNA from soil interred remains results from the presence of metals and humic/fulvic acids (Matheson et al., 2009; Tuross, 1994; Kim et al., 2008). These soil components can have a very dynamic relationship with each other, the bone and DNA.

Humic and fulvic acids are a large group of poorly understood and ill-defined, biological substances that result from the degradation of plant matter; humic acids are insoluble at acidic pH (<2) and soluble at higher pH, whilst fulvic acids are soluble in water at all pH (Calace et al., 1999). Humic acids have been shown to be part of the weathering of minerals in the natural environment and can bind with the metals present in the minerals in the soil parent material. Low-grade technology has been developed that uses humic acids to remove contaminating metals from polluted soil, but the efficiency was pH dependant. Of particular note was that humic acids were observed to remove calcium from calcite, which is also found in bone and this phenomenon could present as part of the bone degradation pathway. (Baker, 1973; Brown et al., 2000; Kerndorff and Schnitzer, 1980; Petrovic et al., 1998).

The removal of the humic acid compounds is of great importance to those working with soil DNA and substantial efforts have been made to develop strategies to remove them (Tebbe and Vahjen, 1993; Tsai and Olsen, 1992; Wang et al., 2009; Zhou et al., 1996), these methods enabled bacterial DNA to be successfully extracted and amplified, using different extraction and purification methods. However, the bacterial DNA did not suffer from the levels of degradation often seen in ancient or thermally old bone samples, and by applying these methods worked for some degraded bone samples but not for others. A variety of new methods that removed the humic substances, but tried to protect and retain the DNA or prevent it from being lost have been devised, with some success (Kalmar et al., 2000; Kim et al., 2008; Loreille et al., 2007; Sutlovic et al., 2007; Yang et al., 1998).

2.1.8. Nanophotometry

The nanophotometer uses the wavelength of a target molecule to determine the quantity of substances. It has been routinely used to assess levels of DNA (260nm) in samples (Hummell, 2003), but it has also been used to detect the common soil inhibitors fulvic and humic acids (Turross,1994), the DNA overlapping the same wavelength as the inhibitor (260-280nm), resulting in the prospect of a false positive or negative for the presence of DNA.

2.1.9. Chapel House Farm Ancient DNA Study

At Chapel House Farm cemetery there was substantial grave re-use observed, with interments being disturbed to make way for new burials, with many skeletal components being pushed to the surface with little soil to cover them making them vulnerable to the harsh environmental conditions of wet and drying cycles, temperature fluctuations, freeze-thaw cycles, bone weathering, and breakage due to trampling by grazing animals. Burials commonly appeared to be rather shallow and some human bones could be seen emerging from the topsoil, this is in sharp contrast to successful amplification of DNA from skeletal remains such as those of Richard III who was discovered under 1.5 m of soil (University of Leicester, 2012). The soil conditions at Poulton posed considerable challenges for ancient DNA analysis; but the soil parent material of boulder clay along with an alkaline pH may have provided enough protection for some DNA fragments to survive in the teeth, if not the bones. The dentine of teeth generally yield only mtDNA as the pulp cavity contents degrade, giving only limited maternal line information, but it can provide a protective environment that could generate some nuclear DNA. The bone contains both nuclear and mtDNA. Challenges that face DNA analysis included contamination by soil products along with inhibition of the PCR and substantial degradation of the DNA that no useable strands would amplify. Understanding the condition of the DNA may assist in the development of protocols that may help improve the prospect of amplifying old soil contaminated, degraded DNA. To date no DNA work has been completed using the skeletal remains at Chapel House Farm cemetery.

2.2. Materials and Methods

2.2.1. Chapel House Farm Cemetery Soil Studies

2.2.1. (i) Soil Spiking Test

A previous experimental aim was to devise an improved DNA extraction from bone samples that had previously shown a very high level of inhibition during PCR due to the presence of soil components. A new purification system was tried (Qiagen, QIAamp DNA Micro kit) to see if this could improve the removal of the soil inhibitors, and therefore the amplification of DNA during qPCR, however, this resulted in further failure; but it was difficult to judge whether this was due to DNA degradation or persistent contamination by soil contaminants. These findings led to the requirement for a method for assessing the effect of soil inhibitors on a known sample of modern genomic DNA, using a spiking test. Soil contains a cocktail of potential PCR inhibitors one of the most potent of these inhibitors are the humic acids, others include metals and fulvic acid.

2.2.1. (ii) Humic Acid Extraction

During the decalcification step of adding EDTA buffered to pH 8/8.4 with 1% NAOH while processing the bone for DNA study a brown supernatant commonly formed (Figure 6). It was noted that the colour seemed to be darker in the supernatant from the trabecular bone compared to the cortical.

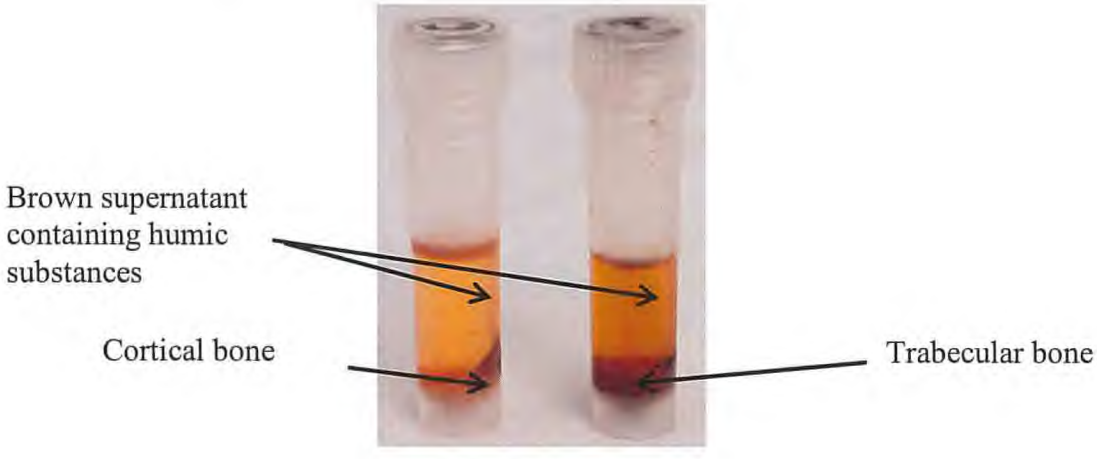


Figure 6: Humic substances released during bone processing.

1 ml of extraction buffer was added directly to the 0.5 g of soil samples previously collected from Chapel House farm (for location see maps see Figures 4 & 5 Chapter 3) in a 2 ml cryo-tube to see if the humic acids could be precipitated out in the same way (Figure 7 & 8). The tubes were then placed on a rocker at room temperature over the weekend to incubate. As can be seen in figures 7 and 8, the results show varying degrees of colouration. Due to the Boulder Clay parent material of the soil containing high levels of Fe this gave some of the supernatant a reddish hue, making the humic acid content difficult to determine using colourimetric analysis. One micro litre of the supernatant was taken from 4 cemetery (grave contexts: 1942, 1956, 1958, 2089) and 3 control (C1, C2, C3) soils and subjected to nanophotometry at 260 nm.

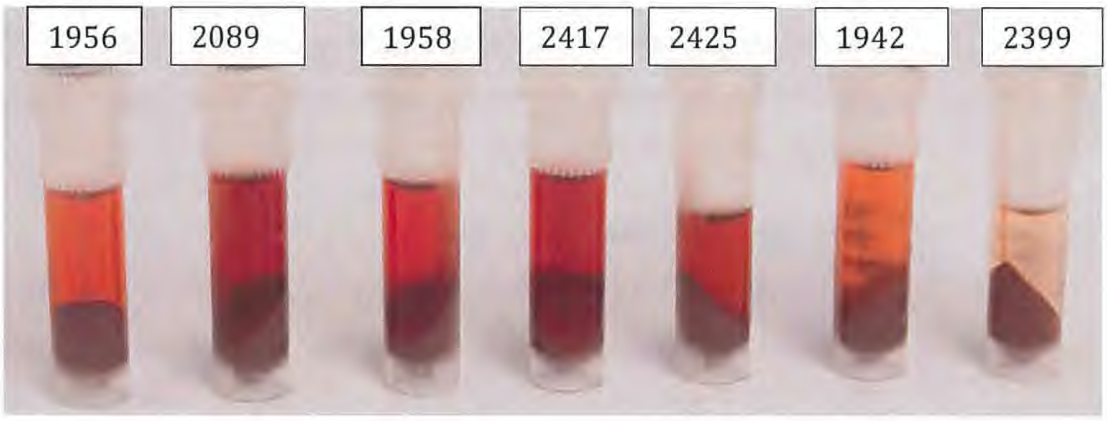


Figure 7: Cemetery grave soil samples (numbers denote grave context numbers) collected from Chapel House farm (some of the supernatent had been removed for analysis).

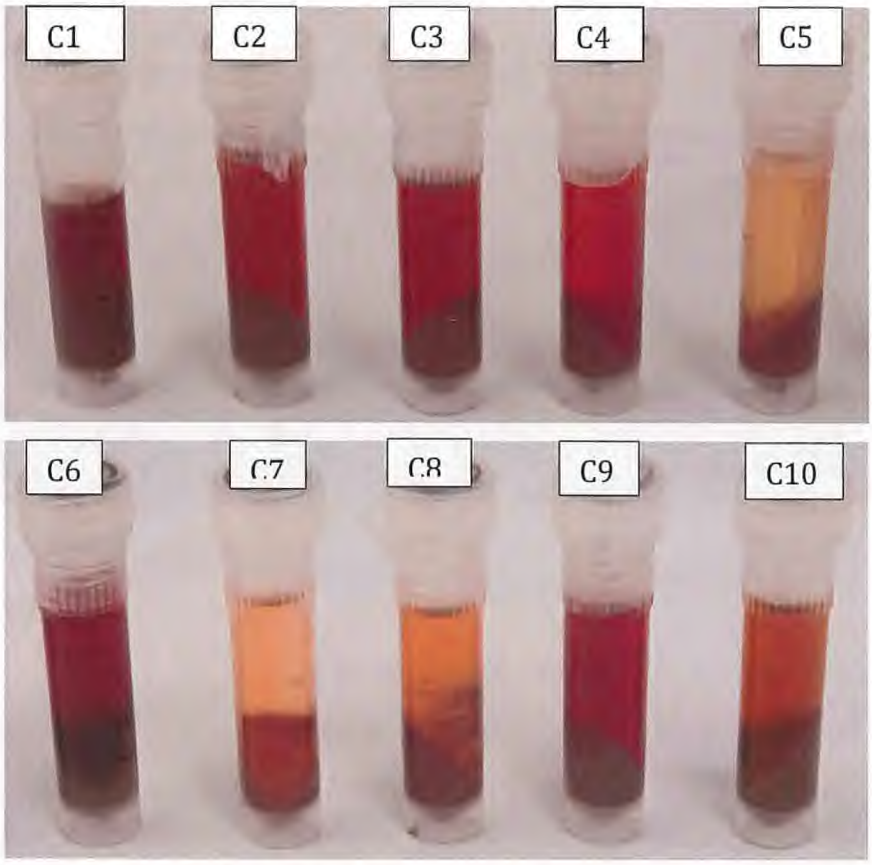


Figure 8: Control soil samples (C) from the perimeter of Chapel House Farm cemetery.

2.2.2. Soil Preparation

2.2.2. (i) Soil Spiking Test:

Five soil samples from Chapel house farm were selected to study the potential effects on modern contemporary DNA:

- C1 and C2 from the control group of soils (Figure 8)
- 1958, 1956 and 1942 context soils from inside the cemetery (Figure 7)
- A sample of the elution from a wormery was used in order to use a sample that contained no soil, just the liquid from the biological breakdown of plant matter.

A 5g sample of soil was placed in a small pot with 5 ml of ultra-pure water and shaken to produce a thick sludge; this was then poured into a small glass test-tube, vortexed using a small plastic hair pestle to mix well. The mixture was left for 1 hour to settle before decanting 1.5 ml into an Eppendorf tube and centrifuging at 13,000 rpm for 3 minutes (Figure 9). The supernatant was aliquoted into fresh Eppendorf tubes ready for use.

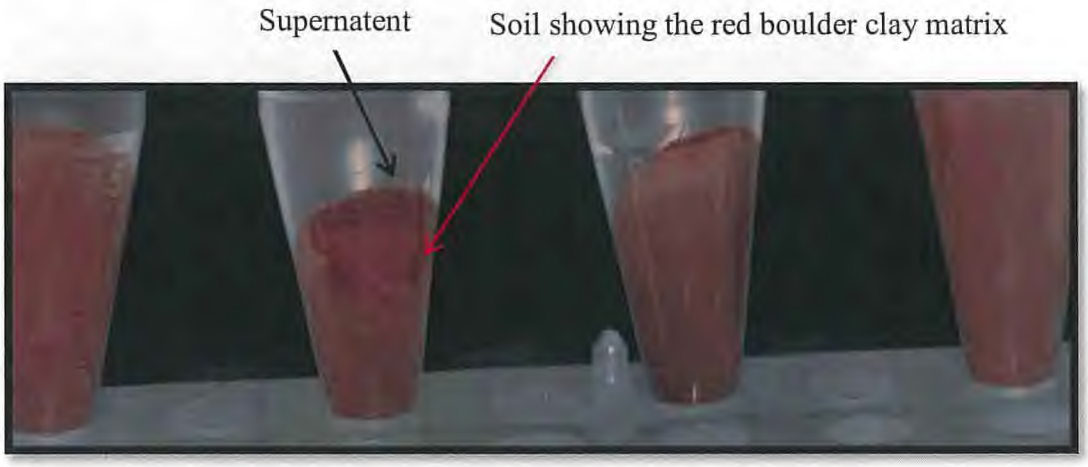


Figure 9: Soil samples prepared for analysis.

2.2.2. (ii) Extracted Humic Acid Spiking Test:

The effects of the soil components were tested using a series dilution of: 1:1, 1: 10, 1: 100, 1: 1000 and 1: 10,000 concentrations of control cemetery soil 1and 2, and grave soil context numbers 1958 (Soil 1), 1956 (Soil 2), 1942 (Soil 3), and worm elution (WE). 1ul of contemporary human DNA was added to each reaction tube and amplified using the following human DNA primers.

Primers:

Oligo Name	Forward Sequence	Reverse Sequence	Amplicon length
KH3	AAACTGGAACGGTGAAG	CCCTCTAAGGCTGCTCAA	624
BW1	GGTTTCTACCCTGCGGA	GCCCACTTCTGGAAGGTT	116

(a). QPCR Sample Preparation:

For each sample in the primer set:

2 µl genomic contemporary DNA

0.4 µl 50x SYBR GREEN

0.16 µl forward primer

0.16 µl reverse primer

10 µl 2x Master Mix

7.3 µl aliquot of each series dilution was used. The reaction mix was made up in bulk adding each dilution series sample to it. Negative and positive controls were included in the analysis:

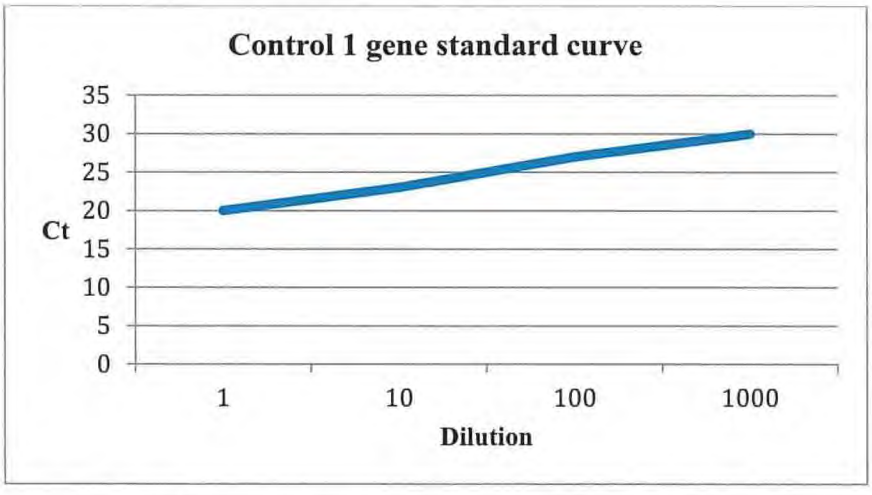
- Negative controls ensure that no extraneous contamination has occurred from either the operator or the consumables. This sample is run with ultra-pure water only.
- Positive controls ensure that the processes are all functioning correctly, and if there is no amplification in the experiment, it is not due to chemical failure.

2.2.2. (iii) PCR Efficiency:

As the soil contaminants were added to the PCR reaction, the probability of inhibition of the PCR was unknown. Ordinarily the rate of amplification (Figure 10) would be the target not the rate of inhibition. The PCR efficiency was calculated using two standard methods in order to double check the best method for calculating the rate of inhibition or amplification of the PCR. The optimum target amplification was 2 (100%), where there is a doubling of the target molecules with each PCR cycle. This efficiency can be calculated using the standard efficiency equation devised by Pfaffl (2001), or a software calculator (Thermo Fisher Scientific).

1. The online PCR efficiency calculator, calculates the qPCR reaction based on the slope of the standard curve (Thermo Fisher Scientific). The result is expressed as a percent.
2. Pfaffl's calculation (Pfaffl, 2001), based on the efficiency standard equation:

$$E = 10[-1/slope]$$



Slope = 3.331

Intercept= 19.8

$R^2 = 0.99$

E = 2.00

Figure 10: An example of an optimum reaction efficiency result.

2.2.3. Poulton Bone Sampling for DNA Analysis

Preliminary study:

A piece of stored disarticulated bone (no detail/information available) was extracted using silica columns (Method 1 below). The flow-through was evaluated using 2µl of the sample and the nanophotometer, The nanophotometer demonstrated that there was no obvious genomic DNA present in this stored bone sample. It was decided that future analysis should use only recently excavated bone in order to maximise the probability of obtaining useable DNA. Four disarticulated, recently excavated adult bones (5 months post excavation) were acquired for analysis, no other details or information about the bones was available:

1 femoral head with a small section of the shaft in place
1 9cm distal humerus
1 complete atlas vertebra
1 cranial fragment

All bone samples were prepared using the same preparation method to ensure the risk of contamination was reduced to the absolute minimum. Mask, gloves, eye protection and lab coat were worn at all times and changed regularly, all equipment and consumables were either heat treated using a microwave or UV light treated in a crosslinker for 20 minutes, or sterile disposables were used. The only water used throughout was UV treated ultra-pure (ddH_20). The bone was washed in ultra-pure water with cloths that had been steam sterilised in the microwave for 6 minutes - having been wetted and placed in microwaveable food grade vegetable steam bags. This was to ensure as much soil was removed from the surface of the bones as possible. The target area for drilling was marked with a fine permanent marker pen and placed on aluminium foil. The foil and bone were UV light treated for 20 minutes each side moving the bone to ensure all areas were exposed to the light in order to eliminate any surface contaminating DNA. Following the completion of the UV light treatment the bone was wrapped in the foil without touching it.

As a specific aDNA lab was not available to use, a strategy had to be employed that reduced the risk of contaminating the samples with modern contemporary DNA. A laboratory was made available that had not processed tissue or had any biological

experiments performed in it. Drilling was performed on a specially treated surface. Tests showed that treating surfaces with 2% hypochlorite (Milton) was a satisfactory way of eliminating rogue DNA (Hulme, K.; Town, N. unpublished data). The surfaces that needed treating were the Dremel drill bit and the work surface. Three sterile pots were prepared containing a solution of 2% hypochlorite, ddH_20 and absolute alcohol, the drill attachment was rinsed in each of them in turn between each use, and before and after. The work surface was cleared and given a general good clean to remove surface dirt and dust. All surfaces were then cleaned using the 2% hypochlorite, ddH_20 and abs. alcohol using a sterile cloth (UV treated then wetted with ultra-pure water and steam microwaved for 5 minutes in a food bag). Clean plastic was placed on the surface to create a disposable surface and cleaned as per the work surface using a new sterile cloth. This surface was then covered in clean paper.

The bone was brought to the prepared surface and unwrapped. The first 2mm of the bone surface removed using the Dremel drill and disposed of. The foil was replaced with a fresh sheet that had been UV treated. A piece of bone roughly 1.5 cm^3 was sawn off using a Dremel drill with a small carbon steel rotary blade, and then immediately placed into a sterile pot. The bone fragment was weighed and recorded and the bone was loosely wrapped in foil and gently crushed in a pestle and mortar. The bone was then ground to a fine powder using a liquid nitrogen freezer mill, following the instructions in the manual, and then tipped into a sterile container. The bone was used immediately or stored at -80°C until needed.

2.2.4. Bone DNA Extraction Techniques

All bone was prepared as above prior to being subjected to the DNA extraction methods. These methods were trialled in order to assess the best method of DNA extraction for the Poulton DNA.

(i). Method 1: Silica Columns:

The bone was purified using a revised protocol (Per. Comm. Louhelainen 2009). The kit used was the QIAamp DNA Micro kit (Qiagen, U.K.).

NOTE: Contact between the QI column and the flow-through should be avoided. Some centrifuge rotors vibrate during acceleration, resulting in flow-through that may

contain ethanol, coming into contact with the column. Care should be taken when removing the column and collection tube from the rotor, so that contact can be avoided.

About 100mg of powdered bone was placed into a 1.5 ml Eppendorf microcentrifuge tube and 360µl Buffer ATL, 20µl Quigen Proteinase K, were added along with 1µl carrier RNA (1µg/µl) - as long as it did not interfere with downstream applications, and incubated at 37°C overnight. Following incubation the temperature was reset to 70°C ready for further incubation. The tube was briefly centrifuged to remove droplets in the lid. 300µl Buffer AL was added, the lid secured and mixed using pulse-vortexing for 10 s, this ensured that the sample and the buffer were thoroughly mixed to yield an homogeneous solution. (A white precipitate may form, this is harmless and will dissolve during incubation). The tube was placed in the water-bath at 70°C, for 10mins, with pulse-vortexing for 10s every 3mins to improve lysis (alternatively a thermomixer or orbital incubator could be used, shaking at 900rpm for 10mins), the tube was then centrifuged at full speed (20,000x g; 14,000 rpm) for 1min. The supernatant was then transferred to a new 2ml Eppendorf tube and 100µl ETOH was added and left to stand at room temperature for 5mins, this was to help improve the binding conditions for the very small fragments, as the sample material was very old and the DNA may be degraded. This was transferred to the QIAamp MinElute column without wetting the rim, and centrifuged at 500 g for 1min to help the binding of the DNA to the column, then centrifuged at 6000 g (8000 rpm) for 1min (if the lysate has not fully passed through the column membrane then centrifuge at a higher speed until the column is empty). The column was transferred to a clean 2ml collection tube, and the old tube containing the flow-through discarded. 600 µl of Buffer AW1 was added without wetting the rim, then centrifuged at 6000 g (8000 rpm) for 1min, the collection tube containing the flow-through was discarded and the column was placed in a clean 2ml tube. 600 µl Buffer AW2 was carefully added without wetting the rim, and centrifuged as before, discarding the tube and flow-through. The column was dried by centrifuging at full speed (20,000 g, 14,000 rpm) for 3mins, as ethanol carry-over could affect downstream applications, the collection tube and flow-through were then discarded as before. The column was finally placed in a clean 1.5ml micro-centrifuge tube, and 35 µl room temperature Buffer AE applied to the centre of the membrane to ensure complete elution of the bound DNA, and left to incubate for

5mins to help increase the DNA yield, and then centrifuged at full speed for 1min. The resulting flow-through was either used immediately or stored frozen at -80°C until required.

(ii) Method 2: Phenol Chloroform:

*It is very important to ensure that a fume hood is used when working with a phenol and chloroform.

With this method there is the potential for DNA to be found in both in the supernatant and the residual bone powder, therefore both are retained, but only the bone is described here. Phenol chloroform extraction is very commonly used in degraded bone samples and involves separating the DNA into the aqueous phase.

Decalcification: 200 mg of bone powder was incubated in 1.5 ml of extraction buffer 50 mM EDTA (Ethylenediaminetetraacetic acid) buffered to pH 8.0, 10 mM TRIS buffered to pH 8.0, 100mM NaCl, 0.5% SDS and incubated for 24 hours at room temperature or gentle heat (37 °C) on a rocker under constant agitation, the tubes sealed with parafilm. It was then centrifuged at 5,000 rpm for 10 minutes. The supernatant was removed to a new sterile, labelled Eppendorf and the bone powder was retained (at this point the tubes can be stored at -80 °C if not needed immediately). 5 µl of 20 mg/ ml proteinase K, was added along with and any other components that were necessary to potentially increase the yield, such as PTB (Phenacylthiazolium bromide), DMSO (Dimethyl sulfoxide) or DTT (Dithiothreitol) and incubated for 24 hours at 37 °C, under constant agitation in a rocker. The proteinase K was deactivated by incubating at 80 °C for 5 minutes.

Following incubation 500 µl of 25:24:1 phenol chloroform isoamyl alcohol was added and agitated on a rocker for 10 minutes, followed by centrifuging at 4 °C for 10 minutes at 13,000 rpm. The top aqueous layer was decanted into a fresh sterile Eppendorf tube, being careful not to include any of the interphase or phenol (Figure 11), then 6 µl of co-precipitant and 700 ul of 95% ETOH, were added then vortexed lightly to mix and centrifuge at 4 °C for 25 minutes at 13,000 rpm. The DNA pellet could be visually identified stuck to the bottom of the tube, and removal of the supernatent would allow further washes with 70% ETOH to clean any remaining phenol, great care needed to be taken as this could risk losing DNA with each wash.

The DNA pellet was then diluted in TE buffer or ultra-pure water ready for use or was frozen at -80 °C for long-term storage.

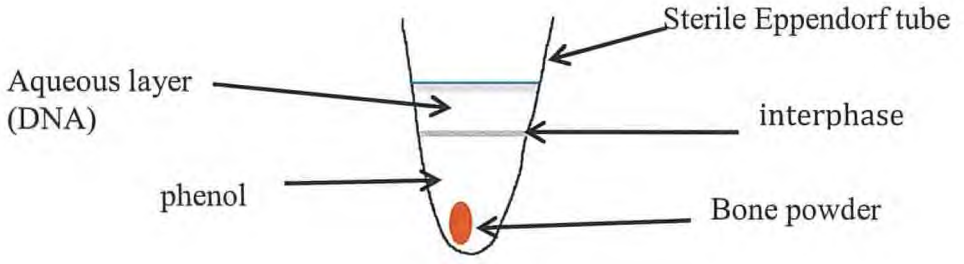


Figure 11: Phenol Chloroform treatment demonstrating the different phases during DNA extraction.

(iii) Method 3: Chelex:

Chelex is a useful technique for extracting DNA from tissue, but its effectiveness for old degraded archaeological bone has not been widely tested. It is easy to use with no complicated secondary steps, with the added benefit of chelating the contaminating polyvalent metals (Walsh et al., 1991).

200mg of bone powder was added to 500 µl of 20% Chelex made up with x1 TE buffer or TRIS in a sterile Eppendorf tube. The Chelex is made up of tiny resin granules which can settle quickly and it was advised to keep stirring gently to ensure adequate is taken up. The sealed Eppendorf tube was placed in a heat block set to 95°C for 10 minutes to separate the double strands of DNA into single strands (and hopefully cleave any bonds with contaminating ions). It was removed and cooled quickly on ice, then centrifuged for 3 minutes at 13,000 rpm to ensure the resin granules were settled well in the bottom of the tube. The supernatant was gently aliquoted off into a fresh sterile Eppendorf tube, being careful not to take up any resin granules as these are potent PCR inhibitors. It was used immediately as Chelex extracted DNA is prone to degradation in storage.

(iv) Method 4: Trizol:

This method is commonly used to prepare RNA as it uses guanadinium thiocyanate and phenol chloroform to separate the RNA in the top phase with the DNA retained in the bottom phase. As before, all work must be carried out in a fume hood.

The bone was decalcified as per the phenol chloroform method, both the bone and supernatant were retained, each being treated separately.

A 200 mg sample of bone powder/supernatant was homogenised in an Eppendorf tube with 800 µl of Trizol under vortex then incubated for 15 minutes vortexing halfway through, then centrifuged at 4 °C for 15 minutes at 13,000 rpm. In this extraction the DNA was in the lower organic phase therefore the two upper phases were removed (the RNA is retained in the aqueous phase). The DNA was precipitated in ETOH and pelleted by centrifuging at 4 °C for 20-25 minutes at 13,000 rpm.

2.2.5. Nanophotometer DNA Quantitation

The DNA concentration was quantified using Implen Nanophotometer (Munchen, Germany). The DNA peak can be visualised at a wavelength of 260 nm (Figure 12).

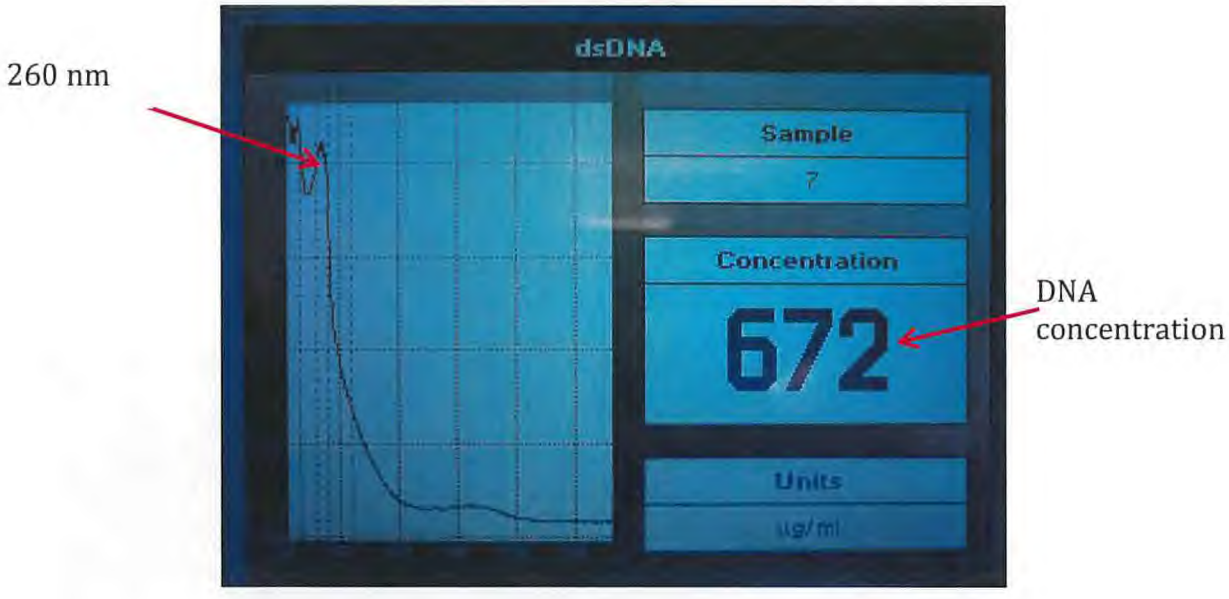


Figure 12: Nanophotometer reading, showing the DNA peak at 260 nm.

2.2.6. DNA Extraction of Recently Excavated Disarticulated Bone

(i) Method 1:

No DNA was successfully extracted from the recently excavated, disarticulated Poulton bones using the silica columns. The test demonstrated that the protocols had worked and that there was no contamination of the samples with exogenous DNA from the consumables or the operator. Whilst the method may have removed the soil inhibitors it may have also retained the small fragments of degraded DNA and a less robust method may have to be employed to try and retain as much of the DNA as possible without compromising on the removal of the soil contaminants.

(ii) Method 2:

The phenol chloroform method enabled the DNA pellet extracted from the recently excavated, disarticulated Poulton bone to be visually identified (Figure 13), and confirmed with nanophometer as:

- 116 µg/ml from the bone powder and 182 µg/ml from the supernatent for the femoral shaft.
- 62 µg/ml for the bone powder and 144 µg/ml for the supernatent from the distal humerus.
- No DNA detected in the cranium and vertebra bone samples.

The sample was diluted with 20 µl of DNA-free, ultra-pure water and stored at -80°C ready for PCR.

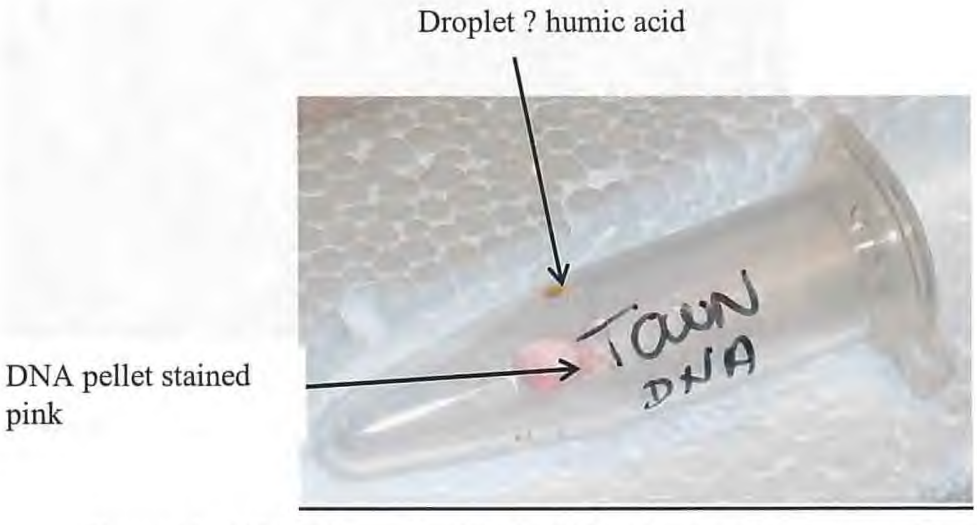


Figure 13: DNA pellet stained pink, with a droplet of co-extracted humic acid.

(iii) Method 3:

Soil contaminants may have prevented the Chelex from working, or no useable DNA was obtained from the bone samples.

(iv) Method 4:

DNA results extracted using Trizol, resulted in:

- 18 µg/ml from the distal humerus
- 80 µg/ml from the femoral shaft.

The phenol chloroform extraction method yielded the most DNA from the femoral head and distal humerus, quantified using the nanophometer. The DNA from these two bones was used for the qPCR.

2.2.7. QPCR Preparation of Extracted Bone DNA

For each reaction:

Master Mix: SYBR GREEN	10 µl
Forward primer	0.7 µl
Reverse primer	0.7 µl
DNA sample	2 µl
BSA	2 µl
ddH_2O	4.6 µl

Primers:

Oligo Name	Forward	Reverse	Amplicon Length
Amelo JL	TGACCAGCTTGGTTCTA	CARATGAGRAAACCAGG	123
16144/16400	TGACCACCTGTAGTACAT	GTCAAGGGACCCCTATCT	376/309
15971/16400	TTAACTCCACCATTAGGC ACC	GTCAAGGGACCCCTATCT	364/309
16144/16322	TGACCACCTGTAGTACAT	TGGCTTTATGTACTATGTAC	376/343
FAM 5/6	CTAGGAGATCATGTGGGT ATGATT	GCAGTGAATAAATGAACGA ATGGA	82/402
FAM 7/8	TTAATGAATTGAACAAAT GAGTGAG	GCAACTCTGGTTGTATTGTC TTCAT	29/693

The programme used was set at Stage 1 - 50 °C for 2 minutes 1 repeat

Stage 2 - 95 °C for 10 minutes 1 repeat

Stage 3 - 95 °C for 0.15 minutes 40 repeats, then 60 °C for 1 minute

Stage 4 – dissociation 95 °C for 0.15 minutes, 60 °C for 1 minute, 95 °C for 0.15 minutes, 60 °C for 0.15 minutes

2.2.8. Inhibitor Test

Quantification was performed using real time qPCR and a dilution series of the flow-through from the stored bone sample was used: 1:5, 1:25 and 1:100, positive and negative controls were included. 1µl of contemporary modern DNA was added to each test sample. The qPCR was set for 45 cycles. This was repeated for the 3 chosen primers. The results of the qPCR showed that there was no inhibition of the modern contemporary DNA and that Method 1(Silica columns) had effectively removed the soil inhibitors.

Oligoname	Forward	Reverse	Amplicon Length
MT1	TGATTTCACGGAGGATG	AACCCCCTCCCCATGCTT	440
KH1	AAATCTGGCACCACACCTTC	CCACTCACCTGGGTCATCTT	121
AMELO JL	TGACCAGCTTGGTTCTA	CARATGAGRAAACCAGG	123

2.3. Results

2.3.1. (i) Soil Spiking Test

Using both Pfaffl's qPCR efficiency calculation and the online qPCR calculator (Thermo Fisher Scientific) from materials and methods 2.2.1(b), the efficiency (E) of the qPCR reactions could be analysed. The mean expected values were derived from positive controls where each reaction was spiked with ultra-pure water instead of soil extraction. This enabled each reaction in dilution to be compared to the expected value to assess the degree of inhibition.

Primer BW1: The mean expected Ct values for primer BW1= 16.83 (std err = 0.015) (derived from positive controls).

(i) Cemetery soils:

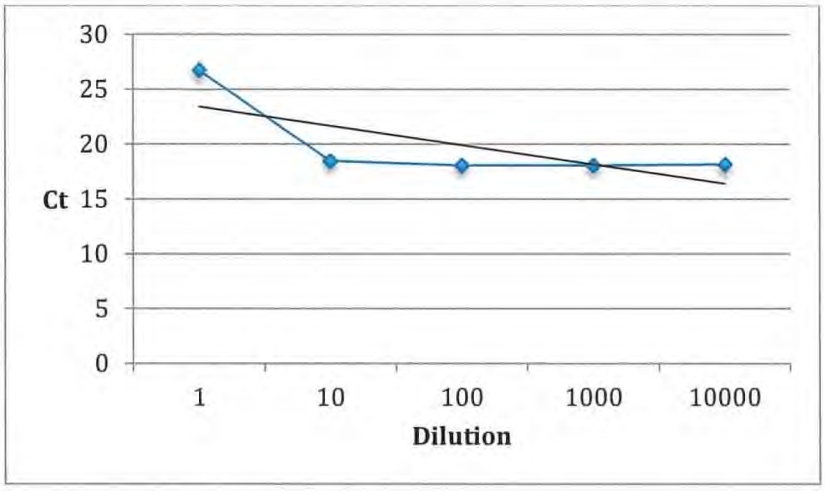


Figure 14: Standard curve and efficiency for Poulton medieval cemetery Soil 1, where slope = -0.0003, Pfaffl (E) = 39296; Thermo Scientific (E) = infinity; R^2 = 0.08.

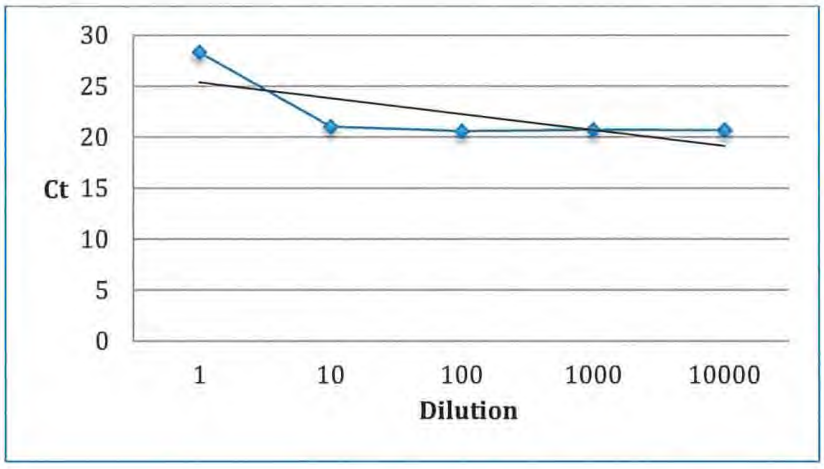


Figure 15: Standard curve and efficiency for Poulton medieval cemetery Soil 2, where the slope = 0.0002; Pfaffl (E) = 43663.8; Thermo Scientific (E) = infinity; $R^2 = 0.09$.

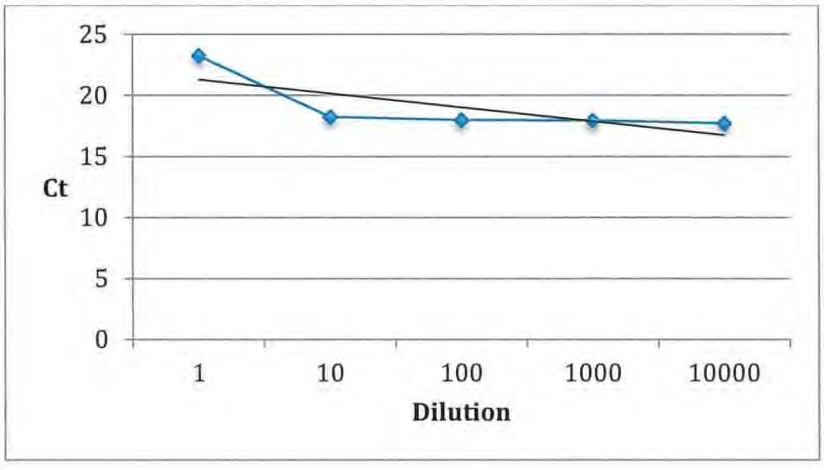


Figure 16: Standard curve and efficiency for Poulton medieval cemetery soil 3, where the slope = -0.0002; Pfaffl (E) = 52996; Thermo Scientific (E) = infinity; $R^2 = 0.12$.

Each of the Poulton cemetery soils (Figures 14-16) have a slope that is near to 0, a negative standard curve, infinite (undetermined) efficiencies, and highly negative regressions. There appears to be only a small amount of inhibition of the PCR even at lower dilutions. The greatest difference in the Ct values was at the 1/1 dilution compared to the expected levels, whilst the rest of the dilutions showed stabilisation. Soil 2 had the highest Ct values, with the greatest inhibition of the PCR, compared to the expected Ct value 16.83.

(ii) Wormery elution:

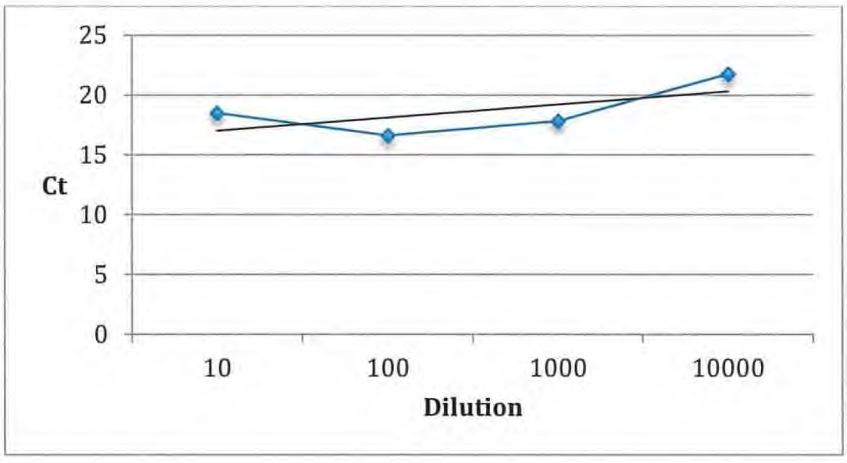


Figure 17: Standard curve and efficiency for wormery elution, where the slope = 0.0004; Pfaffl (E) = -23536.6; Thermo Scientific (E) = infinity; $R^2 = 0.87$.

The elution from the wormery (Figure 17) resulted in a slope of nearly 0, a slight positive standard curve, an infinite (undetermined) efficiency, and a positive regression. The result demonstrated that there was a slight increase in the amount of cycles with increasing dilution, this may have been due to initial inhibition of the PCR (the difference between cycle numbers is slight in 1/10, 1/100, and 1/1000) with an increase in replication of the target molecules at 1/10,000. Compared to the expected Ct value of 16.83, at 1/1 dilution the Ct was undetermined and may have affected the slope.

(iii) Control soils:

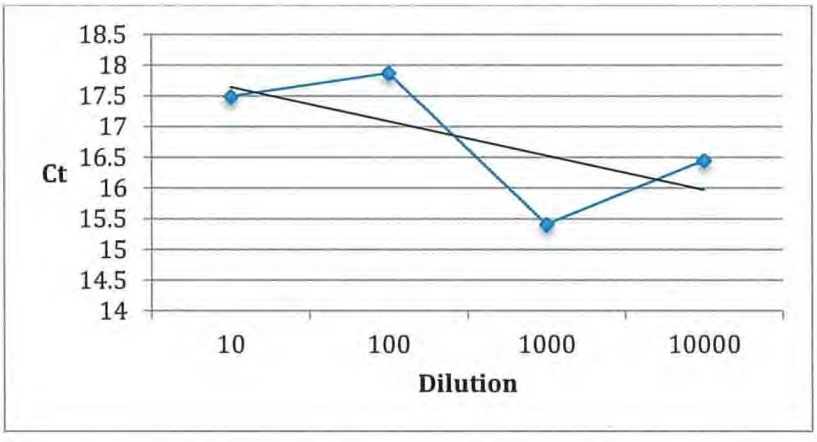


Figure 18: Standard curve and efficiency for Poulton medieval cemetery Control Soil 1, where the slope = -6.887; Pfaffl (E) = 1.45; Thermo Scientific (E) = 39.7%; $R^2 = 0.09$.

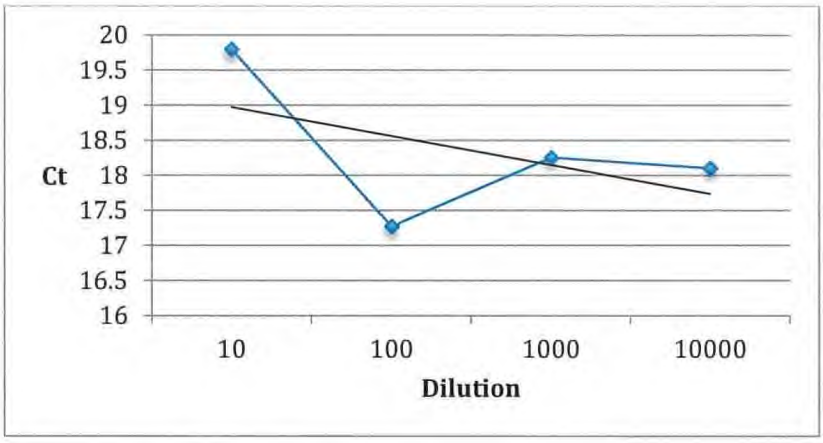


Figure 19: Standard curve and efficiency for Poulton medieval cemetery control 2, where the slope = -3.92; Pfaffl (E) = 2.55; Themo Scientific (E) = 79.8%; R^2 0.03.

The Poulton control soils had negative standard curves and regression (Figures 18 & 19). The efficiency was able to be determined using the standard calculation methods but were still less than optimum. The Ct values were close to the expected values (16.83) at greater dilutions, but at 1/1 dilution the Ct value was undetermined, which may have affected the slope.

Primer KH3: Mean expected Ct values = 29.29 (std err 0.043)

(i) Cemetery soils:

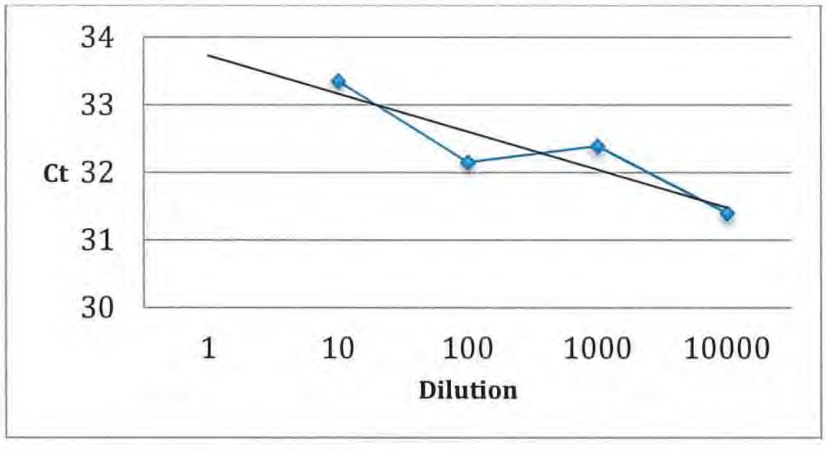


Figure 20: Standard curve and efficiency for Poulton medieval cemetery soil 1, where the slope = -32.685; Pfaffl (E) = 76548; Thermo Scientific (E) = infinity; $R^2 = 0.62$.

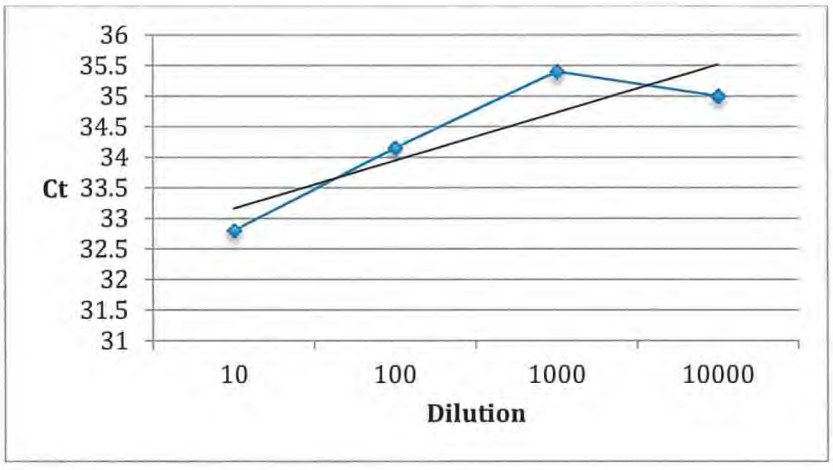


Figure 21: Standard curve and efficiency for Poulton medieval cemetery soil 2, where the slope = -34.03; Pfaffl (E) = -91661.6; Thermo Scientific (E) = infinity; $R^2 = 0.21$.

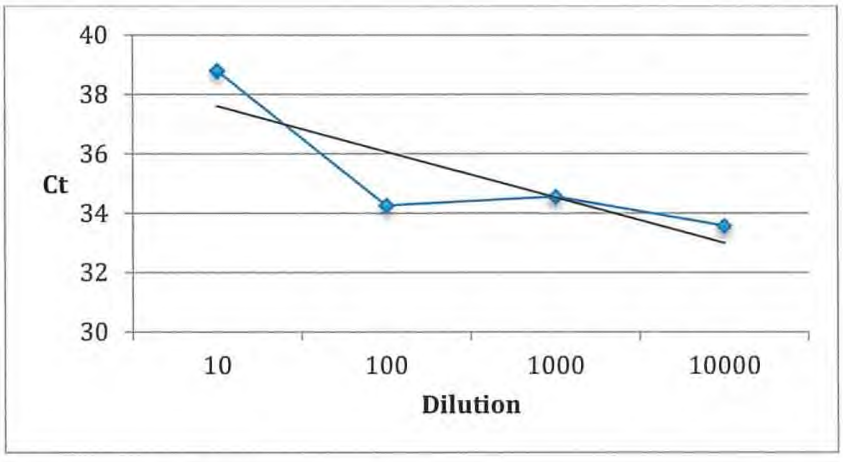


Figure 22: Standard curve and efficiency for Poulton medieval cemetery soil 3, where the slope = -0.0003; Pfaffl (E) = 38954; Thermo Scientific (E) = infinity; $R^2 = 0.27$.

The cemetery soils 1 and 3 demonstrate negative curves (Figures 20 & 22), whilst cemetery soil 2 demonstrated a positive curve (Figure 21). The slopes were nearly 0. All of the soil samples had high Ct values compared to the expected values (29.29), commonly seen in contaminated samples where the PCR struggles to replicate efficiently, or low loading doses of DNA. The Ct value was undetermined at 1/1 concentration in the 3 cemetery soil samples, and this may have affected the slope. The efficiency could not be calculated using the standard calculations, with a very large Pfaffl's value and was undetermined using the Thermo Scientific calculator.

(ii) Wormery elution:

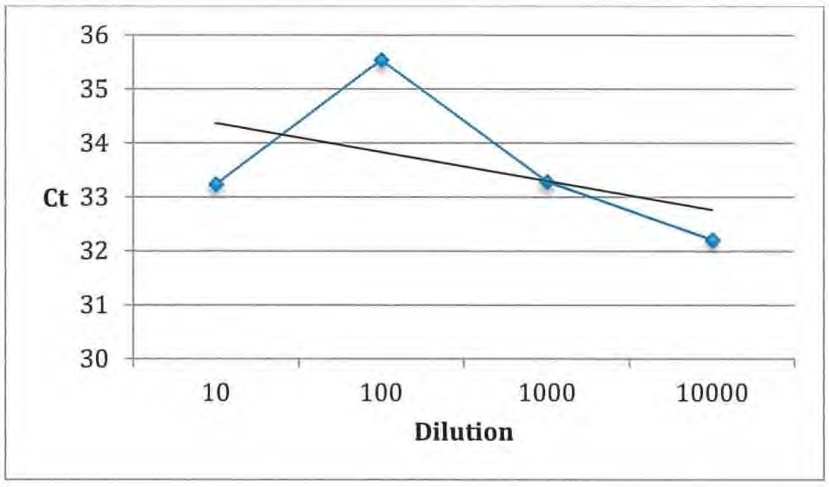


Figure 23: Standard curve and efficiency for the wormery elution, where the slope = -0.0002; Pfaffl (E) = 51165.7; Thermo Scientific (E) = infinity; $R^2 = 0.13$.

The wormery elution demonstrated a slope of nearly 0, very small negative standard curve. There was very little change in the Ct value with dilution, but they were greater than the expected values (29.29) at higher concentrations. The Ct value was undetermined at 1/1. The efficiency was not able to be determined using the standard techniques.

(iii) Control soils:

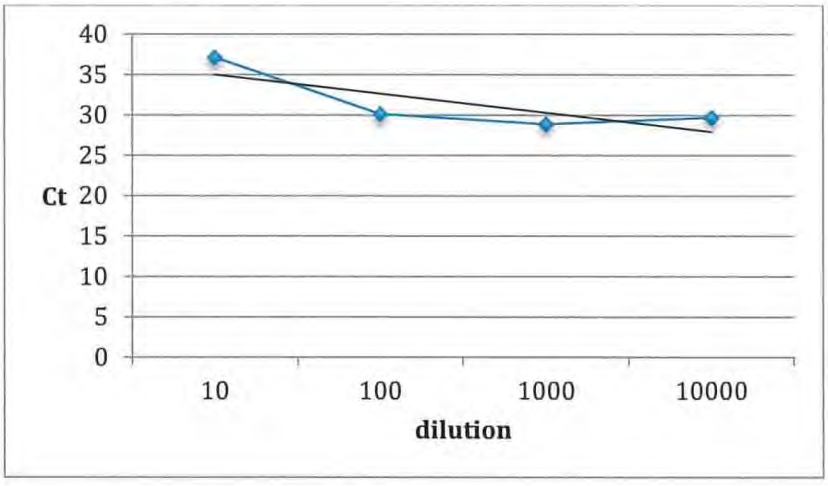


Figure 24: Standard curve and efficiency for Poulton soil control 1, where the slope = -0.0003; Pfaffl (E) = 34958.4; Thermo Scientific (E) = infinity; R^2 0.13.

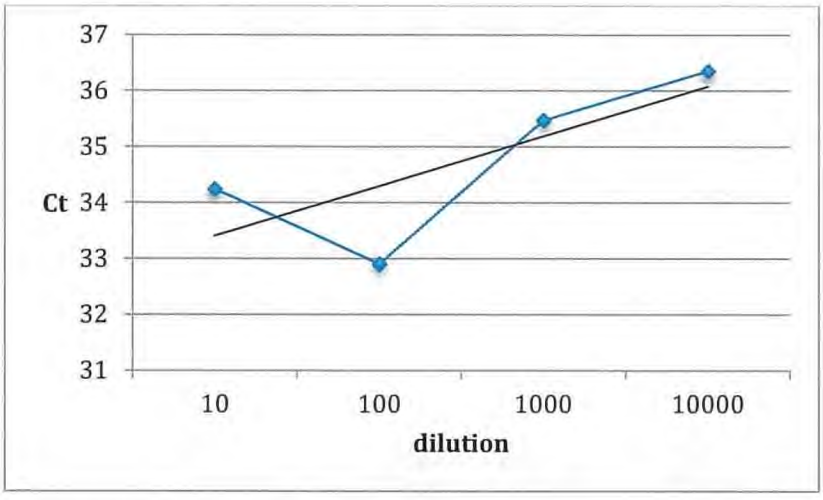
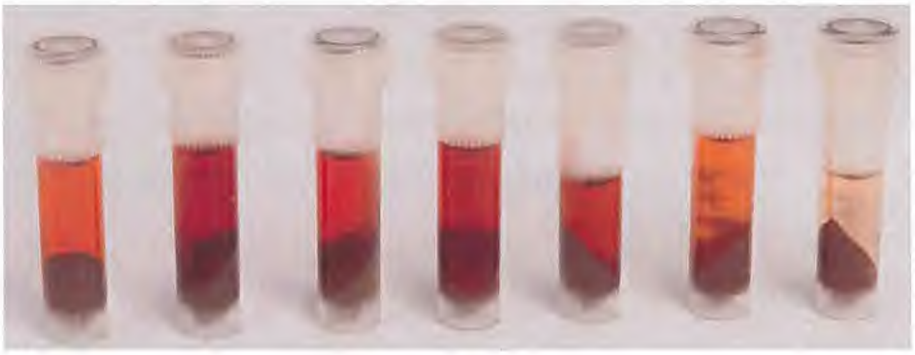


Figure 25: Standard curve and efficiency for Poulton soil control 2, where the slope = 0.0002; Pfaffl (E) = -42156; Thermo Scientific (E) = infinity; $R^2 = 0.58$.

The control soils had slopes of nearly 0, soil control 1 (Figure 24) had a slight negative curve, soil control 2 (Figure 25) had a slight positive curve. The Ct values could not be determined at 1/1 concentration. Soil control 1 had Ct values that met expected values (29.29) at >100/1 dilution. Soil control 2 had higher than expected Ct values compared to the expected levels.

2.3.1. (ii) Humic Acid Nanophotometry of Soils

The humic acid concentration of the soils using nanophotometry, was not related to the visible colour of the supernatant due, in part, to the influence of other elements such as Fe that contribute to the soil's colour (Figure 26 & 27). The attempt to use visual identification of humic acid concentration is not a reliable method. Spectrophotometric analysis was trialled, but was unsuccessful in obtaining any useable data.



1956 2089 1958 N/A N/A 1942 N/A

C1 C2 C3 N/A N/A

Figure 26: Cemetery (numbers denote grave context numbers) and control soils (C) used for nanophotometry.

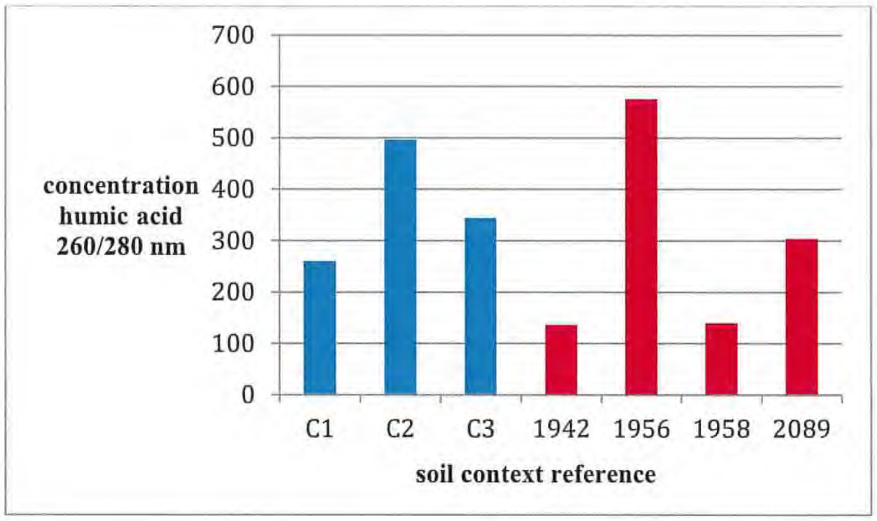


Figure 27: The concentration of humic acid from Poulton Medieval cemetery and control soils at 260/280 nm using nanophotometry.

2.3.1. (iii) QPCR DNA Amplification

Figure 28 shows the dissociation curves for the qPCR reactions using the humerus and the femoral bone powder. The qPCR proved no amplifiable DNA present in either the humerus or the femur using any of the primer sets, this would have been seen as defined peaks at >80°C (black vertical line), but peaks under 80°C can be seen where the formation of primer-dimers occurred due to there being no useable target DNA available. There appeared to be a very small amount of contaminating contemporary DNA in two of the negative controls, this was not deemed significant as it was very small and appeared at cycle 38 out of 45 cycles.

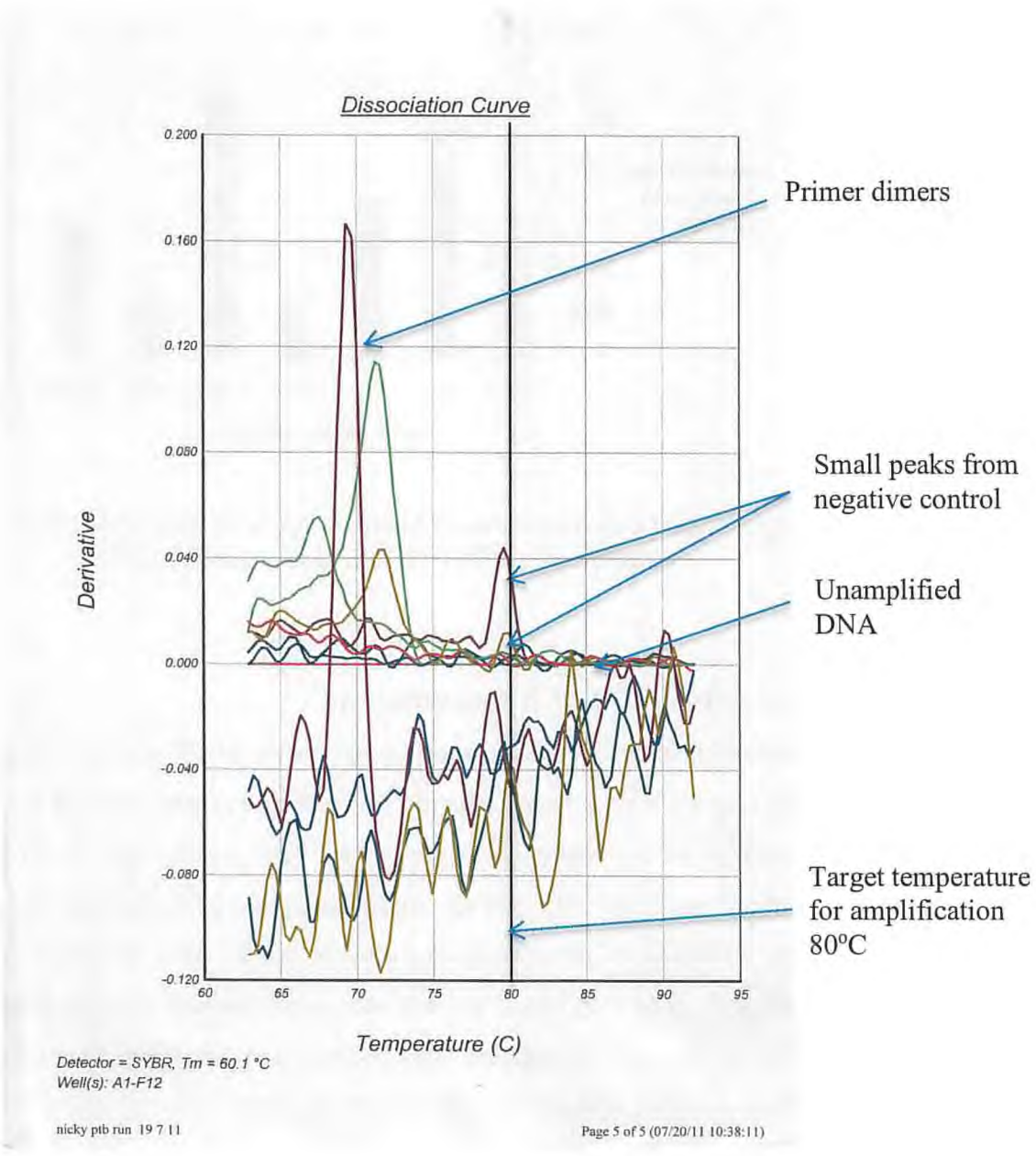


Figure 28: Dissociation curve for the bone extracted DNA. Each colour represents a reaction sequence.

2.4. Discussion

Humic acids (HA) have been considered to be a potent inhibitor of the PCR, and their removal vital to the success of DNA amplification of soil interred bone. These large aromatic compounds have been identified as a brown colouration in the bone decalcification buffer, commonly EDTA. The EDTA and humic acids both share a great affinity for metals, but the high concentration of the EDTA compared to the humic acid allows the humic acids to precipitate into solution, colouring the supernatant brown. This can be seen in the EDTA treated soil intruded bone samples (Figure 25), the cemetery and control soil samples (Figures 26 & 27). The spongy trabecular bone appeared to contain more soil, as the open texture acted as pockets to collect and retain the soil components, and as a result the supernatant appeared slightly darker and more concentrated than the cortical bone supernatent. Using visual quantification of the humic acids in EDTA treated bone or soil is an not accurate method, supernatant that may appear darker and have more humic acids may prove not to be the case. The presence of iron from the Till and Sandstone can give a dark red hue to the supernatant, giving a misleading indication of the level of humic acids. When comparing the cemetery and control soil sample supernatant colours (Figure 26) to the nanophotometer results (Figure 27) it soon becomes clear that visual assessment of the humic acid content is not useful. Grave context soils 1942 and 1958 contain the lowest concentration of humic acids (136 and 140 μg/ml, respectively) but they were very different in colour, grave context 1942 being much lighter in colour than grave context soil 1958. Grave context soil 1956 contained the highest concentration of humic acid (576 μg/ml) but was similar in colour to grave context 1958. Grave context soil 2089 was darkest in colour but contained only half the concentration of humic acids (304 μg/ml) compared to grave context 1956. The use of the nanophotometer demonstrated an effective method of assessing the humic acid concentration of the supernatant of the soils, however this method would be less useful for assessing the humic acid concentration of DNA extractions due to the DNA occupying the same wavelength (260/280 nm). By taking a sample of the soil from next to the bone, during excavation, it would be possible to assess the concentration of humic acids that the bone may be vulnerable to and use this to plan the decalcification and extraction buffers that may be most suited to maximising the likelihood of a successful DNA extraction and amplification.

The extraction buffer components such as EDTA, SDS, and TRIS can be potent PCR inhibitors (Gallup and Ackermann, 2006), and therefore the brown supernatant extracted from treatment with EDTA extraction buffer could not be used to inoculate the DNA for the spiking test. It was hoped inoculation of the PCR with the dissolved components, using purified water, in a series dilution using sensitive house-keeping genes could demonstrate the effects that these soil components had on the PCR. In order to fully evaluate this effect on the PCR it was necessary to calculate the efficiency of the series dilution. In most PCR reactions gene expression is the target, not the rather arbitrary inhibiting effect of contaminants. During a normal gene expression dilution series it would be expected that it would take a linear increase in the amount of cycles to reach a maximum amount of DNA molecules with increasing dilution. With PCR inhibitory contaminants, the opposite would be expected, as the effect of the contaminants was reduced with each dilution, it would be expected to see a reduction in the cycles needed to reach a maximum amplification of the DNA molecules. With the primer BW1 cemetery soil samples (Figures 14-16), the higher cycles can be seen at 1/1 concentration with some inhibition of the PCR being seen, but this cannot be seen in the rest of the dilutions. The dissolved components appear to have very little inhibitory effects on the PCR in these samples, given the fact that they contained varying concentrations of humic acids (Soil 1 = context 1958; soil 2 = context 1956; soil 3 = context 1942). The effects of the wormery elution (Figure 17) on the PCR gave a result that was hard to explain, with very little change in the amount of cycles needed to maximize PCR amplification, given that this was a compound that contained no other soil components, only the plant derived humic acids. Nanophotometry of the wormery elution would enable the humic acid content to be quantified, and may shed some light on the outcome of the PCR. The control soil samples allowed the PCR efficiencies to be calculated unlike the cemetery soils and the wormery elution, although neither were optimum amplifications. The humic acid concentrations of the control soils 1 and 2 (Figures 18 & 19) contained concentrations similar to the cemetery soils, with lower PCR cycles at dilution, demonstrating the lack of inhibitory effects of the dissolved components. With Primer KH3 the dissolved cemetery soil components resulted in the 1/1 being undetermined (>40 cycles) as the PCR ultimately struggled to progress, at greater dilution the effects became more apparent, with soil 1 (Figure 20) being low in humic acids, demonstrated a slight decrease in the cycles (33-31), this was not very different to the expected 29 cycles.

Cemetery soil 3 (Figure 22) also being low in HA, also demonstrated an undetermined PCR (>40 cycles) at 1/1 concentration along with a slight decrease in number of cycles with greater dilution (34-38), although the PCR just about managed to proceed with overall higher cycling rates. Soil 2 (Figure 21) being higher in humic acids, also had undetermined (>40 cycles) at 1/1 concentration, but demonstrated only a slight increase in the number of cycles at greater dilution (33-35). The wormery elution containing humic acids demonstrated the same trend as the cemetery soils with undetermined cycling rate at 1/1 concentration, slight decrease in the cycling rates (35-32) with a spike at 1/100 dilution (Figure 23). The control soil samples (Figures 24 &25) demonstrated conflicting results, whilst both suffered an undetermined PCR (>40 cycles) at 1/1 concentration, soil control one (C1) demonstrated some inhibition with increased cycling at 1/10 dilution (38 cycles), this plateaued out at higher dilution with cycling similar to expected levels (30 cycles). Soil control 2 (C2) demonstrated a pattern similar to cemetery soil 2 with little difference between the cycle rates with dilutions, but demonstrated relatively high cycle rates (33-36). By using a positive control it was possible to generate an expected cycle rate for each primer BW1 = 16.83, KH3 = 29.29, this was taking into consideration the effects of the reaction without the soil contaminants. One explanation for this could be that some DNA primers are more resistant to the effects of humic acids and the dissolved soil contaminants than others. By knowing the PCR cycle expected rate, comparisons to the dilutions can be made. By using this premise it was possible to demonstrate that BW1 showed more resistance to the effects of spiking than KH3, particularly at higher concentrations.

The use of water-dissolved components may not have made all of the soil components available to the DNA spiking test as they remained chemically bound to the soil components such as clay, or humic particles, and were not released into the water solution. By improving the humic acid and other soil component extraction techniques that do not involve the use of PCR inhibiting chemicals such as EDTA, then the full impact of the soil components may be able to be explored. The EDTA may be able to be precipitated out by altering the buffer solution pH. The PCR reactions contained very little inhibition at greater dilutions, which ultimately affected the efficiency calculations; this lack of linear amplification was due to the slope results being near to 0, rather than the required 3/-3. The role of PCR inhibition is very important for the

continued study of ancient DNA studies and whilst progress has been made in the assessment of soil component PCR inhibition, focus remains on gene expression studies, rather than soil contaminated DNA (Gallup and Ackermann, 2006). The use of EtBr gel fluorescence remains the preferred method of visually assessing the amplification of soil contaminated and degraded DNA (Higuchi et al 1993), and whilst this has its uses, it must be remembered that humic acids also fluoresce with EtBr, which could skew the results and give a false positive enhanced result.

There are effective methods for the removal of humic acids, following decalcification, but they risk losing valuable DNA either in the discarded supernatant or chemically bound up with the extracted humic acids (Tebbe and Vahjen, 1993; Tsai and Olsen, 1992; Wang et al., 2009; Zhou et al., 1996; Kalmar et al., 2000; Kim et al., 2008; Loreille et al., 2007; Sutlovic et al., 2007; Yang et al., 1998). The loss of DNA can be an acceptable risk if there is DNA of good quality even in very small quantities. The four extraction techniques used yielded DNA in different quantities, the silica columns and chelex resin were used to effectively remove the humic acids, but they were too indiscriminate and whilst they were very effective at binding the humic acids, they also held the DNA onto the membrane or granule surface. This may indicate that the DNA strands were very small and probably too degraded to amplify successfully. The Trizol successfully extracted 18 ug/ml from the humerus and 80 ug/ml from the femoral shaft. Trizol is routinely used for the extraction of RNA from tissue samples, and its use in the extraction of DNA from bone may not have removed the all the impurities, resulting in the co-extraction of the soil components, metals, and humic acids, even in small amounts. There was also a substantial difference in the quantity of DNA extracted from the bones with the femoral shaft yielding more than four times that of the humerus. The phenol-chloroform method resulted in the best DNA extraction rate of the four methods used with nearly 50% more being extracted from the femoral shaft supernatant (182 µg/ml) than from the bone powder (116 µg/ml). The humerus extraction yielded more than twice the amount from the supernatant (144 µg/ml) than the bone powder (62 µg/ml). As the supernatant contains the humic acids and other co-extracted soil components, as well as the DNA strands, it is difficult to assess how much of the DNA is chemically bound to these impurities as they occupy the same nanophotometer wavelength.

The qPCR amplification resulted in no useable DNA being detected in the studied bone samples from Chapel House Farm cemetery (Figure 28). There was some contamination of the negative controls but this was only small and at higher cycling, the use of positive controls (to indicate that the reactions were otherwise sound) was not advised due to the risk of cross-contamination. The cleaning and sterilising protocols devised in the absence of a "clean" laboratory worked well with the minimum level of contamination observed. The DNA that was able to be extracted could have come from a variety of sources given the level of soil intrusion, it was not possible at this stage, to determine if the extracted DNA was from soil derived microorganisms, plant or animal material, or human in origin.

2.5. Conclusion and Further Work

The improvement in extraction of the soil components could enable the further understanding of how these components interact with contemporary DNA. Further identification of the soil components into their unique families would also help to understand the impact that each component has on the DNA as well as their combined effect. Further analysis of the rate of inhibition of the qPCR, rather than amplification of the DNA would make great strides in quantifying the PCR inhibition rather than using the subjective, and sometimes inaccurate, skills of EtBr gel fluorescence.

The type of bone used for DNA extraction in this study was a very important consideration, with the femoral shaft yielding more DNA than the humerus. The cranium and vertebral extractions did not yield any DNA. All the bones used came from disarticulated fractured bones from disturbed previous interments, and were not considered important for other studies and could therefore be presented for trial DNA analysis. The use of only one bone of each type restricted the full analysis of each bone type, but it was not possible to extend the study without being able to provide evidence of the usefulness of further bone destruction. As these bones were not the best in the collection, it may have had a significant bearing on the lack of DNA yield and PCR amplification, and further study using the best quality, recently excavated bones/teeth, removed under aseptic conditions may improve this situation, as well as increasing the number of bones used. Taking a small sample of soil directly next to the

femoral bone at the site during excavation would enable further accurate soil and bone DNA analysis.

Optimizing the DNA extraction to release as much DNA that may be chemically bound could be enhanced by employing soil analysis, as the parent material and soil components can vary widely at each cemetery. Knowledge of the impact of these soil components at the bone surface may allow improve the extraction efficiency. The interaction between clay, metals and humic compounds with bone DNA has been under explored, as has the efficiency of using iron and other metal tolerant polymerases.

Chapter 3. Chapel-House Farm: Understanding the Relationships Between Bone Preservation and Burial Environment.

Objectives

The objectives of this study were to:

- Analyse the preserved bone to assess the potential intrusion of soil components that may help to explain bone condition, as well as soil contamination status for DNA studies using non-destructive X-ray fluorescence (XRF).
- Analyse the soil from both inside and outside the Medieval cemetery to assess the soil components and the rate of mobility of bone components in the soil using XRF and soil pH.
- Use light microscope observations to assess the degree of anthropogenic activities as well as the type of parent material of the cemetery soil to explain the bone condition.

3.1. Introduction

Bone and tissue preservation is of considerable interest to those involved with archaeological bone studies. Little work has been done to assess the quality of bone condition and contamination prior to analysis, until problems arise. Contamination of bone with soil products has been noted to cause problems with C^{14} dating and DNA amplification technology (Gillespie, 1989; Hedges and Klinken, 1992; Kalmar et al., 2000; Tuross, 1994). The treatment of the bone pre and post mortem, along with the interaction with its environment can be represented by the preservation and condition of the bone, but the principles behind bone preservation are poorly understood.

Human bones from the Medieval cemetery located at Chapel House Farm, Poulton, Cheshire, UK were chosen for study due to their general good state of preservation; however some skeletal remains in the graveyard were discovered in a poor condition without an obvious environmental explanation.

3.1.1. Bone Physiology

Bone develops from either endochondrial or intramembraneous ossification (Gilbert, 2003). This development begins in the embryo and continues into maturity. Live human bone consists of a complex mineral and organic composite material that gives both structural as well as tensile strength (Burr, 2002; Currey, 2002; Collins et al.,

1995; Trueman and Martill, 2002), (Figure 1). Normal healthy bone consists of approximately (by weight) 70% mineral (50% volume), 20% organic collagens (36-50% volume) and 10% water (Collins et al., 2002; Pate and Hutton, 1988; Reiche et al., 1999). The function of bone moves beyond just providing structural support for the muscular system, it also provides protection for delicate organs such as the lungs and brain, and the marrow contained in the medullary canals of long bones produces red and white blood cells vital for the healthy function of the cardio-vascular and immunological systems. It can also function as a reservoir for mineral calcium and phosphorus involved in metabolic processes (Weiner and Wagner, 1998).

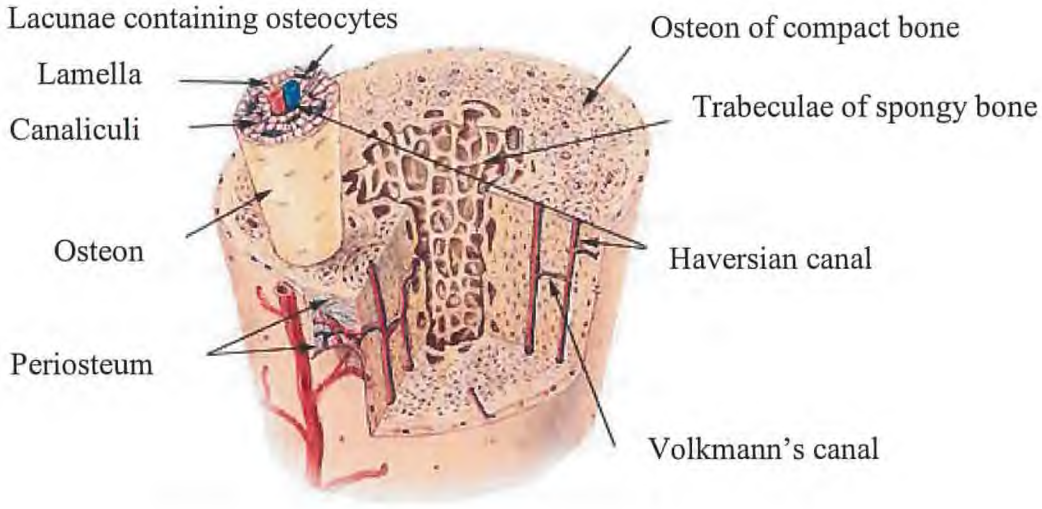


Figure 1: Structure of normal healthy bone (National Cancer institute ©Seers Training Group).

Bone falls into two main types: cortical or cancellous. The tough solid cortical (compact) bone lies in direct contrast to the open textured cancellous (spongy/ trabecular) bone. The external surface is covered by the protective periosteum, a tough, thin, fibrous tissue that has the appearance of parchment when dried. Bone components are highly hierarchical in structure in order for it to maintain its structural integrity (Tzaphlidou, 2008). The osteons of compact bone lie in longitudinal fibres supported by a crystalline mineral matrix. The mineral component of bone primarily consists of the apatite group, calcium phosphate with substitutions of chlorine, fluorine and hydroxyl channel filling ions; apatites also commonly exist within the normal soil geology (Currey, 2002; Wopenka and Pasteris, 2005). The chemical formula for bone

apatite is usually written as Ca_{10} $(PO_4)_6(OH)_2$, assuming here that there is an hydroxyl (OH) component. The rate at which ionic substitution takes place during the life of the organism would depend on the ingested minerals in the available sources of water, and in the case of dead bone in the surrounding water and sediments (Trueman and Tuross, 2002). The dynamic mineral component undergoes regular remodelling throughout the healthy individual's life, through a highly vascularised blood flow system via the Haversian and Volkmann's canals, with the mature trabecular bone undergoing a 32% (percentage mass Ca annually) turnover rate compared to 4.3% for cortical bone (Carvalho et al., 2004).

It is thought that the non-collagenous proteins including the phosphoproteins, osteopontin, sialoprotein, osteonectin and osteocalcin, that form part of the organic component, may function as part of the regulatory system of the mineral part of bone (Gillespie, 1989; Jae-Young et al., 1998; Triffitt, 1987). It is thought that osteocalcin is responsible for the binding of the hydroxyapatite to collagen (Turner-Walker, 2008).

The organic component of live bone consists of Type 1 collagen and proteins. Collagen is a complex organic polymer, comprising of long chain alpha and short chain beta chains that form a triple helix structure; the precise chemistry varies depending on the form and function. There are many types of collagen, the most common type to be found in bone is Type I, and is arranged into long fibrils that are protected by a network of apatite plates (mineralisation) in a trimeric form (Collins et al., 1995; Turner-Walker, 2008). In fibres the collagen polymer is thought to be thermally stabilised by the "polymer in box" theory, in that the collagen polymer is constrained by the adjacent molecules (Miles and Ghelashvili, 1999). As collagen forms the organic part of the bone matrix and is responsible for giving bone its tensile strength, heating and bone collagen extraction methods change its form, making investigation of its properties more challenging (Nielsen-Marsh et al., 2000).

3.1.2. Bone and Soil Component Interactions

The soil-interred corpse's soft and hard tissues decompose depending on the temperature and hydrology of the environment (Collins et al., 2002; Nielsen-Marsh et al., 2006; Turner-Walker, 2008). In extremes of temperature the preservation of soft tissues can be increased due to either permanent mummification due to dehydration

(Galloway et al., 1989) or freezing in the permafrost (Willerslev et al., 2004; Schwarz et al., 2009). This condition is usually expressed as the "thermal age" (Collins et al., 2002), some bones can be thermally young compared to their chronological age, such as those frozen in permafrost whilst others such as recently cremated remains may be thermally much older than their chronological age. On some occasions the bone degraded and dissolved completely leaving only a silhouette in the soil to denote the presence of an interment (Beard et al., 2000). Under temperate conditions the soft tissues degraded leaving the hard bone and teeth exposed to the environment and these in turn can undergo a series of changes depending on the environmental conditions; including soil parent material, pH, rate and frequency of water table incursion into the grave, high environmental temperatures, action of microorganisms, scavenging animals, surface weathering and plough damage. The depth of burial can be vital for preservation, as the soil can provide a protective and stable environment, due to the effects of temperature variation being buffered between 25-50 cm deep in the soil (Florides and Kalogirou, 2004; King et al 2014). Shallow burials located near to the surface will not only be exposed to the effects of the temperature and weather variation but also may lie in close contact with the most dynamic part of the soil horizon containing the humic acids.

The age and health of the buried individual can also affect the preservation, with juvenile bone being less able to withstand adverse environmental conditions than adults (Gordon and Buikstra, 1981). Favourable conditions for bone preservation such as those from Chapel House Farm would have to include several important factors to buffer the negative effects of the burial environment; the main pathways for bone degradation include the chemical degradation of the bone mineral, collagen, and the effects of microorganisms (Collins et al., 2002).

Bone mineral that is in contact with the soil water environment can be preserved by the buffering nature of localised dissolution of the hydroxyapatite structure and clogging of the vascular pores in the bone by very small soil components such as clays (providing the water flow is limited), as the hydraulic action of repeated wetting and drying cycles can be particularly destructive (Hedges and Millard, 1995a; Nielsen-Marsh and Hedges, 2000a; Turner-Walker, 2008). Bone that remains in a permanent pH neutral/alkaline water-logged environment can demonstrate better preservation

than those that do not (Forbes et al., 2004; Gordon and Buikstra, 1981). Water of neutral or slightly alkaline pH (>pH6) is also vital to prevent dissolution of the hydroxyapatite structure, however, drier alkali environments can be detrimental through the formation of calcite crystals and hydrolysis of the collagen, conversely wet acidic environments can cause leaching of the mineral components of bone through the formation of water soluble brushite (Collins et al., 1995; Gordon and Buikstra, 1981; Piepenbrink, 1989). The soil parent material can play an important role in the preservation of bone, with a neutral pH, along with small particle size clay or sediment soil with limited water movement demonstrating much improved preservation than the wet acidic open textured sandy soils (Crowther, 2002). The presence of well mineralised bone, even when it has been in contact with the soil environment can still exhibit most of the properties of intact bone, this preservation has been quantified using several methods: porosity, histology, crystallinity, carbonate and calcite content (Gutierrez, 2001; Hedges and Millard, 1995b; Smith et al., 2005). The preserved mineral component of soil interred bone can also be assessed using the birefringence properties of bone under polarised light microscopy (Piepenbrink, 1986; Piepenbrink, 1989; Turner-Walker, 2008), allowing the bone structure and integrity to be seen directly, in order to define good or poor histological preservation of a sample. Good histological preservation however, is not an indication that the bone would have good collagen or DNA preservation, as bones in good histological condition can suffer degradation of the collagen and DNA especially if stored at room or fluctuating temperatures for long lengths of time following excavation (Hummel, 2003; Pruvost et al., 2007). Applying quantitative rather than qualitative values to bone preservation provides a better overall perspective especially when applied to each aspect of bone assessment, whether that is histological, DNA or collagen studies (Hedges and Millard, 1995b; Nielsen-Marsh and Hedges, 1999; Paabo et al., 2004).

The movement between soil and bone components is dynamic and complex, with hydrology being the core principle at work through dissolution, precipitation, mineral replacement, recrystallization and ionic substitution (Hedges and Millard, 1995a; Nielsen-Marsh and Hedges, 2000a; Turner-Walker, 2008); and these processes will themselves be under the influence of soil pH, parent material and temperature (Janos et al., 2011; Pate and Hutton, 1988). The type of soil that bone is interred will have a direct effect on its preservation, as the bone is entirely dependent on its environment

for survival. It is becoming increasingly important to conduct studies of the soil environment of skeletal remains in order to fully understand not only the preservation mechanisms, but the potential soil intrusion into the bone material for analytical technologies such as C^{14} dating, collagen and DNA studies, to ensure accuracy and clarity of results. Soil analysis prior to excavation may be able to predict the outcome of well-preserved skeletal remains being found at a location, or if extra care may need to be taken during excavation of delicate remains. Acidic soils tend to have increased bone and soil ionic exchange mobility, especially under the effects of free draining soil and hydrology (Jans et al., 2002; Nielsen-Marsh et al., 2007). Bone can sequester metals during the lifetime of the living individual or through soil contamination during the time spent in the burial environment (Budd et al., 2000; Hu et al., 1998; Lambert et al., 1985a; Muller et al., 2011; Pike and Richards, 2002; Rabinowitz, 1991; Reiche et al., 1999). The rate at which ionic movement between the bone and the soil occurs is fundamentally under the influence of hydrology as this is the primary agent by which most endogenous and exogenous elements move (Hedges and Millard, 1995a). Water moves by either precipitation percolating downwards through the soil horizons or by moving upwards via the ground water table, either as a permanent or temporary feature (Forbes et al., 2004; Gordon and Buikstra, 1981; Hedges and Millard, 1995a). The ionic exchange of elements will depend on the total ions available, and this in turn will also have to include other factors such as the condition of the skeletal remains at the time of the interment and environmental factors. Interred remains from infants, the elderly, or those in chronic poor health may be prone to accelerated rates of decomposition and leaching of bone components, increasing the elemental ionic availability (Gordon and Buikstra, 1981; Mays, 1996). The geochemical conditions of the burial environment such as soil pH, organic matter content, parent material, particle size, temperature range, microbial activity, duration of interment, soil solution fluoride and carbonate concentration can all influence the rate of ionic exchange between the calcium phosphate lattice in bone and the soil components (Nielsen-Marsh et al., 2007; Pate and Hutton, 1988).Whether an element is chemically bound to bone mineral or just forced through the bone pores under the action of hydraulic water pressure will largely depend on the solubility of the particular element or compound, and influence of the environmental pH (Hedges and Millard, 1995a; Lambert et al., 1985c; Pike and Richards, 2002).

Biological attack on bone has been well documented, but the causes remain unclear as to whether it is as a result of microbial or fungal action in terrestrial environments, these linear/longitudinal areas of mineral erosion (microscopic focal destruction - MFD) were first observed by Wedl in the Nineteenth century (Child, 1995a; Child, 1995b; Hackett, 1981; Jans et al., 2004). Microorganisms form a significant part of the soil matrix being more abundant in the organic component, as they are an essential part of the decomposition of biological matter. The characterisation of soil bacteria is very poorly understood due to their dynamic and complex nature, as they have very mobile genetic components and can exchange DNA between different species and genera making classification difficult (Lejj et al., 1993). The microbes involved in the decomposition pathways of human remains vary depending on the taphonomic stage; those that form part of the initial putrefaction process will differ to those involved in the more advanced stages (Child, 1995a; Child, 1995b). Some collagenase producing microorganisms have been identified and cultured under laboratory conditions, and their effects reported, but it is not understood that they exist in any great numbers to have a significant detrimental effect on soil interred archaeological bone (Balzer et al., 1997; Child, 1995b; Dixon et al., 2008). Microbial penetration into bone may be possible via pores in the surface, but this may not be enough to cause severe degradation as the large collagenase molecules would not be able to penetrate due to their size, and fully mineralised bone would be robust enough to render it resistant to this form of erosion (Collins et al., 1995; Trueman and Martill, 2002). Microbes may have an indirect effect on the degradation process, through metabolic oxidizing pathways, releasing soil acidifying carbon dioxide, which could be responsible for bone mineral dissolution, facilitating access to the collagen (Brock, 1976; Bromfield, 1954). Post mortem alteration of bone in the burial environment can be superficial or deep into the bone tissue (Reiche et al., 1999), making destructive analysis necessary in order to assess the degree of diagenesis within the bone. Efforts have been made to predict or model bone diagenesis pathways, with some success, but given the huge variety of variables, the preservation of any given bone, at any given site, under the huge variety of conditions that may exist within that site, there is still very little certainty about the true preservational state of bone samples presented for analysis (Hedges, 2002). Some techniques can require relatively large quantities of bone to be destroyed and this may present difficulties with precious samples, those samples with restrictions due to ethical reasons, or those that are of small size (Kaestle and

Horsburgh, 2002; Nielsen-Marsh and Hedges, 1999; Nielsen-Marsh and Hedges, 2000b; Katzenberg, 2001; Matheson et al., 2009). Sectioning of bone for histological examination requires specialist skill and knowledge to prepare, making this technique difficult to routinely apply to bone samples without specialist training. Deciding on the best assessment of bone has been difficult, as the destruction of human forensic and historical samples tends to, and should be, avoided where possible but little research has been done to identify those analyses that provide the best information weighted against the bone destruction necessary.

Unmineralised collagen is particularly sensitive to changes in temperature and hydration, and can easily begin to degrade under adverse conditions, but mineralised collagen, under the right conditions, can remain preserved for extended periods of time (Ortner et al., 1971; Turner-Walker, 2008). Stabilisation of collagen occurs during mineralisation when bound to the hydroxyapatite, which ensures a resilience to changes in hydration and temperature (Nielsen-Marsh et al., 2000) It is debated whether initial dissolution of the mineral phase is necessary before the collagen begins to degrade, however, it would seem that degradation of the collagen either through hydrolysis or microbial attack is the primary source of bone diagenesis (and a major cause of weight loss in bone), which can then lead to catastrophic mineral dissolution (Hedges, 2002; Nielsen-Marsh et al., 2007).

3.1.3. Soil and Human Bone X-Ray Fluorescence (XRF) Analysis

X-ray Fluorescence uses short wave length electromagnetic radiation to excite the atoms in a bulk target sample. The excited atoms become ionized, and if enough energy is released to dislodge a tightly held inner electron from its shell, the atom then becomes unstable and an outer shell electron moves to replace the missing inner electron, energy is then released as the inner shell electron is more strongly bound than the outer one. The emitted energy is in the form of radiation and has lower energy than the primary initial incident X-rays and is termed "fluorescence". These energy differences between electron shells are characteristic and are used to determine the elements that are present in any given sample (Shackley, 2011).

XRF can only analyse the heavier elements of the periodic table, from sodium onwards, and was not able to give results for other elements such as carbon, but they

would be represented as a "balance". The "balance" can be of great importance when assessing a given sample as it can help to establish other qualities in the sample, such as an estimation of the water content. XRF can be performed using semi or full quantitative analysis and the results are represented as a proportion of 100%. For semi-quantitative analysis a block of metal is used as a standard (usually aluminium), along with an internal standard. The internal standard should be as closely related as possible to the target sample, as a result a soil standard that consisted of a high proportion of clay was used. This enabled the analysis to give precision to the result, whilst using a triplicate sample analysis would ensure accuracy. The semi-quantitative analysis "scans" the sample and will present the results in reference to the standard, but caution was used with those elements that appeared in smaller quantities as they could be confused with other elements, and the accuracy reduced, however the curves can be double checked for accuracy. For this reason the results for those elements being studied that appeared in larger quantities would be used and elements < 0.010% (100 ppm) would be disregarded.

Semi-quantitative analysis was used to demonstrate the rate of soil intrusion into the bone. Samples of contemporary anatomical teaching human bone and cleaned pork bone were used for comparison of the elements expected. The benefits of this technique include being well suited to establishing chemical composition of bone and soil samples; it is easy to use and non-destructive (although powdering of the sample may be useful). XRF has been successfully used on a wide range of archaeological samples including animal and human bone (Carvalho et al., 2004; Craig et al., 2007; Janos et al., 2011; Mantler and Schreiner, 2000; O'meara et al., 2001). A quantitative analysis that could fully quantify elements could be performed using inbuilt software and calibrated using a certified standard reference sample, but it would not quantify those elements it was not asked for, whether they were present or not.

3.1.4. Chapel House Farm Cemetery Geology

The bedrock in the area consists of Early Triassic Kinnerton formation with superficial deposits of Devensian Irish Till. Till is commonly known as Boulder Clay, as it contains aggregate within it, and is glacial in origin. The parent material consists of Lower Mottled Sandstone. The cemetery site is located 16 m OD (above sea level)

3.1.5. Chapel House Farm Cemetery Weather

The minimum temperature range for Chester (1981-2010) was 1.4 - 11.9 °C, and the maximum was 7.9 - 21.0 °C with a mean annual rainfall of 726.2 mm (Metoffice, 2013). The weather patterns cannot be extrapolated for the whole duration of the cemetery site, and there is no doubt there have been climatic changes throughout the centuries, with fluctuations in the seasonal temperatures and precipitation.

3.1.6. Chapel House Farm Cemetery Soil pH

The pH is a measure of the activity of H^+ ions using an inverse logarithmic scale, ranging from 1 (the most acidic) to 14 (the most alkaline). Most soils range from pH 2-12, with 6-7 being neutral (Ellis and Mellor, 1995). Most measurements are taken using a 1: 2.5 weight to volume ratio of soil to deionised water (or a calcium chloride solution). The measurement involves using either an electrometric probe or colourimetric indicator strips.

The pH of the soil is an important part of routine soil analysis; and one of the vital investigations into the degradation dynamics of human remains (Carter et al., 2010; Fiedler and Graw, 2003; Forbes et al., 2004; Gordon and Buikstra, 1981; Pate and Hutton, 1988; Turner-Walker, 2008). The interaction of bone with the burial environment is vital to understanding the preservation of the Poulton human skeletal remains. Soil analysis techniques use well established methods that are non-destructive to bone that can be employed to investigate the mobility of bone and soil elemental ions in the immediate environment of the bone.

3.1.7. Chapel House Farm Cemetery Soil Microscopy

The use of light microscopy observations on each soil sample enabled the visual identification of the soil matrix for any artefacts including bone that could provide additional explanation of the soil chemistry (Canti, 2003; Jakes and Sibley, 1982; Williams, 1977). Visual examination of the soil can provide useful information about the nature of the soil composition (sand, clay, silt, quartz) and previous use or contamination of the land. This information may help to explain the preservation of the remains, and the movement or retention of individual elemental ions in the soil. This analysis is informative, quick and inexpensive to perform. These observations can provide invaluable evidence of past use of the land in the form of artefacts that that are resistant to the effects of degradation in the soil environment at least in the

short term. Anthropogenic activities such as previous land use or modification may be seen as small artefacts in the soil matrix, these artefacts can be placed historically by noting their presence in the soil horizons. It is possible to see the effects of cultural practices such as coal or charcoal burning that may have been involved in the cremation of human remains, or cooking of animals. As the site had been occupied for over five thousand years the effects of domestic waste deposition, farming practices, industrial activities, rebuilding or land dwelling clearance may be evident from the soil matrix. Activities such as dumping wood or coal ash onto the soil can have short and long-term effects; coal ash causes acidification of the soil due to the presence of sulphur dioxide, and wood ash has a liming (alkaline) effect that can increase the pH of the soil considerably (Egeback et al., 1984; Flues et al., 2002; Ohno and Erich, 1990). These practices may provide some explanation of the preservation of the skeletal remains.

3.1.8. Chapel House Farm Cemetery Skeletal Excavation Techniques

A skeleton was described as "a skull with associated bones" and assigned a skeleton number; the disarticulated remains were given a grave-fill context number. All remains were excavated in accordance with English Heritage guidelines, and the terms of the license.

Excavation of remains included the removal of as much surrounding soil from the skeletal components as possible, they were then photographed with and without associated skeletal remains or other artefacts, measurements recorded and then lifted. The skeletal components were placed in labelled plastic bags ready for processing, and then washed in tap water using a standard domestic toothbrush. When clean, the individual bones were laid out on newspaper-lined plastic seed trays to dry, and returned to the plastic bags for storage. The remains were either lifted and processed by under-graduates and then stored at Liverpool John Moores University, or by trained volunteers and stored in porta-cabins at the site. Any skeletal remains that had not been lifted had in the past been covered with corrugated iron sheets, or tarpaulin, but recently this had been changed to re-interment to protect the bones from the effects of the weather. The skeletal remains were not routinely stored in temperature stable environments, particularly in the porta-cabins where temperatures could fluctuate considerably during the year.

There had been substantial grave reuse at the site, with previous interments being disturbed to make way for new ones (Figure 2 & 3). Some parts of the skeletons seemed to be more robust than others, with juvenile remains being generally more vulnerable than adult. Figure 2 shows the smaller bones of the hands and feet, and how the undeveloped bones suffered more degradation than the developed robust bones such as the long bones of the axial skeleton. This damage can be seen more clearly once the non-associated bones had been removed (Figure 3), part of a bone from a burial that preceded the child burial can be seen beneath the juvenile femur.

Routine soil sifting is not routinely done at this site, due to the heavy clay soil material. Excavated soil was stored in temporary mounds at various locations around the perimeter of the cemetery, but outside of the boundary ditch.

Figure 2: A child couple (SK 183 and 184) buried holding hands, with associated disarticulated skeletal material.

Figure 3: Child couple with most of the disarticulated bone removed.

In some areas of the cemetery the bones were in a very poor condition, in contrast to others that were excavated in excellent condition, this wide variation in preservation is currently not understood, but those remains excavated from the Roman midden area demonstrated greater degradation than those from other areas of the cemetery. On occasion remains were excavated in a poor condition while those in close proximity were excavated in good condition, without an obvious reason. Whilst it is accepted that the fragile juvenile bones would be more vulnerable to decomposition, some of the adult remains suffered very advanced decomposition, sometimes leaving just a powdered and fragmented shadow in the soil. Figure 4 shows this kind of preservation, the well preserved remains of an adult burial contrast sharply with the powdered adult cranium and associated bones, the robust long bones having the best preservation despite being very degraded and fragile. Whether this was due to the individuals being in poor osteological health or due to some environmental cause was impossible to determine without further investigation.

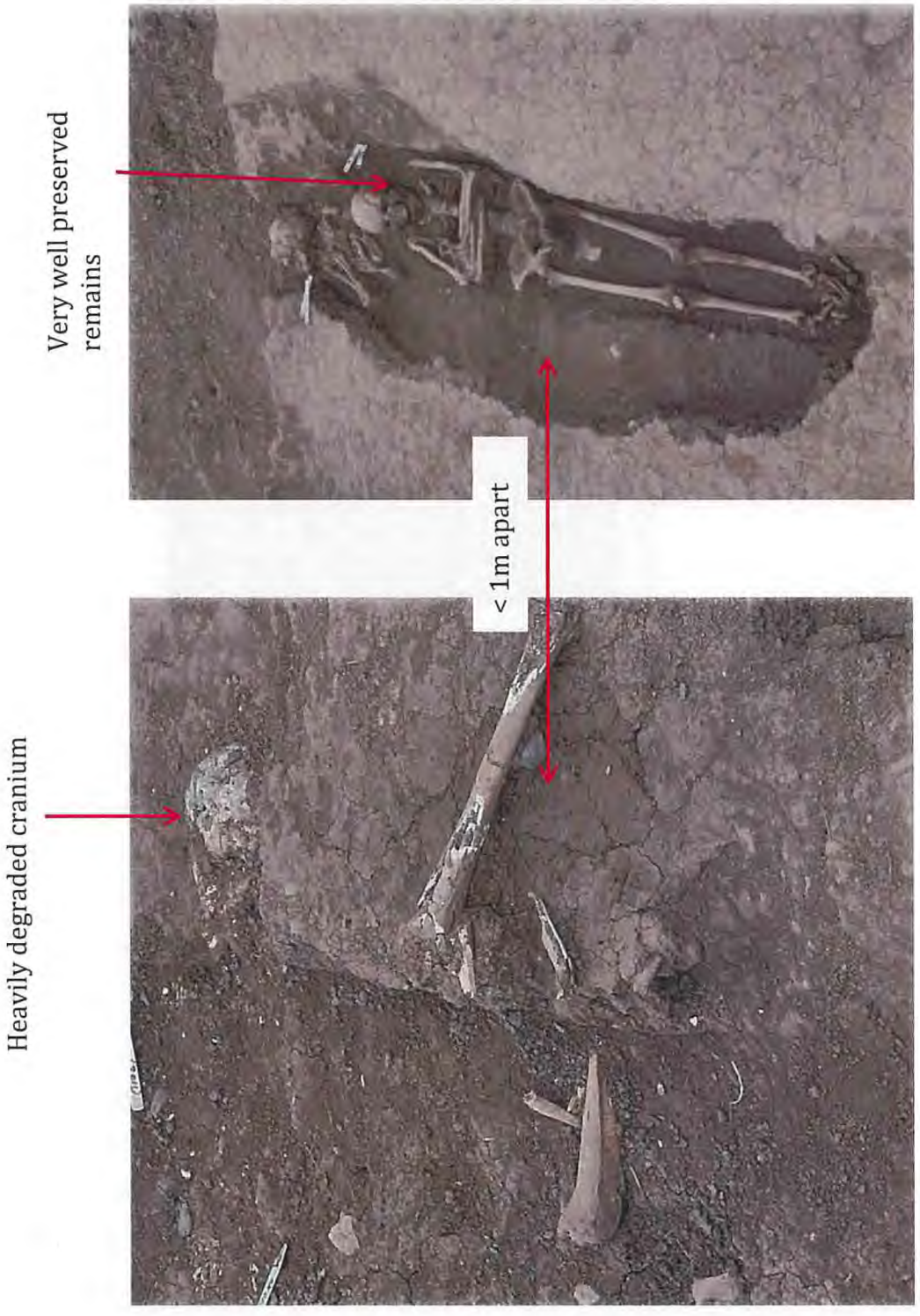


Figure 4: Adult remains in contrasting states of preservation from graves in close proximity.

3.2. Materials and Methods

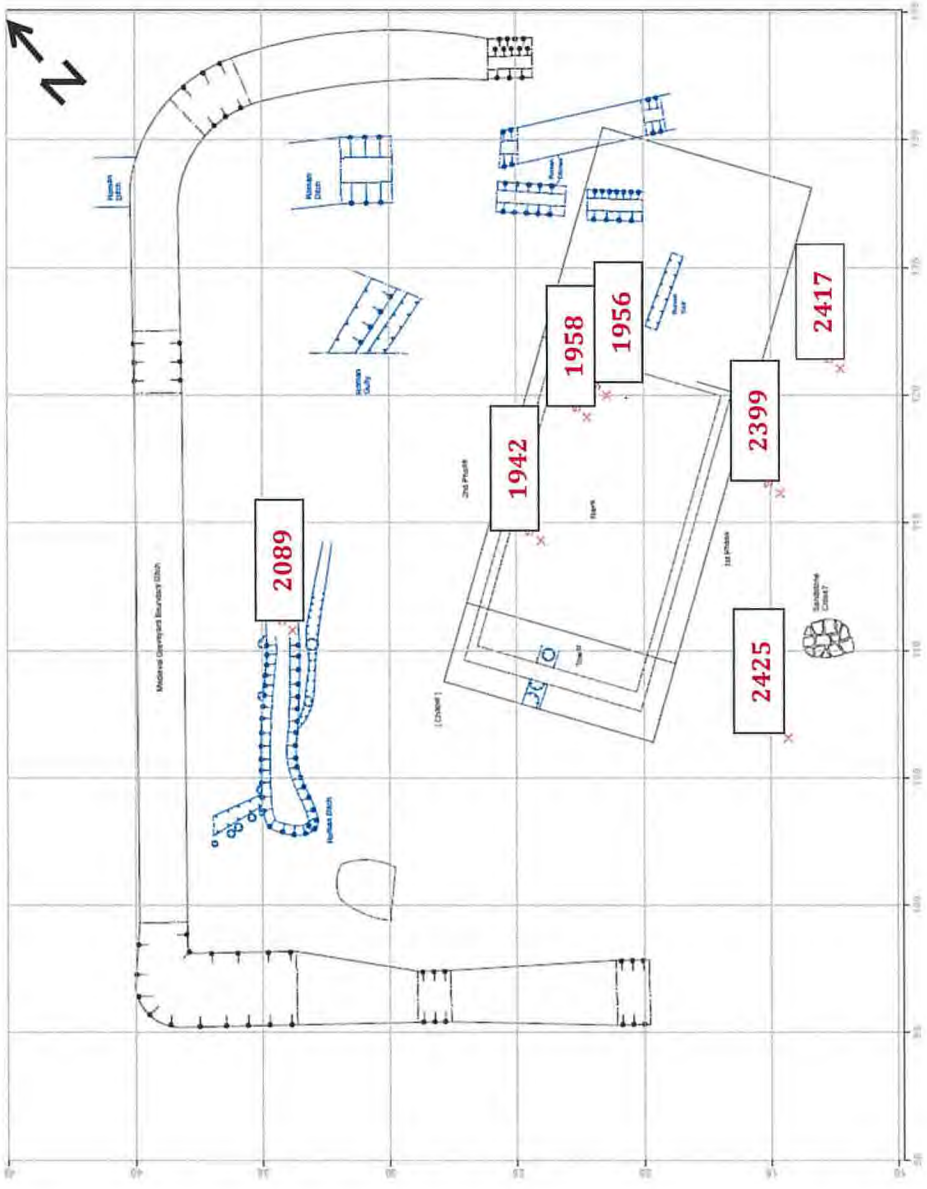
3.2.1. Chapel House Farm Cemetery Soil Sampling

The cemetery has a long history of occupation and some of the historical features that preceded the building of the chapel remain in situ today, the chapel itself under went modification during its life-time and prior to becoming derelict. Soil samples were collected from inside and outside of the cemetery. Each soil sample from within the cemetery was assigned a unique context number, related to a skeleton number(s) if complete remains had been excavated. The samples from outside the cemetery would be described as the control samples and assigned letter "C" and a number C (n). The control samples were collected from around the perimeter of the cemetery excluding the hedge-line that was difficult to access. Additional samples were collected due to the excavated cemetery soil being stored intermittently around the perimeter. Each soil sample was recorded on a scale map (Figures 5 & 6).

Cemetery soil samples = 7
Control samples = 10

Approximately 300 g of cemetery soil was taken from the surface immediately underneath excavated skeletal remains; it was not possible to determine the depth that the sample was taken from due to removal of the surface soil prior to excavation. During excavation soil was removed from the surface using heavy earth moving equipment making the depth of burial difficult to determine, however measurements of the head, pelvis and feet in relation to sea-level were routinely taken and could be compared to the initial cemetery site survey measurements taken before excavation began in 1995. This enabled the depth of burial to be approximated. The depth of the soil was estimated by taking the original landscape surface surveying height, and subtracting the height of the interred pelvis. Each cemetery soil sample was placed in a labelled plastic container and located on a cemetery map (Figure 5).

The control samples were taken from around the perimeter of the cemetery and the location recorded on a map of the cemetery (Figure 6). The depth of each sample was measured and recorded. Efforts were made to sample at least 30 cm deep.



Scale: 1 square = 5m

Figure 5: Cemetery soil sampling locations denoted by x (map courtesy of R Carpenter and M Emery 2012).

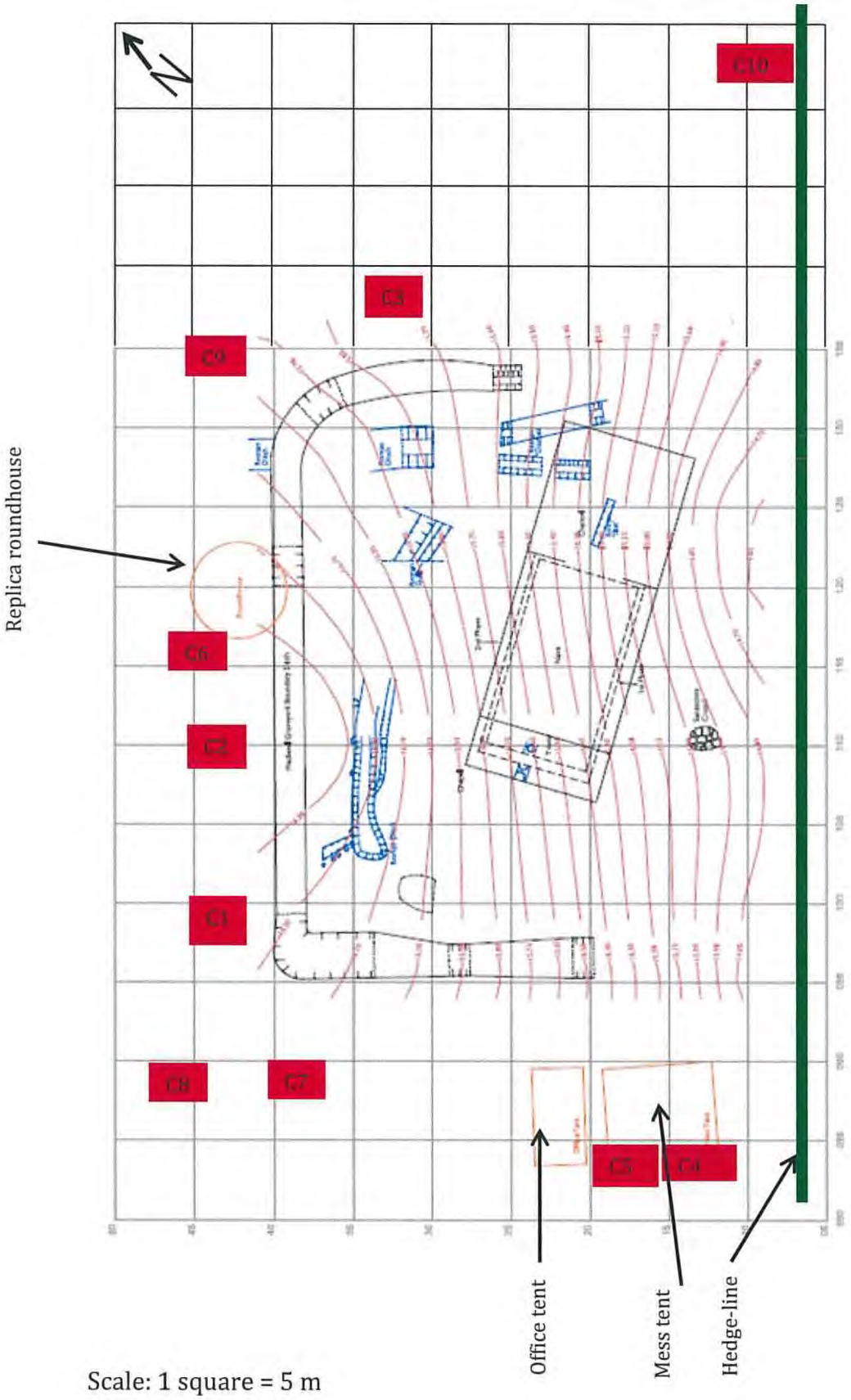


Figure 6: Control sample locations in association with cemetery area landmarks.

3.2.2. Soil Preparation

Each 300g soil sample was air-dried, crushed, and then oven dried at 37 °C. Any visible stones were removed and along with any plant roots, as the presence of these components could skew the XRF results. The soil was lightly crushed further with a pestle and mortar to a fine powder, and sieved with a clean 1mm metal soil sieve (washed with warm free running water and lightly scrubbed with a scouring sponge between samples to prevent contamination). The powder was returned to the labelled box and stored to await analysis.

3.2.3. X-Ray Fluorescence Analysis

Cemetery and control soil samples from the original labelled storage boxes were used, making sure that an homogenous mix was obtained first. Approximately 4g of soil was placed into a 31mm open-ended x-cell cuvette (Spex Certiprep, Middlesex) that had been covered with 4 micron prolene X-ray film (Chemplex, Florida), tamped down with a small flat bottomed pestle, ensuring the even and complete spread of soil, and then capped.

XRF was performed using an EDX-720 Energy X-ray dispersive spectrometer (Shimadzu, Japan) with EDX software: DXP-700E version 1.00 Rel. 017 (Shimadzu, Japan). The soil samples were subjected to a semi-quantitative analysis, each grave and control sample was performed in triplicate. This analysis uses an internal calibrated soil standard, with correction facility. A semi quantitative analysis was performed in order to quantify all the elemental components of the soil. The results were represented as a percentage of the total elements detected, however the XRF cannot individually detect the lighter elements of C, H, N, O, but these are represented as a combined total. The results for all the elements >0.01% were recorded, apart from lead for which all results were recorded. The soil lead was recorded in order to assess the soil content to compare to the bone lead which can be accumulated and retained during the individual's lifetime.

The results from inside and outside the cemetery were subjected to statistical analysis using SPSS17.

3.2.4. pH Analysis

The pH of the soil samples was performed using 10g of sieved homogenised soil and 25 ml of deionised ultra-pure water, stirred and agitated well with a clean spatula and left to equilibrate for 15 minutes in a small plastic beaker. The pH was performed in triplicate using a pH checker and probe (Hanna Instruments, SIS, Wilford, Nottingham, NG11 7EP, UK). The results from inside and outside the cemetery were compared, using statistical analysis using SPSS 17.

3.2.5. Soil Microscopy

Approximately 0.5g of soil was taken from the labelled box and place on a plastic petri dish, shaken to disperse to a thin layer and examined using a Stemi DV4 light microscope (Carl Zeiss, Gottingen, Germany), the results for bone, pottery, plant, charcoal, coal and parent material were recorded and represented graphically. The presence of pottery or other alien objects could skew the XRF results, and it was important to remove these prior to analysis .

3.2.6. Bone Analysis

Archaeological Bone Preparation:

In order to assess the interaction between the soil and the bone, 20 human bones were allocated by a supervisor. It was also important to assess the interaction between different types of bone, and also the effects of the length of time in storage especially in the elemental composition. The selection of bone included:

5 cranial fragments from extended storage.
5 long bone fragments from extended storage.
5 cranial fragments recently excavated.
5 long bones recently excavated.

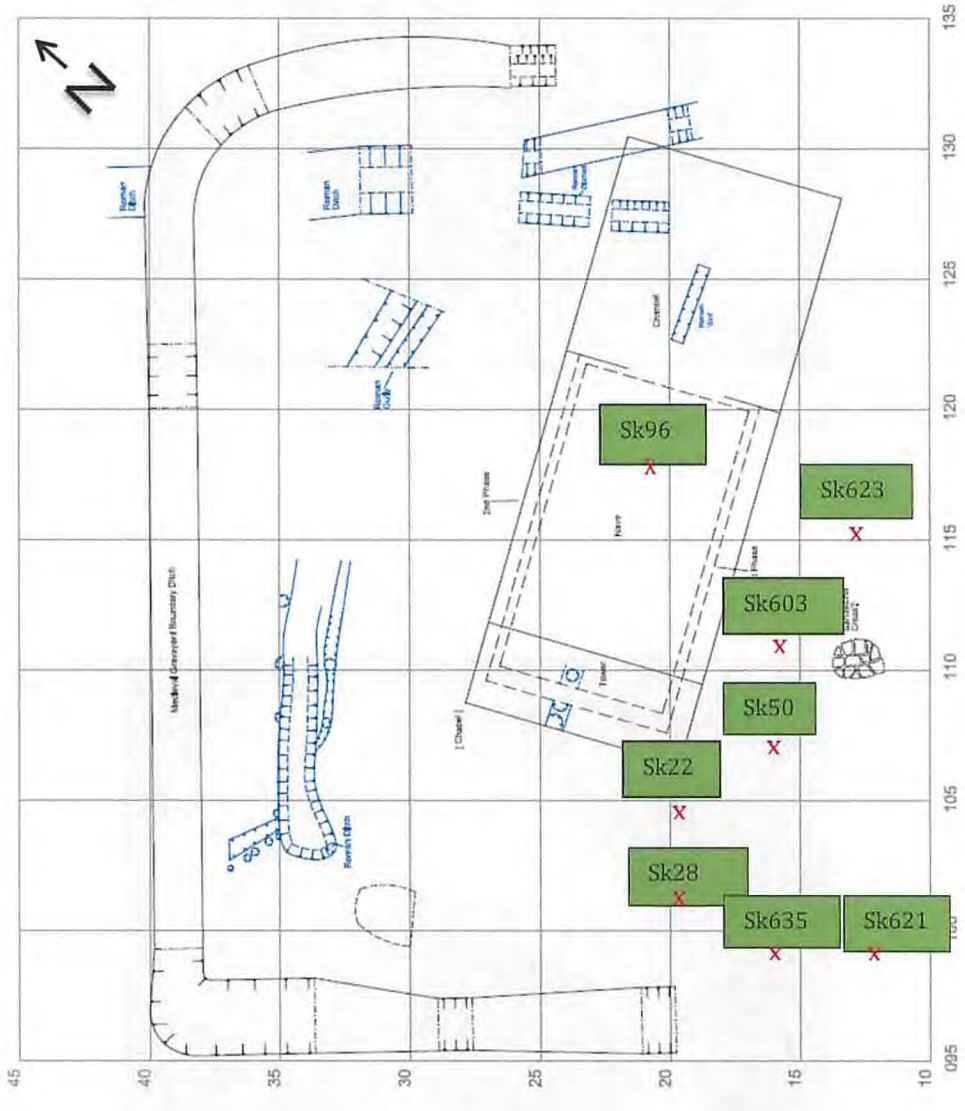
Some of the fragments were obtained from the same individual or grave context (for disarticulated bone fragments). The locations of the bone fragments were recorded on a map of the cemetery (Figure 7). The bone fragments were photographed using a Nikon D300 camera with AF-S Micro-Nikkor 60mm lens and excavation details were also recorded (Table 1). Damage to the bone fragments was kept to a minimum, but some had to be cut with a small hand hacksaw and the cut surfaces dusted to ensure no metal contamination adhered to the bone, this enabled them to be photographed in

cross section to help demonstrate any visible soil intrusion, and to fit in the XRF cuvette. As can be seen from the bone images (Figures 8 & 9), the bone suffered from considerable soil intrusion.

Not recorded:

Sk26: Trench 1, a generic area south west of the chapel.

1010: Generic context area south of the chapel.



Scale: 1 square = 5 m

Figure 7: Location of bone samples within the cemetery.

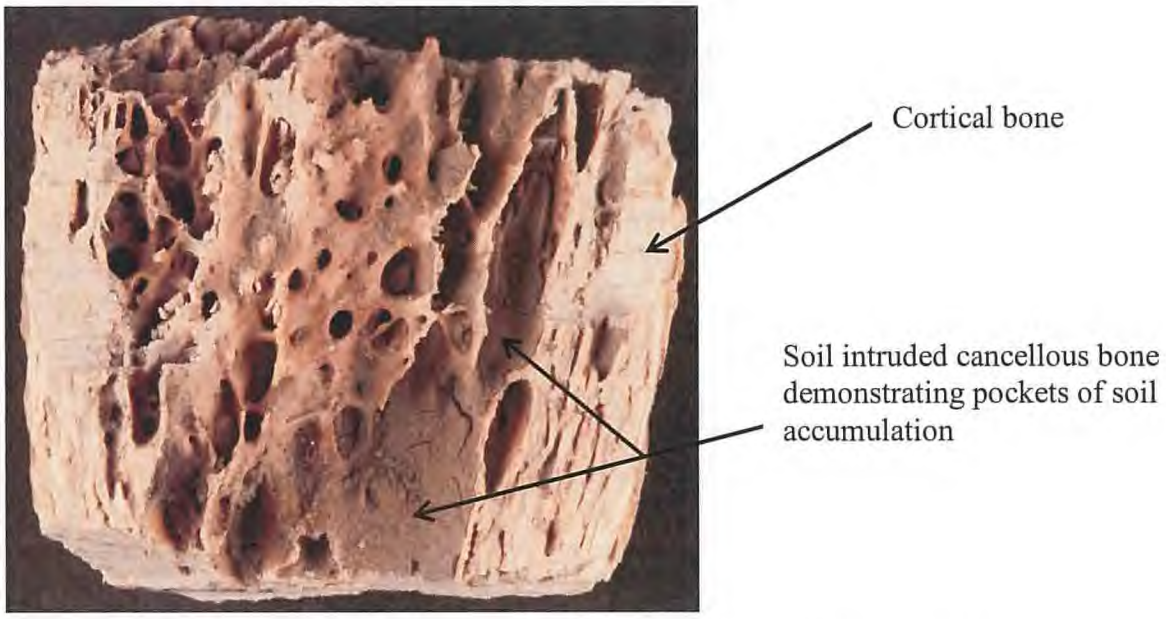


Figure 8: Photograph of soil intruded bone section (bone sample 5b, see Table 1 below).

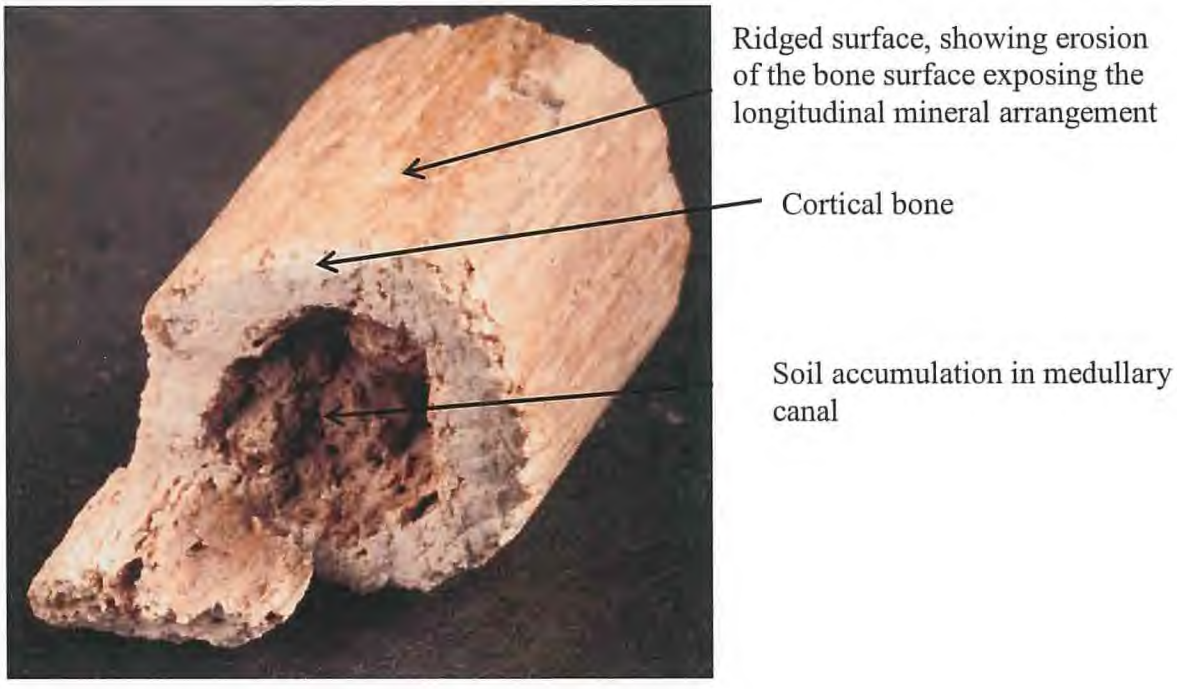


Figure 9: Photograph demonstrating soil accumulation within the medullary shaft and erosion of the bone surface (bone sample 4b, see Table 1 below).

3.2.7. Control Bone Sample Preparation

Control bone was required to ensure that a comparison could be made between soil interred bone and contamination-free bone. Contemporary human bone is difficult to acquire due to ethical and legal issues, however four small hand bones from two anatomical skeletons (>80 years, not recent) held at Liverpool John Moores University Anthropology Department, were made available for testing as to their suitability to be used for control. One pisiform metacarpal and a set of three phalanges from a finger were analysed, along with four samples of pork bone shards, from four separate individuals (Table 5). The anatomical bone fragments were analysed whole.

Animal studies have shown that whilst dog bone is the closest in anatomy to human, pork is an acceptable alternative bone as the gross components, anatomy and micro structure are very similar (Aerssens et al., 1998; Pearce et al., 2003). The adult (> 32 weeks), traditional breed (Saddleback) pork bone was obtained from a local butcher, washed in warm tap water, with the marrow removed using a wooden spatula and the cavity scrubbed with a nylon bottle brush until all residue had been removed. The outer surface was scraped clean until all surface tissue was removed. The bones were placed in labelled glass containers and dried at 37° C for several days in a drying oven. The bone was fragmented using a clean Proctor compactor and placed in a labelled bag. 10g of the bone was broken up further and then powdered in a Fritsch mortar grinder (Fritsch Pulverisette 2, Industriestr 8, 6580, Idar-oberstein, West Germany), this was to investigate the consistency of results from powdered compared to whole bone. The archaeological bone would be analysed whole rather than powdered due to the small size of the fragments, and to minimise bone destruction.

3.2.8. Control Bone XRF

The anatomical human bone was not suitable for comparison analysis due to the preparation methods used: the metacarpal bone contained a large quantity of chlorine demonstrating that it had been bleached, and the three phalanges were contaminated with soil components indicating exposure to a soil environment prior to being used as an anatomical specimen. It was decided to use only the pork bone shards for comparison analysis. Pork bone has been described as having a similar bone composition, anatomy, remodelling, and lamellar structure to human bone, and has

been routinely used in the past for study in the absence of human bone (Pearce et al., 2007; Aerssens et al., 1998).

3.2.9. X-ray Fluorescence (XRF)

(for further details see p78) The powdered bones were analysed as per the soil. The bone fragments, were placed in the cuvette whole and positioned using the inbuilt camera. The bone components were analysed using an oxide setting to help to account for some of the oxygen from the "balance". All analyses were done in triplicate.

Table 1: Archeological Bone Samples

Stored Cranial Bones

No	External	Internal	Reference	Type	Excavation Date	L x W x H (mm)	Burial Depth (cm)
1a			SK 96	cranium	2002	22 x 12 x 1.4	N/A
2a			SK 50	cranium	1998	18.7 x 13.9 x 7.9	N/A

3a	 	SK 22	cranium	1997	18.7 x 14.6 x 11.2	N/A
4a	 	SK xxvi	cranium	1997	22.6 x 17.4 x 3.2	N/A

5a			TR1	cranium	2002	22.7 x 14.6 x 3.7	N/A	

Stored Long Bones

No	External	Internal	Reference	Type	Excavation Date	L x W x H (mm)	Burial Depth (cm)
1b			SK xxii	upper limb	1997	19.3 x 13.2 x 4	N/A

2b			SK xxvi	long bone	1997	14.6 x 10.4 x 2.7	N/A
3b			SK 28	fibula	1996	12.8 x 11 x 2.	N/A
4b*			SK 96	ulna	2002	17.9 x 12.9 x 10.3	N/A

5b			SK 50	ulna	1998	16.7 x 14.4 x 5.2	N/A

Note: * denotes whole bone section

Recently Excavated Cranial Bones

No	External	Internal	Reference	Type	Excavation Date	L x W x H (mm)	Burial Depth (cm)
1c			2466*	cranium	2012	17.1 x 6.8 x 3.9	2 cm

2c	 	1010*	cranium	2012	14.8 x 13.2 x 5.1	N/A
3c	 	2466*	cranium	2012	15 x 14.3 x 5.2	2 cm
4c	 	2520*	cranium	2012	17.7 x 14.2 x 6.3	9 cm

5c			2520*	cranium	2012	17.3 x 14.6 x 4.9	9 cm

Note : * denotes grave context number (bones not affiliated with a skeleton number SK)

Recently Excavated Long Bones

No	External	Internal	Reference	Type	Excavation Date	L x W x H (mm)	Burial Depth (cm)
1d			2512	tibia	2012	20.2 x 10 x 4.8	18cm

2d			2512	tibia	2012	13.5 x 13.3 x 5.5	18cm
3d			2546	tibia	2012	17.7 x 11.3 x 6.9	13cm
4d			1010*	tibia	2012	17.2 x 11.2 x 4.2	N/A

5d			1010*	tibia	2012	11.9 x 8 x 2.5	N/A

Note: * denotes grave context number (bones not affiliated with a skeleton number SK)

Table 2: control bone samples

Image	Type	Origin	Soil Interred	Age Since Death	L x W x D (mm)
	Phalange	Human	Yes	>80 years	29.14 x 5.74 x 6.04
	Phalange	Human	Yes	>80 years	13.76 x 7.03 x 5.35
	Phalange	Human	Yes	>80 years	11.36 x 5.12 x 4.86

	Metacarpal	Human	Unknown	>80 years	13.08 x 9.1 x 8.3
	Femur	Porcine	No	Contemporary	12.46 x 11.02 x 2.83
	Femur	Porcine	No	Contemporary	15.52 x 5.93 x 5.23
	Femur	Porcine	No	Contemporary	15.47 x 6.14 x 3.17

Femur	Porcine	No	Contemporary	10.07 x 9.33 x 4.05

3.3. Results

For statistical output please see Appendix: Chapter 3, 1-8

3.3.1. (i) Soil XRF

The soil from inside the cemetery was compared to the control samples using SPSS 17. Non-parametric testing was applied due to some of the data violating normality, and resisting transformation. A Mann-Whitney U Test (the nonparametric equivalent of the Independent – samples T-Test), was applied to the data to test for significance.

A total of 17 samples were analysed:
7 from the cemetery (see Figure 5)
10 controls from outside the cemetery (see Figure 6)

Silicon:

There was no significant difference between the levels of silicon from inside Poulton cemetery and the control samples (Figure 10). P = 0.674, n = 21, 30, Z value = -0.421, 2 tailed. Although the difference may not be significant the graph shows the silicon levels to be slightly higher from within the cemetery compared to the controls.

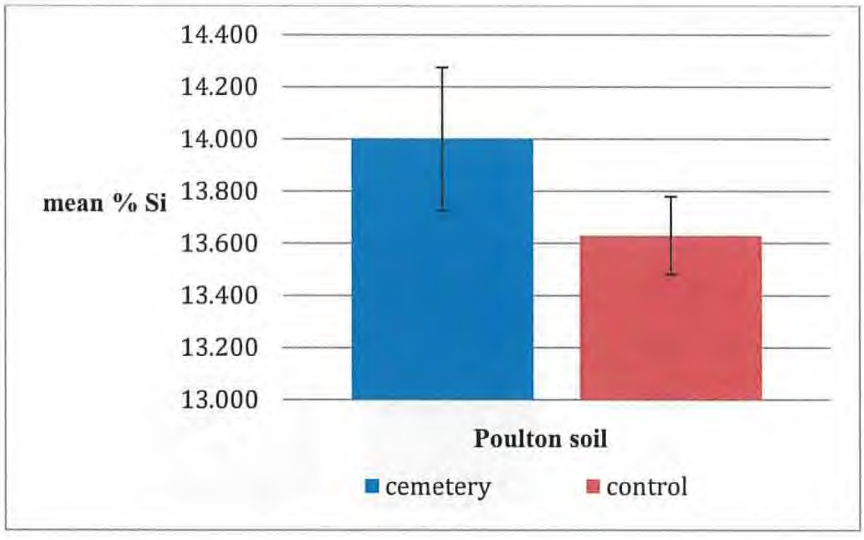


Figure 10: Mean levels of Si from inside the cemetery compared to the controls.

Aluminium:

There was a significant difference between levels of aluminium from inside Poulton cemetery and the control samples (Figure 11). P = <0.0001, n = 21, 30, Z value = -3.866, 2 tailed.

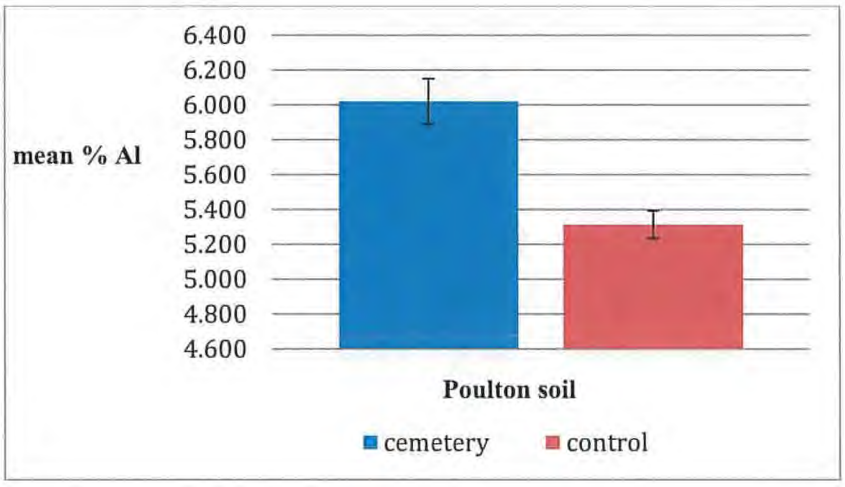


Figure 11: Mean levels of Aluminium from inside the cemetery compared to the controls.

Iron:

There was a significant difference between the levels of iron from Poulton cemetery and the controls (Figure 12). P = 0.006, n = 21, 30, Z value = -2.775 2 tailed.

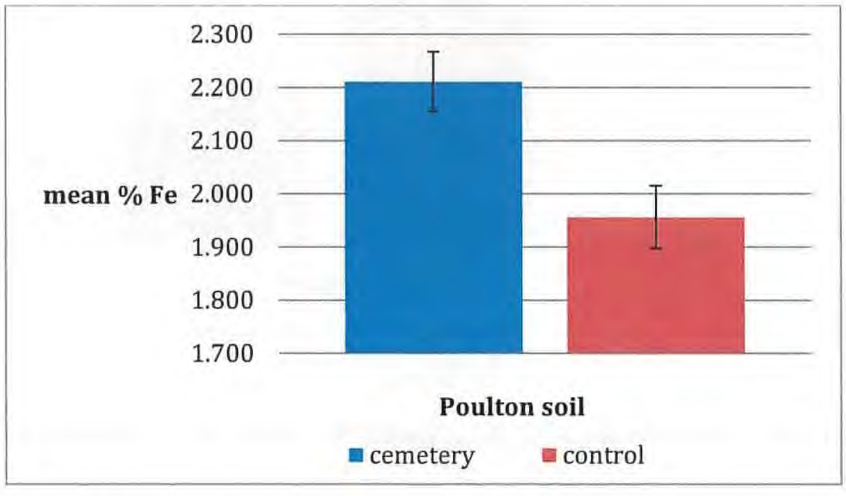


Figure 12: Mean levels of iron from inside the cemetery compared to the controls.

Potassium:

There was a significant difference between the levels of potassium from Poulton cemetery and the control samples (Figure 13). P = <0.0001, n = 30, 21, Z value= -4.709, 2 tailed.

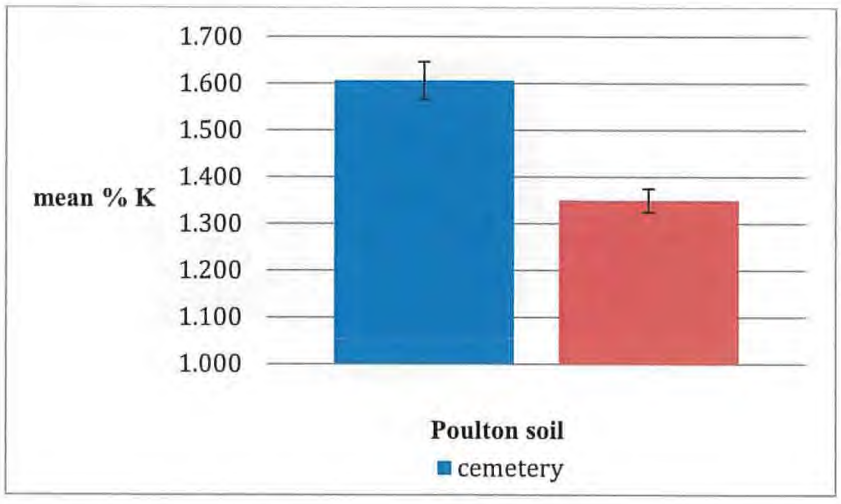


Figure 13: Mean levels of potassium from inside the cemetery compared to the controls.

Magnesium:

There was a significant difference between levels of magnesium from Poulton cemetery and control samples (Figure 14). P = <0.0001, n = 21, 30, Z value= -4.689, 2 tailed.

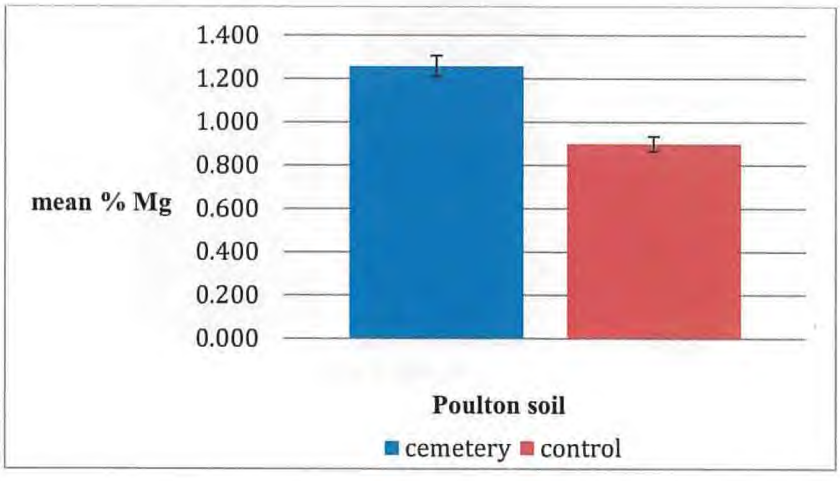


Figure 14: Mean levels of magnesium from inside the cemetery compared to the controls.

Calcium:

There was a significant difference between the levels of calcium from Poulton cemetery compared to the control samples (Figure 15). P = <0.0001, n = 21, 30, Z value = -5.397, 2 tailed.

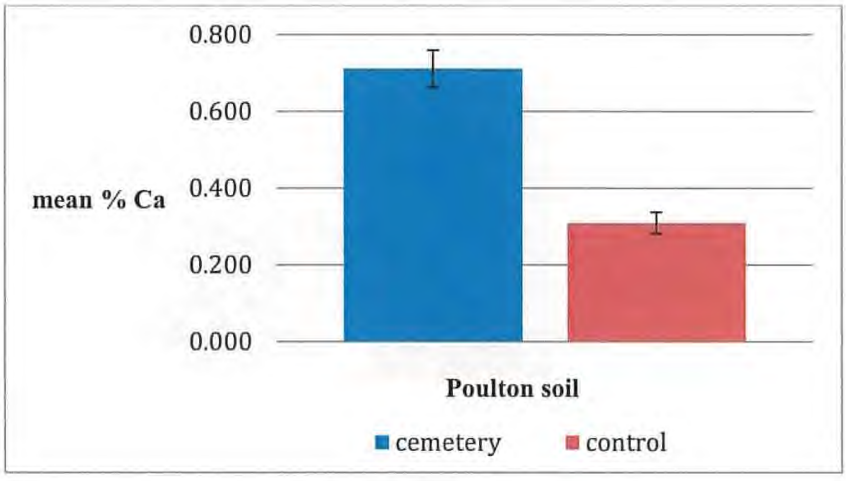


Figure 15: Mean levels of calcium from inside the cemetery compared to the controls.

Titanium:

There was no significant difference between levels of titanium from Poulton cemetery compared to the control samples (Figure 16). P = 0.128, n = 21, 30, Z value = -1.522, 2 tailed.

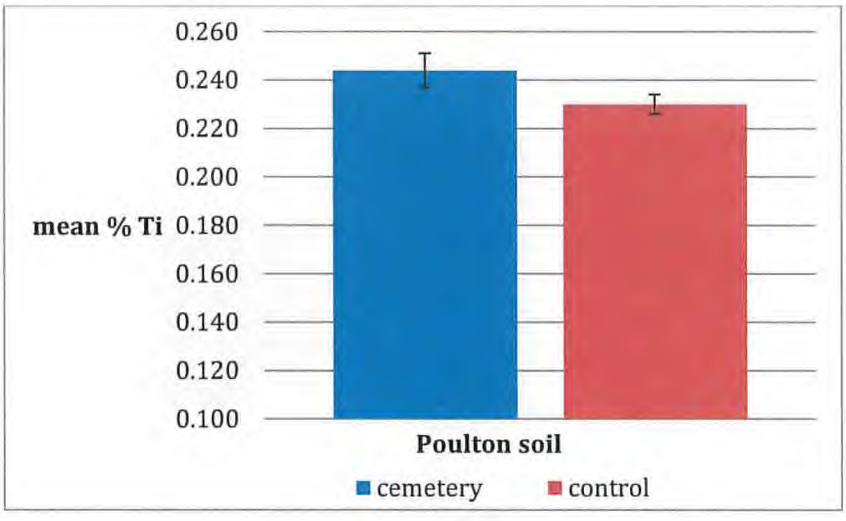


Figure 16: Mean levels of titanium from inside the cemetery compared to the controls.

Barium:

There was a significant difference between the levels of barium from Poulton cemetery compared to the control samples (Figure 17). P = 0.003, n = 21, 30, Z value = -2.933, 2 tailed. Here the control samples had higher levels than the cemetery samples.

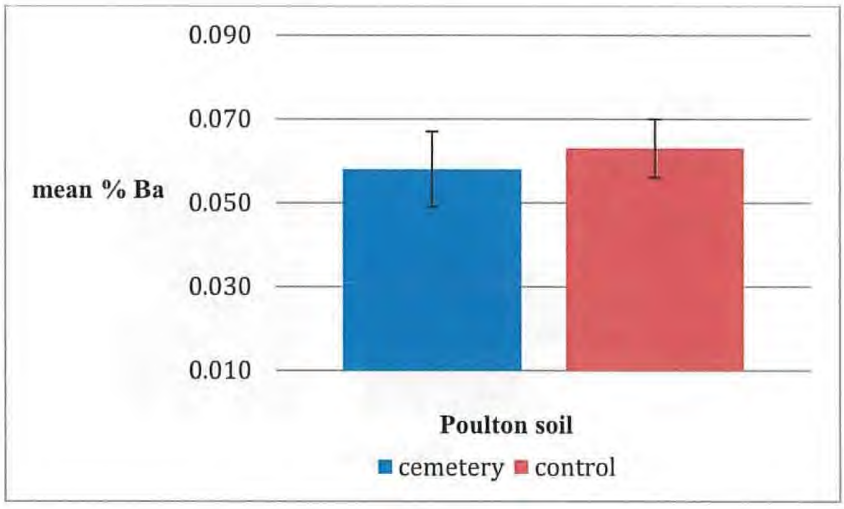


Figure 17: Mean levels of barium from the cemetery compared to the controls.

Phosphorus:

There was no significant difference between the levels of phosphorus from Poulton cemetery compared to the control samples (Figure 18). P = 0.502, n = 21, 30, Z value = - 0.671, 2 tailed. Although the levels of P were not statistically significant, the levels of P from the cemetery would appear to be slightly higher than the controls.

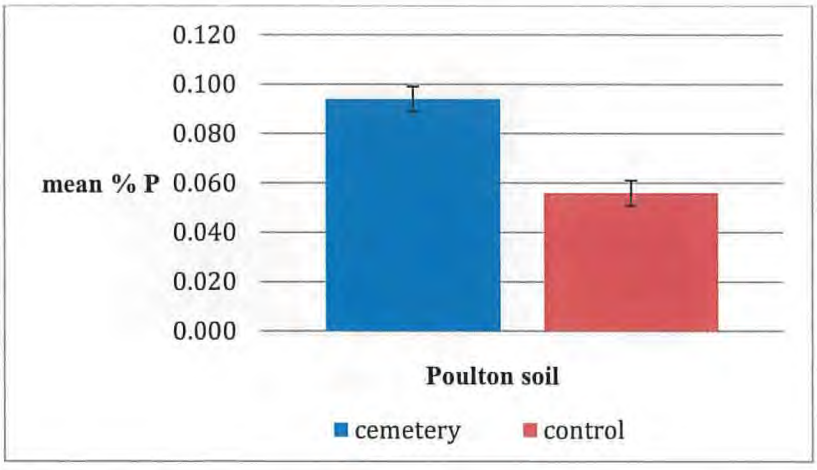


Figure 18: Mean levels of phosphorus from the cemetery compared to the controls.

Sulphur:

There was a significant difference between the levels of sulphur from Poulton cemetery compared to the control samples (Figure 19). P = 0.001, n = 21, 30, Z value = -3.462, 2 tailed. The levels from outside the cemetery are higher than inside, this could be due to the influence of contamination from RAF Poulton.

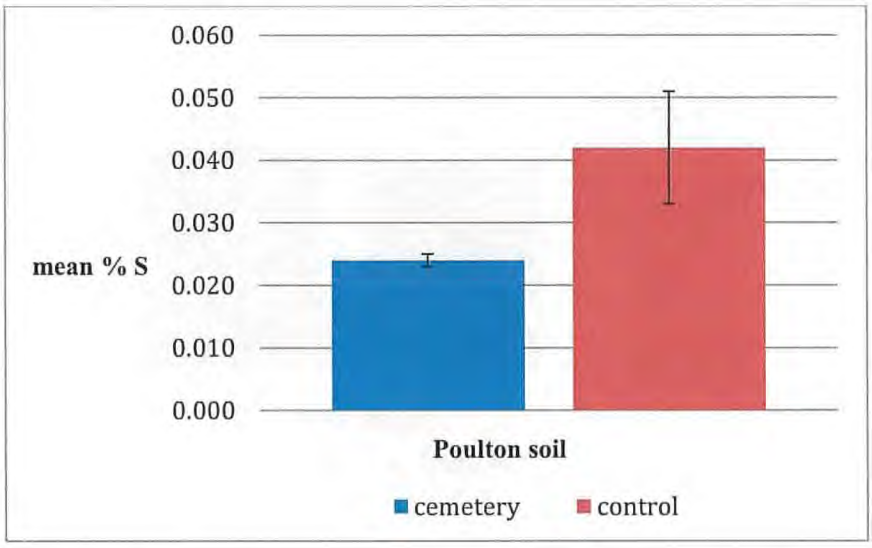


Figure 19: Mean levels of sulphur from the cemetery compared to the controls.

Manganese:

There was no significant difference between the levels of manganese from Poulton cemetery compared to the control samples (Figure 20). P = 0.148, n = 21, 30, Z value = -1.447, 2 tailed.

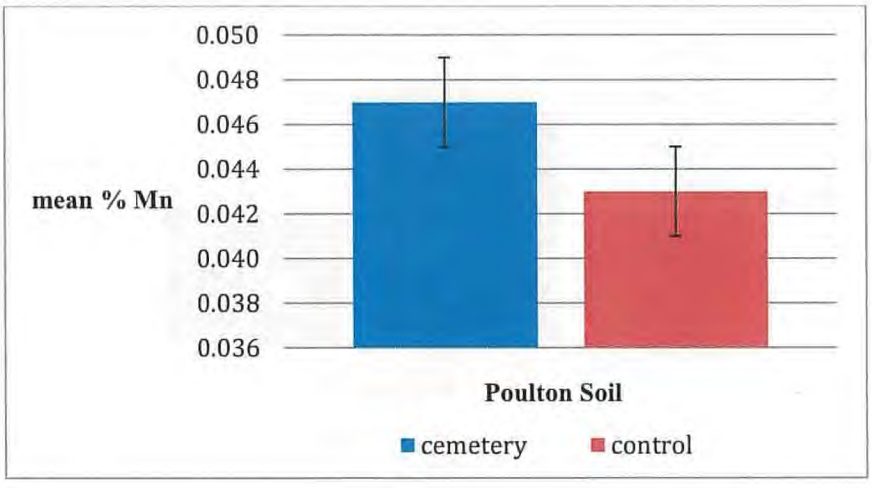


Figure 20: Mean levels of manganese from the cemetery compared to the controls.

Lead:

There was no significant difference between the levels of lead from Poulton cemetery compared to the control samples (Figure 21). P = 0.174, n = 21, 30, Z value = -1.358, 2 tailed.

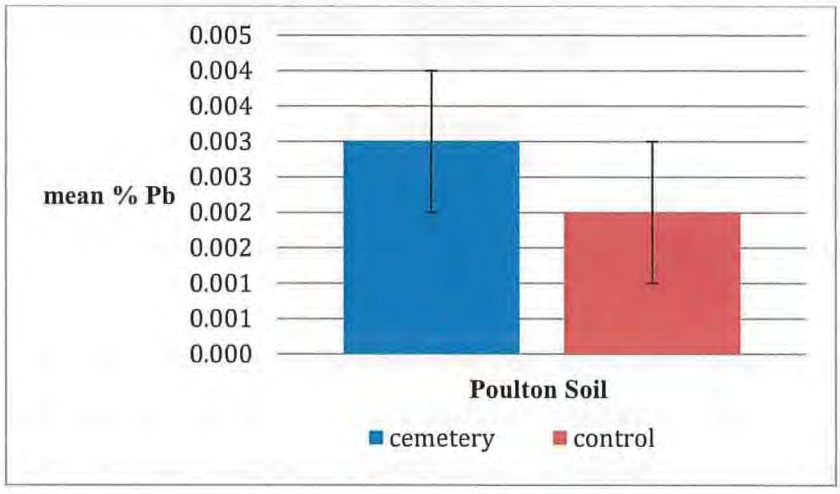


Figure 21: Mean levels of lead from the cemetery compared to the controls.

3.3.1. (ii) Soil pH

The data from the soil pH analysis of both the cemetery and the controls was statistically analysed using the non-parametric Mann-Whitney U test, the equivalent of the Independent–Samples T-test, (due to the data violating normality and resisting transformation).

There was no significant difference between the pH of the Poulton cemetery soil compared to the control samples (Figure 22). P = 0.133, n = 21, 30, Z value = -1.503, 2 tailed. However there is a much smaller standard error for the control soil than the cemetery, demonstrating a more consistent pH across the control soil than the cemetery.

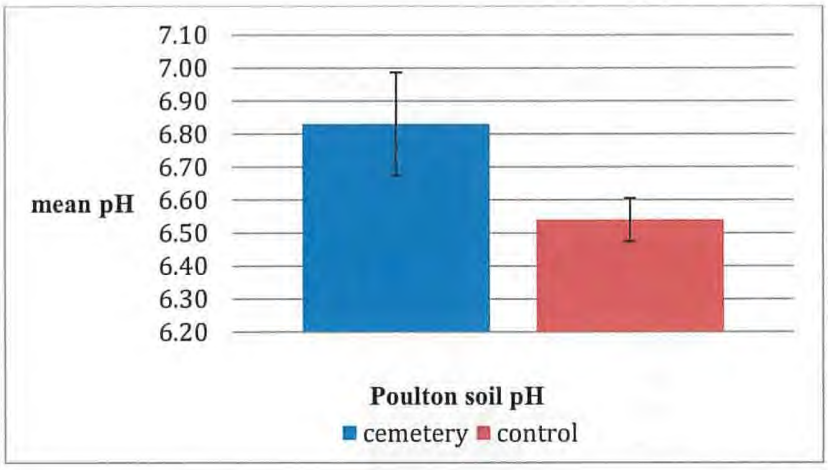
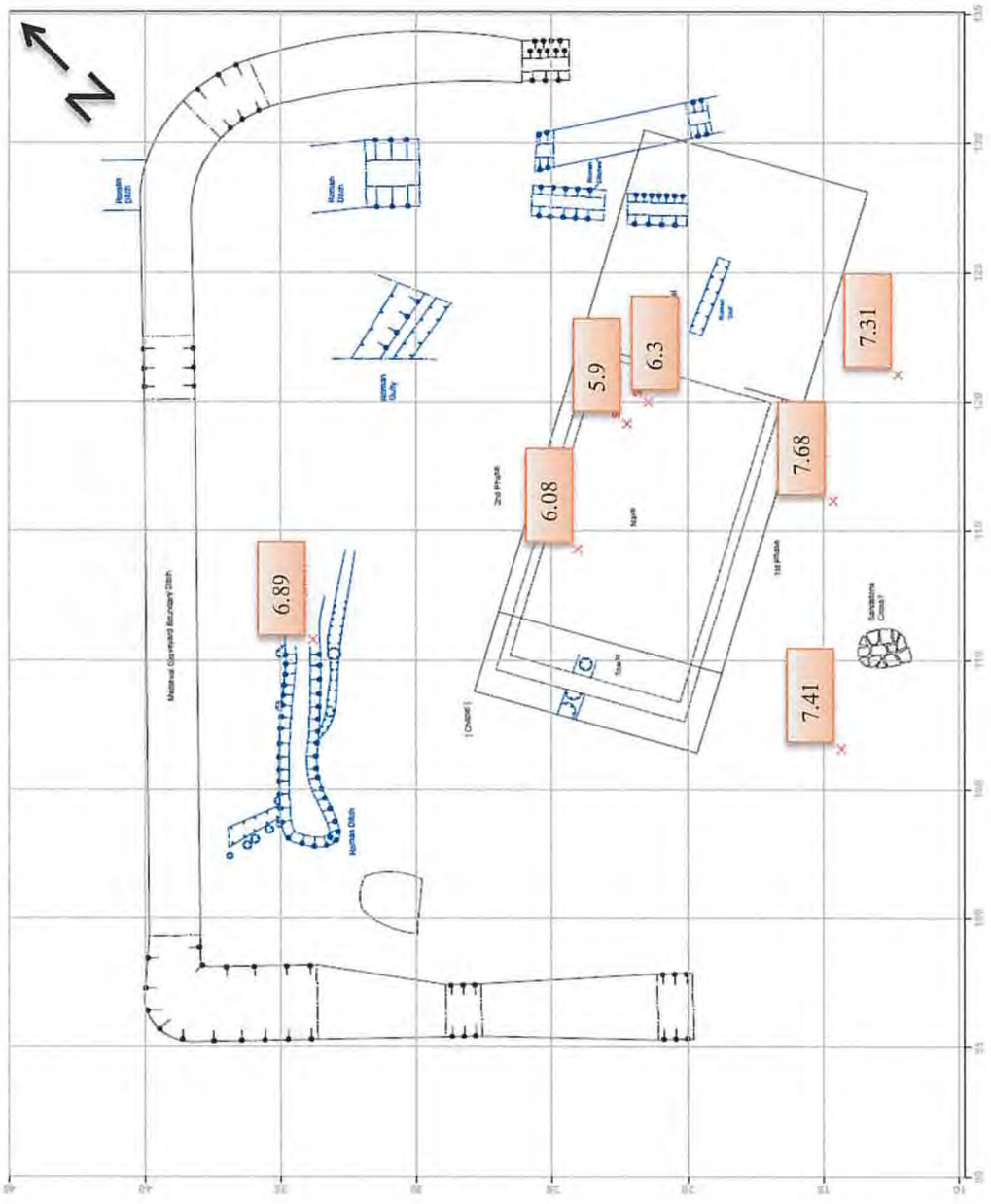


Figure 22: Mean soil pH results for Poulton cemetery and controls.

The pH results can be plotted on a map of the cemetery and used to compare to the level of bone preservation. This information could result in the development of pH soil maps which in conjunction with bone preservation information, soil intrusion and contamination maps could help to build a picture of bone condition across the whole cemetery (Figure 23). This has not been done to date due to time constraints, but would be ideal for future work.



Scale: 1square = 5 m

Figure 23: Cemetery soil pH locations denoted by x (Map courtesy of R. Carpenter and M. Emery 2012).

3.3.1. (iii) Soil Microscopy

The microscopy results demonstrate that wood burning was the main activity, not coal burning. Pottery finds are frequent from within the cemetery boundary, as this is the area subjected to excavation, but the results demonstrate that they can be present in the soil outside the cemetery boundary. The presence of bone fragments in the soil may prove helpful in assessing the fragility of skeletal remains (Figure 24).

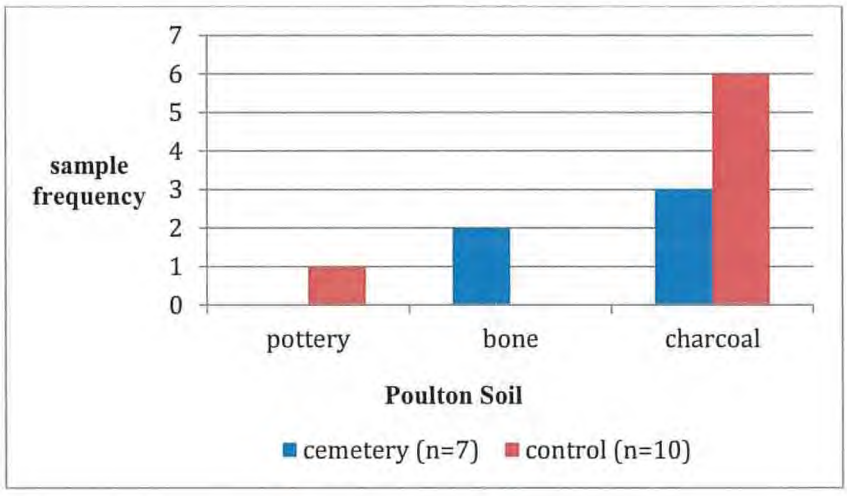


Figure 24: Anthropogenic activity levels from the soil from Poulton cemetery.

The parent material from Poulton has been described geologically, but it was necessary to verify this microscopically. Not all the soil samples contained the Till, but the quantity of sample used was very small. The soil contained a large quantity of clay. There were only a few superficial root fragments in some of the samples due to the presence of turf and not trees. The cemetery has a hedge line on the South-East side (Figure 25).

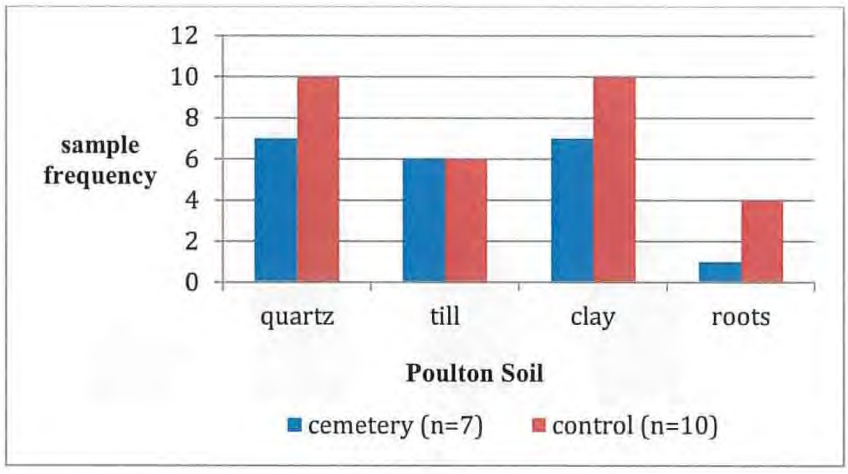


Figure 25: Parent material from Poulton cemetery.

3.3.1. (iv) Bone Soil Intrusion (XRF)

An elemental comparison was made of the stored and recently excavated bone fragments (n=3 of each, in triplicate= 15), (Figure 7). The some of the data were not normally distributed and resistant to efforts to transform them to normality, therefore a Mann-Whitney U test for independent samples was used for all the data (the non-parametric equivalent of an independent samples t-test). Tables for significance can be found in Appendix 3.

Stored and Recently Excavated Cranium:

The mean percent levels for Ca, P, Fe Zn, Pb and Sr were significantly different ($p=<0.05$). Si, Al, Mg, K, Ti, S, Mn, and Sr were not significantly different ($p>0.05$). (Figures 26 - 28).

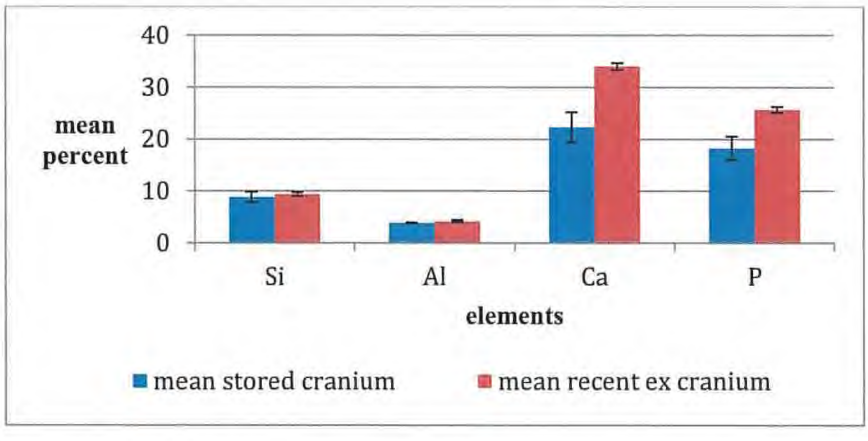


Figure 26: The mean levels of Si, Al, Ca and P from the stored and recently excavated cranial fragments.

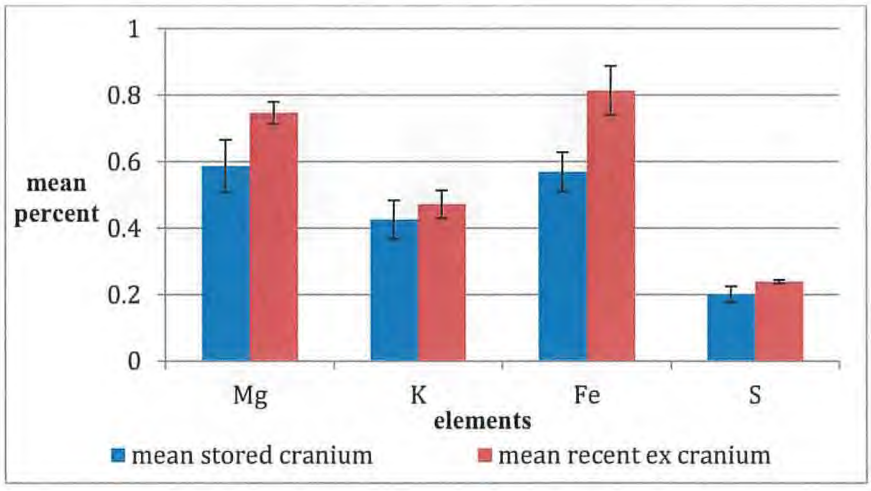


Figure 27: The mean levels of Mg, K, Fe and S levels from the stored and recently excavated cranial fragments.

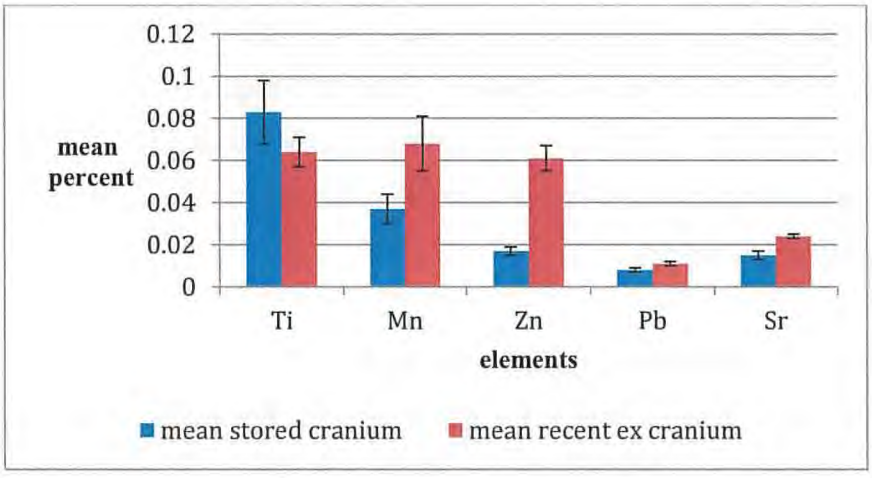


Figure 28: The mean levels of Ti, Mn, Zn, Pb and Sr from the stored and recently excavated cranial fragments.

Long bones:

The mean percent levels for Ca, P, Fe, S and Sr were significantly different ($p=<0.05$). The levels for Si, Al, K, Mg, Ti, Mn, Zn, and Pb were not ($p>0.05$). (Figures 29 - 31)

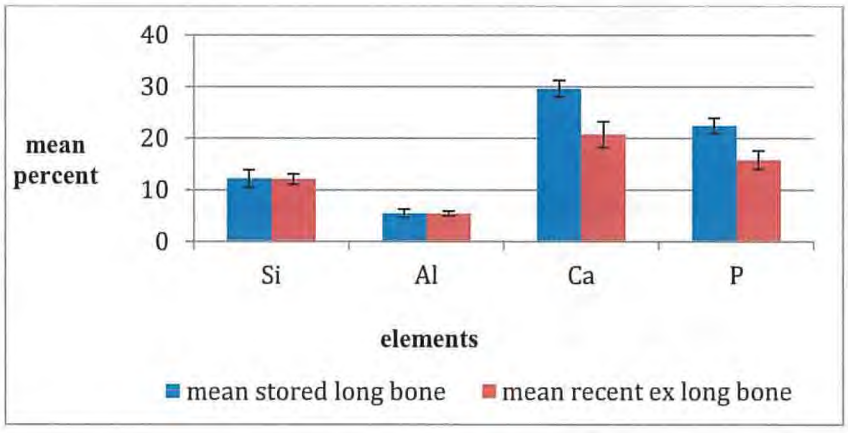


Figure 29: The mean levels of Si, Al, Ca and P from the stored and recently excavated long bones.

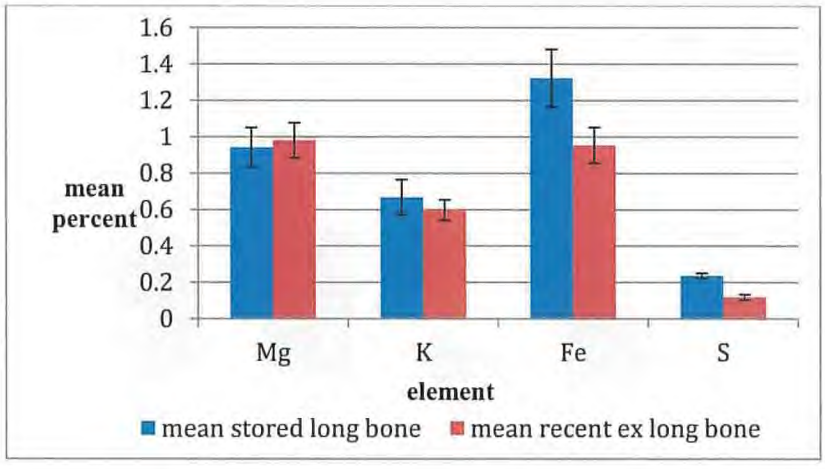


Figure 30: The mean levels of Mg, K, Fe and S from the stored and recently excavated long bones.

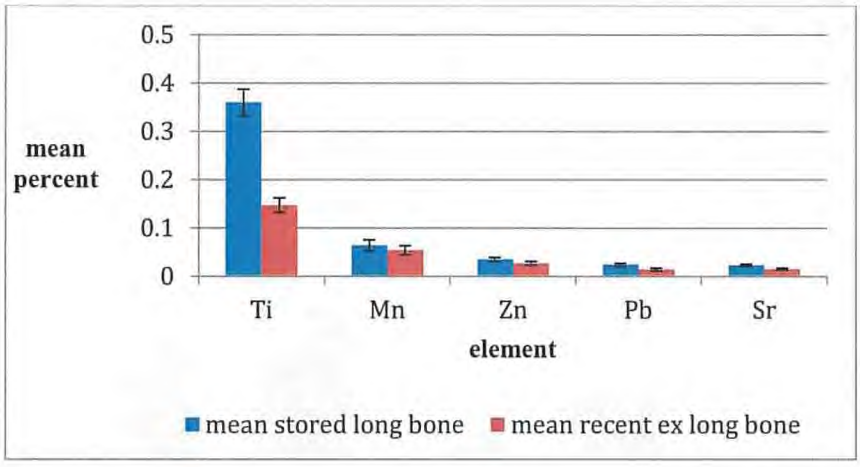


Figure 31: The mean levels of Ti, Mn, Zn, Pb, and Sr from the stored and recently excavated long bones.

Cranium vs. long bones:

The mean percent levels of the elements from the stored cranial fragments were compared to the levels of the stored long bones. Mg, Fe, Ti, Zn, Pb and Sr were significantly different ($p=<0.05$), but not Si, Al, Ca, P, K, S, and Mn ($p>0.05$). (Figures 32 - 34).

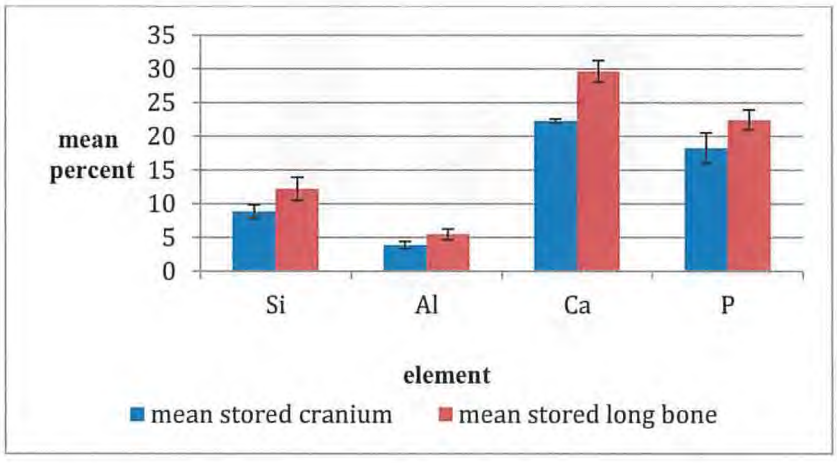


Figure 32: The mean levels of Si, Al, Ca, and P from the stored cranium and long bones.

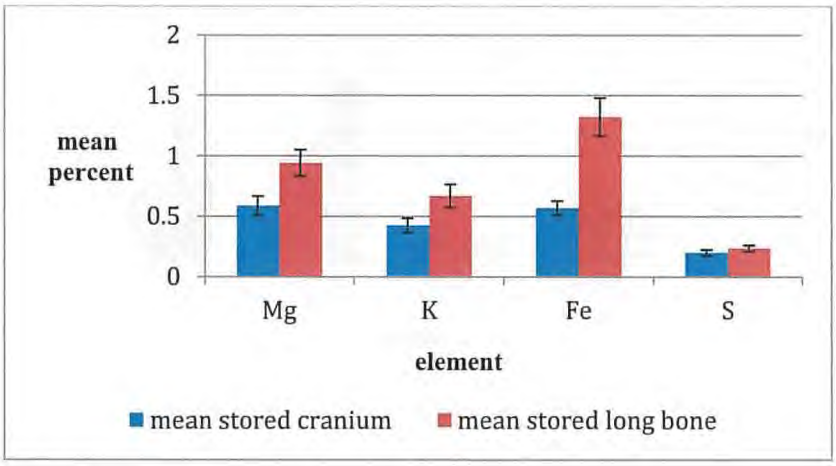


Figure 33: The mean levels of Mg, K, Fe and S from the stored cranium and long bones.

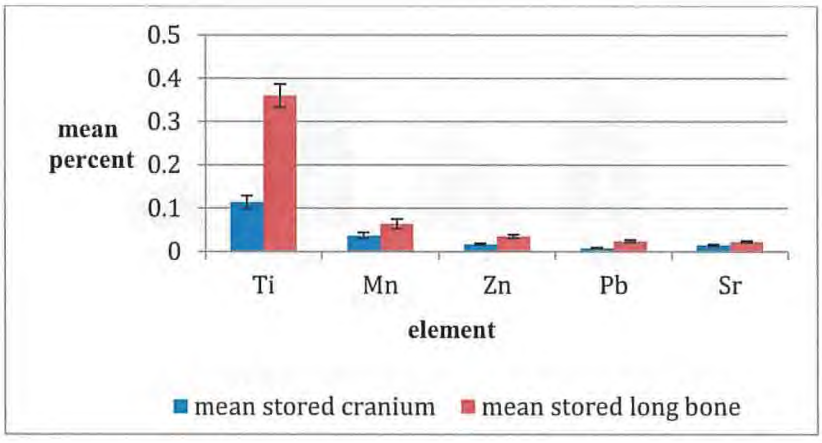


Figure 34: The mean levels of Ti, Mn, Zn, Pb and Sr from the stored cranium and long bones.

Recently excavated crania and long bones:

The elemental mean percent levels from the recently excavated cranium and long bones were compared. Si, Al, Ca, P, Ti, S, Zn, Sr were significantly different ($p=<0.05$). Mg, K, Fe, Mn, and Pb were not significantly different ($p>0.05$) (Figures 35 - 37).

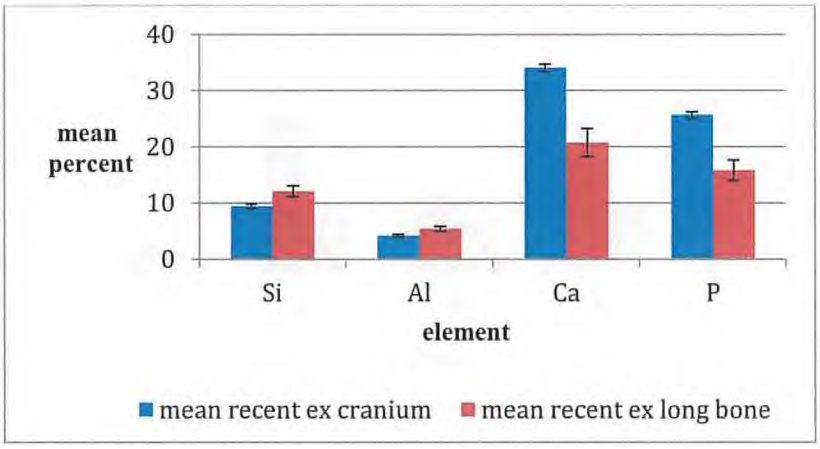


Figure 35: The mean levels of Si, Al, Ca and P from the recently excavated cranium and long bones.

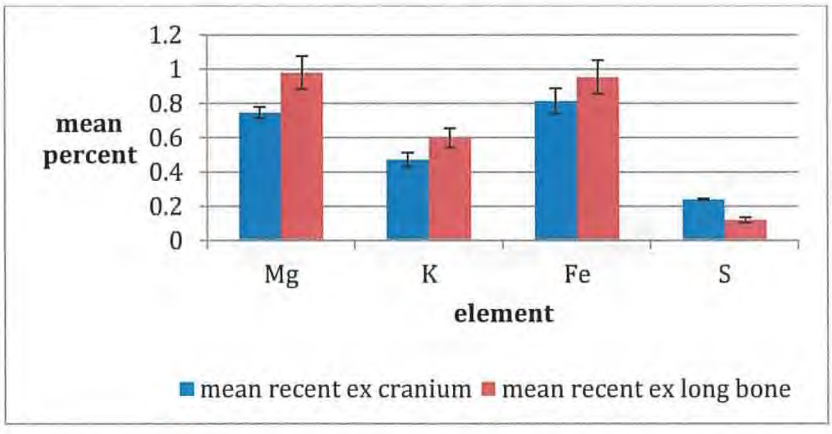


Figure 36: The mean levels of Mg, K, Fe and S from the recently excavated cranium and long bones.

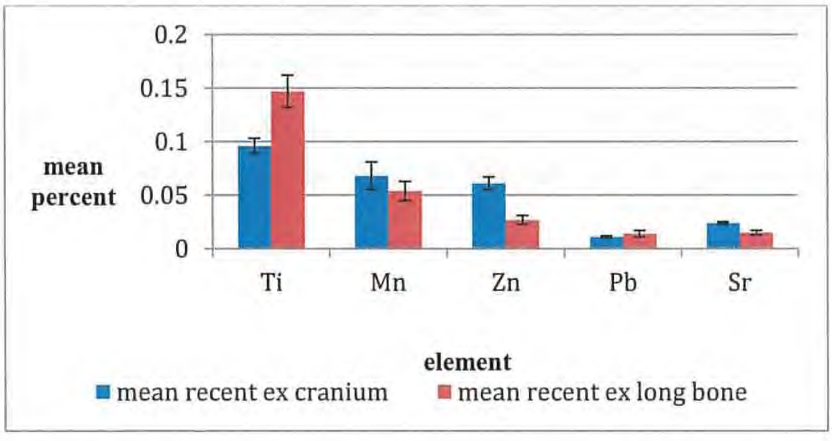


Figure 37: The mean levels of Ti, Mn, Zn, Pb and Sr from the recently excavated cranium and long bones.

Stored bones vs. control:

The mean percent elements that were significantly different were Si, Ca, P, Mg, K, Fe and S ($p =< 0.05$). The elements Al, Mn, Pb, Ti, were not statistically analysed due to the fact that these elements were not present in the pork control bone. The elements Zn and Sr were present in the in such low amounts in control bone (<=0.01%) as to make the levels unreliable for statistical testing (Figures 38 - 40).

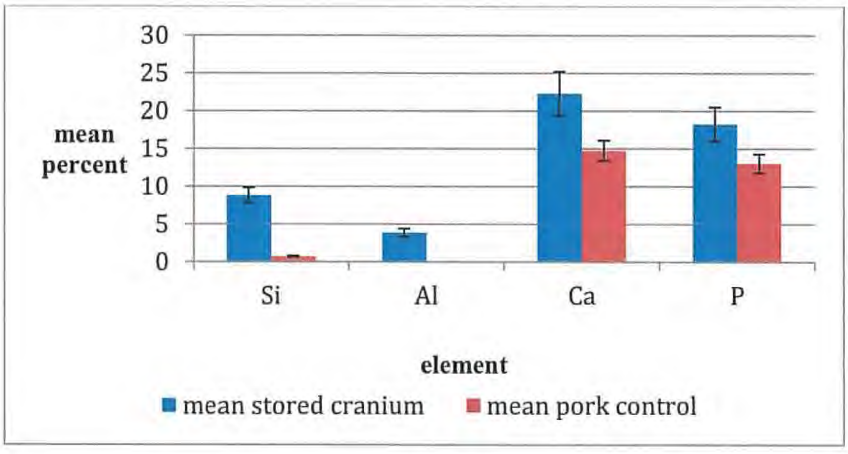


Figure 38: The mean levels of Si, Al, Ca, and P from the stored cranium and pork bone control.

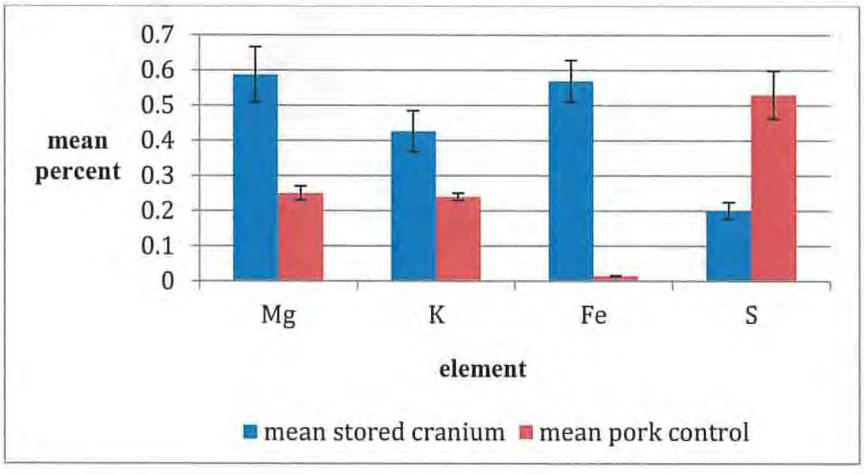


Figure 39: The mean levels of Mg, K, Fe and S from the stored cranium and pork bone control.

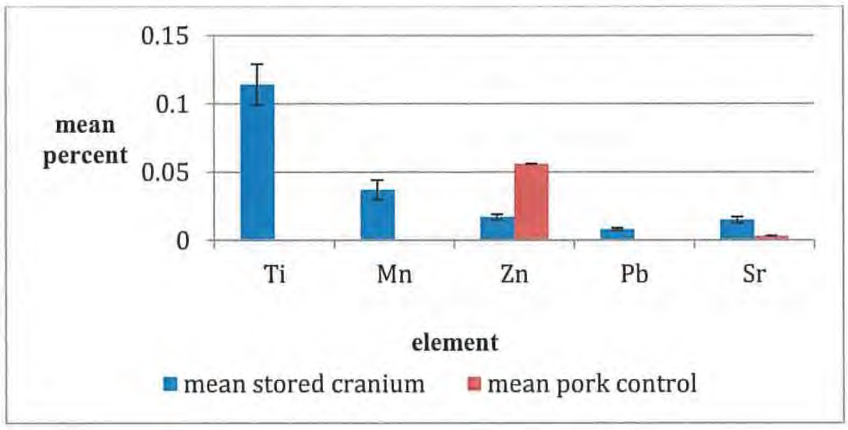


Figure 40: The mean levels of Ti, Mn, Zn, Pb and Sr from the stored cranium and pork bone control.

Stored long bone and pork control bone:

The mean percent levels of Si, Ca, P, Mg, K, Fe and S were all significantly different in the long bone compared to the control pork bone ($p<=0.05$). The pork bone control contained no Al, Mn and Pb, and very low levels of Sr (Figure 41 - 43)

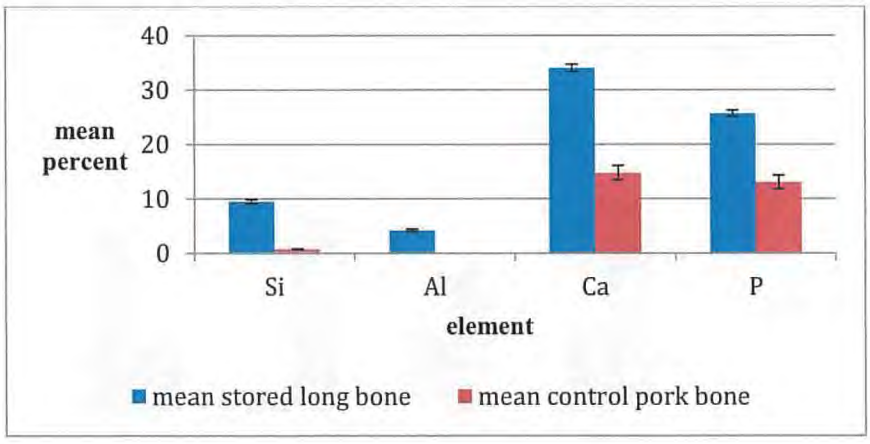


Figure 41: The mean levels of Si, Al, Ca, and P from the stored long bone and the pork bone control.

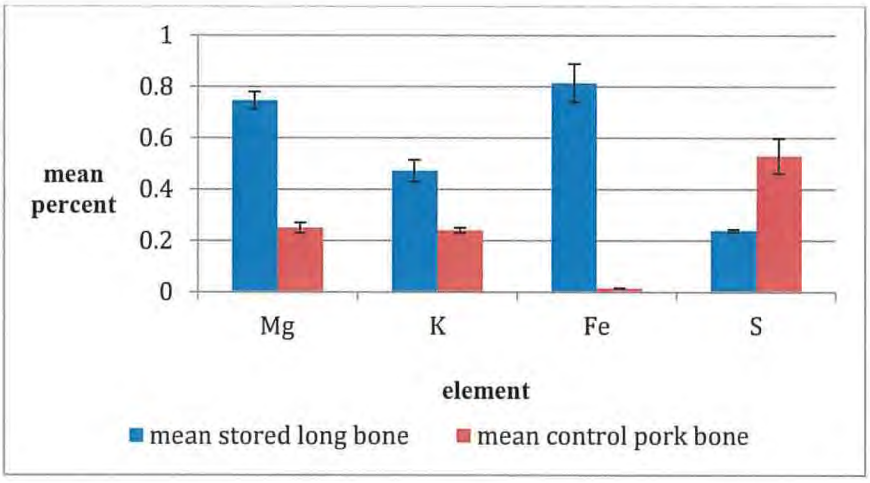


Figure 42: The mean levels of Mg, K, Fe and S from the stored long bone and the pork bone control.

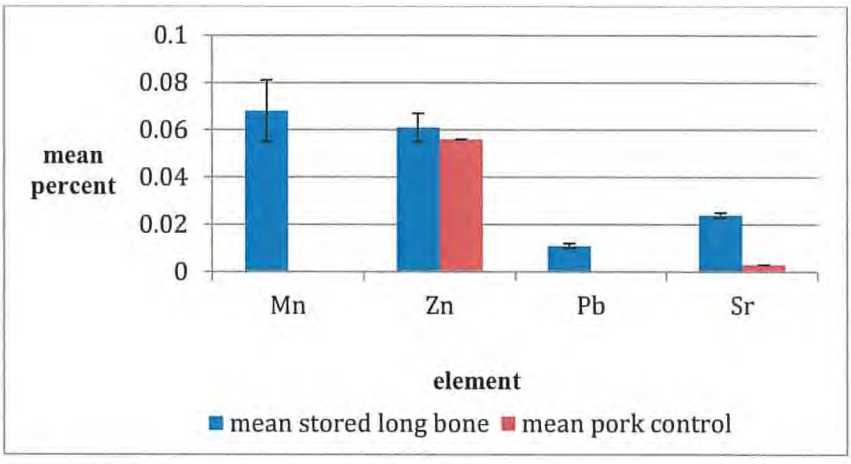


Figure 43: The mean levels for Mn, Zn, Pb and Sr from the stored long bone and the pork bone control.

Recently excavated cranium bone vs. control:

The mean percent levels for Si, Ca, P, Mg, K, Fe, and S were significantly different for the recently excavated cranium and pork control bone ($p<=0.05$). As before there was no Al, Ti, Mn or Pb in the control bone and very low levels of Sr (Figures 44 - 46).

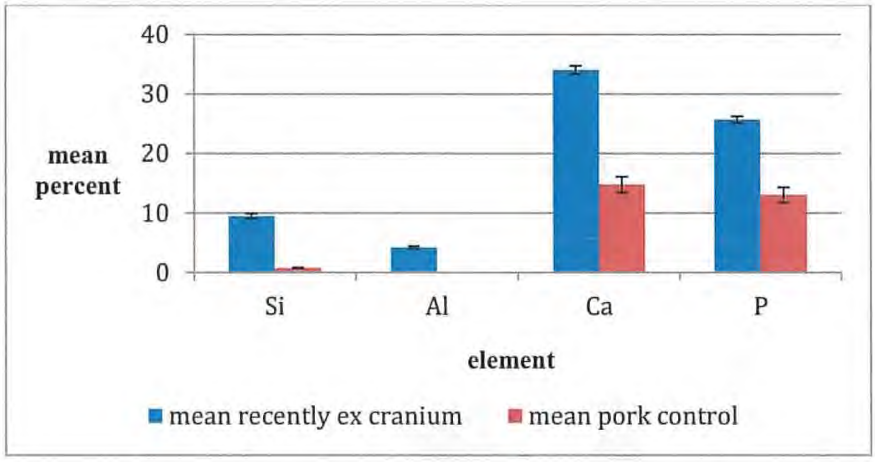


Figure 44: Mean levels of Si, Al, Ca and P from recently excavated cranium and pork control bone.

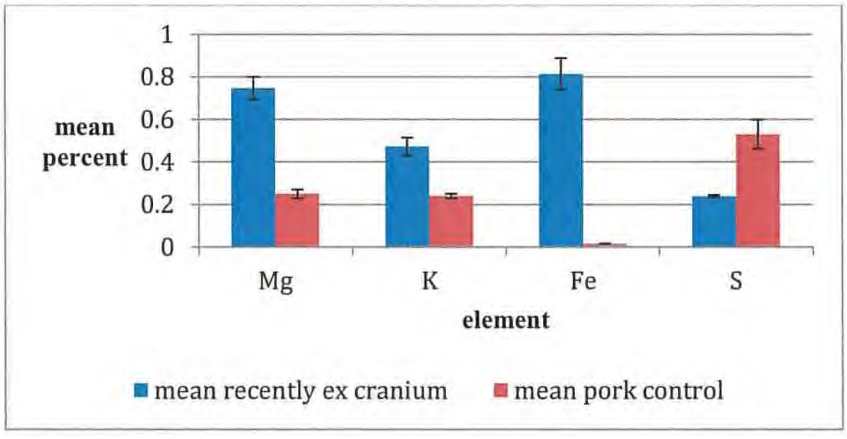


Figure 45: Mean levels of Mg, K, Fe and S from recently excavated cranium and pork control bone.

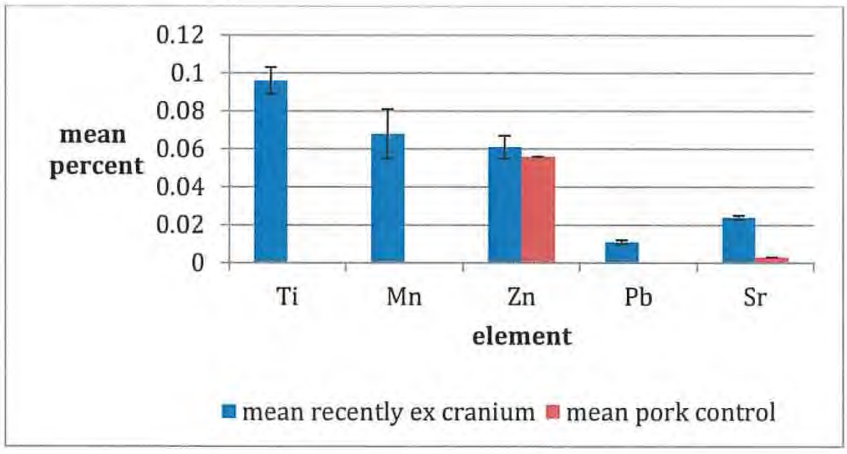


Figure 46: Mean levels of Ti, Mn, Zn, Pb and Sr from recently excavated cranium and pork control bone.

Recently excavated long bones and pork control bone:

The mean percent levels of Si, Ca, P, Mg K, Fe and S were significantly different between the recently excavated long bones and the pork control bone ($p<=0.05$). The control pork bone did not contain Al, Ti, Mn, and Pb, with very low levels of Sr (Figures 47 - 49).

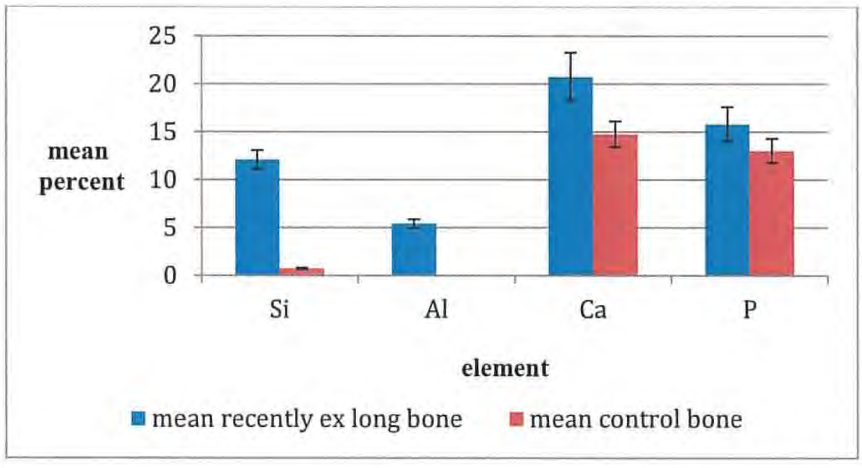


Figure 47: Mean levels of Si, Al, Ca and P from the recently excavated long bone and pork control bone.

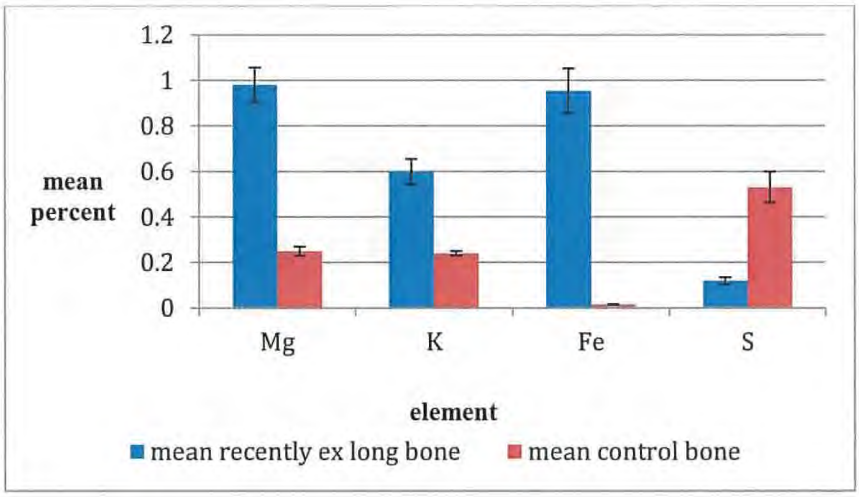


Figure 48: Mean levels of Mg, K, Fe and S from recently excavated long bones and pork control bone.

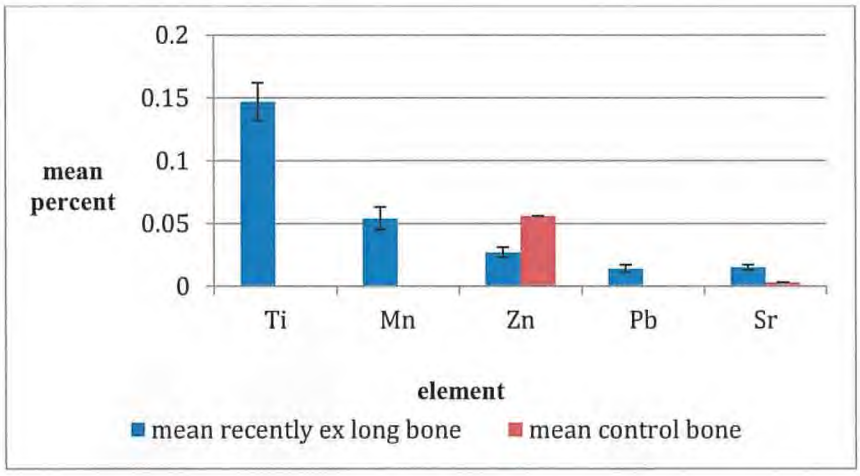


Figure 49: Mean levels of Ti, Mn, Zn, Pb and Sr from the recently excavated long bone and pork control bone.

Powdered Control Bone

The powdered pork bone gave more consistent results with a much decreased standard error than the whole bone used for this study whole bone was used specifically for this study to allow investigation using minimally destructive analysis.

3.4. Discussion

The mean levels of aluminium (Al), iron (Fe), potassium (K), calcium (Ca), barium (Ba) and sulphur (S) from the cemetery soils were significantly different from the mean levels of the control samples analysed with values of Al: 6.02 (cem) 5.31 (con), Fe: 2.21 (cem) 1.96 (con), K: 1.35 (cem) 1.6 (con), Ca: 0.71 (cem) 0.3 (con), Ba: 0.058 (cem) 0.063 (con), S: 0.024 (cem) 0.042 (con), demonstrating that the elements from bone decomposition and anthropogenic activity can potentially persist in the soil for substantial periods of time. Al and Fe are associated with the clay particles as they form part of the composition of clay compounds; however they can also be associated with activities from RAF Poulton. The activities from RAF Poulton could also account for the higher levels of lead (Pb), Ba, K, magnesium (Mg), and S, from the oils, waste and decomposing metals. Human decomposition could account for the significantly higher levels of Ca, Mg, K, and Fe in the soil, their mobility being largely pH dependant, and whilst these elements are commonly found as part of the soil matrix, the increased mean levels in the cemetery compared to the controls would indicate something other than a natural variation in the soil matrix. There is approximately between 35–45 mg/Kg body weight of iron in a normal healthy adult human individual, indicating that in a body weight of 70 kg there would be between 2.45 g and 3.15 g of iron, this would increase the iron in the soil environment considerably at a localised level (Andrews, 1999). It is not understood how these agents would support the persistence of these metal ions in the soil over a long period of time. The presence of metal chelating humic acids in the soil is one of the ways that could assist in this, especially given that the primary source of humic acids is the biological degradation of animal and plant matter, including inhumations and manuring. The land at the Medieval cemetery, has been primarily used as grazing land with sheep and cows dominating the agricultural practice. The Medieval cemetery at Poulton did not have a secure fenced area following its abandonment which would have given animals free range over the site, and was only fenced following the start of excavation work in 1995. The humic acids may have chelated with some metals to form complex compounds that would be retained within the soil and not washed way, however this usually happens at lower pH. The Poulton cemetery pH was generally more neutral (cemetery soil: pH 6.83, standard error 0.156; control pH 6.54, standard error 0.065) (Baker, 1973; Brown et al., 2000; Kerndorff and Schnitzer, 1980; Petrovic et al.,

1998). Quantifying the amount of humic acids would be one way of establishing the potential involvement of humic acids and soil metal retention.

The levels of sulphur were higher in the control samples than the cemetery, the reason for this is not clear but the sulphur may have become more mobile in the cemetery soil and leached out, in this particular study. The same could be said for phosphorus which can form highly complex compounds in the soil environment (usually metals), which can become soluble, but this is governed by soil pH (Larsen, 1967). The levels of phosphorus within the cemetery were not statistically different to the control soil samples and therefore do not explain the higher levels of metals seen in the cemetery environment. If the elemental results were due to the inhumation interaction with the soil components (mainly clay particles) then this could prove to be a useful tool in quantifying the bone/Fe elemental mobility and predicting the bone quality at a given location within the cemetery boundary. It could also assist in defining the cemetery boundary in cases where this was not clear especially where the skeletal remains were in a really advanced state of decay or dissolved away.

There was no significant difference between the pH of the cemetery soil and the control, being about neutral. The cemetery soil pH was slightly higher with greater mean and standard error than the control samples. The pH appears to be lower in the area of the chapel; this could be explained by the reduced amount of burials, or from the low pH sandstone building foundations. It would be helpful if more soil samples were analysed from the within the cemetery in order to clarify this situation. This greater variation in the cemetery pH may be key to answering some of the questions about the bone preservation being better in some areas than others, in this study.

The microscopy results were able to confirm the type of parent material, with the presence of quartz, and clay seen in all of the samples. Till (Boulder Clay) was found in 6/7 cemetery samples and 6/10 control samples. There were some root fragments found in the control samples (4/10), which would have been expected as they were sampled from the unexcavated turfed areas, and limited fragments (1/7) in the cemetery samples, probably due to the extensive excavation work that had been carried out. There were greater quantities of charcoal detected in the control samples (6/10) than the cemetery (3/7), as wood burning seemed to have been prevalent

compared to coal burning, this may have been due to the lack of industrial activity as it was primarily an agricultural area. Coal did not feature at all in the samples analysed. Charcoal burning has the additional benefit of temporarily alkalining the soil (Etiegni and Campbell, 1990), and given that burning of human remains was the preferred cultural practice up until Christianity became the dominant religion, Poulton may have been subjected to many centuries of not only pyre burning human remains, but also for cooking, smelting, and religious festivals. Cremated remains were a frequent find in the grave-fill, but did not feature in the microscopy work. Bone fragments did feature in 2/7 of the cemetery samples (0/10 in the control), and this could prove to be a useful tool in establishing bone diagenesis by observing in situ bone fragmentation. Anthropogenic activities such as pottery sherds and building materials is a common feature during excavation, but the microscopy only revealed 1/10 in the control soil and not at all in the cemetery samples.

The bone XRF results comparing the stored and recently excavated crania demonstrated significant differences in the levels of Ca: 22.3% (stored) 34.1% (recently excavated); P: 18.27% (stored) 25.7% (recently excavated); Fe: 0.57% (stored) 0.8% (recently excavated); Zn: 0.017% (stored) 0.061% (recently excavated); Pb: 0.008% (stored) 0.011% (recently excavated); K: 0.43% (stored) 0.47% (recently excavated) and Sr: 0.015% (stored) 0.024% (recently excavated), (Figures 28-30). The levels for these elements were higher in the recently excavated bone compared to the stored. In the long bones the Ca: 29.65% (stored) 20.75% (recently excavated); P: 22.46% (stored) 15.816% (recently excavated); Fe: 1.32% (stored) 0.95% (recently excavated); S: 0.24% (stored) 0.119% (recently excavated) and Sr: 0.023% (stored) 0.015% (recently excavated) (Figures 29-31) were significantly different, with higher levels in the stored compared to the recently excavated bone. This discrepancy could be due to the effects of the bone drying over time, or the crania beginning to degrade. The results for recently excavated crania and long bones were compared, where the levels for Si: 9.42% (recently excavated cranium) 12.1% (recently excavated long bones); Al: 4.19% (recently excavated cranium) 5.42% (recently excavated long bones); Ca: 34.04% (recently excavated cranium) 20.75% (recently excavated long bones); P: 25.68% (recently excavated cranium) 15.82% (recently excavated long bones); Ti: 0.096% (recently excavated cranium) 0.147% (recently excavated long bones); S: 0.239% (recently excavated cranium) 0.119% (recently excavated long

bones); and Sr: 0.024% (recently excavated cranium) 0.015% (recently excavated long bones) were shown to be significantly different (Figures 35-37). The levels for the elements Si, Al, Ti were higher in the long bones than the crania, demonstrating the high level of soil intrusion into the bone, with its open textured trabecular bone acting as pockets to retain the soil products. The levels for Ca, P, Zn, S and Sr were higher in the crania than the long bones, and as these form the major components of bone they could show either greater soil component intrusion or better rates of preservation and resistance to soil intrusion due in part to containing less trabecular bone. This could have implications for choosing the best bones for future DNA analysis. The Sr levels were higher in the crania than the long bones, which may provide useful information for Sr isotope analysis. In the stored bones the long bones had significantly higher levels of the elements than the crania (Figures 32-34), this fits in with the idea that freshly excavated bones are best for DNA analysis (Pruvost et al., 2007). The Pb and Fe levels were not significantly different, but were higher in the long bone compared to the cranial fragments, this information is important for contamination sensitive analysis such as the polymerase chain reaction used in DNA studies, and for also understanding the rate at which each bone type may sequester and retain lead and other heavy metals during the life-time of the individual. During the processing of healthy bone for DNA study the normal non soil intruded bone components (Ca, P, with low levels of Mg, K, Zn, S, with trace amounts of Fe, Si and Sr) do not cause significant problems with the PCR, therefore these normal components can be used to assess the quality of the bone. Knowing the degree of contamination from soil is important as there can be problems of inhibition of the PCR from soil products, particularly metals and the complex organic humic acids, and can influence the methods used to process the bone sample for DNA study. Methods that remove these contaminants may significantly improve the PCR amplification results (Matheson et al., 2009).

The levels of Si, Fe, and Sr appeared in trace levels in the pork control bone. Zn, Pb, Sr, Al, Mg, Ti and Mn appeared in very low quantities as to make statistical analysis unreliable or unnecessary as the amount = 0% was represented in the sample, Al, Ti, Mn, and Pb did not appear at all in the pork bone control. The Poulton bone suffered major soil intrusion that extended through the bone not just on the surface, accumulating into all the available spaces making removal more challenging (Lambert

et al., 1989). The major components of bone represented in the pork control were Ca: 14.77%; P: 13.04%; Mg: 0.25%; K: 0.24% and S: 0.53%. It was useful to compare the Poulton bone to an uncontaminated control bone in order to assess the degree of contamination or diagenesis (Figures 38-49). The degree of soil intrusion into the Poulton bone included the very small particles associated with the clay parent material, by comparing this bone to the control bone components it is possible to determine the mobility of the major and minor components of bone and soil. Levels of Si, Al, Ca, P, Mg, K, Fe, Ti, Mn, Pb and Sr were higher in the Poulton bone than the control showing that a certain proportion of the elements came from the soil, this differed somewhat from other work done previously, demonstrating the differences that can occur under different environmental conditions (Lambert et al., 1985b). The S and Zn were higher in the control, demonstrating how the bone components can be also be mobilised out of the bone. The higher levels of Pb/Sr in the Poulton bone demonstrated that some were soil derived which could have implications with contamination for future Pb/Sr isotope testing. The differences in the long bones and the crania may be explained by their different development pathways, as the long bones develop via endochondrial ossification and the crania via intramembraneous ossification.

3.5. Conclusion and Further Work

Due to the increased variation in the pH of the cemetery soil it may prove helpful to plot the pH across the whole area of the cemetery and link this to the mobility of the bone elemental ions and to bone preservation.

Bone preservation, including bone soil intrusion could be plotted on a map linked to the pH and elemental mobility to identify the rate of bone diagenesis, but also the best preserved bone samples for future study.

The powdered pork bone gave more consistent results with a decreased standard error than the whole bone used for this study. It may be useful to use powdered bone samples (both analytical and control) where it is feasible to do so, but it may not be possible in all cases where the sample size is very small, precious or precluded from destructive analysis.

Routine XRF analysis of the bone and soil prior to DNA study could help in the assessment and choosing the most suitable bone for study but also the level of soil component contamination within the bone. The routine provision of soil samples surrounding the bone would also allow destructive analysis to be done without harming the bone sample. Studies to evaluate levels of metal ion contamination that would potentially inhibit the PCR would be helpful. Spiking modern contemporary DNA with known concentrations, and choosing the more metal resistant polymerase enzymes for the reactions could assist with this.

There were higher values of the major components of bone in the recently excavated bone compared to the stored bone in the crania and long bones (Si, Al, Ca, P, Mg, K, Fe, Ti, Mn, Pb,S and Sr) bones, and requires much further detailed analysis in order to clarify the role of diagenesis in stored and newly excavated bone. The role of S and Zn in the bone degradation pathways also warrants further investigation. The age, sex, and health of the individual may have had an impact on the different bones, as well as the different anatomical and development mechanisms, this would require further study.

Chapter 4: The Organic and Inorganic Phases of Soil Intruded Bone From Chapel House Farm.

Objectives

The objectives of the study were to.

- Investigate the relationship between the calcium and phosphorus in the cranium and long bones, using a commercial bone standard and contemporary pork bone to compare to both recently excavated and stored bones from Chapel House farm. This analysis could enable the assessment the degree of involvement of these ions in the preservation and degradation pathways of archaeological bone, using XRF (X-ray fluorescence) and FT-IR spectroscopy (Fourier transform infrared spectroscopy).
- Analyse the collagen content of the bone using FT-IR spectroscopy.
- Identify the major soil components from inside and outside the cemetery from Chapel House Farm using FT-IR spectroscopy and attempt to quantify them using a commercial humic acid standard reference sample.
- Assess the condition (splitting factor, calcium and phosphate ratios) of the apatite matrix using FT-IR spectroscopy, by comparing and contrasting the use of the splitting factor and calcium and phosphate ratios.

4.1. Introduction

The survival of organic matter in bone is of crucial importance to the investigation of paleodiets, DNA and C^{14} dating, but can be commonly complicated by contamination of the bone by components from the interment soil. Bone can appear to be in very good condition; particularly histologically, but present difficulties with detecting useable organic bone components (especially those that have been stored at room temperature and above, or subjected to repeated freeze/thaw for some time post excavation). Degradation mechanisms of the organic component of bone are poorly understood, which can make choosing archaeological bone for study difficult as it can be hard to determine which bone is likely to give the best outcome for organic analysis.

Human bone in apparently visibly good state of preservation and soil samples from the medieval cemetery located at Chapel House Farm, Poulton, Cheshire, UK, were used for analysis (20 bone samples as per Chapter 3).

4.1.1. Collagen Composition

Collagen forms the organic part of the bone matrix and is responsible for giving bone its tensile strength, whilst the mineral component provides compressive strength (Trueman and Martill, 2002). Bone develops either by endochondrial growth, at the ends of the bones, such as the long bones of the femur; or by intramembraneous ossification from small islands of bone within muscle, such as the cranial bones (Gilbert, 2003). Collagen accounts for 22% of weight and 36% of volume in compact bone (Collins et al., 2002). It is a complex highly stable protein polymer, comprising of long alpha and beta chains arranged in a helical format; the precise chemistry varies depending on the form and function. This large molecule consists largely of 4-hydroxyproline, the helical structure being held together with hydrogen water bridges to the peptide backbone (Miles et al., 1995). In bone the collagen fibrils form a composite matrix that facilitates the elastic properties required for load bearing and resistance to fracture (Burr, 2002). This strength is derived mainly from the extracellular, intermolecular, covalent crosslinks, in both transverse and lateral formats within the bone matrix. These systems have been shown to change as the bone matures, and have been the subject of recent study due to their role in bone health (Burr, 2002; Knott and Bailey, 1998; Barnard et al., 1987). Collagen is secreted by the osteoblasts and may exist in both mineralised and non-mineralised forms in the healthy body (Jae-Young et al., 1998).

There are many types of collagen, the most common type to be found in bone is Type I, and is arranged into long fibrils that are protected by a network of 50 x 25 x 2-3 nm carbonated calcium phosphate (apatite) plates, known as mineralisation, in a trimeric form (Trueman et al., 2008; Turner-Walker, 2008; Collins et al., 1995). The embedding of collagen in the apatite plate matrix provides a mutually thermal and solubility protection facility in live bone, but renders collagen ultimately sensitive to hydration concentration in both live and dead bone, that can cause degradation from the conversion of insoluble collagen to soluble gelatin (Miles and Avery, 2011; Miles

and Ghelashvili, 1999; Flory and Garrett, 1958). In bone the organisation of the collagen fibres will largely depend on their function; either planar sheets called lamellae that can also form concentric cylinders called osteons (Haversian systems), or indistinct forms resulting in woven bone (Figure 1). These arrangements can be found in both compact and cancellous bone as well as bone that is in transition between the two (Tzaphidou, 2008; Jae-Young et al., 1998). The organic part of the bone also consists of non-collagenous proteins such sialoprotein, osteocalcin, Gla-protein, phosoproteins, osteonectin, proteoglycans, albumin, and a_2 HS-glycoprotein; other non-collagenous proteins exist in very minute quantities, and demonstrate the very complex suite of agents responsible for maintaining healthy robust bone (Triffit, 1987). In fibres the collagen polymer is thought to be thermally stabilized by the "polymer in box" theory, in that the collagen polymer is constrained by the adjacent molecules (Miles and Ghelashvili, 1999).

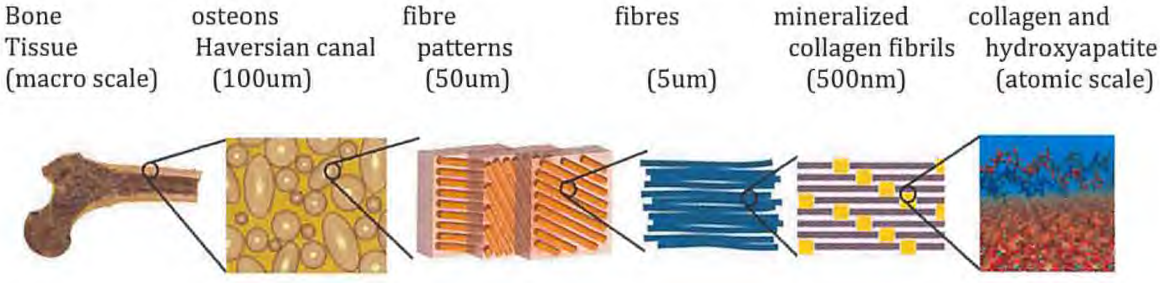


Figure 1. Bone collagen structure and formation (adapted from Nair et al., 2013).

4.1.2. Bone Collagen Degradation

The crystalline apatite and collagen matrix provides mutual protection from degradation in dead bone (Trueman and Martill, 2002). Environmental conditions can result in bone undergoing change at the point of exposure to the decomposition processes. Bone is a highly vascular tissue with a network of blood vessels running through it, following the decomposition of the soft tissues these networks leave a series of pores throughout, creating a pathway for environmental agents to penetrate (Trueman and Martill, 2002).

Warm temperatures, environmental chemistry, pH and animal destruction; the effects of water from both precipitation and water table fluctuations can be responsible for dissolution of the mineral apatite by chemically available water. This water can also cause contamination of the bioapatite and/or rapid bone degradation (Carvalho et al., 2004, Trueman et al., 2004; Kruegar, 1991; Hedges, 2002; Piepenbrink, 1989). Microorganisms are also considered to be responsible for bone damage, the most important of these is thought to be collagenase producing microbes, from environmental bacteria and fungi (Zapata et al., 2006; Jans et al., 2004; Hedges, 2002; Trueman and Martill, 2002; Turner-Walker and Syversen, 2002; Child, 1995a, Collins et al., 1995, 2002; Grupe, 1995; Grupe and Piepenbrink, 1989; Piepenbrink, 1989, 1986; Bond and Van Endt, 1984; Ortner et al., 1972).

Bell et al. (1996) found bone that had been exposed to the environment had undergone micro-structural changes due to the actions of bacteria as little as three months post-mortem. This visible damage was first identified by Wedl using a light microscope in the Nineteenth century taking the form of tunneling and erosion of the bone matrix (Hackett, 1981). This phenomenon was later described as microscopic focal destruction (MFD) and formed part of the Oxford Histological Index (OHI) scoring system for recording bone condition (Hedges, 1995). Microbes were thought to be one of the principle causes of decomposition of bone and collagen (Dixon et al., 2008; Jans et al., 2004; Child 1995a), mostly from the collagenase enzymes that demineralize the bone and degrade the collagen, and by the production of an acidic microclimate. Jans et al. (2004) were able to describe the surface damage seen on bone (Figure 2), and classify it according to the type of features seen. Another hypothesis is that the destruction follows the degradation of collagen into gelatin (Bond and Van Wart, 1984). In vivo experiments have demonstrated the devastating effects of collagenase producing microbes on bone, but it has not been clarified that these would exist in any great numbers to cause significant damage in the natural decomposition environment. The microbial destruction of the collagen has still not been fully understood as it has been considered that the collagen and apatite matrix molecules were too large to allow the collagenase enzymes to penetrate initially and that other agents had to be responsible (Hedges, 2002; Turner-Walker and Syversen, 2002).

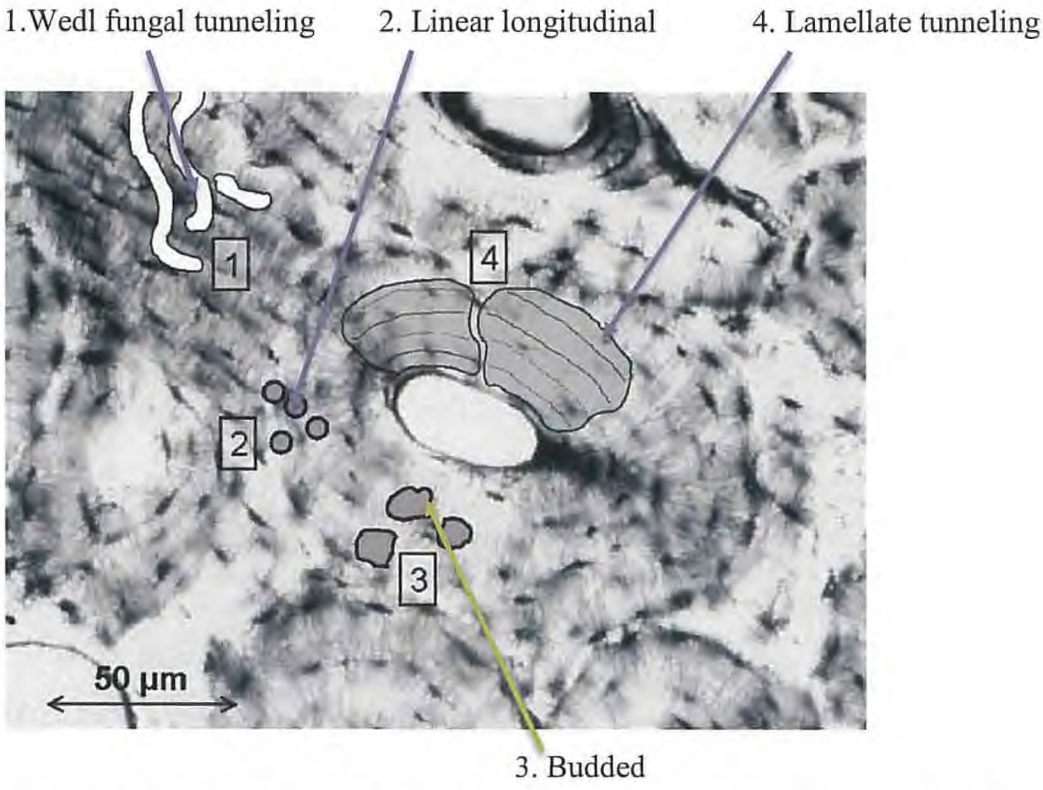


Figure 2: Schematic diagram of the types of MFD observed in bone (adapted from Jans et al 2004).

4.1.3. Humic Acids and Bone Interaction

Humic acids belong to a large a group of complex aromatic chemical compounds that also include fulvic acid and humin. They are derived from the decomposition of biological organisms, including plants, vertebrates, invertebrates, and by the action of microorganisms; they have a high affinity for chelating with metals to form large complex compounds (Perez et al., 2008; Mao et al., 2008; Perez et al., 2004; Haberhauer, 1998; Baker, 1973). Humic acids are thought to have a primary role in the colouration and darkening of the soil (Kumada, 1965). The relationship between bone proteins and humic acids in the burial environment is still not fully understood, except to say that humic acids can enter the bone via the bone-pore/soil-water exchange (Hedges, 2002; Pate and Hutton, 1988). Due to the acidic nature of humic acids, human remains found in humic-rich soils such as peat-bogs can more often than not result in the soft tissues and bone collagen being found in a good state of preservation to the detriment of the dissolved apatite, the bone being reduced to a soft "plastic" state (Pyatt et al., 1991).

Experiments on Type I collagen have shown a pronounced affinity with humic acids, with saturation measured in hours, and some thought to the provision of a degree of protection of the collagen through the formation of chemical cross-links (Van klinken, 1999; Van Klinken and Hedges, 1995; Tuross, 1994). These experiments had been conducted on extracted Type I collagen, not on composite bone, with the extent of the mutual protection of the collagen-apatite matrix not quantified. Few bone studies have considered investigating bone mineral and collagen from a soil contamination point of view, in order to quantify each component and move towards an understanding of how the burial environment interacts directly with the bone mineral and collagen.

4.1.4. Collagen Studies

Past collagen studies have tended to focus on the field of disease research (Miles and Avery 2011; Skrzynski et al., 2009; Tzaphildou, 2008; Fantner, 2004), with a some focus directed at the analysis of forensic and archaeological bone collagen (Trebacz and Wojtowicz, 2005; Lozano et al., 2002, 2003; Nielsen-Marsh et al., 2000; Villanueva et al., 1976). Thermal analysis studies have been used to explore the properties of collagen as well as for quantification and quality assessment. Exposure to heat, the burial environment, and bone collagen extraction methods commonly change its form, making investigation of its properties more challenging (Chadafaux et al., 2009; Neilsen-Marsh et al., 2002; Roberts et al., 2002).

4.1.5. Bone Quantitative X-ray Fluorescence

Quantitative XRF differs from semi-quantitative in that all the elements programmed for will be fully quantified, and expressed as a percentage, but will not quantify elements it was not programmed to look for. The instrument is programmed using an industry-recognised standard and calibration curve, instead of a block of aluminium used in semi-quantitative analysis, making it a very accurate non-destructive analytical tool. Whole, reasonably sized bone samples can be used, but it can be better to use a powdered pressed pellet, as this will provide a consistent homogenous sample. Quantitative XRF has been used for assessing the mineral component of bone and other archaeological samples (Janos et al., 2011, Carvalho et al., 2004; O'meara et al., 2001), and its place in the development and understanding of the bone matrix, especially Ca/P ratios, is under-explored.

4.1.6. FT-IR Spectroscopy

Fourier transform infrared spectroscopy is a routine method of identifying functional chemical groups, which give rise to characteristic absorptions (Housecroft and Constable, 2006). An IR spectrum measures the energy at which a particular molecular group absorbs infrared radiation; this absorption is usually recorded in wave numbers. Each wave number is associated with a vibrational mode. The Fourier transform involves the use of a mathematical algorithm, which transforms the raw data into the spectrum. As the strength of the absorption is proportional to the concentration of the sample being analysed it can be used for quantitative analysis, this is assisted further by the use of an approved fully quantified industry standard.

FTIR spectroscopy has been used in analysis of a variety of composite samples, including archaeological bone and soil samples. It has been used for the assessment of both bone mineral and collagen. The usefulness of this technique lies in the fact that it can be used for multiple assessments as the spectra scans multiple vibrations and requires only a very small amount of the target sample (usually 2-3mg), mixed and pelleted with KBr, making it ideal for minimum destruction of precious or limited samples. The most useful of these investigations have been the splitting factor (SF), also known as the crystallinity index (CI), calcium/phosphate ratios and collagen quantification, but it was also important to examine the extent of humic acid and clay intrusion into the bone from the burial environment to investigate the interaction between the soil and bone components (Tan, 1992). Cooking and exposure to the environment has been shown to change the collagen, and further investigation of the soil components may give some indication of change in the target samples being used (Chadafaux et al., 2009; Neilsen-Marsh et al., 2002; Roberts et al., 2002).

(i) Splitting Factor:

In fresh bone the apatite crystals are very small and plate shaped; during exposure to the soil water solution these crystals dissolve and then recrystallize, but undergo changes to larger needle-like structures with reduced solubility compared to the fresh bone (Berna et al., 2004). The changes to the crystalline structure of the bone are termed the "crystallinity index" (CI) "splitting factor" (SF), or the "infrared splitting factor" (IRSF), depending on the author and are determined by using FT-IR spectroscopy. This can be seen in the FT-IR spectrum as a sharpening of the two

absorbance peaks at 603cm^{-1} and 565cm^{-1}. The splitting factor can vary from about 2.5-2.8 in fresh bone to 7 in severely degraded bone (Berna et al., 2004; Steiner and Kuhn, 1995). The increase in this factor is associated with bones that are in the "recrystallization window" in that they are closer to the dissolution risk (at the boundary between recrystallization and dissolution), and represent the crystals becoming larger and more ordered. At an environmental pH of less than 7 (acidic), bone mineral dissolves (Weiner and Bar-Yosef, 1990). The calculation value is derived from a baseline drawn from 750-495cm^{-1}, the heights of the peaks (A and B) as well as the distance of the baseline to the lowest point between them (C) (Surovell and Stiner, 2000; Wright and Schwarcz, 1996; Weiner and Bar Yosef, 1990), this is illustrated in Figure 3.

Splitting Factor = A+B/C

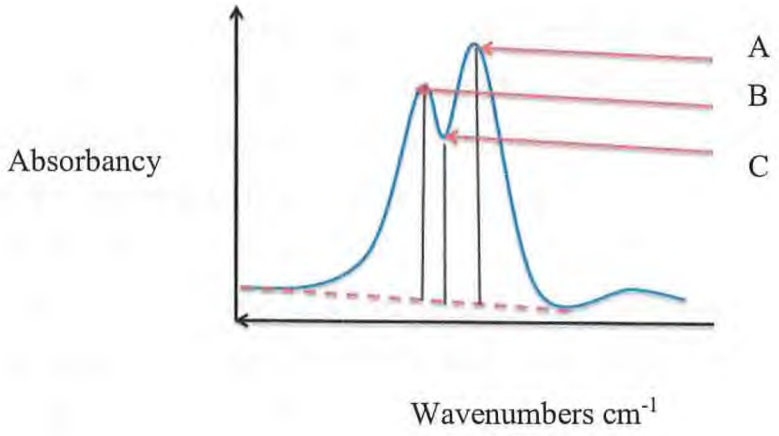


Figure 3: A schematic representation of the FT-IR spectra output, demonstrating the splitting factor calculation (Surovell and Steiner, 2000, Weiner and Bar-Yosef, 1990).

Other authors have used alternative calculations, 1030 cm^{-1}/1010 cm^{-1} (Alvarez-Lloret, 2006), 874 cm^{-1}/565 cm^{-1} (Steiner et al., 1995), 1415 cm^{-1}/874 cm^{-1} (Wright and Schwarcz, 1996), where the mineralised tissue was altered by environmental conditions, or by the use of turtle shell, not bone. The protocol and methodology of Weiner and Bar-Yosef (1996) is still used to calculate the SF in most bone samples,

mostly due to the fact that the 565cm^{-1}/603 cm^{-1} occupies the part of the spectrum least affected by contaminating exogenous elements.

The SF can be affected by degradation of the matrix by factors such as heat, environmental water, uptake of elements from the environment, organic rich soil, sandy soil, and soil component intrusion or preserved by processes such as fossilization, dry environments such as caves, or soils that are more impervious to water penetration such as clays (Lebon et al., 2008; Olsen et al., 2008; Trueman et al., 2008; Roberts et al., 2002; Berna et al., 2004, 2000; Weiner and Bar-Yosef, 1990; Weiner and Bar-Yosef, 1990).

(ii) Calcium and Phosphate Ratios:

The calcium (carbonate)/phosphate ratio has been used to explore the relationship between the organic (collagen) and inorganic (mineral) phases of bone, and in particular in respect of bone condition (Olsen et al., 2008). The ratios can be calculated using either FT-IR spectroscopy or by quantitative XRF (Carvalho, 2004) the ratio for FT-IR is normally 0.31-0.61, decreasing with degradation (Olsen et al., 2008). The calcium carbonate gives peaks at 710, 874, 1415 and 1540 cm^{-1} and the phosphate at 565, 603 and 1035 cm^{-1}. Olsen et al. (2008) used 1415/1035 cm^{-1}, Trueman et al. (2008) used 1540/605 cm^{-1}and 1455/1415, whilst Stiner et al. (1995) used 874/565 cm^{-1}, avoiding the use of 1415 as this peak was associated with amide (collagen) group and could lead to confusion.

FT-IR spectroscopy, allows peaks of the different elements to be uniquely identified, but they can be over-ridden by contaminating elements. The carbonate peak at 710 cm^{1} can be associated with calcium carbonate contaminants, which whilst being helpful for assessing the level of contamination in the bone, would not be chosen for calculating the Ca/P ratio.

Body composition studies have recorded body composition elemental levels, by either weight or by percent (Wang et al., 1992; Forbes et al., 1953). These body composition studies recorded Ca and P ratios as 1.72 by weight and 2.31 by percent. Forbes (1953) would be used for XRF of bone components as these were routinely recorded as a percent.

(iii) Collagen Quantification:

The collagen (protein) content of bone can be quantified using standard extraction techniques, however this can alter the form of the collagen making further study difficult. Things can be further complicated by the contamination with exogenous components. FT-IR has been used to assess the collagen component using the phosphate peak at 1030 cm^{-1}, and amide peaks at 1539 cm^{-1}, 1650-1653 cm-1, and using the ratios of carbonate 1455 cm^{-1} and amide 1415 cm^{-1} (Thompson et al., 2009; Trueman et al., 2008; Berna et al., 2004; Stiner et al., 1995; Weiner and Bar-Yosef, 1990). Curve fitting technology has been used to investigate degraded bone, however a major flaw with previous studies has been the lack of comparison with known levels in contemporary bone, this being essential for the understanding of how the collagen's changes manifest themselves and the understanding of the relationship with the other bone components (Chadefaux et al., 2009; Lebon et al., 2008).

(iv) Humic Acid and Clay Bone Intrusion:

Humic acids and clays have been characterized using FT-IR (Gonzalez-Perez et al., 2008, 2004; Mao et al., 2008; Madjova, 2003; Haberhauer et al., 1998), but little work has been done using soil intruded bone to investigate the effect that overlapping of the curves can have on quantification of the bone elements.

4.1.7. Chapel House Farm Medieval Cemetery

The remains from Chapel House Farm, Poulton, Cheshire, provided a unique opportunity to assess bones from one site that outwardly present themselves in various states of preservation depending where in the cemetery they were interred. Some of the remains have spent a decade or so in storage at room temperature, whilst others had only recently been excavated.

The parent material consists mostly of clays, mainly Irish till and sand, but there was a trench that contained a Roman midden with large volumes of biological material.

4.2. Materials and Methods

4.2.1. Soil Analysis

Soil samples from a previous study of Chapel House Farm were used for study (Chapter 3: Figures 5 & 6). The main components of interest were the humic acids and clays, and their contribution to the FT-IR spectra. Humic acids had been considered to form part of the dark colour contained in the humus of the soil. Chapter 1 had been able to quantify the humic acids using the nanophotometer, but colourimetric studies had not proven useful and it was considered that clarification of the humic acid content of the soil using another method would be appropriate.

2 samples from outside the cemetery boundary (Control soil: Chapter 2, Figure 8):

1 thought to be "low" in humic acids, being paler in colour (Control 4)
1 thought to be "high" in humic acids, being darker in colour (Control 1).

2 samples from inside the cemetery (Chapter 2, Figure 7):

1 thought to be "low" in humic acids, being paler in colour (Cemetery context 2399)
1 thought to be "high" in humic acids, being darker in colour (Cemetery context 2089)

These four samples would able to give a general overview of the basic soil components as well as quantifying major components of interest.

In order to assess the humic acid content of the bone A humic acid Sodium salt standard reference sample H-1, 675-2 (Sigma Aldrich), was obtained and subjected to the same analysis as the soil samples.

4.2.2. Soil and Humic Acid FT-IR

The dry soil and humic acid samples were taken from the original labelled containers. A 2mg sample of soil and 1mg of humic acid was crushed in a pestle and mortar along with 200mg of KBr (potassium bromide has to be kept in a hot oven to remain in a powdered form), and tipped into a 13mm die, and tapped down. This was placed under 10 tonnes pressure for 20 minutes to form an opaque disk. Each disk was wrapped in tissue, labelled and placed in a desiccator until required.

The samples were run using a FT-IR System Spectrum BX (manufacturer: Perkin Elmer), with Spectrum 5 Peakfit curvefitting software. All the peaks were smoothed and normalized. The analysis was performed using 1024 scans, with a resolution of $2cm^{-1}$ and the absorbance plotted in the range 4000-400 cm^{-1}. Random samples were run in triplicate and normalised to ensure consistency, these samples proved to be consistent and the rest were run singly. A background check was run prior to starting any new analysis.

The spectra were examined prior to running the bone samples to identify any peaks that may be at risk of overlapping with the bone components (Figures 4-6). All FT-IR spectra for the humic acid and soil analysis were printed on acetate sheets for direct overlay with the bone spectra to analyse any peaks that would indicate the type and degree of contamination components (See Appendix, Chapter 4).

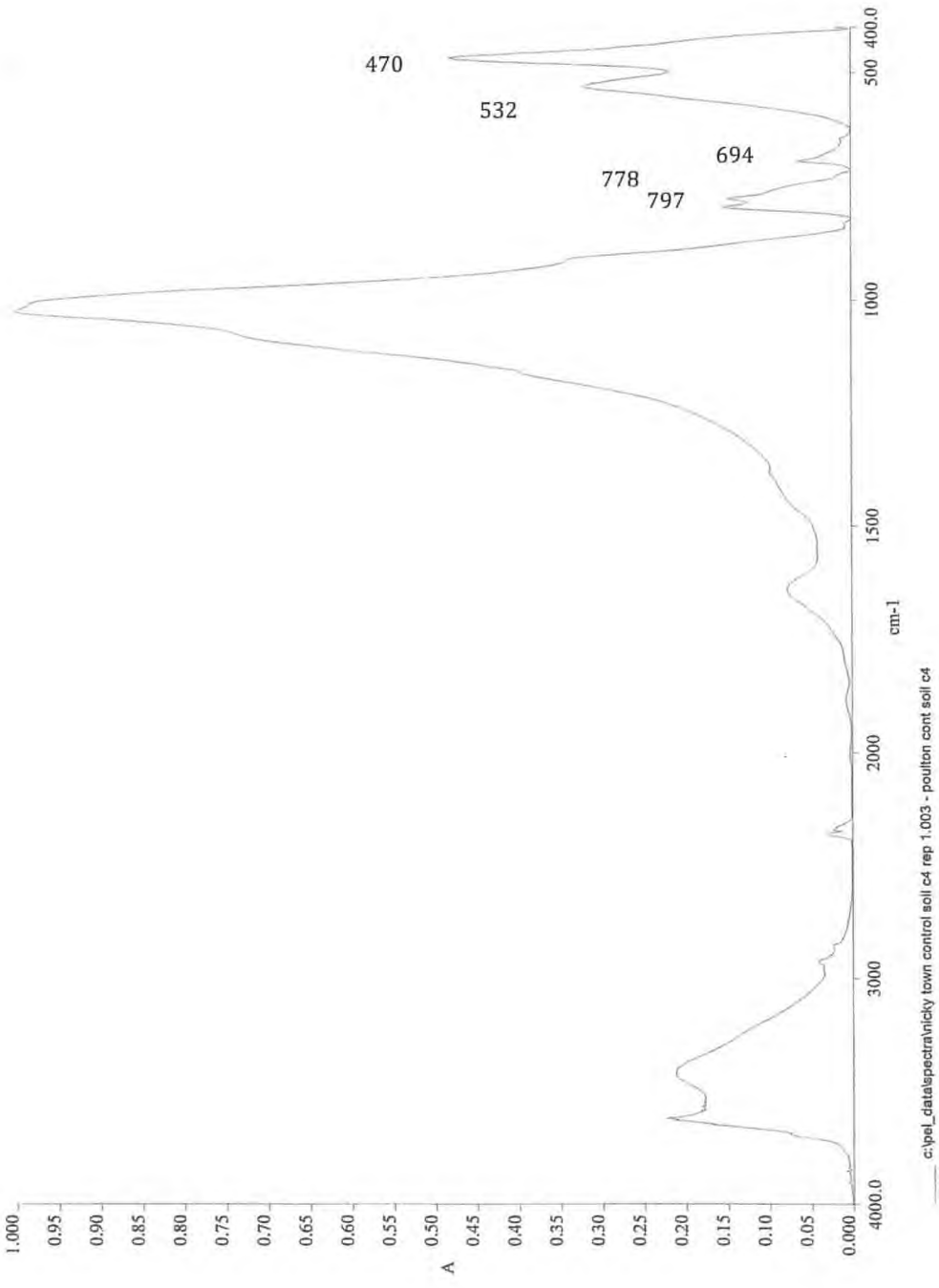


Figure 4: Major FT-IR peaks associated with Poulton control soil sample.

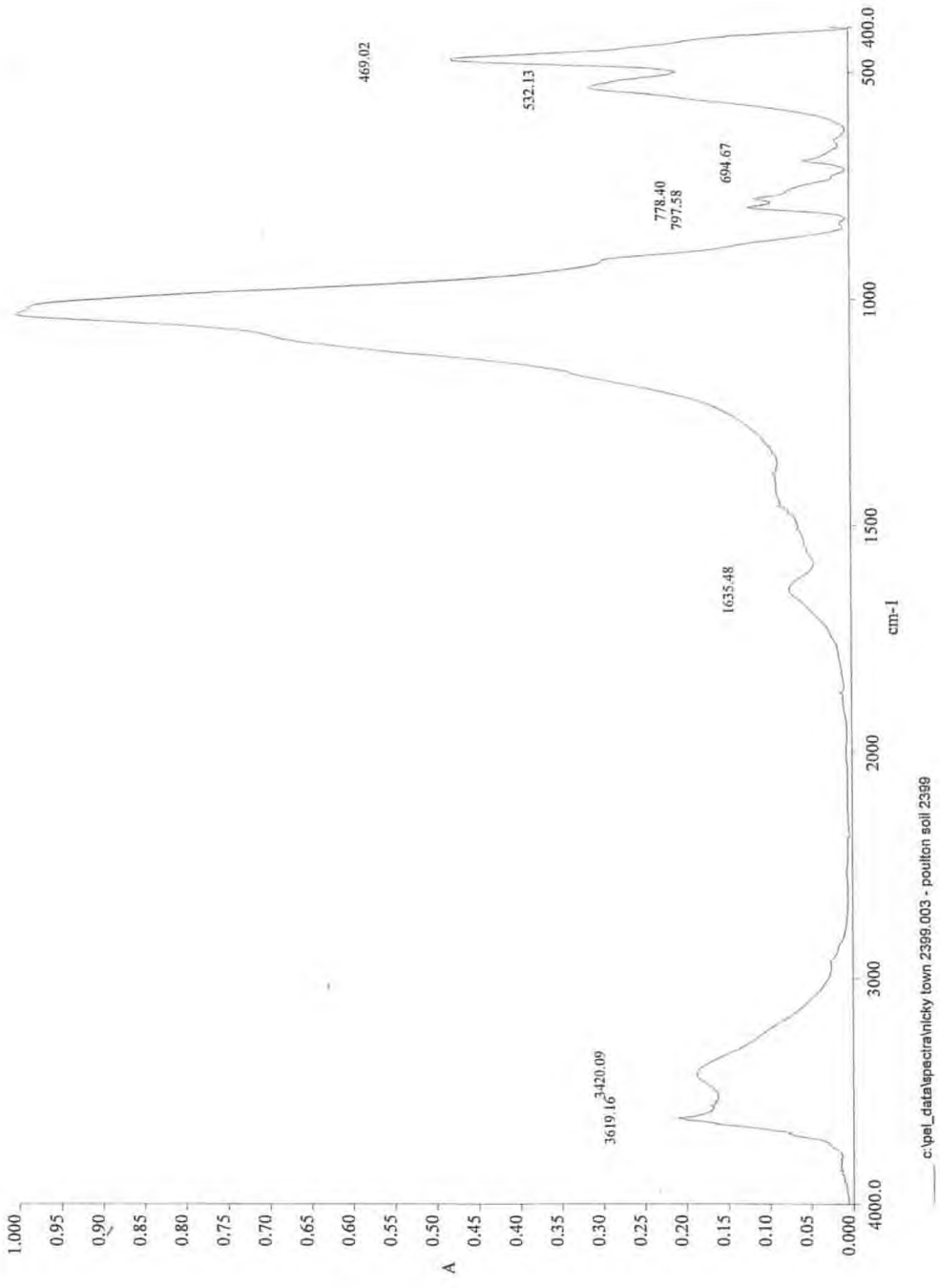


Figure 5: Major FT-IR peaks of a cemetery soil thought to be "low" in humic acids.

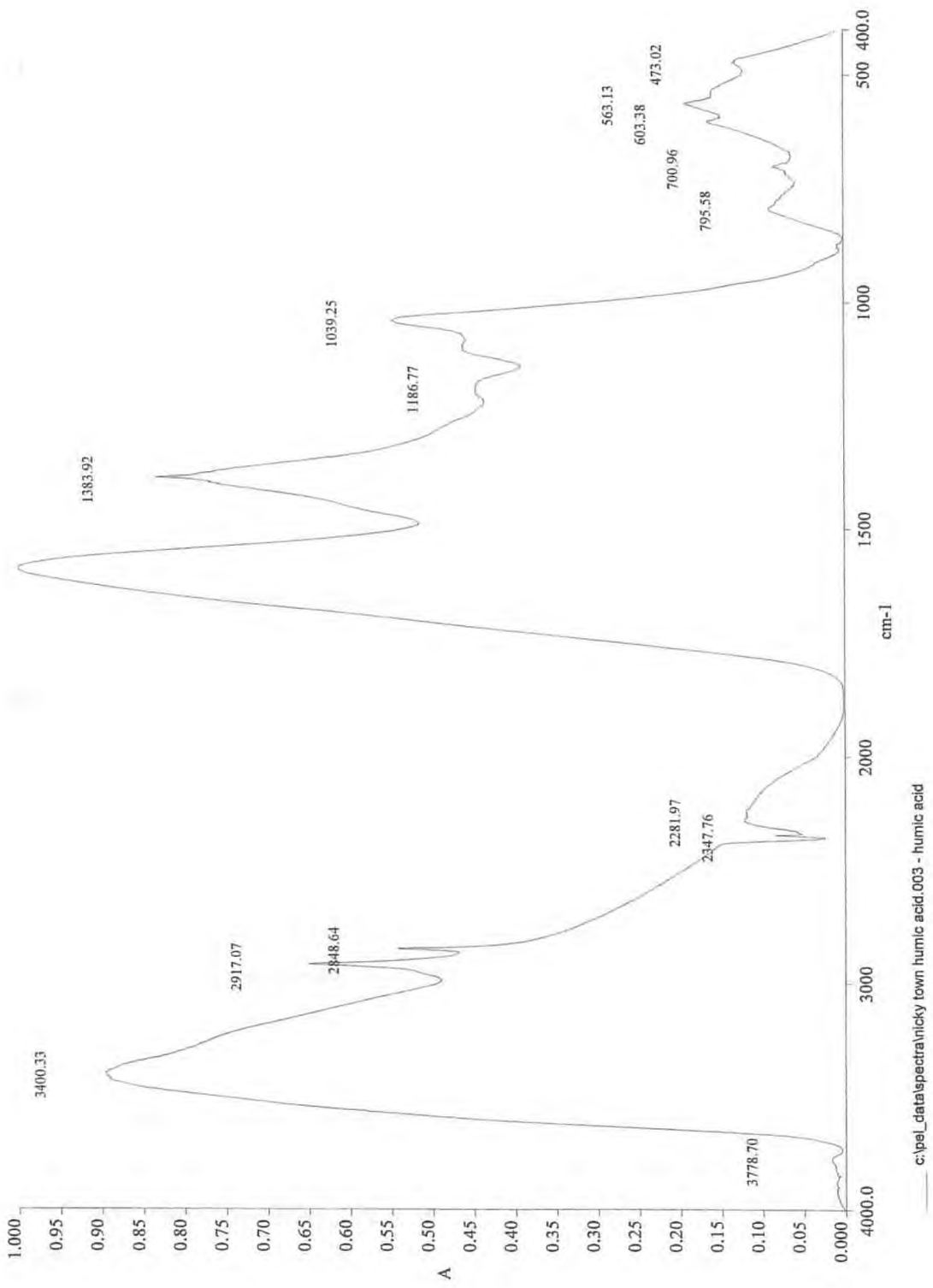


Figure 6: Major FT-IR peaks of a humic acid standard reference sample.

The humic acids reference sample (Figure 6) demonstrated major peaks at 1635, 1383, 1186, 1039 cm^{-1} and minor peaks at 795, 700, 603, 563 and 473 cm^{-1}. The contribution of the humic acids to the Poulton soil samples (Figures 4 & 5) had not been extracted and quantified and were therefore unknown, but did not appear to be represented in any great degree by peak size, except 1039. The only other peak that could have been associated with the humic acid was 694, but this was only a minor contribution. The minor peaks at 603, 563, and 473 cm^{-1}, seen in the humic acid standard reference sample, were only very minor peaks in the Poulton soils and were more likely to have originated from the clays.

The clays appear to be represented in the Poulton soil samples to a much greater degree than the humic acids. The inorganic minerals have been seen to occupy the 790, 690 and 540 cm^{-1} (Mao et al 2008), and 469 cm^{-1}, as the Si-0, and bound to metals particularly Fe, Al and Mg.

4.2.3. Archaeological and Pork Bone Preparation

(i) Archaeological bone:

The analysis of the bone would to attempt to quantify the collagen, assess the splitting factor, and the Ca/P ratios. The skeletal samples used were from the Poulton Medieval cemetery (Chapter 3: Tables 1 & 2). The same 20 bones were used:

5 cranial fragments from extended storage.
5 long bone fragments from extended storage.
5 cranial fragments that had been recently excavated.
5 long bone fragments that had been recently excavated.

A small fragment of the archaeological bone was removed using a small hacksaw, and crushed lightly using a pestle and mortar. then powdered using a Fritsch Pulverisette 2 mortar grinder (Industriestr 8, 6580, Idar-oberstein, West Germany). Each sample of powder was homogenised and placed in a 1.5 ml labelled cryotube.

(ii) Pork bone:

A 10 cm sample of 4 fresh pig femora were obtained from a local butcher, they were cleaned and any adhering tissue scraped away and the cavity scrubbed clean with a bottle brush under warm water. The bone was then dried at 36 °C over the weekend, then cooled. The periosteum was removed (this looks like parchment paper), and the bone was fragmented using a cleaned geological Proctor compactor, then approximately 2 g of fragments were selected and powdered down to <2mm. This was then further finely powdered using the Fritsch Pulverisette 2 mortar grinder (Industriestr 8, 6580, Idar-oberstein, West Germany). Each sample of powder was homogenised and placed in a 1.5 ml labelled cryotube.

(iii) Human bone reference material:

A standard reference sample of human bone NIST SRM 1486 (LGC standards) was obtained in order to calibrate the XRF for elemental quantitative analysis and FT-IR spectroscopy comparison. This material consisted of standardised ground bone, where the elements have been fully quantified. This would enable quantification of the inorganic bone matrix. The preparation technique involved high temperature steam processing drying and grinding, resulting in significant loss of the collagen.

4.2.4. Bone FT-IR

A 2 mg sample of homogenised bone was ground with 200 mg of KBr, in a pestle, tipped into a 13mm die, and tapped down. This was placed under 10 tonnes pressure for 20 minutes to form an opaque disk. Each was wrapped in tissue, labelled and placed in a desiccator until required.

The samples were run using a FT-IR System Spectrum BX (manufacturer: Perkin Elmer), with Spectrum 5 Peakfit curvefitting software. All the peaks were smoothed and normalized. The analysis was performed using 1024 scans, with a resolution of $2cm^{-1}$ and the absorbance plotted in the range 4000-400 cm^{-1} . Random samples were run in triplicate and normalised to ensure consistency, these samples proved to be consistent and the rest were run singly. A background check was run prior to starting any new analysis.

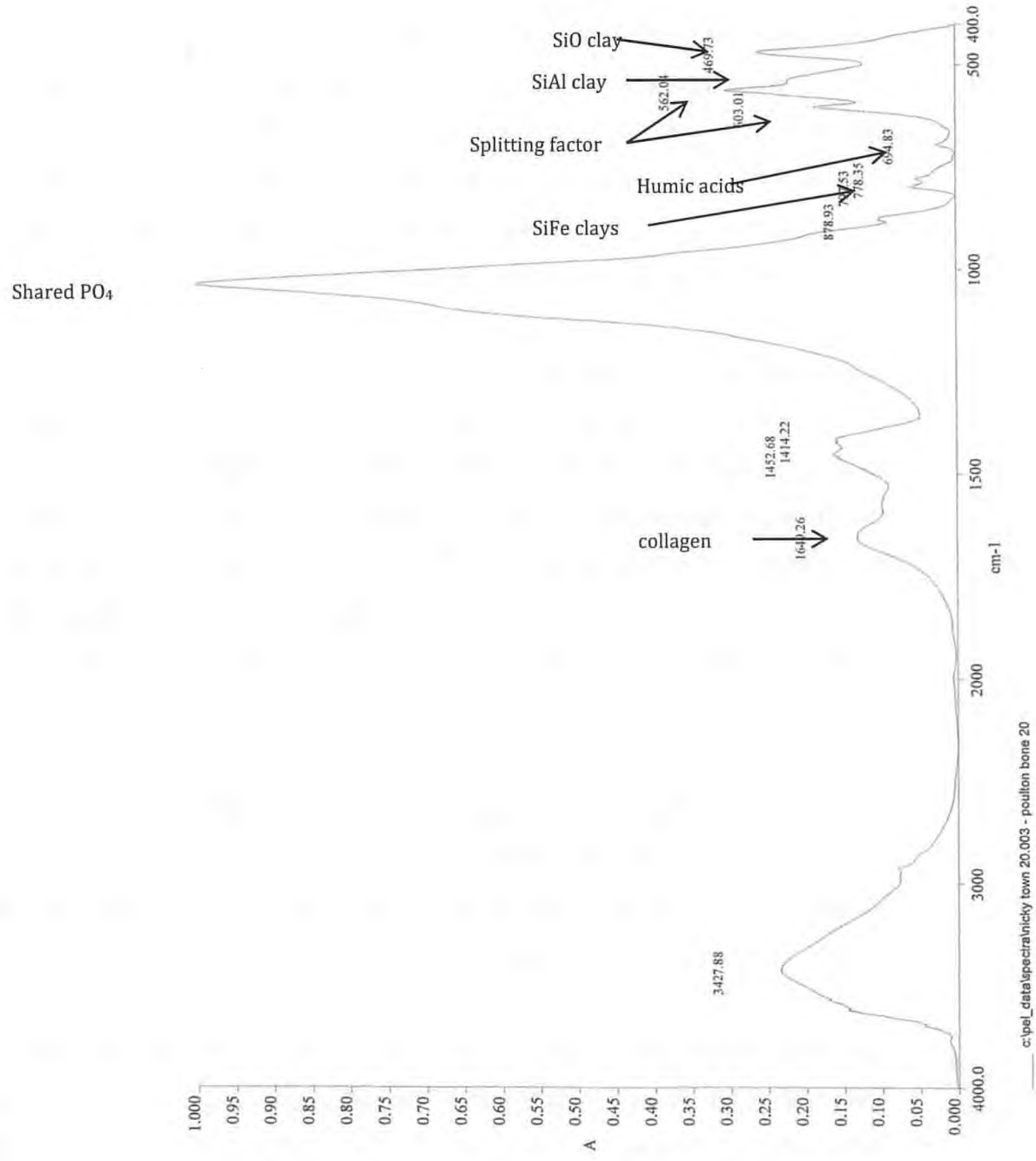


Figure 7: Major FT-IR peaks from an archaeological bone sample.

The main bone analysis would include the soil components, collagen quantification, splitting factor and Ca/P ratio. Figure 7 shows how a soil contaminated bone sample spectra would look, with the peak overlapping mainly the PO_4 peak at 1035.

Absorbance peaks 1654-1652 cm^{-1} seen in the bone standard reference sample (Figure 8) and pork bone (Figure 9) compared to 1635 $cm^{=1}$ in the Poulton soils, would indicate that caution should be applied when using the data to ensure that the peak being used is genuine. The 1650 $^{cm-1}$ peak was associated with the amide band of type I collagen. Peaks at 1550-1540 cm^{-1} and 1450-1455 cm^{-1}, were only observed in greater quantities in the bone samples, and in very small quantities in the soil. The peak at 1417 cm^{-1} could be from decomposition of the 1450-1455 cm^{-1} into two peaks. The preparation techniques used in the human bone reference sample may have resulted in some decomposition of the bone matrix, both the organic and inorganic phases. The peaks at 1239 and 961 cm^{-1} can be clearly seen in the pork bone but are only demonstrated as shoulder peaks in the bone standard and the Poulton soil samples. There was also a sharpening and narrowing of the major 1239-872 cm^{-1}, 1035 cm^{-1} , 694-469 cm^{-1} peaks compared to the fresh pork bone. The peaks at 694, 532 and 469 cm^{-1} were shared with the bone. Peaks at 797 and 778 cm^{-1} were only seen in the soil samples and not in the bone, indicating that these peaks were more than likely from the soil intrusion into the bone from clays.

4.2.5. Calculations

(i) Collagen:

The collagen content was calculated by comparing the result of mean of the combined pork bone collagen to the individual archaeological bone collagen, expressed as a percentage.

$A/B \times 100 = C$

Where A= pork bone collagen, B= archaeological bone collagen, C= percentage of archaeological bone collagen to pork bone collagen.

Contamination of the FT-IR spectra with soil and humic acid components had to be taken into consideration, however when the pork bone spectra 1650 was compared to the soil control spectra the soil contribution was very low and it was felt safe to proceed.

(ii) Splitting Factor:

The splitting factor (or crystallinity index) would be calculated using the equation shown in Figure 3 (Surrovel and Steiner 2000, Weiner and Bar-Yosef 1990).

A (565 cm^{-1}) + B (603 cm^{-1}) / C (the lowest point between them to the baseline)

The calculation value was derived from a baseline drawn from 750-495cm^{-1}, the heights of the peaks (A and B) as well as the distance of the baseline to the lowest point between them (C).

The contribution of the soil components contamination of the archaeological bone demonstrated a close peak at 532 cm^{-1} in the soil and 560 cm^{-1} in the pork bone, but it was considered safe to continue.

(iii) Calcium and Phosphate Ratio:

The calcium/phosphate ratio was calculated using FT-IR peak comparison, and quantitative XRF. Caution had to be taken in regard to the level of soil contamination in the archaeological bone. The contribution of soil derived calcium and phosphorus could be seen in the soil analysis FT-IR spectra, and as a result of this the peaks at 1455, 1415, 874 cm^{-1}, could be used. The peak at 1650 cm^{-1} from the soil was compared to the pork bone and whilst it was shared, the soil contribution was very low. The peak at 1035 cm^{-1} (1024-1030 cm^{-1} , in soil, 1035 cm^{-1} in humic acid and clays) was shared by the soil and bone in almost equal quantities, making the use of this peak questionable, but given the relatively low levels of humic acids a decision would whether to use this would be considered during the archaeological bone analysis. The effect this phosphate contribution would have on the XRF results meant that caution was to be applied.

All the results would be imported into SPSS for statistical analysis.

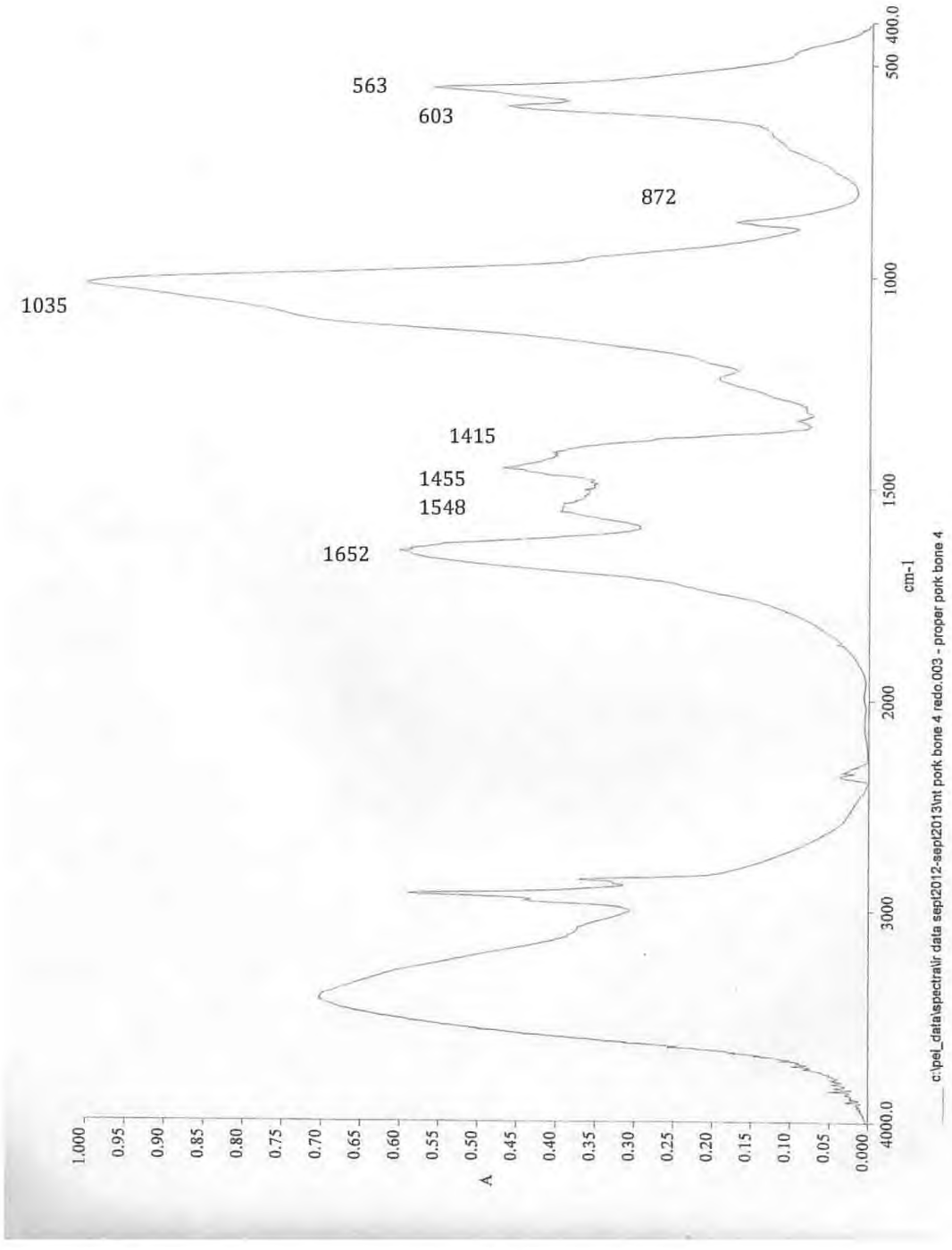


Figure 8: Major FT-IR peaks for fresh pork bone.

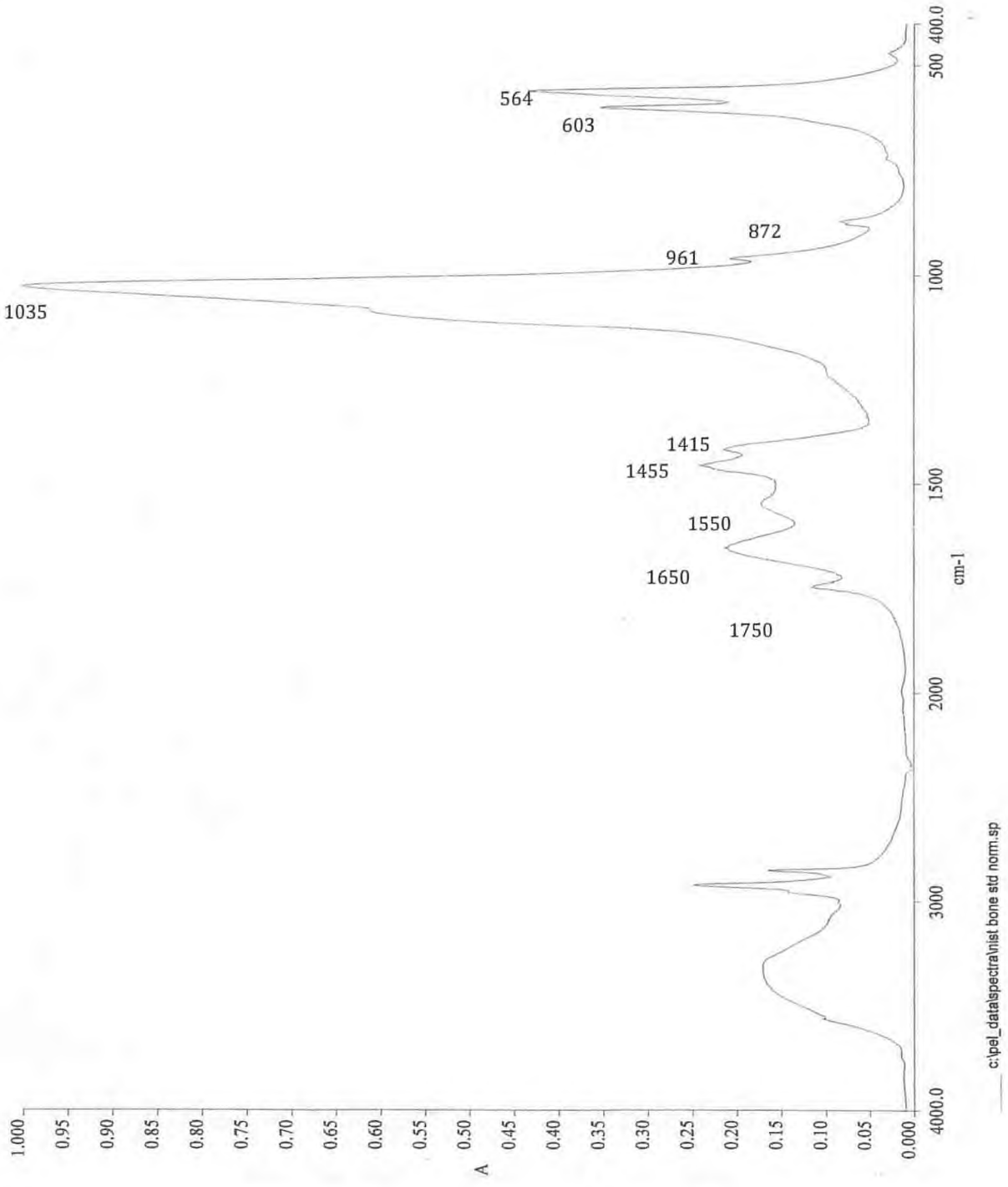


Figure 9: Major FT-IR peaks for human bone reference sample 1486.

4.3. Results

All the FT-IR spectra for the archaeological bone, pork bone, human bone reference sample, humic acid, and soil samples were printed out and the comparison samples were printed separately onto acetate sheets for direct comparison (see Appendix: Chapter 4). As can be seen from the spectra from Figure 7, (Chapter 3, Table 1: 5d, recently excavated tibia), the soil interred bone demonstrated a profile with mixed bone and soil components. This mixed profile meant that the soil and humic acid components that shared peak areas with the archaeological bone could not be used for calculating the Ca and P ratios. Fortunately the peaks used for calculating the splitting factor and collagen did not seem to be affected and it was considered safe to continue.

The data was imported into Excel and subjected to statistical analysis.

4.3.1. (i) Clay Content: Semi-Quantification

The peak absorbance from the soil components that did not occupy the same peak as the pork bone were used to semi-quantify the clay fraction of the soil intruded archaeological bone. This could also be used to assess the accuracy of the collagen content and Ca/P ratios where contamination may be an issue. The absorbance of peaks 470, 532, 694, 778 and 797 cm^{-1} were selected.

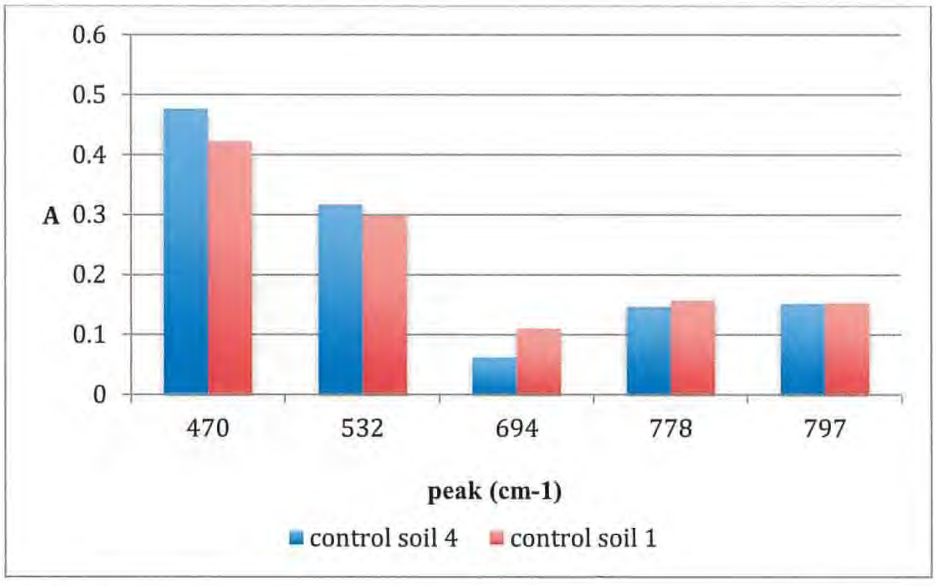


Figure 10: Absorbance peaks of control soils indicating the amounts of clay and humic acid components.

The highest clay peaks in the control soils (Figure 10)were found at 470 cm^{-1} (SiO) and 532 cm^{-1} (Si-Al), with very little variation between the two samples. The quantity of these components can be semi-quantified in the bone samples by comparing the peak absorbance to these soils.

The humic acid peak 694 cm^{-1} demonstrates low levels of humic acids present in the control soils (Figure 10).

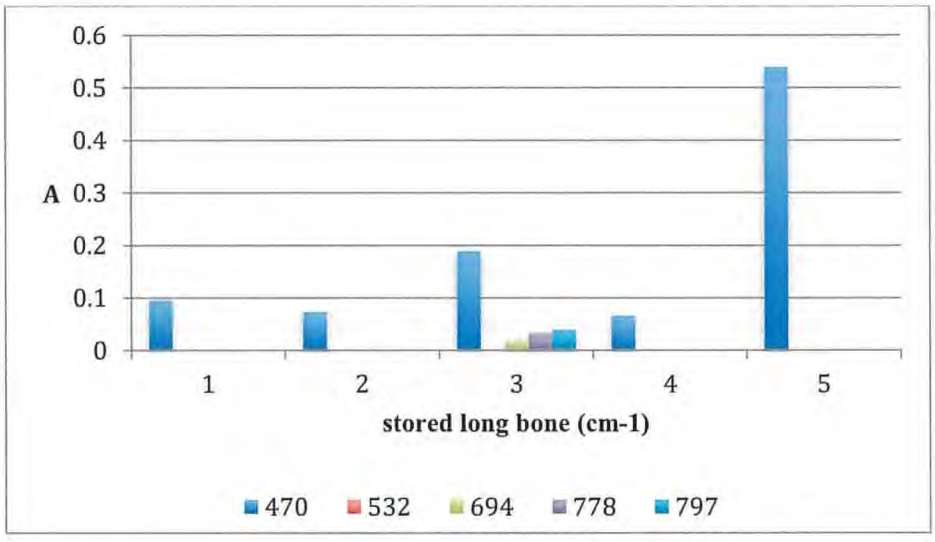


Figure 11: Amount of each clay component in the five stored long bone samples.

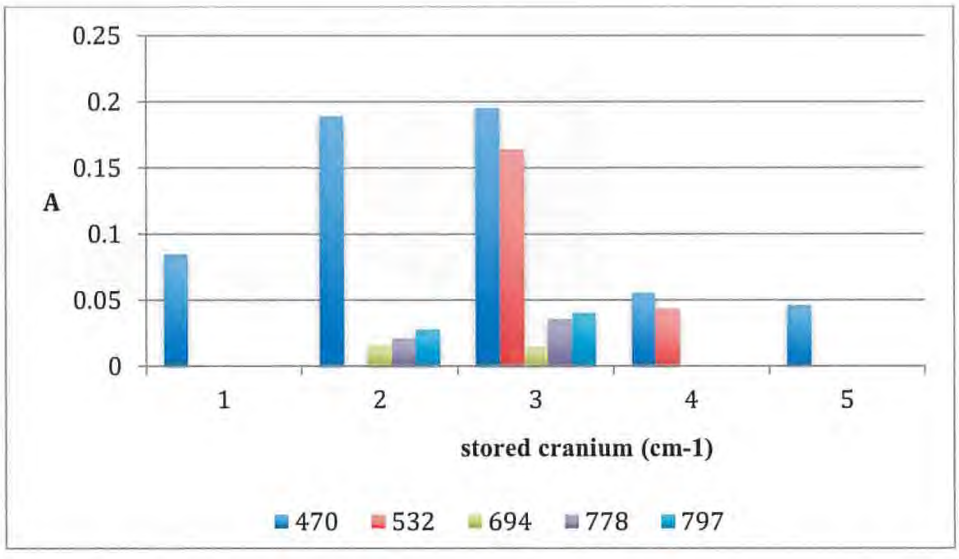


Figure 12: Amount of each clay component in the five stored cranial samples.

The presence of the clay components in the stored bone samples demonstrated higher levels of these components in the stored cranium than the long bones (Figures 11 & 12). Sample 3 in the stored long bones had markedly higher clay components than the rest of the long bone samples, and sample 5 had the highest level of the most commonly found clay at 470 absorbance. Samples 1 and 5 in the cranium had lower clay components than the rest of the cranium samples. The cranial samples had much lower quantities of the most frequently represented 470 (SiO) than the long bones samples.

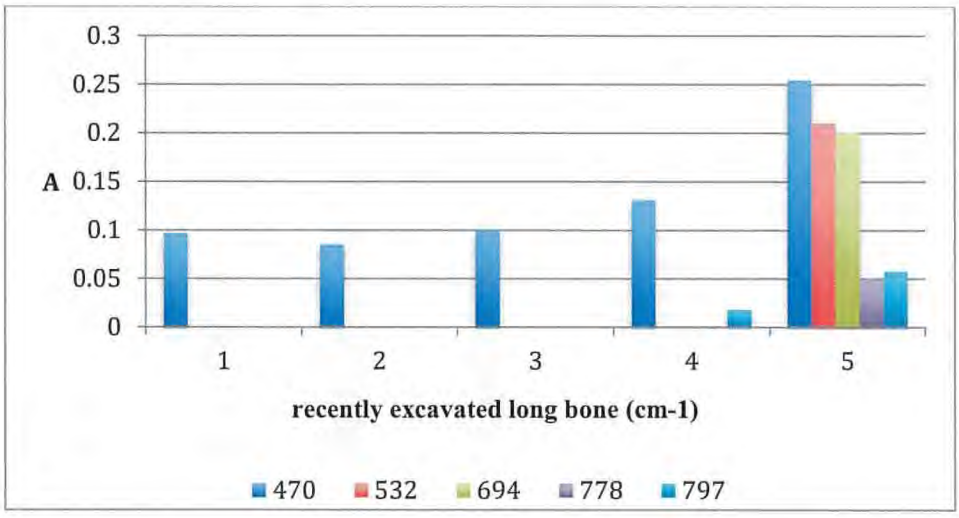


Figure 13: Amount of each clay/humic acid component in the five recently excavated long bone samples.

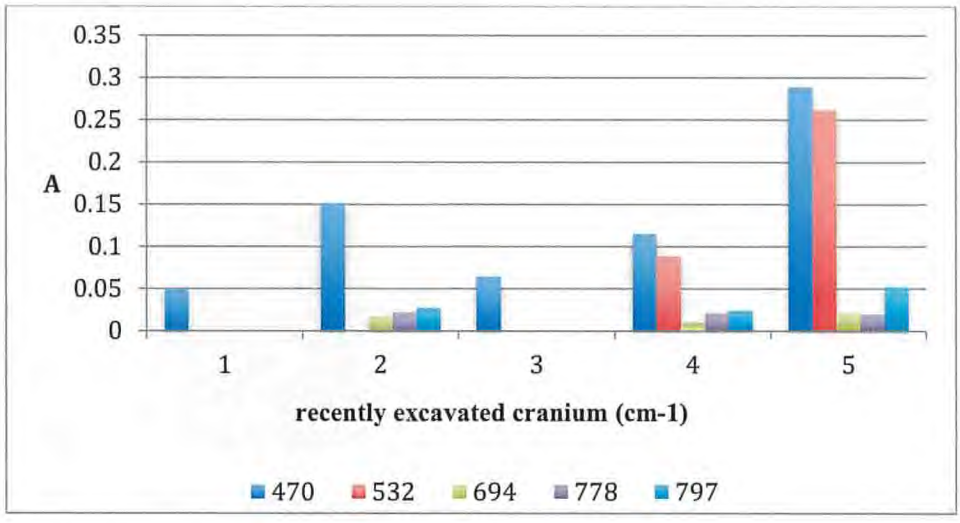


Figure 14: Amount of each clay/humic acid component in the five recently excavated cranial samples.

The presence of the clay components in the recently excavated bone samples demonstrated higher levels of these components in the cranium (Figures 13 & 14). Sample 5 in the long bones contained much higher clay components than the rest of the long bone samples. Samples 1 and 3 of the cranial samples contained fewer clay components than the rest of the cranial samples. The long bones contained much lower levels of the most frequently represented 470 (SiO) than the cranial samples. The cranial samples had higher levels of humic acids.

4.3.1. (ii) Bone Collagen

The peak absorbance 1650 can represent the collagen content of bone. The levels can be represented by a percentage of fresh pork bone.

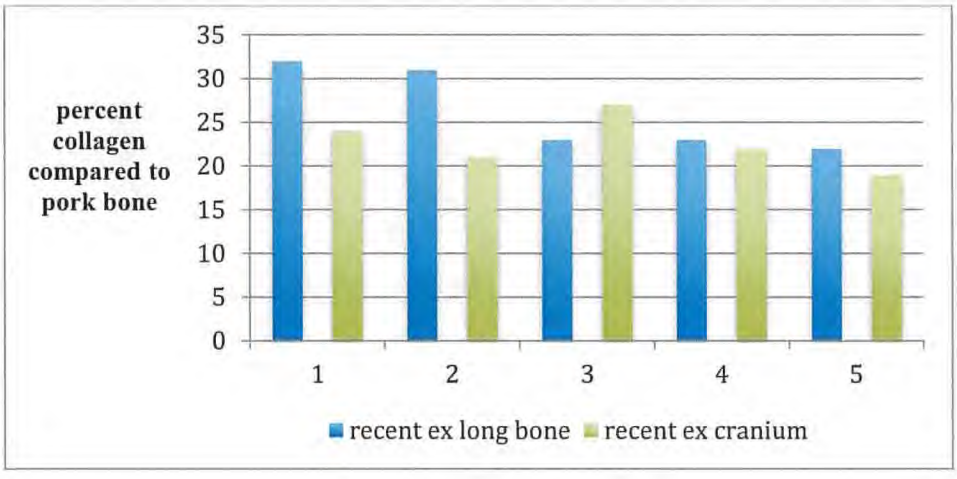


Figure 15: Collagen content of recently excavated archaeological cranium and long bone compared to fresh pork bone.

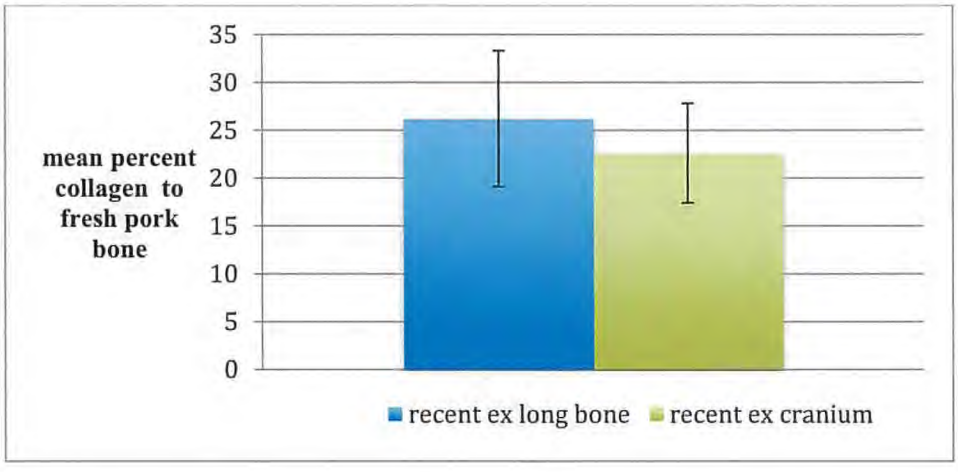


Figure 16: Mean collagen content of the recently excavated archaeological bone.

In the recently excavated bones the collagen content would appear to be higher in the long bones. Statistically there was no significant difference using an Independent-samples T-test, p=0.51, t=1.401, n=5,5.

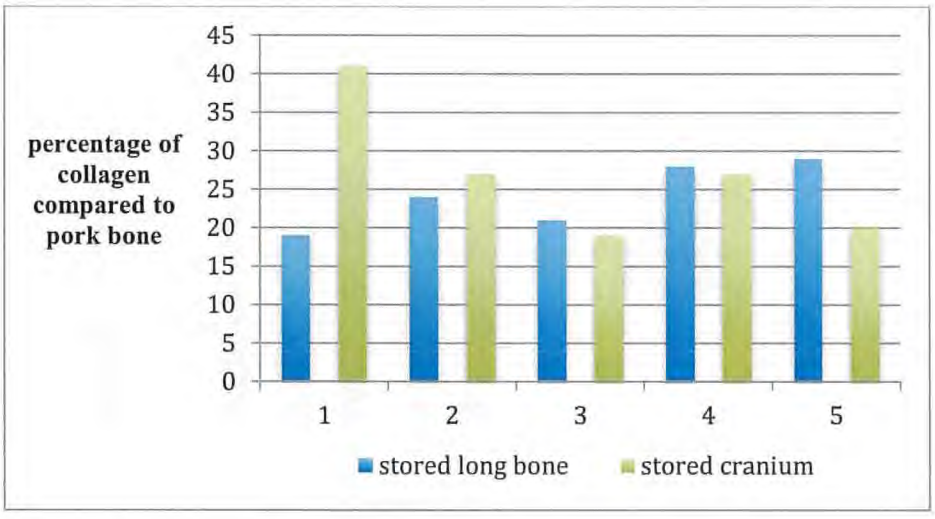


Figure 17: Collagen content of stored archaeological cranium and long bone compared to fresh pork bone.

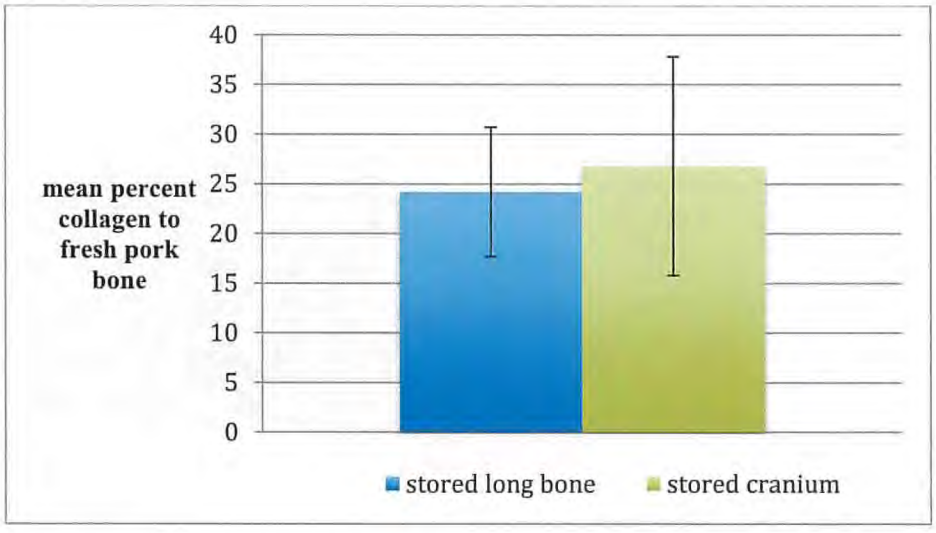


Figure 18: Mean collagen content of the stored archaeological bone compared to fresh bone.

In the stored bone it would appear that the stored cranium had the highest collagen content, but there was one cranial sample that could have skewed the results. There was no significant difference using and Independent-samples T-test p=0.412, t=0.594, n=5,5.

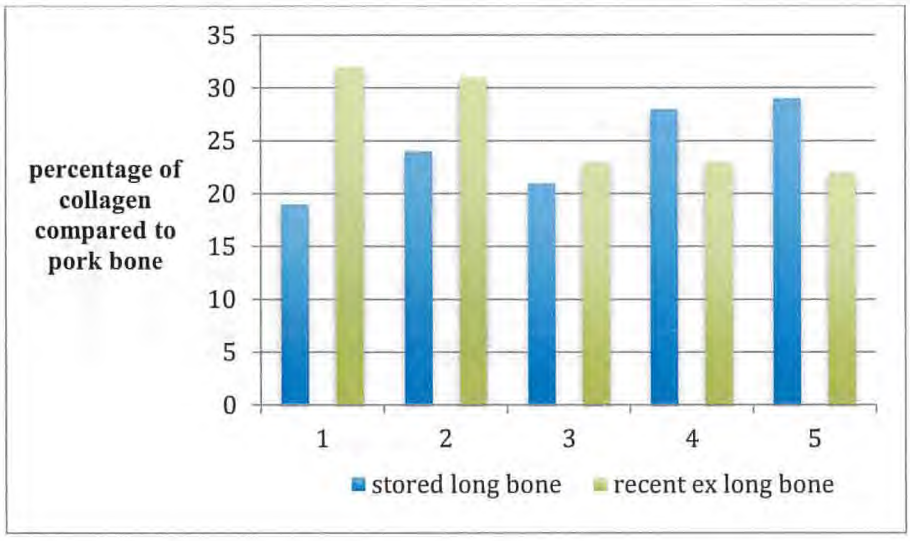


Figure 19: Collagen content of stored and recently excavated archaeological long bone compared to fresh pork bone.

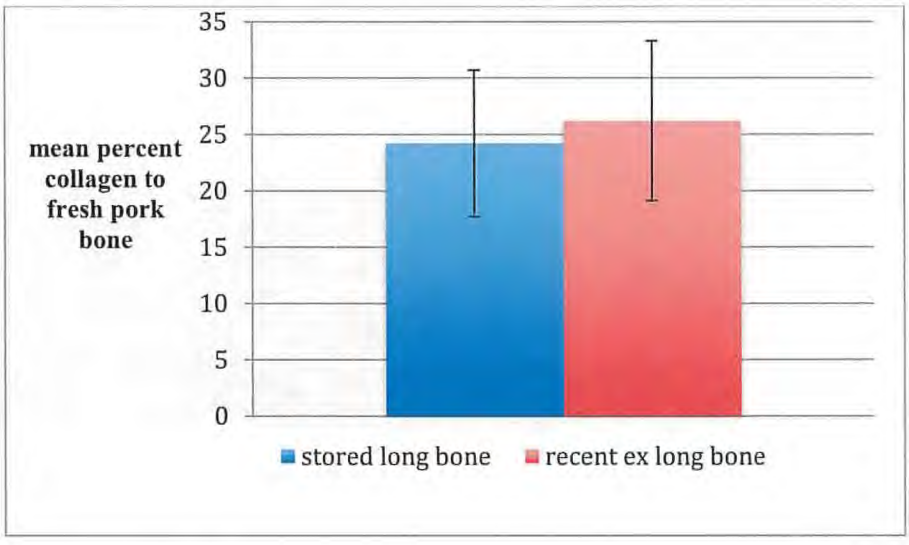


Figure 20: Mean collagen content of the stored and recently excavated archaeological long bone compared to fresh pork bone.

There appears to be a higher mean collagen content in the recently excavated long bone, but this was not statistically different using an Independent-samples T-test, p=0.453, t=0.687, n=5,5.

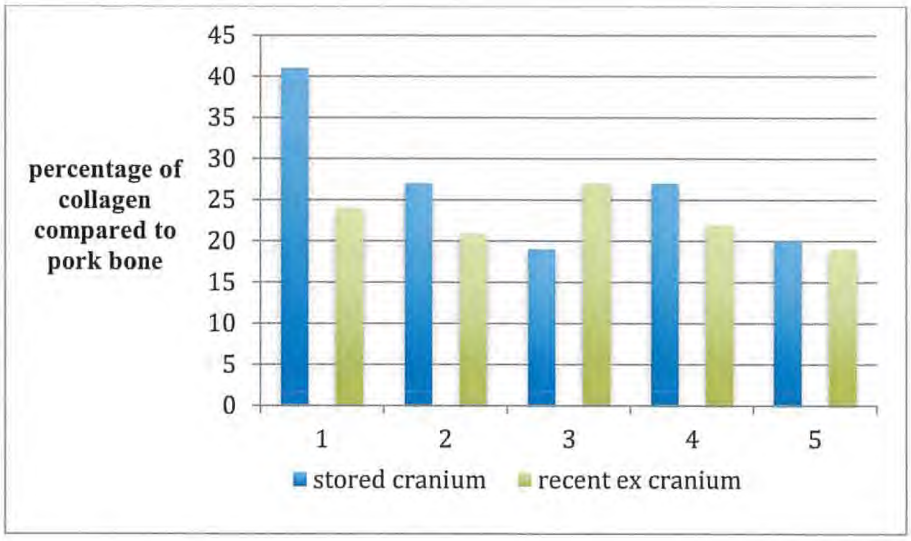


Figure 21: Collagen content of stored and recently excavated archaeological cranial bone compared to fresh pork bone.

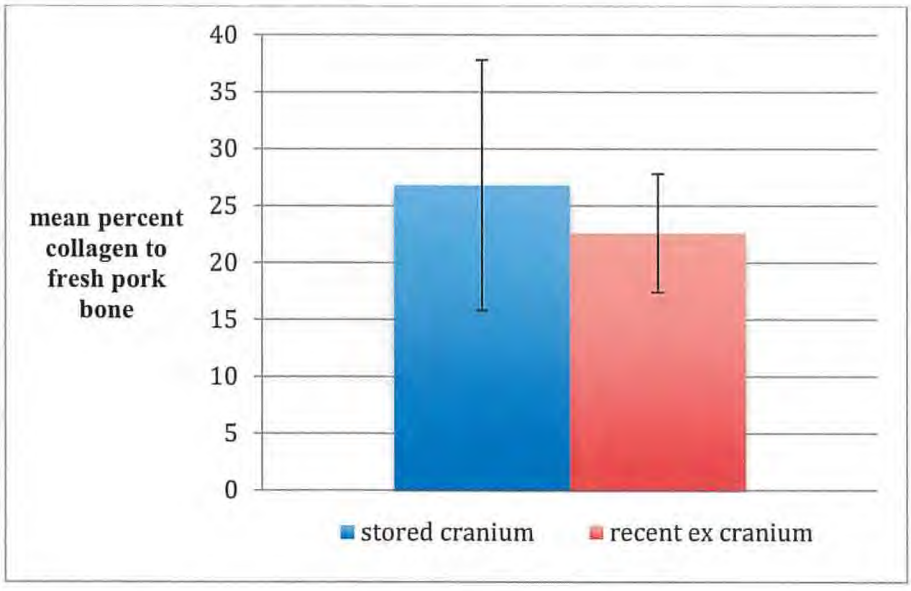


Figure 22: Mean collagen content of the stored and recently excavated archaeological long bone compared to fresh pork bone.

In the cranial samples it would appear that the stored samples contain higher levels of collagen than the recently excavated samples, but this was not statistically different using an Independent-samples T-test, p=0.233, t=1.01, n-5,5. The cranial fragments were taken from different parts of the cranium, and this could explain the results seen.

The ratio of the absorbance of the carbonyl (CO) peak 1455 cm^{-1} to the carbonate (CO_3) peak 1415 cm^{-1} has been used to assess the organic to inorganic components in bone. These absorbance peaks were not present in the soil samples and it was safe to assume that they did not represent soil contamination in the archaeological bone. The use of the 1455/1415 cm^{-1} peak ratio could be a more useful tool in assessing the amide group in bone using a comparison to fresh pork bone. The ratio of 1455 to 1415 cm^{-1} was between 1.0 -1.2 in the fresh pork bone, and this was the same in all of the archaeological bone samples, making this difficult to interpret in a useful way to quantify the collagen content, and as a result this method was not used.

4.3.1. (iii) Splitting Factor (Crystallinity Index)

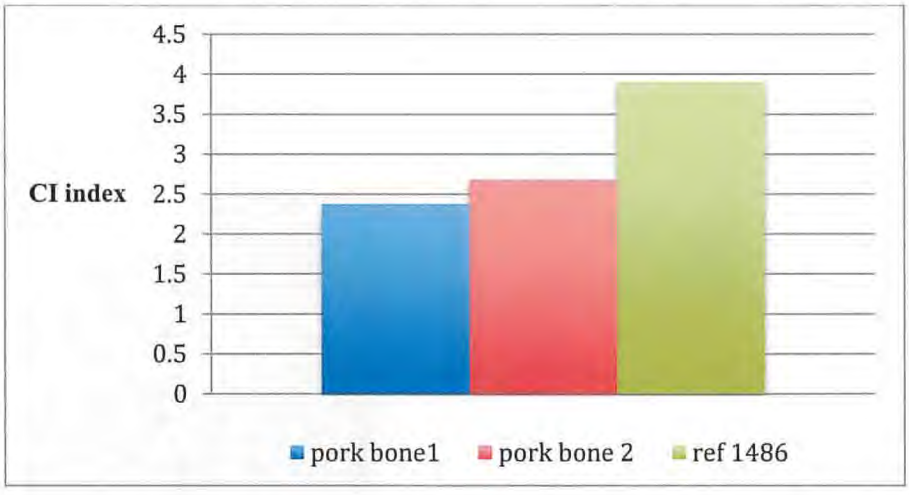


Figure 23: The splitting factor (crystallinity index) values for fresh pork bone and the standard reference material 1486.

The splitting factor for the fresh pork bone samples were within expected levels of 2.5-2.9, however the preparation techniques used during the human bone reference material 1486 had caused degradation of the bone and increased the splitting factor. The pork bone SF was used for the statistical analysis.

The archaeological bone was statistically tested against the fresh pork bone.

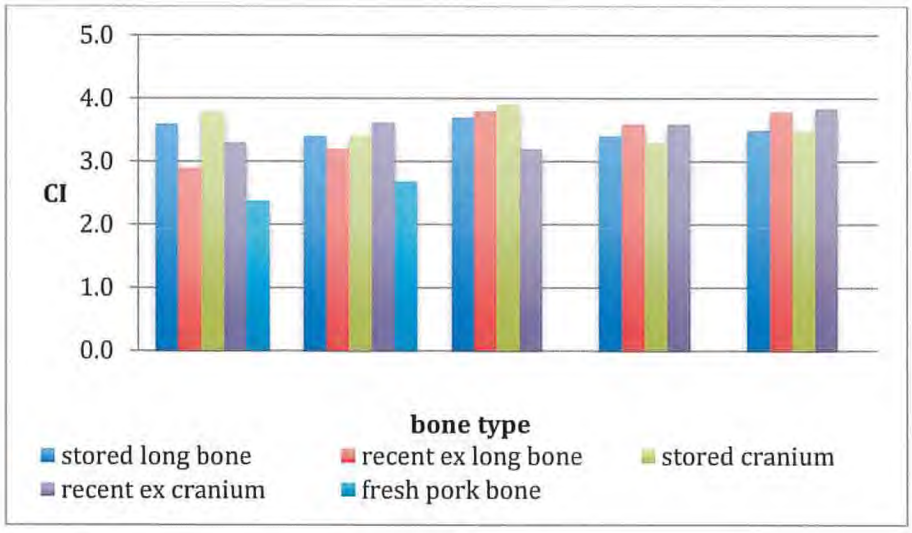


Figure 24: Splitting factor for all bone samples.

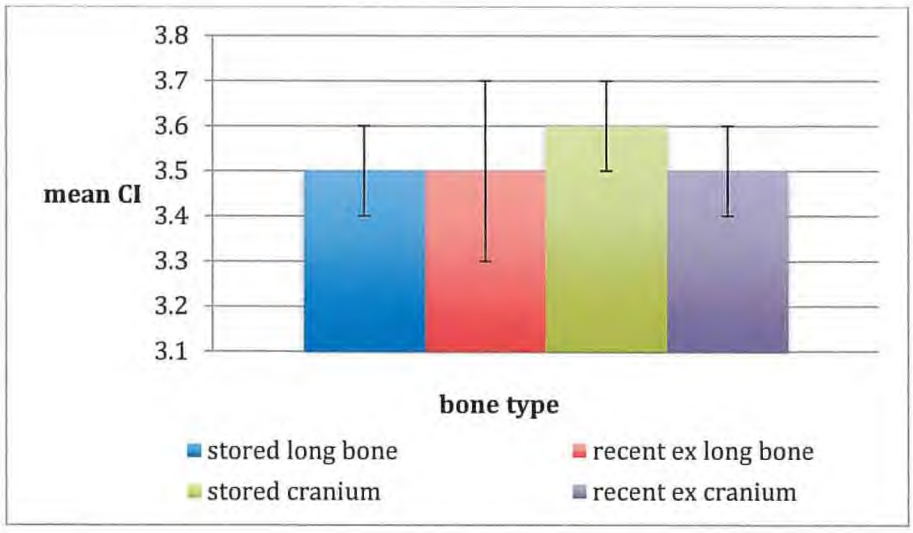


Figure 25: The mean values for the splitting factor (crystallinity index) for the four types of archaeological bone.

There appeared to be a greater increase in the mean splitting factor for the stored cranial samples compared to the stored and recently excavated long bone, and the recently excavated cranium. There was also a greater variation in the splitting factor between the recently excavated long bones.

Independent T-tests revealed no significant difference between each of the groups p=>0.05, df=8. There was a significant difference between the fresh pork bone and the archaeological bone: stored long bone p=0.003, recently excavated long bone p=0.006, stored cranium p=0.002, recently excavated cranium p=0.004.

4.3.1. (iv) Calcium and Phosphate Ratios

(a) X-ray Fluorescence (XRF)

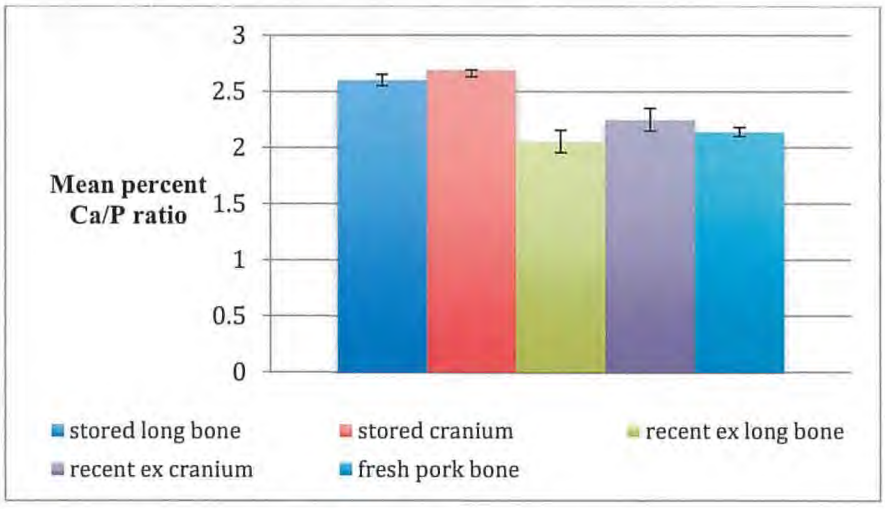


Figure 26: Mean percent calcium and phosphate ratio from stored and recently excavated long bones and cranium compared to fresh pork bone.

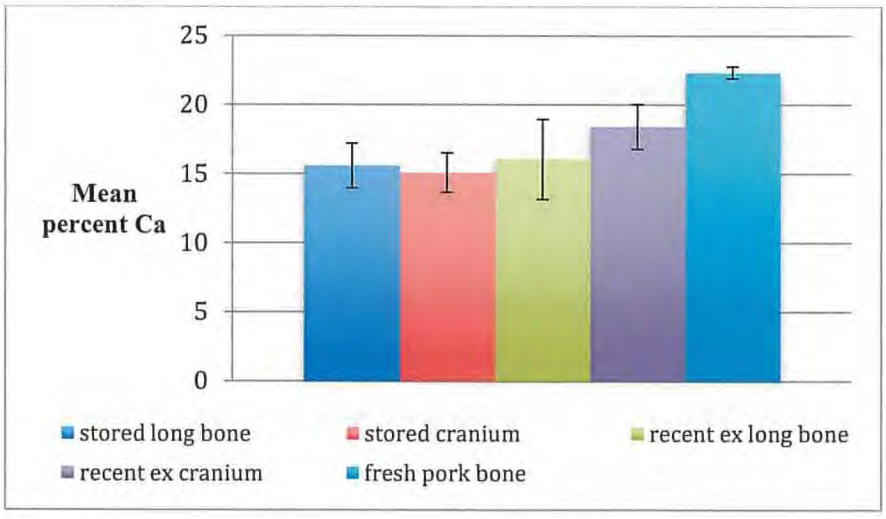


Figure 27: Mean percent of calcium from stored and recently long bones and cranium compared to fresh pork bone.

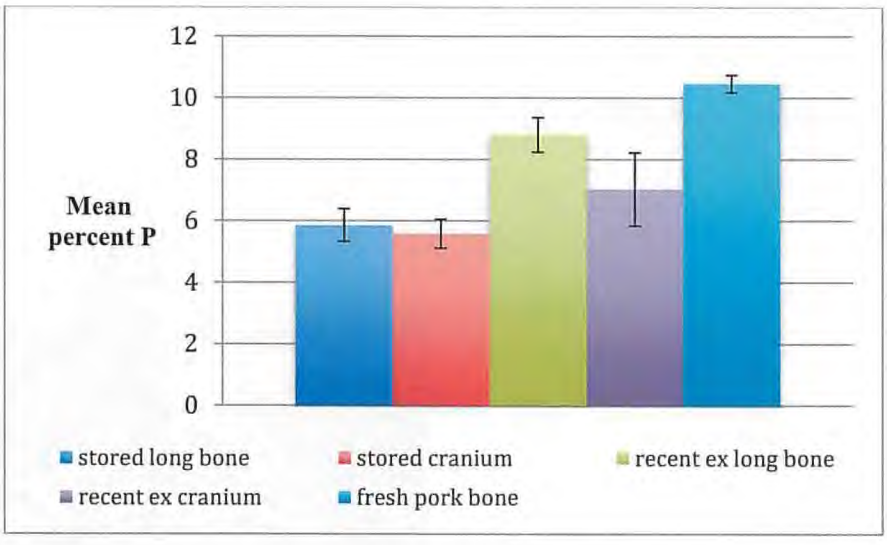


Figure 28: Mean percent of phosphorus from stored and recently excavated long bones and cranium compared to fresh pork bone.

The calcium and phosphate ratio was highest in the stored long bones and cranium compared to the recently excavated long bones and cranium, which show similar values to the fresh pork bone. The ANOVA was significant p=0.0001 F=9.585 df 4,19. The multiple comparisons revealed a significant difference between the stored and recently excavated long bones p=0.003; stored long bones and fresh pork bone p=0.02; recently excavated long bone and stored cranium p=0.001; stored and recently excavated cranium p=0.023; stored cranium and fresh pork bone p=0.005; stored long bone and fresh pork bone p=0.02.

The stored long bones and cranium had similar mean calcium values with the recently excavated cranium having the highest calcium value of the archaeological bones compared to the fresh pork bone. The ANOVA was not significant 0.054 F= 2.862 df 4,18. This result is close to being significant.

There were more distinct differences in the mean phosphorus levels with the stored bones containing markedly less phosphorus than the recently excavated long bones and cranium compared to the fresh pork bone. The ANOVA was significant 0.001 F=8.342 df 4,18. Multiple comparison Tukey HSD results showed the significance as: stored long bone and fresh pork bone p=0.002; recently excavated long bone and stored cranium p=0.034; stored cranium and fresh pork bone p=0.001; recently excavated cranium and fresh pork bone p=0.014; recently excavated cranium and stored long bone p=0.002.

(b) FT-IR Spectroscopy

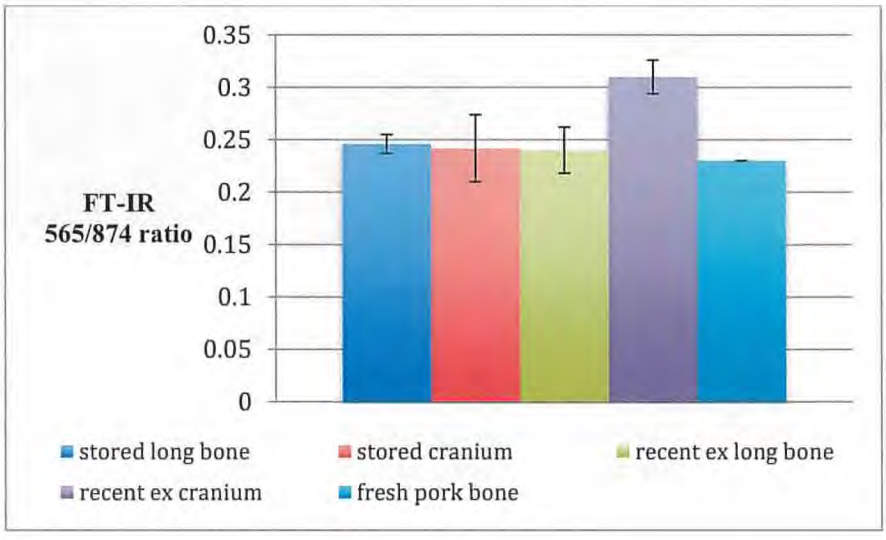


Figure 29: FT-IR 565/874 calcium/phosphate ratio from stored and recently excavated long bones and cranium.

The stored long bones, cranium, and recently excavated long bones shared similar values to the fresh pork bone. The recently excavated cranium had the highest ratio difference to the fresh pork bone.

565/874 cm^{-1} calcium and phosphate ratios: An ANOVA revealed no significant difference, F= 2.264, p=0.105, Tukey HSD p=0.153 n=2,5; multiple comparisons between groups no significant difference p=>0.05.

4.4. Discussion

A major consideration in the investigation of the organic matter in soil-intruded bone has to be the level and the exact nature of these soil contaminants, and their inter-relationships directly or indirectly with the bone. The quality of the bone is of primary importance when considering undertaking costly DNA or C^{14} studies, and submitting a sample that is outwardly in the best possible condition may not be enough if the bone has begun to degrade or has been contaminated by soil components preventing accurate results from being obtained. By taking a small sample (2mg) of five bones from stored and recently excavated long bones and cranium from the Medieval cemetery at Poulton, Cheshire; then using the cemetery soil, contemporary fresh pork bones, as well as a certified bone reference standard, it has been possible to begin to obtain some insight into these complex chemical relationships from a qualitative and quantitative perspective .

The presence of humic acids in the bone has been of interest from a nuisance point of view for DNA and C^{14} studies, its removal being critical due to its potent PCR inhibition and C^{14} contamination, but little consideration had been given to the effects of clay particles on the bone's organic biomolecules. Clays are highly complex silica based amorphous or crystalline compounds that form an important part of the soil's structure (Tan, 1993). They are highly charged particles that can form bonds with a variety of metal species including aluminium, magnesium and iron, commonly seen in the Poulton soil. The ability to be able to assess these components within the bone is of great importance before beginning any studies.

By analysing the Poulton control soil using the peaks absorbances of the FT-IR (Figure 10) the parent soil can be seen to have a high clay (SiO (470 cm^{-1}), SiAl (532 cm^{-1}), SiFe (778, 797 cm^{-1}), with SiO (470 cm^{-1}) dominating each bone sample. There appeared to be relatively low humic acid content (694 cm^{-1}). These peaks were used to investigate the intrusion of these components into the archaeological bone. The stored and recently excavated long bones (Figures 11 & 13) generally had less clay and humic acid than the cranial bone samples (12 & 14). In the recently excavated long bone (Figure 13) sample 5 can be seen to have very high levels of clay and humic acids, comparable to the control soils. This could have resulted from the sample being taken from either an individual with poorer bone health, an area of bone more porous

in nature than the rest of the bone, or from an area in the cemetery where the environmental conditions allowed increased soil intrusion into the bone. The soil at Poulton can vary in that there can be differences in the amount of humus, sand and clay, which can affect the degree of intrusion by these components into the bone. This would indicate that whilst generalisations may be made, it would be more prudent to say that each bone should be treated as an individual sample with its own unique characteristics. Storage of the bone did not seem to alter the clay and humic acid content even after fairly long lengths of time (10 years+). The type of long bone included ulna, tibia and fibula, but the soil intrusion did not appear to be affected by the different types of long bone used.

The collagen results using a comparison with pork bone proved useful. The statistical analysis revealed no significant difference between the stored and recently excavated long bones ($p= 0.453$) (Figures 19 & 20), there appeared to be slightly more collagen in the recently excavated bone than the stored, which would be expected. The analysis of the stored and recently excavated cranium revealed no significant difference ($p=0.233$) (Figures 21 & 22), there appeared to be lower levels of collagen in the recently excavated cranium compared to the stored and much greater standard error within the stored samples, this may have been as a result of the variation in where the fragments were taken from on the cranial vault. There was no significant difference in the stored long bones and cranium results ($p=0.142$) (Figures 15 & 16), but this significance was greatly reduced in the recently excavated long bone and cranium analysis ($p=0.52$) (Figures 15 & 16), where the long bones appear to contain more collagen than the cranium. This may prove useful when deciding which bone fragments to use for collagen analysis.

There appeared to be a greater increase in the mean splitting factor for the stored cranial samples compared to the stored and recently excavated long bone (Figures 24 & 25), and the recently excavated cranium, but here was no significant difference between the different groups ($p>0.05$) There was also a greater variation in the splitting factor between the recently excavated long bones, this may be explained by the high level of soil intrusion in sample 5. The splitting factor had been used to explain the condition of the bone apatite, and had previously been used to score the condition for bones that had been left on the soil surface where they had been exposed

to the environmental conditions, but this may not prove useful for soil interred or stored bone as it does not demonstrate deterioration of the organic biomolecules (Trueman et al., 2008). Bone may appear to be histologically sound, but this may not give a full picture of the useable collagen or DNA. The use of fresh pork bone proved to be a better choice for analysis compared to the human bone standard reference material (Figure 23).

The XRF results for the calcium/phosphorus ratio were able to show a significant difference $p = 0.0001$ between the archaeological bone samples and the archaeological and fresh pork bone (Figure 26). The stored long bone and cranium had a higher ratio than the recently excavated. The fresh pork bone had a ratio of 2.14%, the human bone ratio was 2.31% (Forbes et al., 1953); the long bone had a ratio of 2.1 which was closer to the fresh pork bone ratio, in contrast the cranium that had a ratio of 2.25 which was closer to the human bone ratio. The XRF results were able to identify the differences between the bone qualities of the all the bone samples. In order to clarify this further the results of the calcium and phosphorus were analysed separately. The calcium results revealed no significant difference between the bone samples $p>0.05$, however there was a significant difference in the phosphorus levels between the stored archaeological bones and fresh pork bone and recently excavated archaeological bones (Figures 27 & 28). There was no significant difference between the recently excavated long bones and fresh pork bone. The role that the soil intrusion played in the ratios was unknown and would need to be considered for further study. The use of FT-IR for quantifying the calcium and phosphate ratios proved not to be useful using the 565/874 cm^{-1} absorbances, being statistically not significant (Figure 29). The major phosphate peak (1035 cm^{-1}) could not be used due to it being shared with the soil.

It is useful to analyse all the results together, considering the relationship between the soil intrusion, collagen content and crystallinity index (splitting factor) it is important to ensure that each bone is considered as a unique sample with its own characteristics. It was not considered safe to proceed with any statistical correlation analysis as the bone sample size was too small n=5. The trends seem to appear to show lower collagen content in the most soil intruded stored bones, with higher levels in the recently excavated long bones apart from sample 5 which demonstrated much lower collagen levels in-keeping with its high soil intruded status. The crystallinity index

splitting factor) would appear to show the bones in relatively good condition, (expected levels 2.5-2.8) as the bones were generally within 2.8-3.8 window, but there was a trend to show higher index score in the stored bones.

4.5. Conclusion and Further work

By examining all the samples it is clear that the long bones generally contained lower quantities of soil components, but does raise the very important point that each bone should be seen as an individual and by using the FTIR analysis its soil contamination can be fully explored with only 2mg of sample being required. As only five of each bone type were analysed, this study would benefit from being extended to afford greater clarity of the results. Further analysis of the soil components to clarify the FT-IR results, and to enable a clearer understanding of the soil components by using an extended range of standard reference materials, in particular the clays, sands, humic and fulvic acids. Being able to quantify the humic acid and clay content of the archaeological bone may pave the way for either allowing decisions to be made as to whether to continue with processing the bone sample for DNA or C^{14} studies or employ strategies to remove these components without damaging the organic components of the bone.

The collagen results demonstrated the great variation in the amount of collagen contained in each individual bone, but the recently excavated long bones may prove to contain more collagen than the cranium; extending the study to include more samples would help to clarify this. Quantification of the collagen could be done at the same time as the soil intrusion studies using the FT-IR, as it would require less sample to be destroyed. A fuller understanding of bone and its properties especially at specific sites on each bone would be helpful for choosing samples.

The splitting factor results did not appear to be particularly useful, except to show the stored cranium apatite to be in a slightly higher crystalline state than the other bones, but this was not statistically significant; the role of soil intrusion and the crystallinity would need to be studied further. This analysis can be performed using FT-IR at the same time as soil and collagen analysis, and its inclusion in further work may help to clarify its usefulness in soil-interred bone.

The use of XRF requires 200 mg of bone, which may not be suitable for precious or limited bone sample available, but it is a worthwhile and useful tool especially when used in conjunction with the FT-IR. This method was able to demonstrate the relationship between the organic and inorganic phases of the bone, but the role that the soil intrusion played in the calcium and phosphate ratios would require further study.

Removal of the soil components from the bone to allow a full quantification of the splitting factor, collagen content and calcium and phosphate ratios without degrading the bone and obtain accurate results would be most useful. It is essential to determine to what extent the soil components play in the destruction or preservation of the organic and inorganic phases of the bone.

Chapter 5: The Dissolved Corpse: A Contemporary Liverpool Cemetery Study

Objectives

The objectives of the study were:

- To understand the burial environment of modern, contemporary, interred human remains.
- To further the understanding of bone degradation in the soil environment; and to identify those qualities that might improve assessing bones for DNA study.
- To identify potential forensic applications from the soil study results.

5.1. Introduction

Few studies have been conducted on known soil interred dissolved human remains (Beard et al., 2000). Consent was obtained for the study of two modern municipal cemeteries from Liverpool, UK. The cemeteries were approximately 6.4 km from each other. The cemeteries were established in 1909 and 1856 on land that had been previously used for farming and light industry, respectively. Both West Allerton and Toxteth cemeteries were created in response to the growing need for burial facilities as the population of Liverpool expanded during the Victorian industrial revolution. The cremation of human corpses was illegal until the late Nineteenth century.

5.1.1. Human Body Composition

The human body is made up of chemical elements originating from both the hard and soft tissues, most authors agree on the quantity of the major elements present in greater quantities, but there appear to be some discrepancies about the amounts of minor elements (Table 1). The variation in the quantity of these elements may be dependent on several factors including the age, sex, health and general well-being of the individual concerned (Table 2).

Table 1: Percentage of element in the average healthy human body, by author.

Author	O	C	H	N	Ca	P	K	Na	Cl	Mg	S	Fe	I
Tortora , Grabowski, 2000	65	18.5	9.5	3.5	1.5	1	0.4	0.3	0.2	0.1	0.3	0.1	0.1
Friedman, 1972	65	18	10	3	1.4	1.1	0.25	0.15	0.15	0.05	0.25	0.006	0.000016
Shier et al, 2012	65	18.5	9.5	3.2	1.5	1	0.4	0.2	0.2	0.1	0.3	trace	trace
Marieb, Hoehn, 2007	65	18.5	9.5	3.2	1.5	1	0.4	0.2	0.2	0.1	0.3	0.1	0.1
Martini, Nath, 2009	65	18.6	9.7	3.2	1.8	1	0.4	0.2	0.2	0.06	0.04	0.007	0.0002

Table 2 : Mean percentage of element in the average healthy human body (Friedman, 1972, Marieb and Hoehn, 2007, Martini and Nath, 2009, Shier et al., 2012, Tortora and Grabowski, 2000).

Element	Average	Low	High	SE
Oxygen	65.0	65	65	0.00
Carbon	18.4	18	18.6	0.11
Hydrogen	9.6	9.5	10	0.10
Nitrogen	3.2	3	3.5	0.08
Calcium	1.5	1.4	1.8	0.07
Phosphorus	1.0	1	1.1	0.02
Potassium	0.4	0.25	0.4	0.03
Sodium	0.2	0.15	0.3	0.02
Chlorine	0.2	0.15	0.2	0.01
Magnesium	0.1	0.05	0.1	0.01
Sulphur	0.2	0.04	0.3	0.05
Iron	0.1	Trace	0.1	0.02
Iodine	0.1	Trace	0.1	0.03

5.1.2. The Burial of Human Remains

Little work has been done on the detection of older buried remains, apart from attempts using ground penetrating radar, magnectometry, electrical resistivity, and geophysical methods, with limited results. Techniques such as electrical resistivity has had some improved results, but needs experience to use (Bevan, 1991; Conyers, 2006; Powell, 2004; Pringle et al., 2008). Problems identified with these technologies arose from the fact that clandestine graves tend to be shallow <100cm deep (Figure 1), in contrast to the single cemetery burials of between 100-140cm deep (Manhein, 1996). This resulted in the remains sometimes lying in the most dynamic part of the soil horizon, that can be affected by animal scavenging and scattering as well as the effects of environmental conditions (Haglund et al., 1988; Komar, 1998). Older historical (and sometimes forensic) skeletal remains can to be brought to the surface, fragmented and scattered as a result of agricultural and animal activities.

The burial of human remains can be either cultural, as a means of disposing of dead corpses and accompanied by relevant rituals and laws in a known designated area, in contrast to the forensic graves that are used as a way of hiding either a single or even large quantities of corpses. How an individual is buried in a cemetery, is governed by central government and local laws and detailed information is recorded and available

for inspection. In the case of clandestine or forensic graves a single interment is often done hurriedly, often just enough to dispose of the corpse resulting in a shallow burial. Mass graves can be very deep, especially where heavy earth moving machinery can be used to construct excavated pits in order to dispose of a large quantity of corpses, and soil bulldozed over the top (Haglund et al., 2001; Ruwanpura et al., 2006).

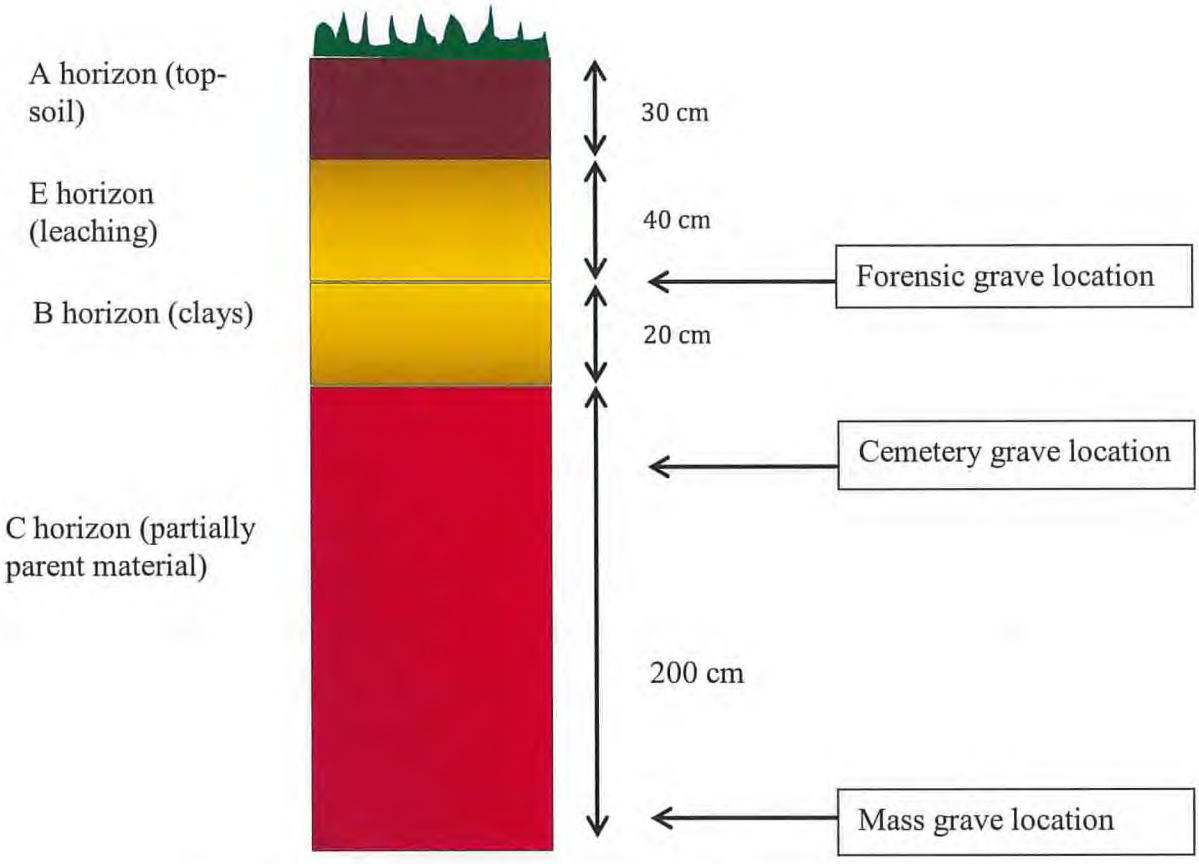


Figure 1: A disturbed soil horizon from different types of burial.

5.1.3. Bone Decomposition

Bone consists of both organic and inorganic compounds, the inorganic mineral component provides bone with its supportive matrix, whilst the organic collagen provides tensile strength (Turner-Walker, 2008). Factors that influence bone preservation include environmental stability, hydrology, depth of burial, exposure to the elements, temperature, pH, soil parent material, the age of the individual, wrappings and animal activity. Buried bone interacts with the environment differently than bone left on the surface and is wholly dependent on its environment for survival; bone can survive for centuries or decompose within a relatively short period of time

depending on the soil type and environmental conditions (Balzer et al., 1997; Behrensmeyer, 1978).

The action of microorganisms can assist in the degradation of the bone collagen by the production of collagenase enzymes (Balzer et al., 1997; Child, 1995a; Child, 1995b; Dixon et al., 2008; Hedges, 2002; Trueman and Martill, 2002). This degradation can begin once the mineral phase of bone has begun to dissolve under the action of water, as the pores in the intact bone structure are too small to allow the collagenase enzymes to penetrate (Nielsen-Marsh et al., 2006).

The role of water (hydrology) is fundamental to the preservation of interred bone (Carter et al., 2010; Hunter and Cox, 2005). The effects of environmental water are compounded by the influence of temperature and pH. When exposed to the effects of water, the bone mineral components can degrade into both soluble (brushite) and insoluble (calcite) compounds, depending on the pH (Piepenbrink, 1989). Removal of water from remains during exposure to hot, dry temperatures (natural mummification) can result in the preservation of bone and tissue for considerable lengths of time (Galloway et al., 1989; Wheeler, 1995). Conversely, remains can be preserved in the permafrost for centuries due to the water being locked up in ice crystals, resulting from the low environmental temperature, and where there would be a significantly reduced destructive impact from microorganisms (Willerslev et al., 2004). Bone that remains in alkaline water permanently can be better preserved than bone that is exposed to a fluctuating water table; similarly juvenile bone can degrade faster than adult bone due to its porous and fragile nature, especially at lower pH values (Gordon and Buikstra, 1981). The presence of higher water pH can directly affect the preservation of human remains with the formation of adipocere (Fiedler and Graw, 2003; Forbes et al., 2004). These often well preserved waxy remains are found in cool, moist, anaerobic soils with poor drainage, consisting mainly of clay or loamy parent material from interaction between the acidic body fat and the alkaline water in the interment environment.

5.1.4. Liverpool Cemetery Soil and Bone Interaction

The impact of the composition of the soil parent material and bone, has only been discussed in relation to hydrology, as it is the soil response to the precipitation and water table that governs the stability of the interred bone components. The role of weathering by parent material without the action of hydrology has not yet been explored, but the effects with hydrology have, especially wet sandy soils, where the decomposition was observed to be more advanced (Crowther, 2002; Carter et al., 2010; Nielsen-Marsh and Hedges, 2000; Turner-Walker, 2008). Soils that contained larger particle sizes such as coarse sand and gravel could yield bone in a considerably poorer condition than those with a smaller particle size such as clay. When these conditions are applied to bone material in the form of frequent temporary water table rises that intrude into the grave area, low soil pH, shallow burial, along with fluctuating soil temperatures, with an open textured parent material such as sand, the complete decomposition of both the organic and mineral components of dead bone can occur within a few years (Balzer et al., 1997; Carter et al., 2010; Crowther, 2002; Nielsen-Marsh and Hedges, 2000; Pate and Hutton, 1988; Trueman and Martill, 2002). Crowther (2002), and Pate and Hutton (1988) discussed the ionic exchange relationship between bone and soil, and how this exchange could continue until the bone was fully incorporated into the soil matrix. It is this leaching of bone components into the soil matrix that could assist in furthering the understanding of bone decomposition path-ways, as well as locating the presence of older skeletal remains. Soil related analysis on human remains has tended to focus on the post-mortem interval (Balzer et al., 1997; Mann et al., 1990; Vass et al., 1992; Turner and Wiltshire 1999; Vass 2001) or the location of the recently interred corpse by studying the biological components of decomposition, (Carter et al., 2007; France et al., 1992; Larson et al., 2011; Owsley, 1995; Rodriguez and Bass, 1985; Statheropoulos et al., 2007; Van Belle et al., 2009).

5.1.5. Soil Microscopy

Light microscopy observations on each soil sample enable the visual identification of the soil matrix for any artefacts other than bone that could provide additional explanation of the soil chemistry. The examination of soil using the light microscope has formed one of the basic soil investigation techniques after examination of soil

characteristics using the naked eye (Canti, 2003; Jakes and Sibley, 1982; Williams, 1977). Visual examination of the soil can provide useful information about the texture of soil composition (sand, clay, silt, quartz) and previous use or contamination of the land. This information may help to explain the decomposition of the remains, and the movement or retention of individual elemental ions in the soil. This analysis is informative, quick and inexpensive to perform. These observations can provide invaluable evidence of past use of the land in the form of artefacts that that are resistant to the effects of degradation in the soil environment at least in the short term. Anthropogenic activities such as previous land use or modification may be seen as small artefacts in the soil matrix, these artefacts can be placed historically by noting their presence in the soil horizons (apart from grave reuse from subsequent burials, common with family plots). It is possible to see the effects of coal or charcoal burning, domestic waste deposition, farming practices, industrial activities, rebuilding or land dwelling clearance, from evidence in the soil matrix. Activities such as dumping wood or coal ash onto the soil can have short and long-term effects; coal ash causes acidification of the soil due to the presence of sulphur dioxide, and wood ash has an alkaline effect that can increase the pH of the soil considerably (Egeback et al., 1984; Flues et al., 2002; Ohno and Erich, 1990). The cemeteries chosen for this study have been subject to human occupation for centuries and the use of microscopy may yield some information about the previous use of the land and human activity on the sites that may provide additional evidence for understanding bone decomposition.

5.1.6. Liverpool Cemetery Soil X-Ray Fluorescence (XRF)

(For further details see p 78) XRF can only analyse the heavier elements of the periodic table, from sodium onwards, and is not able to give results for other elements such as carbon, but will represent these as a "balance". The "balance" can be of great importance when assessing a given sample as it can help to establish other qualities in the sample, such as an estimation of the water content. XRF can be performed using semi or full quantitative analysis and the results are represented as a proportion of 100%. For semi-quantitative analysis a block of metal is used as a standard (usually aluminium), and also be programmed to analyse oxides. The semi-quantitative analysis "scans" the sample and will present the results in reference to the standard, but caution must be used with those elements that appear in smaller quantities as they

can be confused with other elements, however the curves can be double checked for accuracy. Those elements being studied appear in larger quantities and elements < 0.010% (100 ppm) would be disregarded.

The benefits of using XRF include being well suited to establishing chemical composition of soil samples; it is easy to use and non-destructive. One important consideration was the difference that occurs between different soil matrices in close proximity, one soil sample can significantly differ from another just a few centimetres away. However, XRF has been successfully used on a wide range of archaeological samples including bone (Carvalho et al., 2004; Craig et al., 2007; Janos et al., 2011; Mantler and Schreiner, 2000; O'meara et al., 2001).

5.1.7. Liverpool Cemetery Soil pH

The pH of the soil is an important part of routine soil analysis; and one of the vital investigations into the degradation dynamics of human remains (Carter et al., 2010; Fiedler and Graw, 2003; Forbes et al., 2004; Gordon and Buikstra, 1981; Pate and Hutton, 1988; Turner-Walker, 2008). The interaction of bone with the burial environment is vital to understanding the preserved state or destruction of excavated bone. Soil analysis techniques use well established methods that are non-destructive to bone that can be employed to investigate the mobility of bone and soil elemental ions in the immediate environment of the empty grave, decaying remains or bone.

5.1.8. Liverpool Cemetery Study

Consent was given to take soil samples from two cemeteries from Toxteth and West Allerton, Liverpool, Merseyside, UK (Figure 2). Both cemeteries were municipal, and contained burials from a wide variety of religions, these being placed in separate areas of the cemetery. The cemetery also contained separate areas for cremation burials.

The grave-diggers from both cemeteries had observed that very little of the corpse, including teeth and bone, remained within 20-30 years post interment, depending on the size of the adult, even if the coffin remained intact. It was also noted that there was often a high, fluctuating, water table. These temporary water table rises were observed to intrude into the upper soil horizons, and in some cases cause mild flooding of the

surrounding area; this caused the cemetery graves to be subjected to water intrusion during sustained wet weather. There were old streams running through both cemeteries, these are not visible today (Earp and Taylor, 1986; Warrington et al., 1980).

Figure 2: Location of Toxteth and West Allerton cemeteries, Liverpool (Google maps © all rights reserved), the two cemeteries sampled in this study.

The decomposition of these remains were investigated using a multi-disciplinary approach in order to be able identify a range of biochemical and physical changes in the soil from adult graves ranging from 10 to 150 years post interment.

5.1.9. Liverpool Cemetery Geology

It was important that both cemeteries shared the same bedrock geology and environmental conditions in order for them to be able to be used for consecutive analysis. The parent material consisted of Early Triassic Chester Pebble beds, sand,

siltstones, sandstone and Till beds (Earp and Taylor, 1986; Warrington et al., 1980). Both cemeteries had old subterranean streams running through them, and along with sustained rainfall, caused the water table to rise and fall frequently.

West Allerton is situated 30-40 m above sea level (OD- above ordinance datum) and Toxteth is situated 60 m above sea level OD (taken from 1994, OS Landranger map 1:50,000, sheet 108, Liverpool and surrounding area).

5.1.10. West Allerton Cemetery (1909)

The previous use of the land has important implications for the cemetery studies, as contamination could be demonstrated in the results. It was important to obtain as much accurate information about the sites as possible, including maps and land use historical records.

The West Allerton site has a long history of human occupation, demonstrated by the presence of the Neolithic Calder standing stones, located nearby. Early maps (Ordnance Survey First Series1803-1863, taken from Vision of Britain 2004) show the area dedicated to farming, with woodland and fields (Figure 3). Later descriptions of the area identify it as still being an area with lots of green spaces, for the more wealthy residents of Liverpool (Farrer and Brownhill, 1907). This map shows the area before the land was acquired by the local council for the cemetery, as part of the large farm estate of "The Hall". The later map (1925) shows the redevelopment that took place over the next century, with the fields and farms replaced with houses and parks (Figure 4). The area is still known as "Springwood" due to the presence of the high water table.

Figure 3: West Allerton cemetery, marked in red,(Ordnance survey first Series 1803-1864, taken from Vision of Britain, 2004).

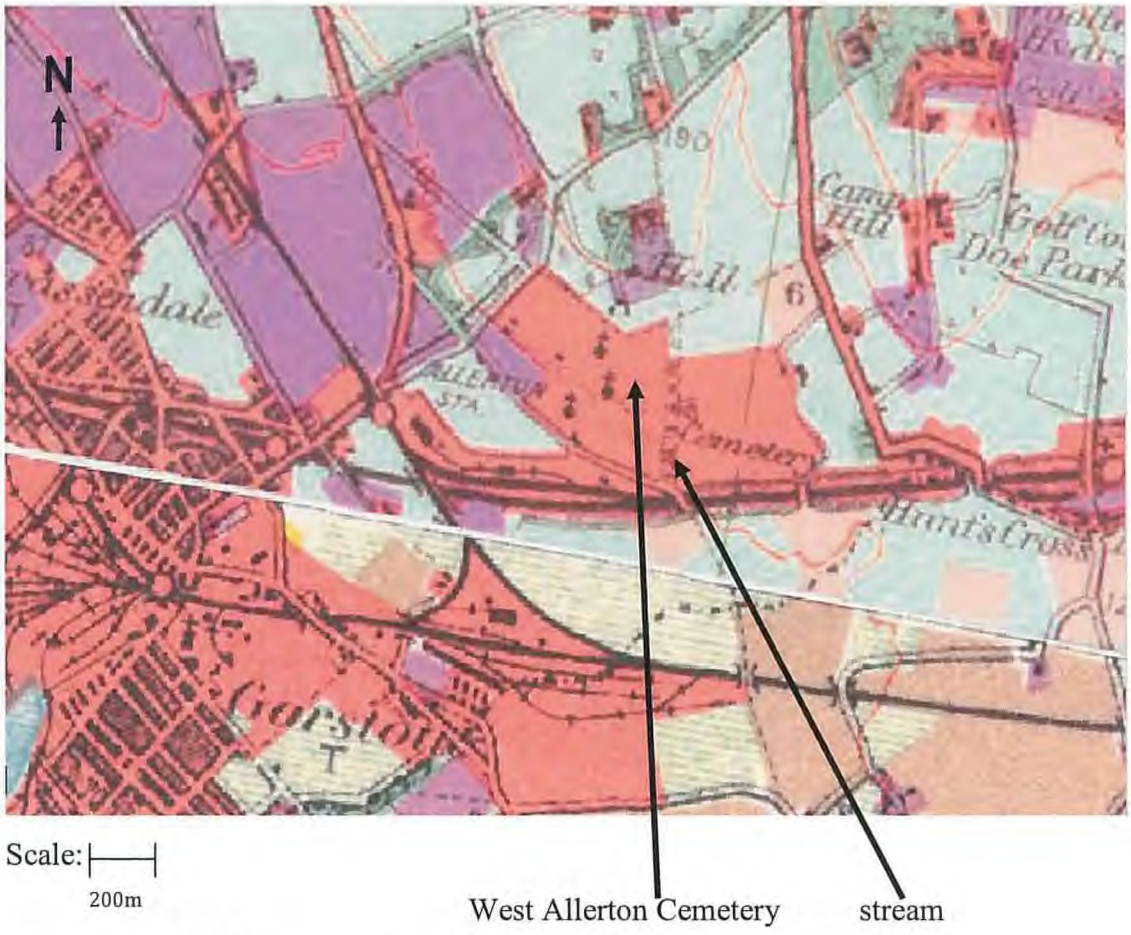


Figure 4: West Allerton cemetery land utilisation map 1925-1945 (take from Vision of Britain, 2004) Note: For map key see Figure 7.

1.2.11. Toxteth Cemetery (1856)

The Ordnance survey map of the Toxteth cemetery site (First Series 1803-1864, taken from Vision of Britain 2004) identifies the Toxteth cemetery as being located near the rope walks site, with the cemetery lodges occupying the demolished rope walk factory building site; Smithdown Road can be seen above it. There was a tannery close by, along with the rope works factory (Figure 5). The land was bought from the Earl of Sefton in the 1850's in response to the needs of the city for burial outside of the urban areas, due to the increasing population of Liverpool. Prior to this, the area had been seen as a quiet hamlet having been a royal park for over 400 years, losing this status in the Seventeenth century. With the influx of workers to the increasingly industrialised Liverpool, the area quickly became a notorious slum, with several demolition episodes before the Twentieth century (Farrer and Brownhill, 1907).

Industry close to the cemetery site included the copper extraction works, built by Charles Roe in 1776. This site was converted into a pottery in 1794, becoming the Herculaneum ceramic works until 1833, subsequently converting into a ship dock that bore its name (Figure 15). These maps were of the earlier series, as they were able to show the location of the Herculaneum works and the land use prior to being used for the cemetery, in 1856. The copper works were approximately 5 km from the cemetery site. The land use map 1925-1945 (Figure 6) shows the area as being completely urbanised into part of the city of Liverpool, but some redevelopment created parks and limited green spaces. There was an open stream running through the cemetery that later became subterranean, and can still be seen on contemporary aerial images.

Figure 5: Toxteth rope walk site prior to its redevelopment as a cemetery, marked in red (Ordnance survey First Series 1803-1864 taken from Vision of Britain, 2004).

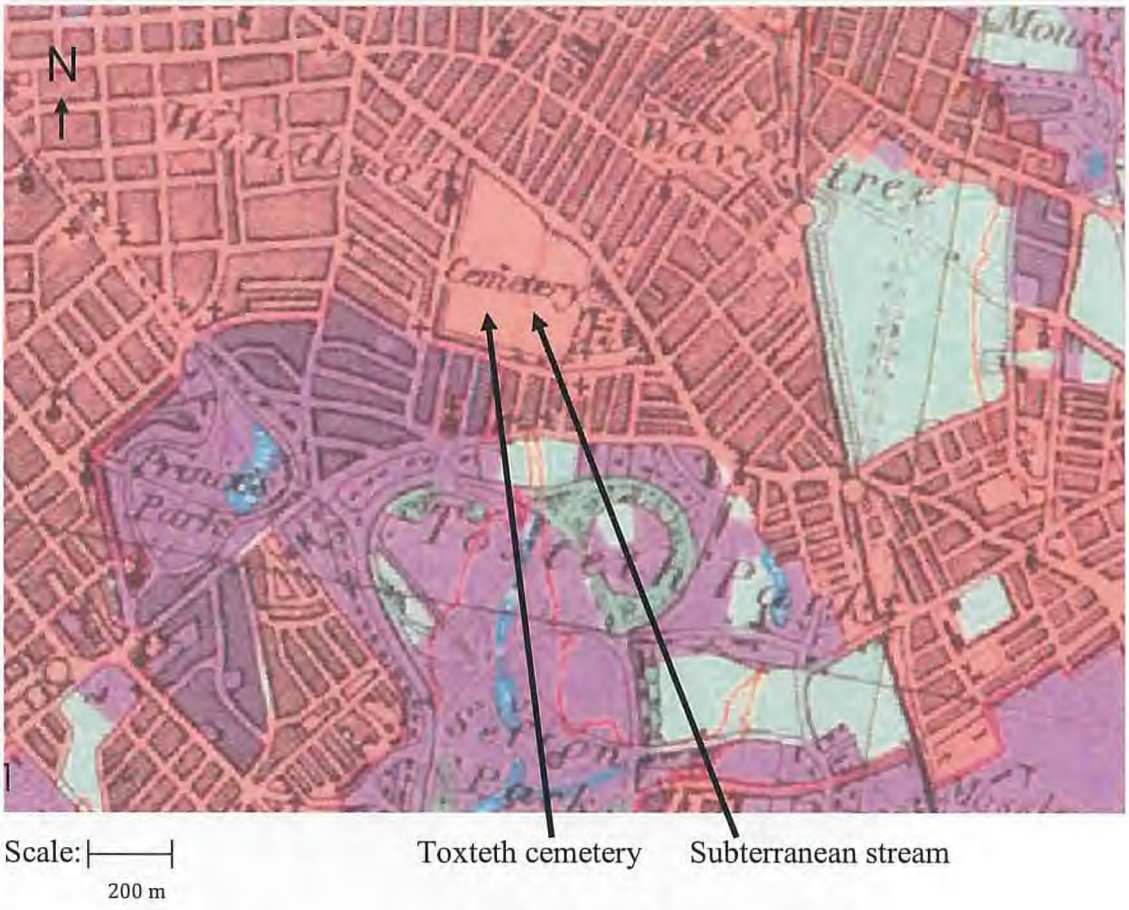


Figure 6: Toxteth Cemetery: Land Utilisation map 1925 -1945 (taken from vision of Britain, 2004) Note: For map key see figure 7.

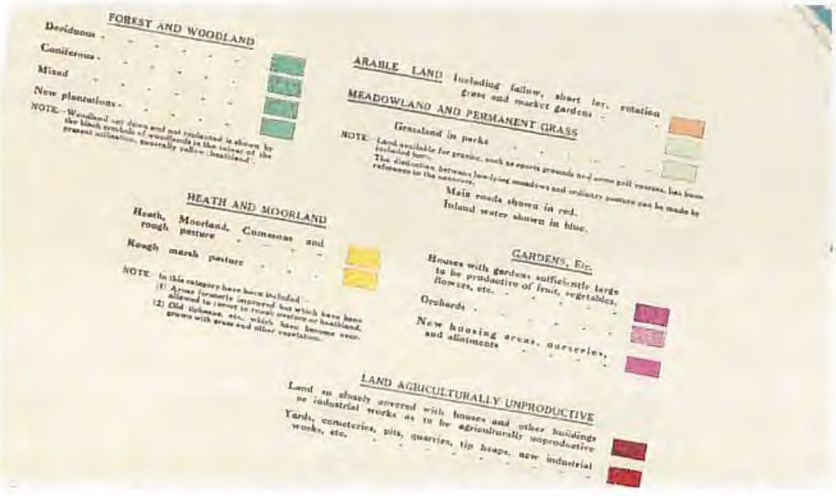


Figure 7: Land utilisation map key 1925-1945 (taken from Vision of Britain). Key for map 4&6.

5.2. Materials and Methods

5.2.1. Ethical Considerations

Consent was agreed with the Manager of the municipal cemeteries, and an agreement drawn up in relation to ethical issues that could arise from working in a sensitive public area (see Appendix: Chapter 5). The use of soil analysis avoided any ethical concerns, as the grave itself remained undisturbed at all times.

5.2.2. Grave Sampling Strategy

The graves from Liverpool are routinely dug for four individuals unless requested otherwise, but this can differ between cemeteries. Jewish and Muslim graves were always single burials, but the Jewish grave areas were difficult to access as the grave was often lined with concrete and a slab placed on top, each slab butted to the adjoining one making it impossible to sample the soil in between. A single grave could vary between 100-140cm in depth; the coffin being normally 45-50 cm deep. The soil that was excavated from the grave was not necessarily the same that would be returned to fill in following the coffin interment, especially if many individuals planned on attending the graveside interment, as the soil was taken away to a central storage area. Following interment the grave was filled in and was topped up periodically to avoid an uneven surface as the soil settled, until it was level and could be turfed over.

It was important to consider that the soil horizons being sampled were disturbed, and were considered in this context (Figures 1 & 8b). The soil had also suffered reworking from the interment resulting in a disturbed soil horizon that would respond to both the environmental conditions and the interned coffin and corpse. It is this relationship between the soil, environment and interment that could provide useful information about the decomposition of bone in the burial environment, both from a forensic as well as an historic perspective, as they both suffer disturbance of the soil horizons.. The soil would eventually reach a state of equilibrium, however the time taken for the soil to reach this state was not known.

The soils from two adult graves (n=2) were sampled from the left and right side of the grave cut from 7 time periods (n=14) 2000, 1980, 1960, 1940, 1920, 1900 and 1850 using a 7cm Edelman combination hand soil auger, at 4 depths: 0-50 cm, 50-70 cm, 70-90 cm, and 90-110cm (Figures 8a & 8b). The Edelman auger was used, as it was

considered more suitable to sampling soils that contain aggregate, or more difficult to work.

Each time period: 2 graves, left and right, at 4 depths = Total of 16 samples
West Allerton: (2000, 1980, 1960, 1940, 1920) = Total of 80 samples
Toxteth: (1900, 1850) = Total of 32 samples
5 control samples from West Allerton = 20 samples
2 control samples from Toxteth = 8 samples
Total studied samples = 140

Each grave was located on a map of the cemetery (Figures 10 & 11). The general condition of the graves was recorded and included the number of inhumations interred, level of subsidence and number of inhumations to the left and right, if known (Table 3).

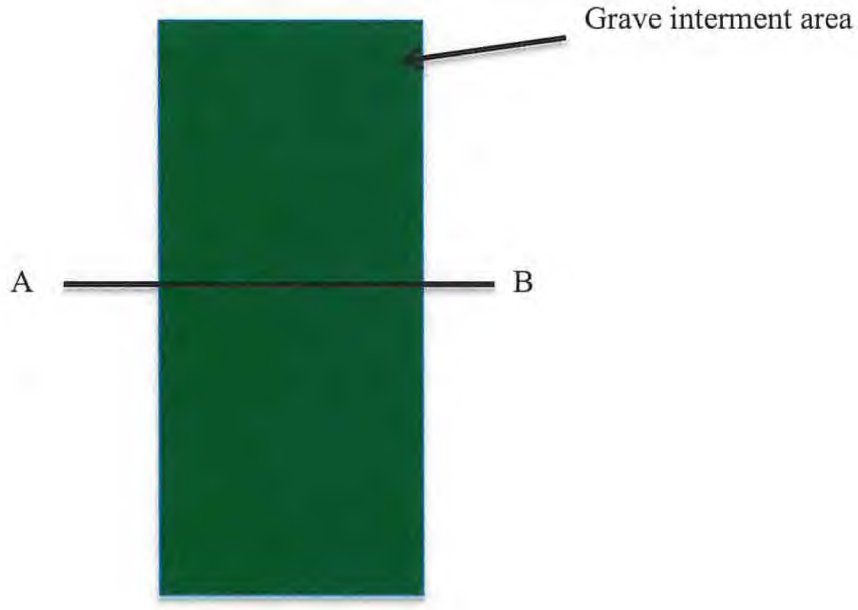


Figure 8a: Sampling method either side of the grave interment area, A and B denote where the auger was used to take the soil sample.

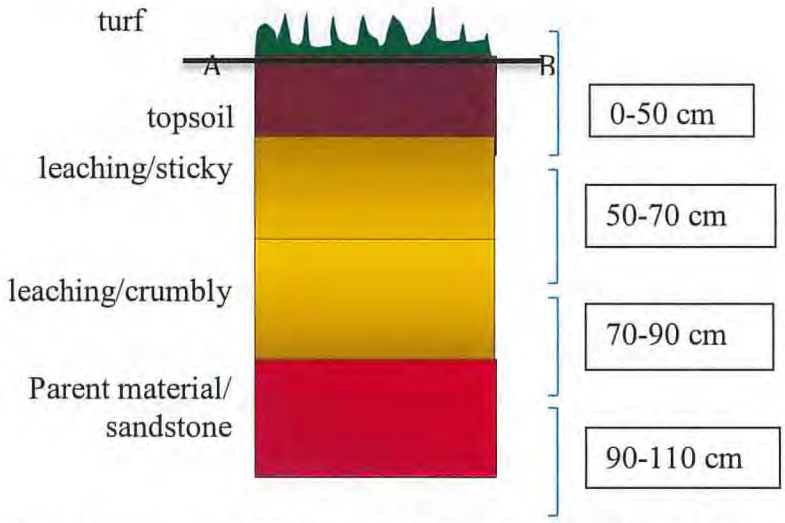


Figure 8b: Sampling interval depths used to sample the soils, in relation to the Liverpool cemeteries soil horizons.

In order to avoid the grave area and contact with any human remains that could be present, a 20 cm distance was taken each side of the headstone (A and B on Figures 8a 8b), or 20 cm outside of the grave subsidence outline, if present, was used. The area for sampling was measured equi-distance from the headstone and foot of the surface of the grave. Whilst each grave would not generally be the exact year, they were still considered as part of a time period (which could have a small discrepancy). Care was taken to select graves that only contained one inhumation but it was sometimes necessary to use graves that contained more than one individual. These graves would only be sampled for older burials or where there was a substantial period of time between the burials. These coffins would be placed on top of the previous inhumation with approximately 0.75 m of soil separating them. Multiple graves would be very much deeper with the predetermined number of interments specified, the most recent known to be 100-140 cm deep. The deeper graves could penetrate into the hard sandstone parent material, with the grave digging staff utilising heavy drilling machinery.

Sampling the soil at 110cm would intrude into the first few centimetres of the area adjacent to either side of the coffin itself. The soil was extracted from both the left and right side of the grave cut using the auger and a separate 400g from each side and depth was taken, this was mixed thoroughly at the site, and placed in a plastic box.

Each boxed sample was given a unique code and any surplus soil was returned to the sampling hole when the total sampling was completed. This was done in order to dispose of the surplus soil and to prevent trip injury. A plug of turf was then placed into the surface soil to disguise the area. Advice was taken from the cemetery staff to ensure that areas that were observed to have frequent surface flooding were avoided. Graves sampled from 2000-1920 were obtained from West Allerton (opened in 1909), and 1900-1850 from Toxteth (opened 1856). Some of the graves contained hard sandstone parent material, which made sampling very difficult, and on occasion had to be abandoned. Soil samples were taken from the predetermined depths, crushed, homogenised and dried to prevent the growth of microorganisms and fungi that could affect the chemistry results.

The control samples were taken from the verges across the road from any graves, approximately 10m away, making sure there was no incline, but within the cemetery perimeter itself. As with the grave sampling it was not always possible to go down to the full 110cm with all samples. Each core sample interval demonstrated its own unique characteristics that could distinguish it from the next. A typical soil core sample is illustrated in Figure 9.

Table 3: Grave characteristics:

Time period	No. inhumations	Subsidence	Burials to R	Burials to L
West Allerton				
2000 (2000)	1	None	1970, 1991	road
2000 (1998)	1	Slight	2002	2001, 2003
1980 (1982)	1	Slight L	1972	unknown
1980 (1978)	2	Minor	1955, 1965, 1969	1972, 1987,1999
1960 (1959)	2	None	1939, 1988, 2008	1939, 1951, 1967, 1974
1960 (1958)	1	Slight	1948, 1944, 1946, 1955	unknown
1940 (1943)	1	Severe	1945, 1965	1946
1940 (1942)	1	Severe R	1942, 1950	unknown
1920 (1924)	1	None	Unknown	1924, 1941
1920 (1922)	1	None	Unknown	unknown
Toxteth				
1900 (1899)	3	Minor	1895, 1862, 1857, 1858	unknown
1900 (1895)	3	None	Unknown	unknown
1850 (1862)	3	None	1861,1879,1894,1899,1902	1860,1870,1875,1878,1892
1850 (1857)	1	moderate	Masonic memorial	unknown

General soil sample descriptions:

- **0-50 cm:** The dark topsoil, usually humus rich, and crumbly in texture, this was usually about 20-30 cm deep. This dark soil then proceeded down into the first part of the leached horizon that was sticky in consistency.
- **50-70 cm:** The leached horizon continued down and remained sticky in consistency.
- **70-90 cm:** The sticky consistency became more dry and crumbly in texture.
- **90-110cm:** The dry texture continued through to the dark soil and parent material

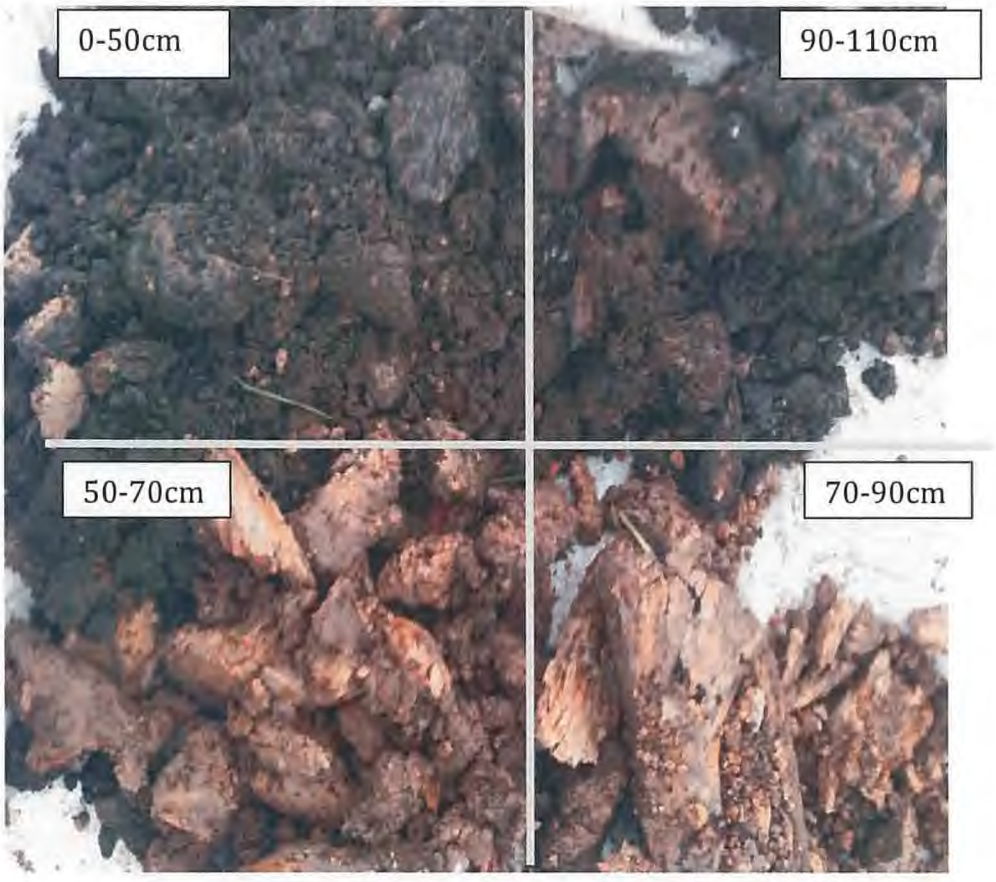


Figure 9: Typical cemetery soil core samples taken at each pre-determined depths.

5.2.3. Grave Maps

Each grave was located on a map (Figures 10 & 11). Each grave and control from West Allerton was limited to the top of the cemetery due to the surface flooding that affected the lower part.

5.2.4. Soil Preparation

Each soil sample was oven dried at 37˚C, having broken up any clumps, and removed any aggregate and plant material. Each soil sample was then ground as fine as possible before being put through a cleaned, scrubbed and rinsed 1mm metal soil sieve and then returned to the original storage box. Decomposing domestic bone china, and building material from the demolition of the old factory was noted in the Toxteth cemetery control samples.

5.2.5. Microscopy Observations

The soil sample was first examined using the naked eye and any larger pieces or artefacts were removed, identified and recorded. Approximately 0.5g of well homogenised soil was placed on a plastic petri dish and examined using a Stemi DV4 light microscope (Carl Zeiss, Gottingen, Germany), the results recorded and represented graphically.

KEY

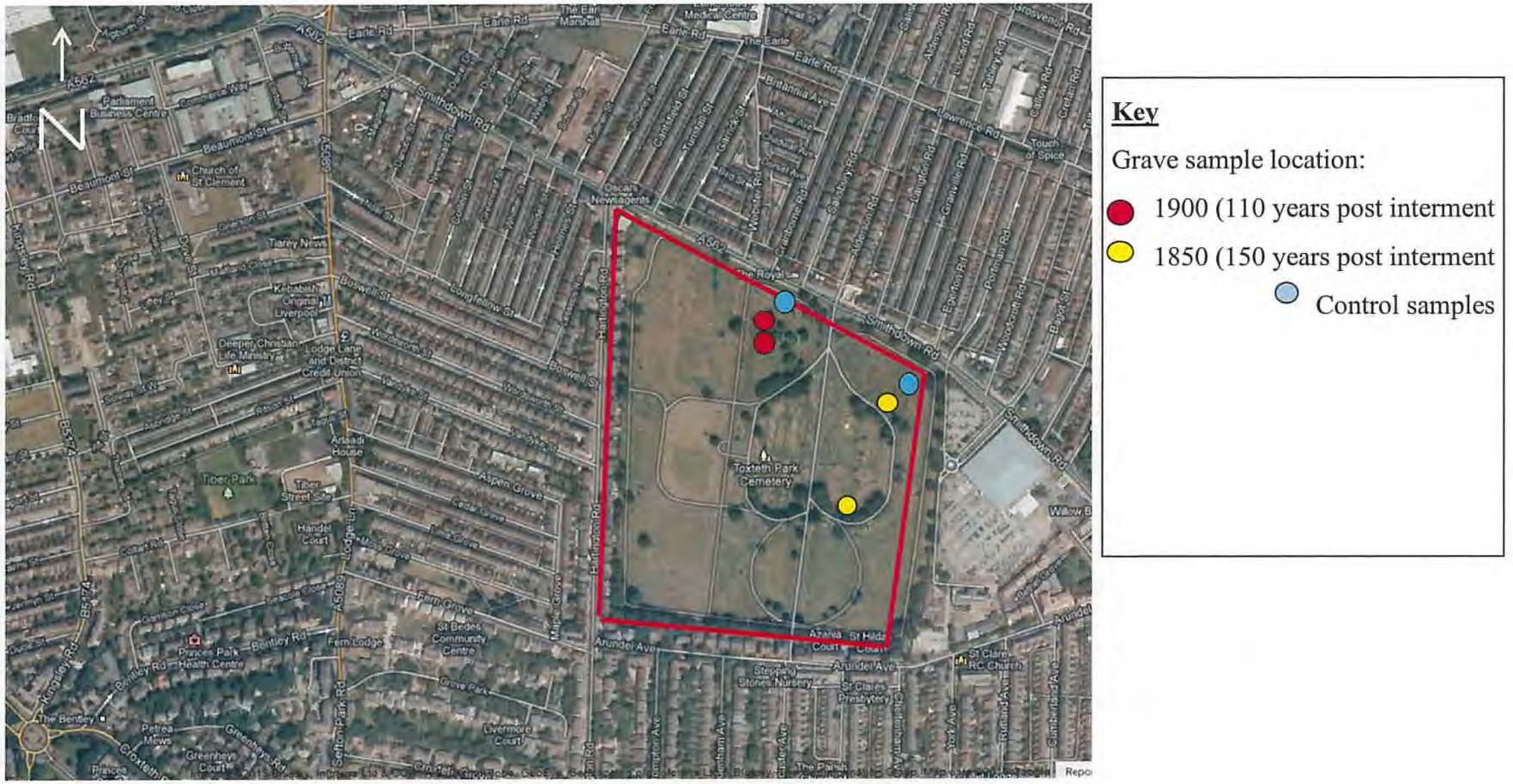
Grave sample location:

- = 2000 (10 years post interment)
- = 1980 (30 years post interment)
- = 1960 (50 years post interment)
- = 1940 (70 years post interment)
- = 1920 (90 years post interment)

Control samples =

Scale: 200m

Figure 10: West Allerton grave and control soil sampling locations (Google Earth).



Key

Grave sample location:

- 1900 (110 years post interment
- 1850 (150 years post interment
- Control samples

Scale: 100 m

Figure 11: Toxteth grave and control soil sampling locations (Google Earth).

5.2.6. X-Ray Fluorescence

Soil from the original storage box was re-used, making sure that an homogenous mix was obtained first. Approximately 4g of soil was placed into a 31mm open-ended x-cell cuvette (Spex Certiprep, Middlesex) that had been covered with 4 micron prolene X-ray film (Chemplex, Florida), tamped down with a small flat bottomed pestle, ensuring the even and complete spread of soil, and then capped.

XRF was performed using an EDX-720 Energy X-ray dispersive spectrometer (Shimadzu, Japan) with EDX software: DXP-700E version 1.00 Rel. 017 (Shimadzu, Japan). The soil samples were subjected to a semi-quantitative analysis, each grave and control sample were performed in triplicate. A semi quantitative analysis was performed in order to quantify all the elemental components of the soil. The results were represented as a percentage of the total elements detected, however the XRF cannot individually detect the lighter elements of C, H, N, O, but these are represented as a combined total. The results for five elements (Fe, Ca, Mg, P, K) associated with decomposition were extracted and subjected to statistical analysis.

5.2.7. pH Analysis

The pH of the soil samples was performed using 10g of sieved homogenised soil and 25 ml of deionised ultra-pure water, mixed well and left to equilibrate for 15 minutes in a small plastic beaker. The pH was performed in triplicate using a pH checker and probe (Hanna instruments, SIS, Wilford, Nottingham, NG11 7EP, UK).

5.2.8. Data Analysis and Processing

The chemical element data obtained was imported to Microsoft Excel and SPSS version 17 (IBM, USA) for statistical analysis.

5.3. Results

For statistical analysis output please see Appendix: Chapter 5, 1-4

5.3.1. (i) Microscopy

The results from each of the cemeteries were combined for each year group and depth. As there were two graves from each year group (n=2) and there were samples from the left and the right of each grave (n=2), this would result in a total of 4 samples for each year group. As previously stated, where sampling was difficult the number of samples could be fewer and this is stated in the sample total for each result. Each of the year group graves could be located on the maps shown in Figures 10 and 11.

Each of the control samples were represented individually, therefore n=1, although there were five control samples in total. This enabled the use of the maps from Figures 10 and 11 to be used comprehensively.

Clay, Sand and Quartz

0-50 cm:

Quartz and clay dominate the soil structure, with some sand being seen (Figures 12 & 13).

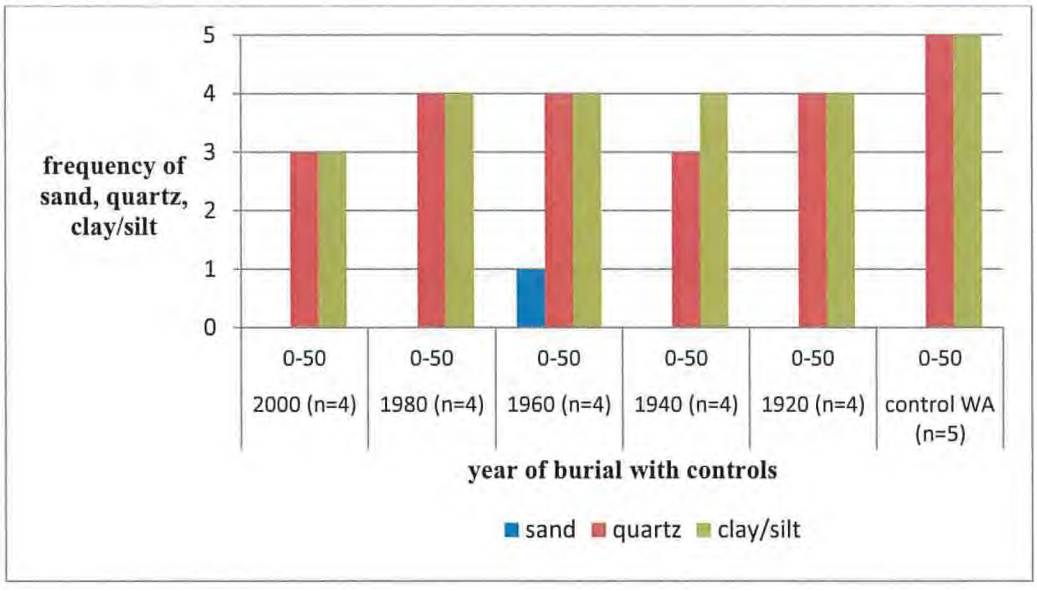


Figure 12: Frequency of sand, quartz, clay or silt 0-50 cm from West Allerton cemetery.

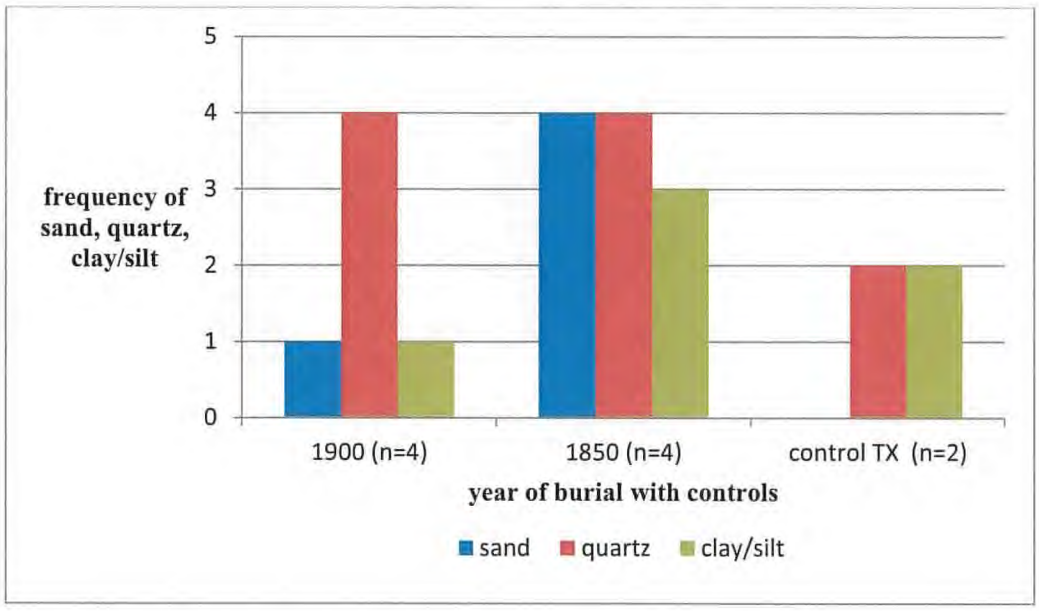


Figure 13: Frequency of sand, quartz, clay or silt 0-50 cm from Toxteth cemetery.

50-70 cm:

Quartz and clay/silt continue to dominate the soil structure (Figures 14 & 15).

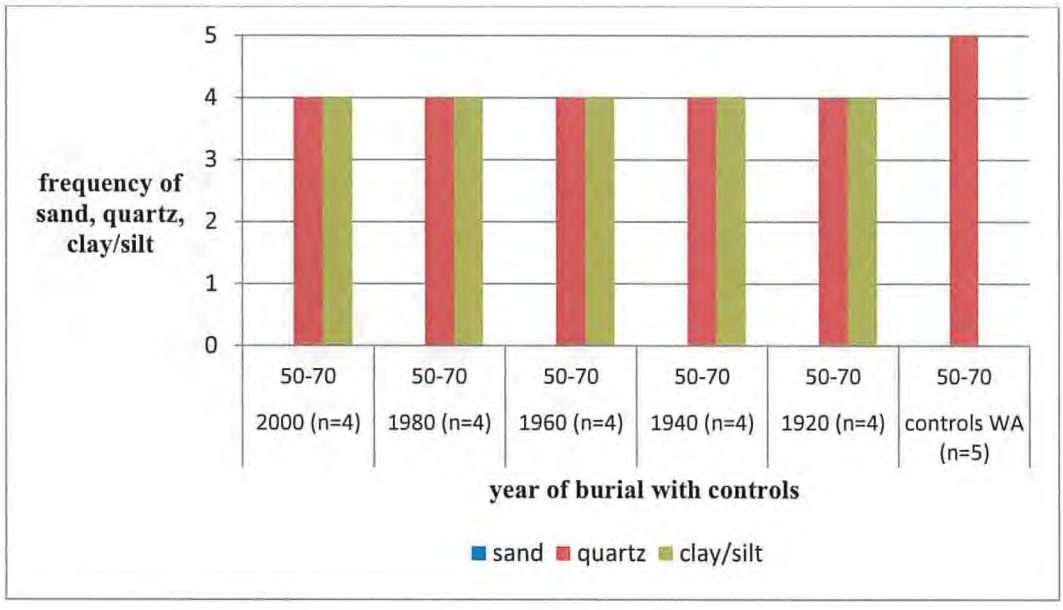


Figure 14: Frequency of sand/quartz/clay or silt 50-70 cm from West Allerton cemetery.

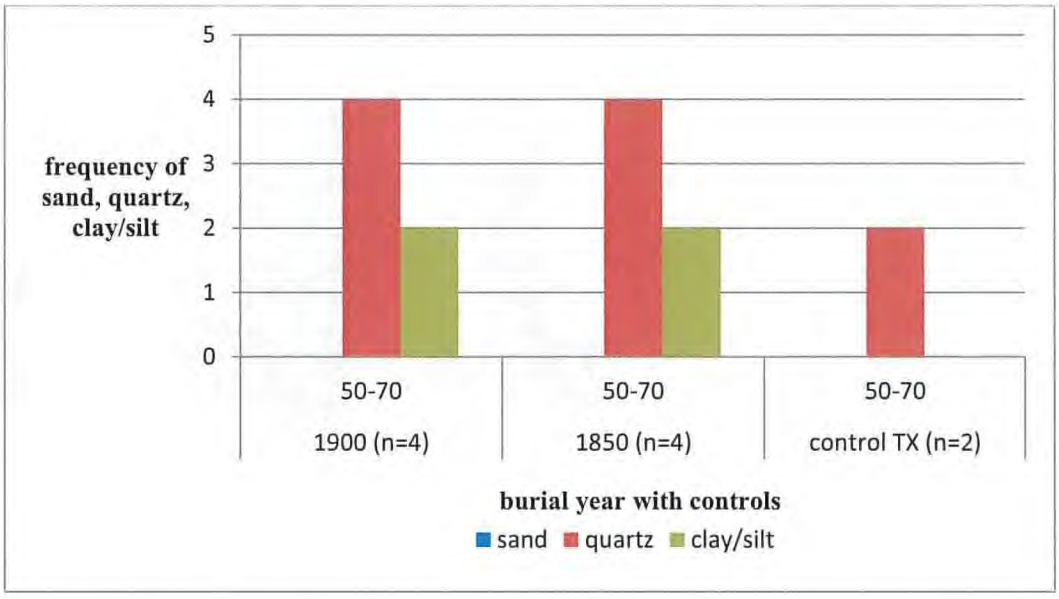


Figure 15: Frequency of sand/quartz/clay or silt from Toxteth cemetery 50-70 cm.

70-90 cm:

Quartz, clay/silt continues to dominate the soil structure, with sand being seen occasionally (Figures 16 & 17)

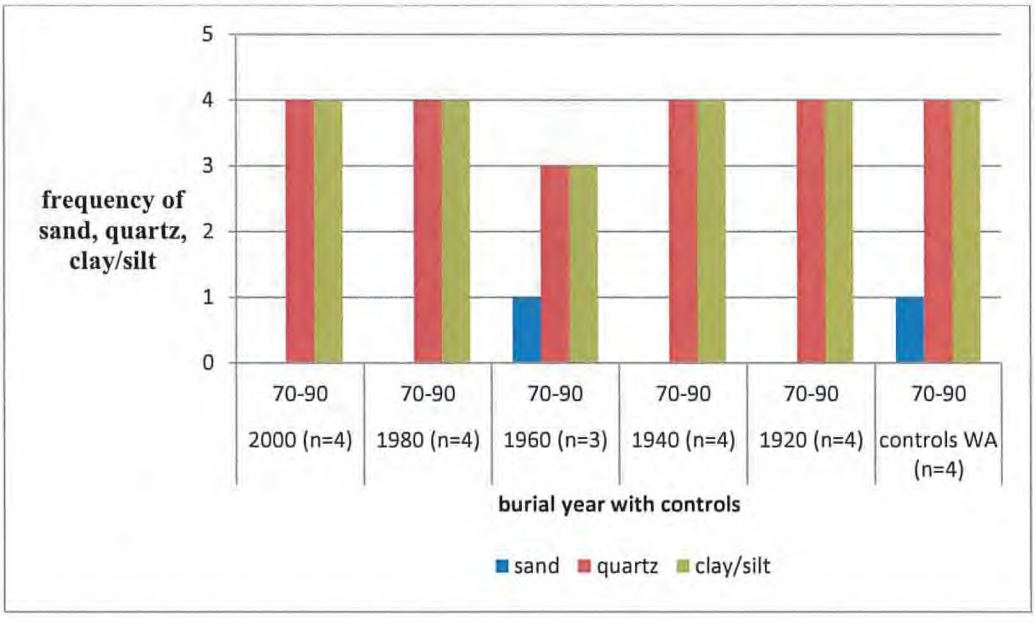


Figure 16: Frequency of sand/quartz/clay or silt 70-90 cm from West Allerton cemetery.

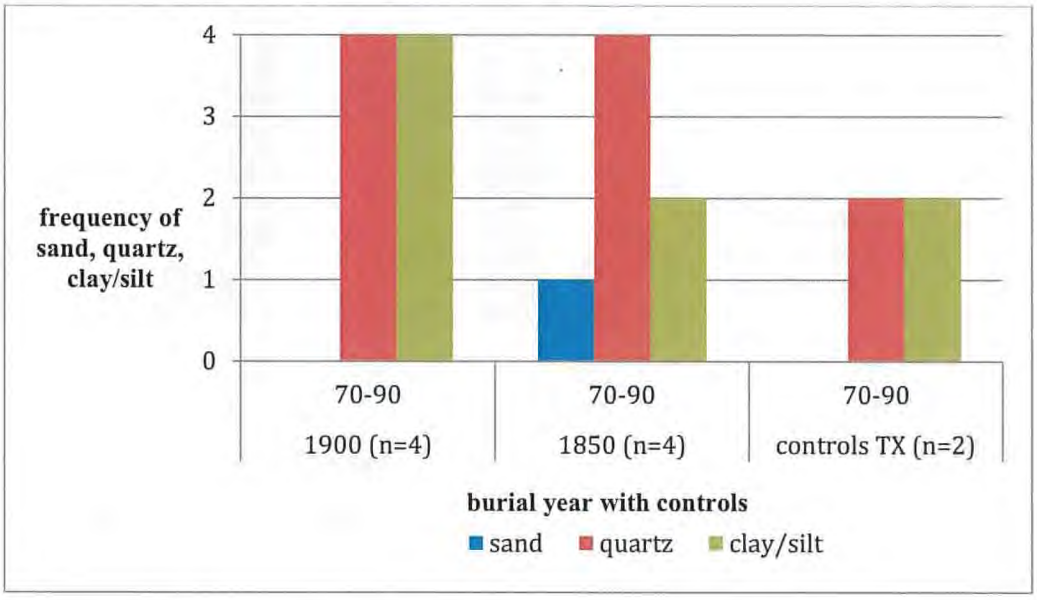


Figure 17: Frequency of sand/quartz/clay or silt 70-90 cm from Toxteth cemetery.

90-110 cm:

Quartz and clay/silt remained the dominant feature of the soil structure (Figures 18 & 19).

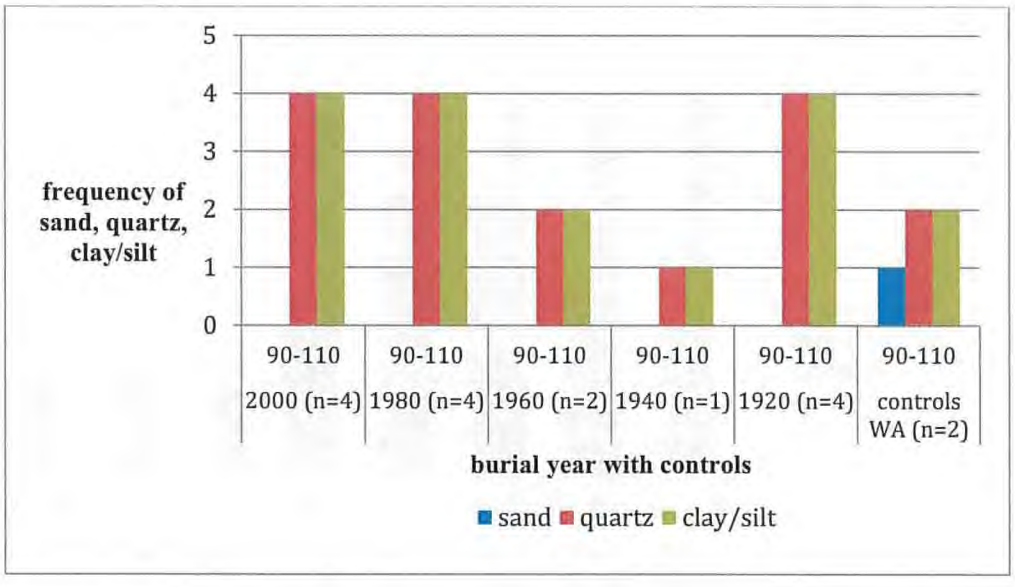


Figure 18: Frequency of sand/quartz/clay or silt 70-90 cm from West Allerton cemetery.

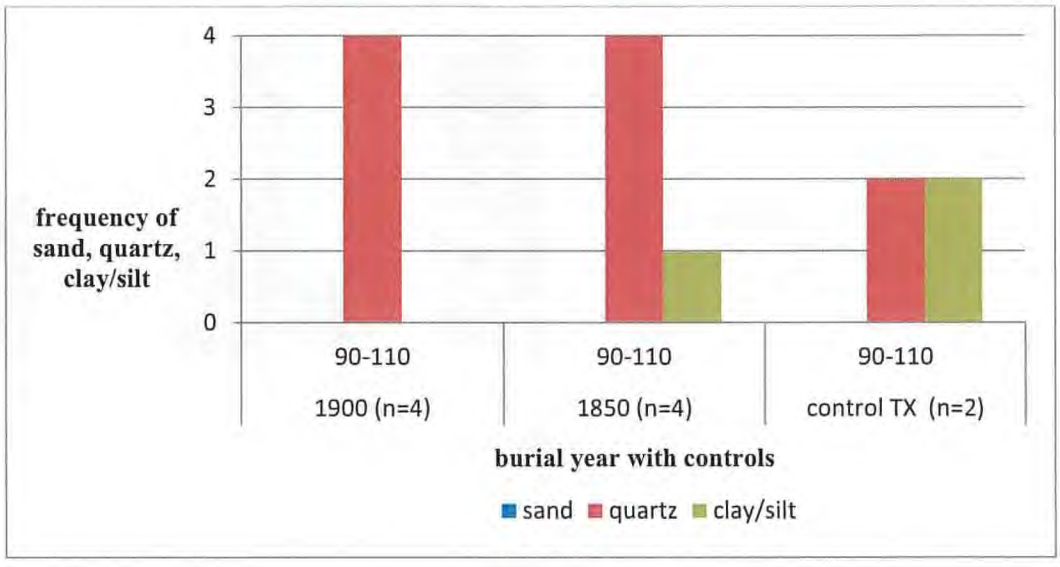


Figure 19: Frequency of sand/quartz/clay or silt 70-90 cm from Toxteth cemetery.

Coal and charcoal

0-50 cm:

Coal fragments appeared in all soil samples apart from one from Toxteth cemetery. This was in sharp contrast to the presence of charcoal, which did not appear in any of the control soil samples or Toxteth cemetery soil samples. Between ½ - ¾ of West Allerton soils contained charcoal (Figures 20 & 21).

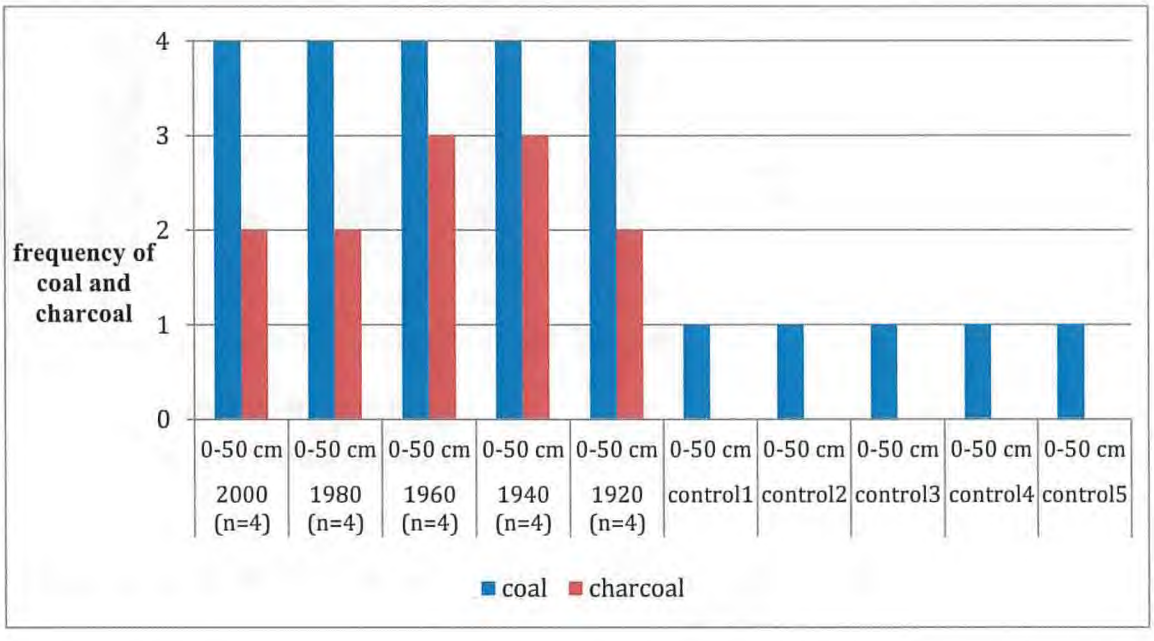


Figure 20: Frequency of coal and charcoal at 0-50 cm from West Allerton cemetery.

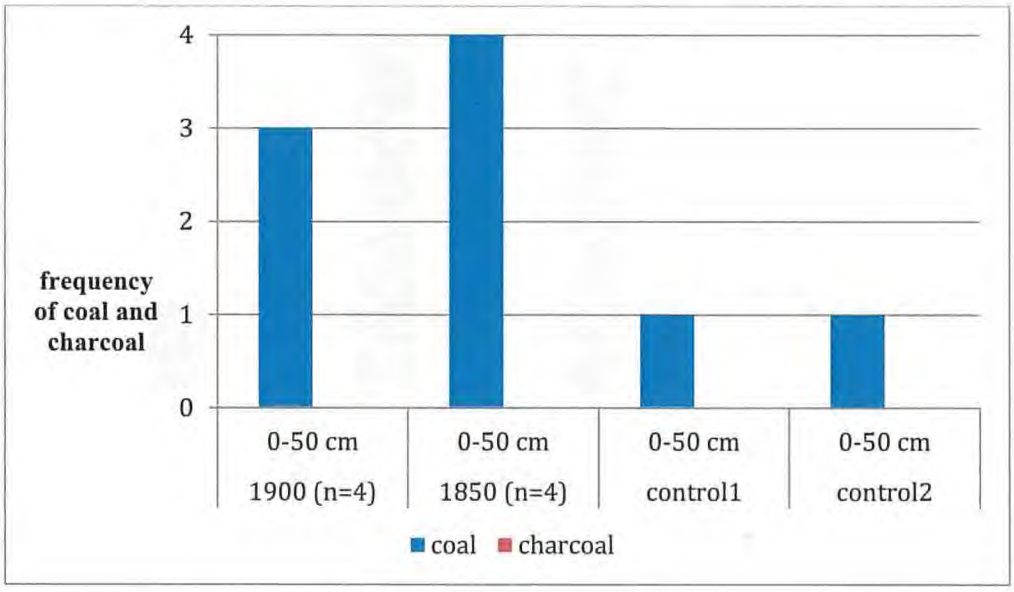


Figure 21: Frequency of coal and charcoal at 0-50 cm from Toxteth cemetery.

50-70 cm:

The presence of coal remained the same in both cemeteries. Charcoal was found in more soil samples, including one of the controls in West Allerton. Charcoal was still not found in the Toxteth samples (Figures 22 & 23).

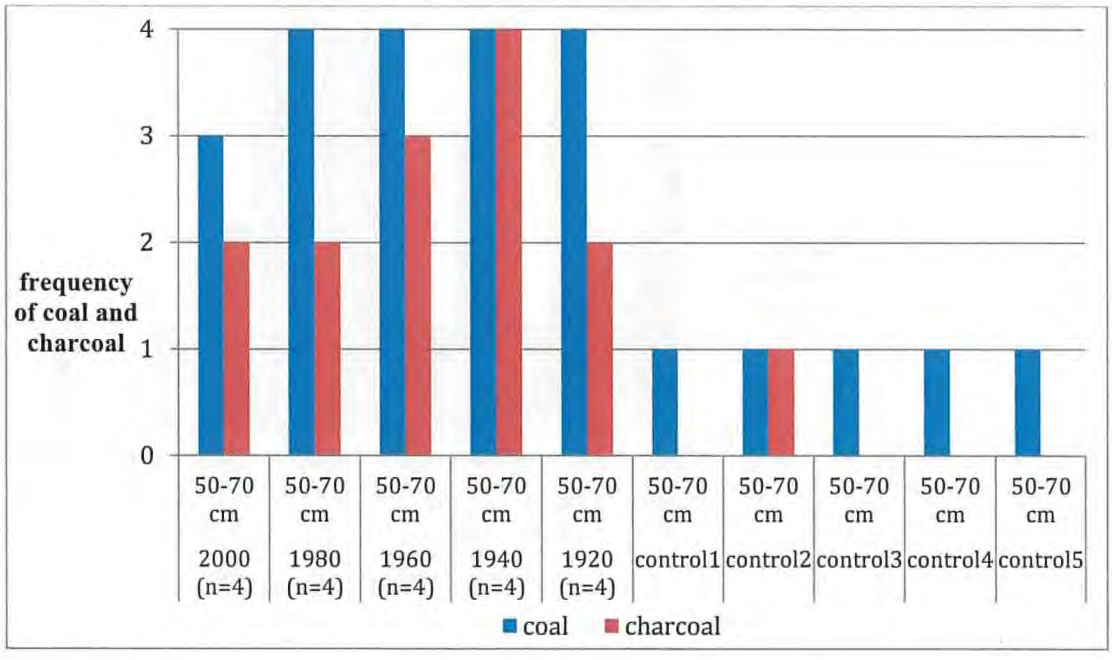


Figure 22: Frequency of coal and charcoal at 50-70cm from West Allerton cemetery.

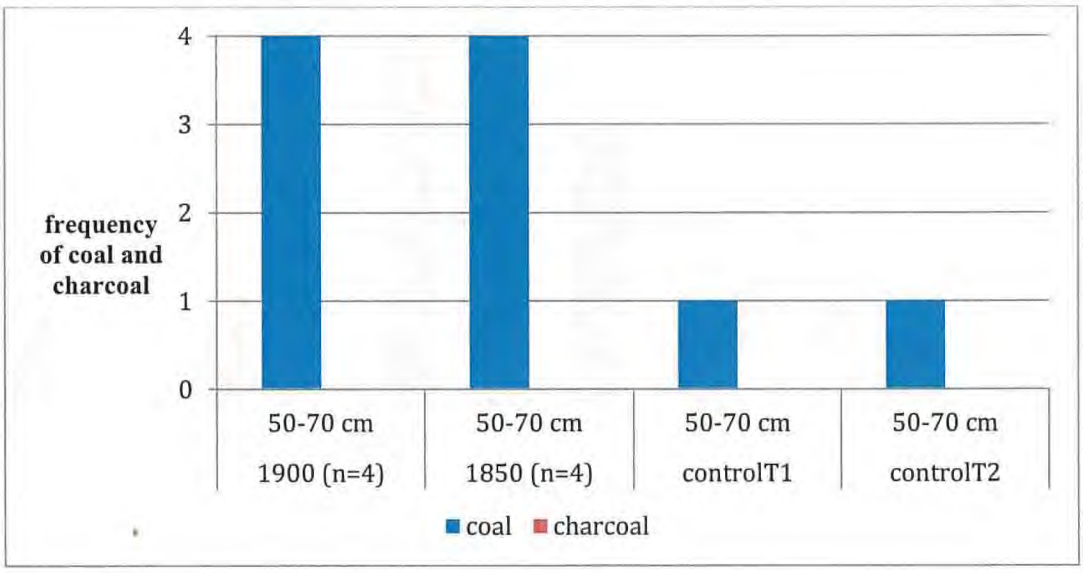


Figure 23: Frequency of coal and charcoal at 50-70 cm from Toxteth cemetery.

70-90 cm:

Coal remained the dominant feature of the soil samples for both cemeteries. There was less charcoal in the West Allerton soil samples, but was seen for the first time in the one of the Toxteth cemetery samples (Figures 24 & 25).

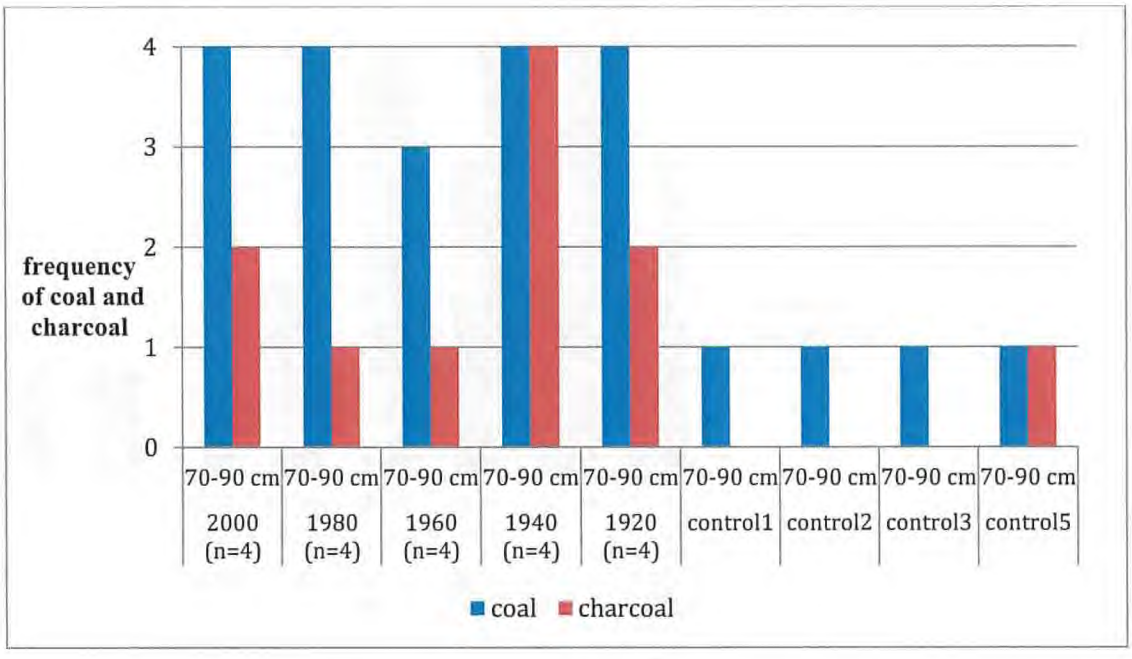


Figure 24: Frequency of coal and charcoal at 70-90 cm from West Allerton cemetery.

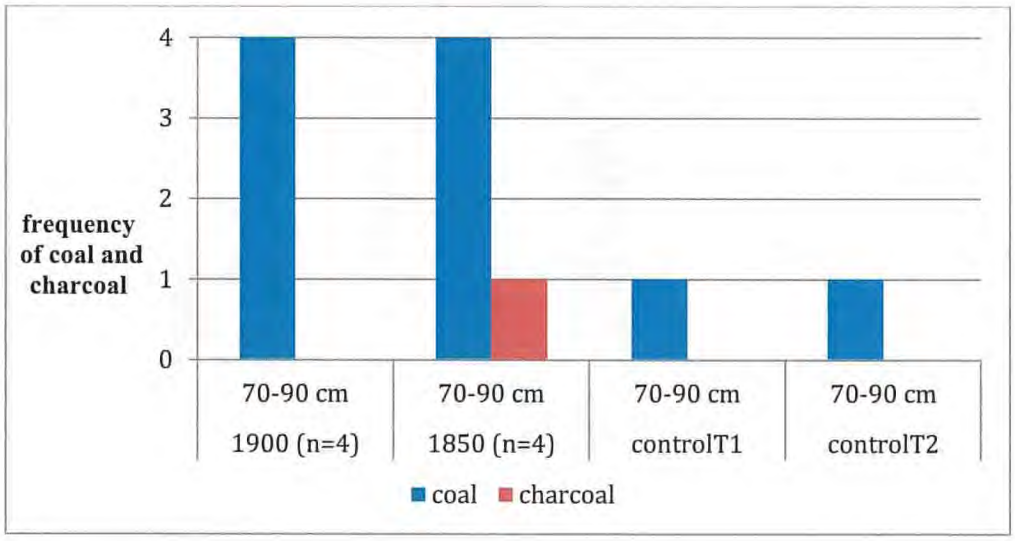


Figure 25: Frequency of coal and charcoal at 70-90 cm from Toxteth cemetery.

90-110 cm:

There was less coal in the West Allerton cemetery samples, but Toxteth remained the same. The presence of charcoal was greatly reduced in the West Allerton soil samples (Figures 26 & 27).

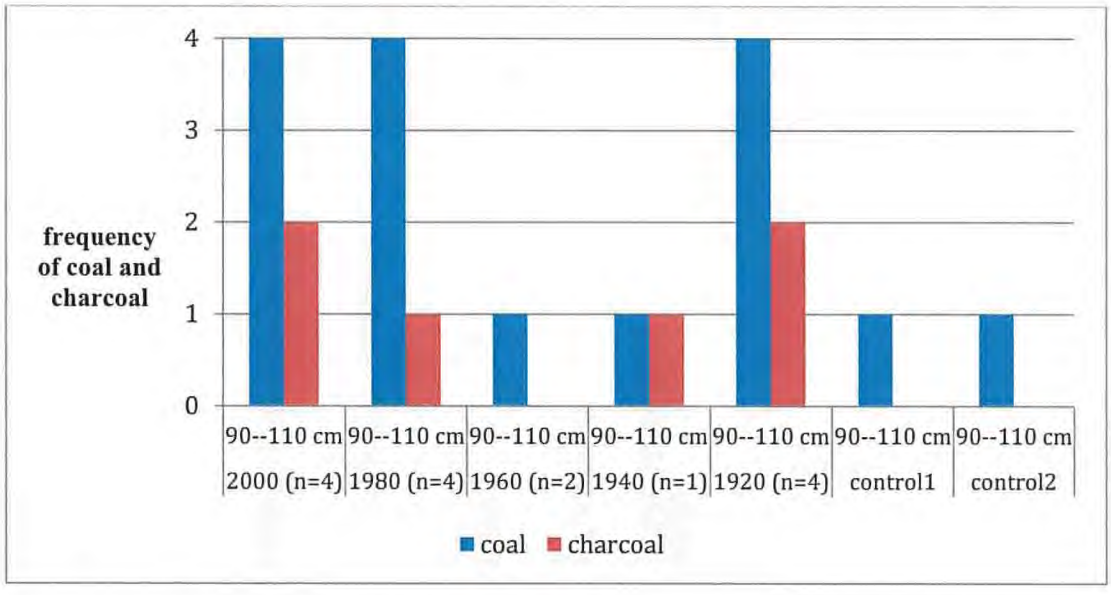


Figure 26: Frequency of coal and charcoal 90-110 cm from West Allerton cemetery.

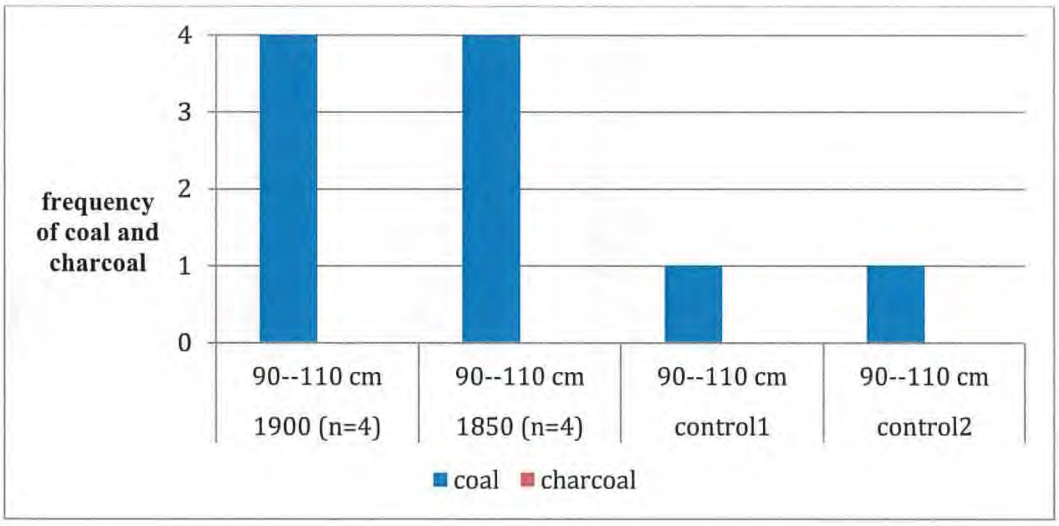


Figure 27: Frequency of coal and charcoal at 90-110 cm from Toxteth cemetery.

Plant and root

0-50 cm:

The presence of plant and root material in the soil samples was an important measurement of plant intrusion into the soil matrix. Most of the plant material consisted of small leaves, twigs or husks. This material could be expected to be found in the first few centimetres of soil due to the action of weathering, previous disturbance of the soil and the action of invertebrates. Both West Allerton and Toxteth had plant and root fragments represented in their soil samples (Figures 28 & 29).

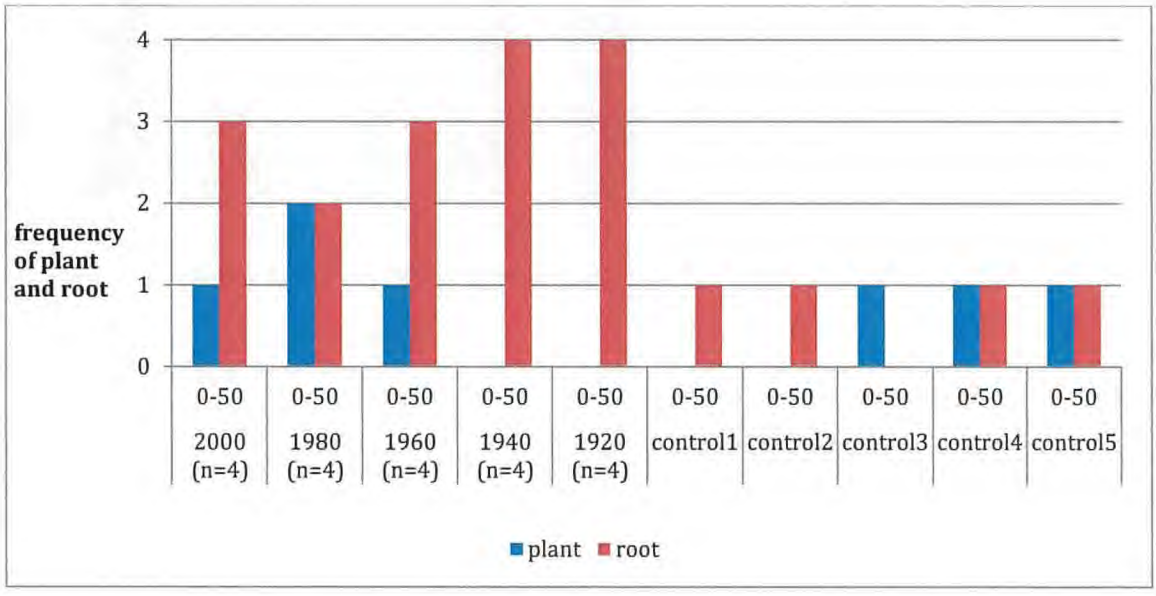


Figure 28: Frequency of plant and root material at 0-50 cm from West Allerton cemetery.

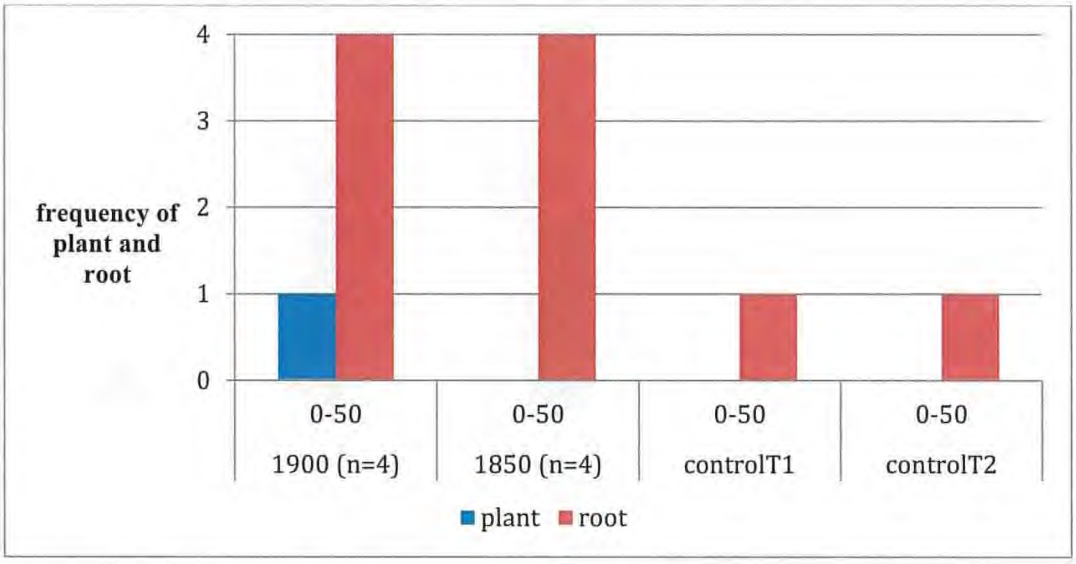


Figure 29: Frequency of plant and root material at 0-50 cm from Toxteth cemetery.

50-70 cm:

Plant and root fragments were seen less in both cemeteries. Plant fragments were seen more in the West Allerton control samples. This may have been due to the tree-lined verges that the samples were taken from, with more humus being represented at this horizon (Figures 30 & 31).

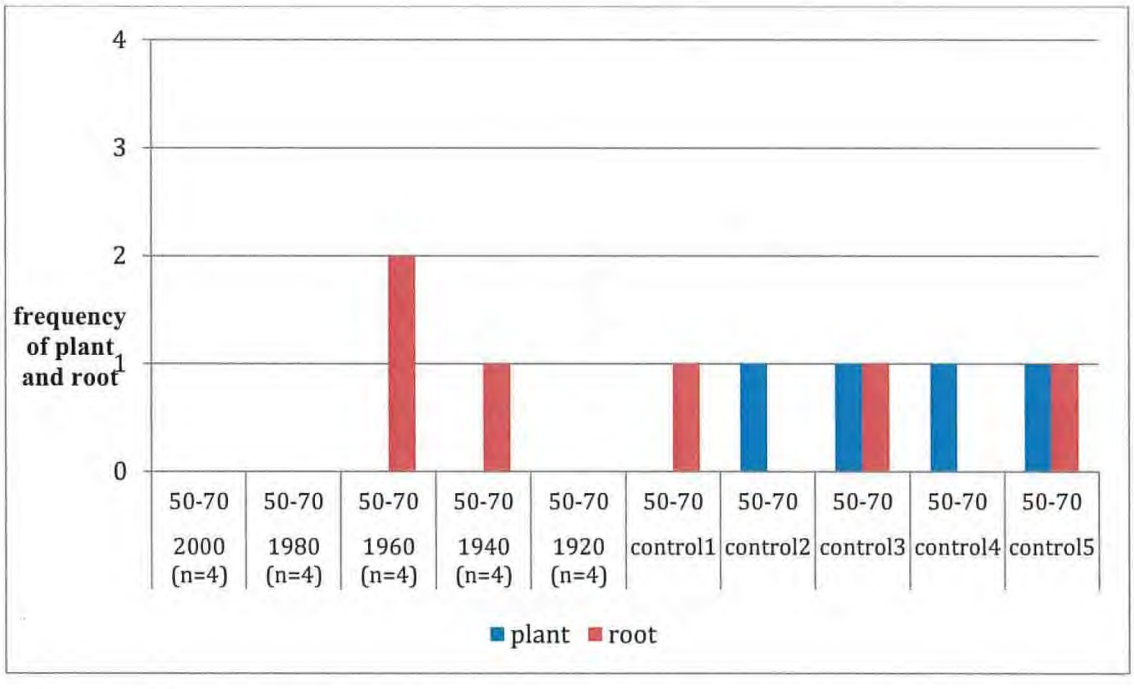


Figure 30: Frequency of plant and root material 50-70 cm from West Allerton cemetery.

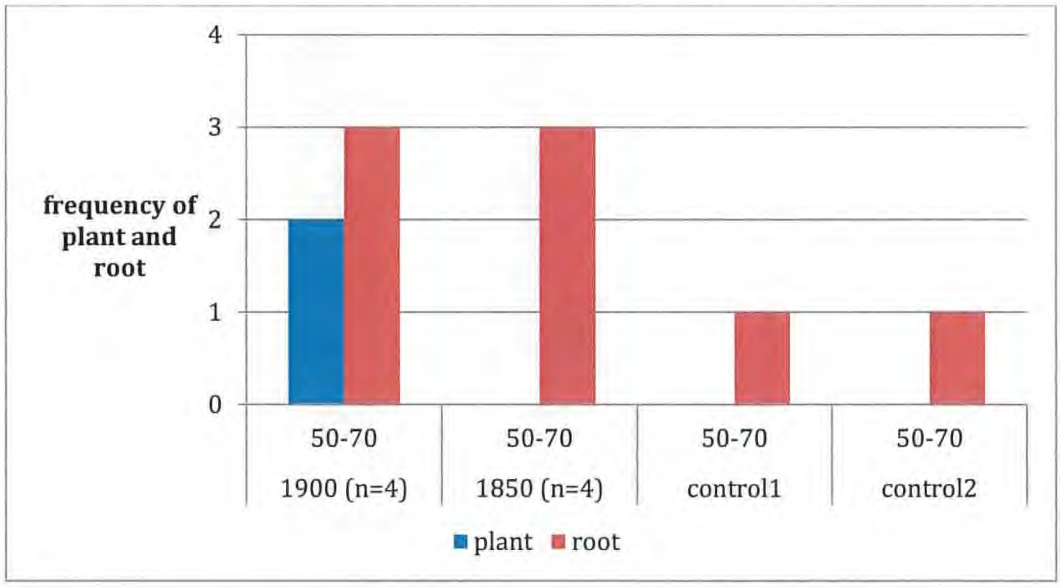


Figure 31: Frequency of plant and root material at 50-70 cm from Toxteth cemetery.

70-90 cm:

The presence of plant and root material declined substantially in both cemeteries (Figures 32 & 33).

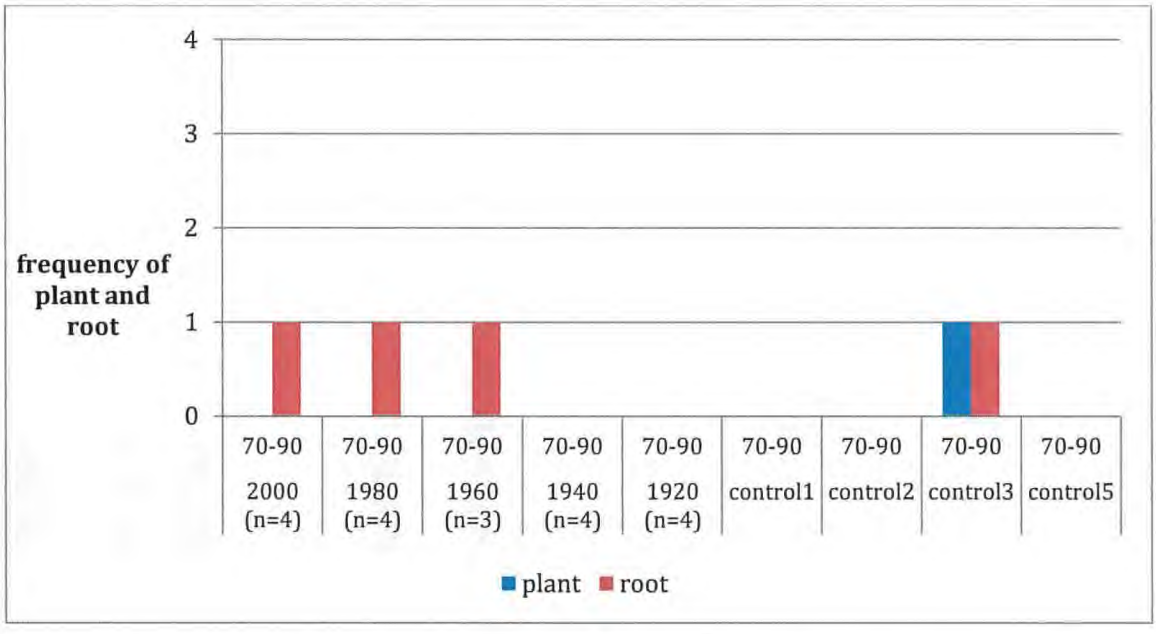


Figure 32: Frequency of plant and root material at 70-90 cm from West Allerton cemetery.

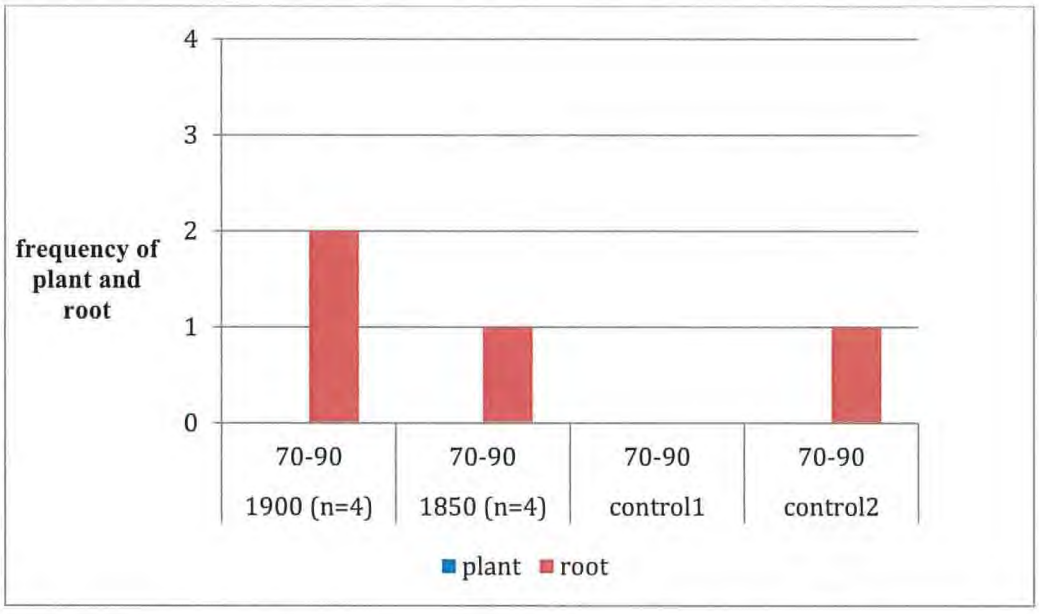


Figure 33: Frequency of plant and root material at 70-90 cm from Toxteth cemetery.

90-110 cm:

Plant and root material have almost completely disappeared from the soil samples from Toxteth cemeteries (Figure 34), and were not observed at all in the West Allerton.

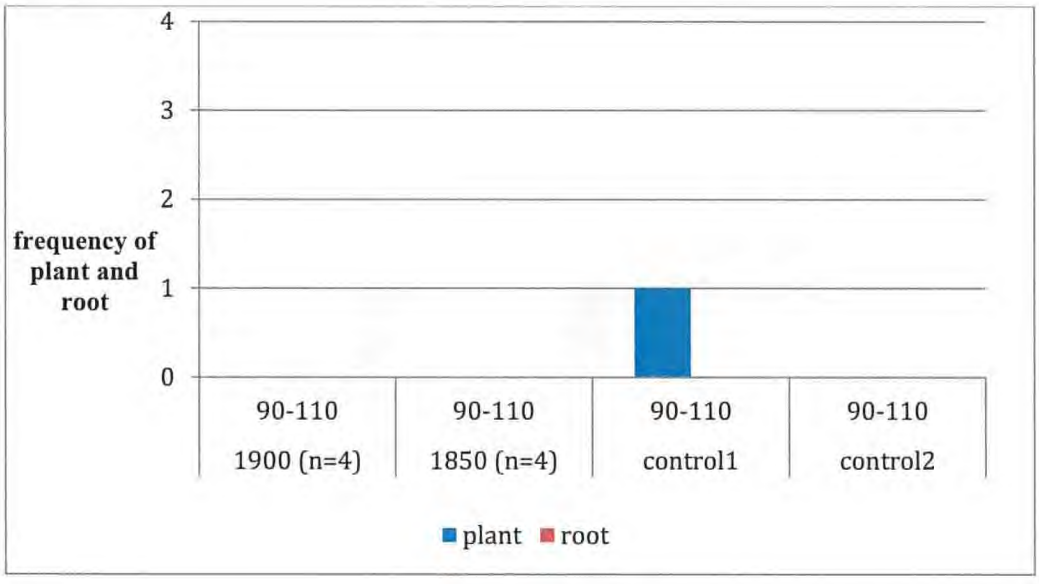


Figure 34: Frequency of plant and root material at 90-110 cm from Toxteth cemetery.

Domestic waste

0-50 cm:

Shards of glass and brick were a rare find from West Allerton and Toxteth (Figures 35 & 36).

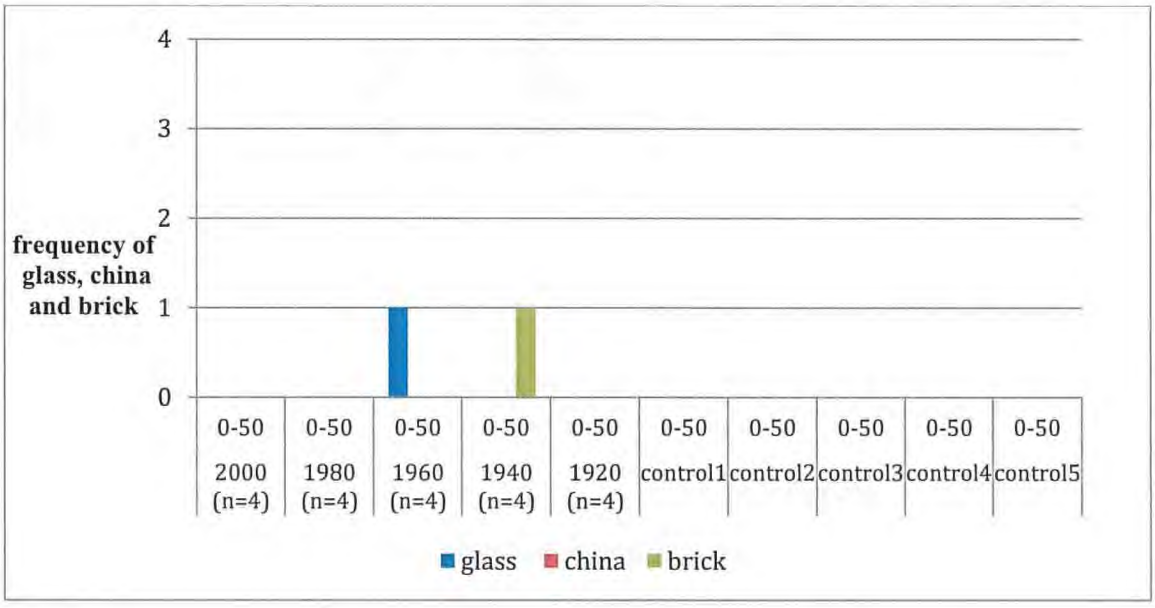


Figure 35: Frequency of domestic waste at 0-50 cm from West Allerton cemetery.

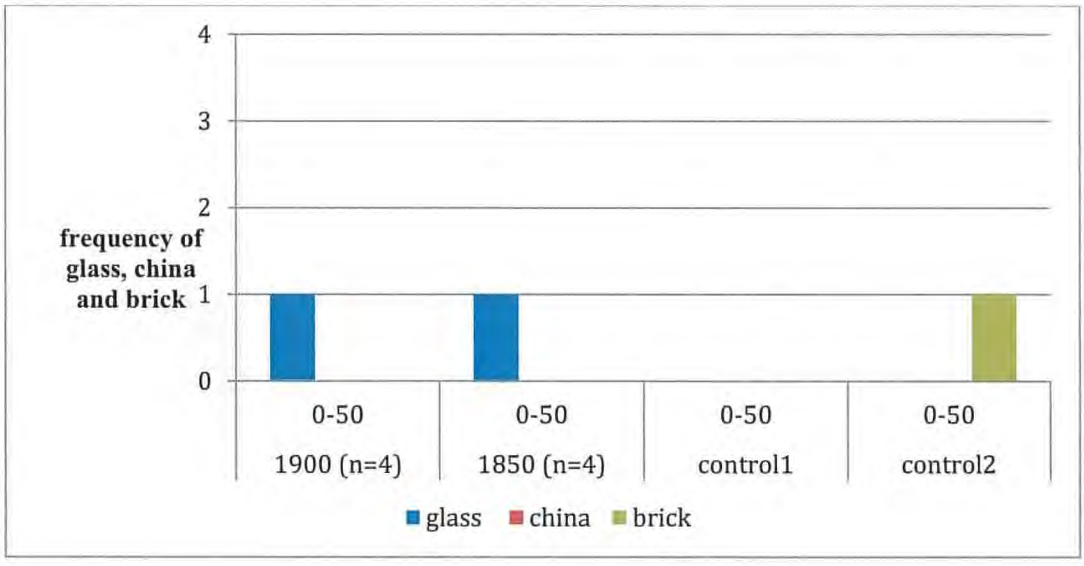


Figure 36: Frequency of domestic waste at 0-50 cm from Toxteth cemetery.

50-70 cm:

Glass and brick remained a rare find at both cemeteries (Figures 37 & 38).

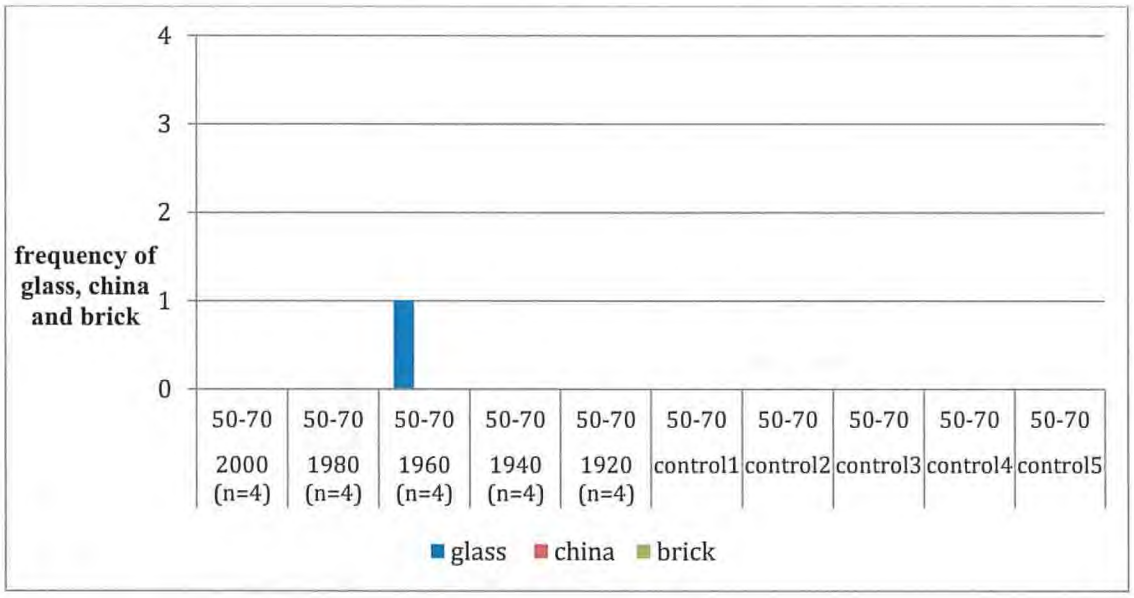


Figure 37: Frequency of domestic waste at 50-70 cm from West Allerton cemetery.

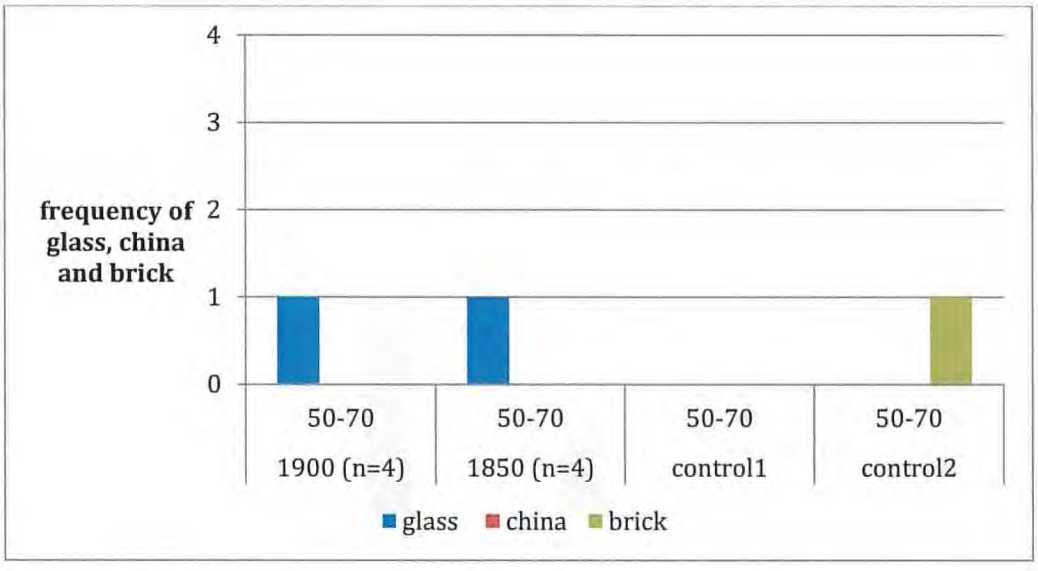


Figure 38: Frequency of domestic waste at 50-70 cm from Toxteth cemetery.

70-90 cm:

Glass and brick remain rarely represented in the soil from either cemetery (Figures 39 & 40).

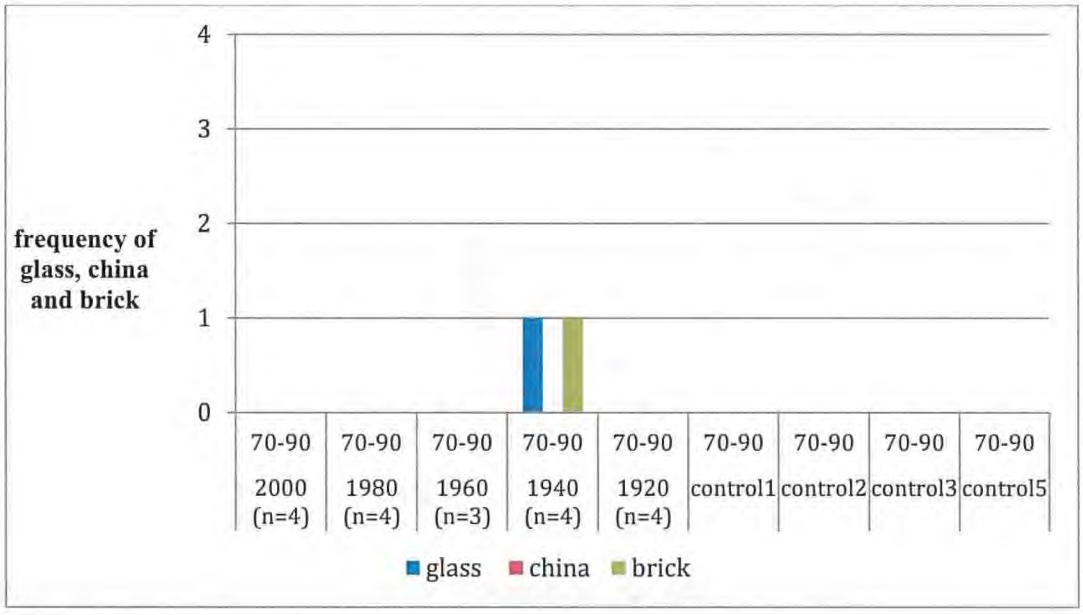


Figure 39: Frequency of domestic waste at 70-90 cm from West Allerton cemetery.

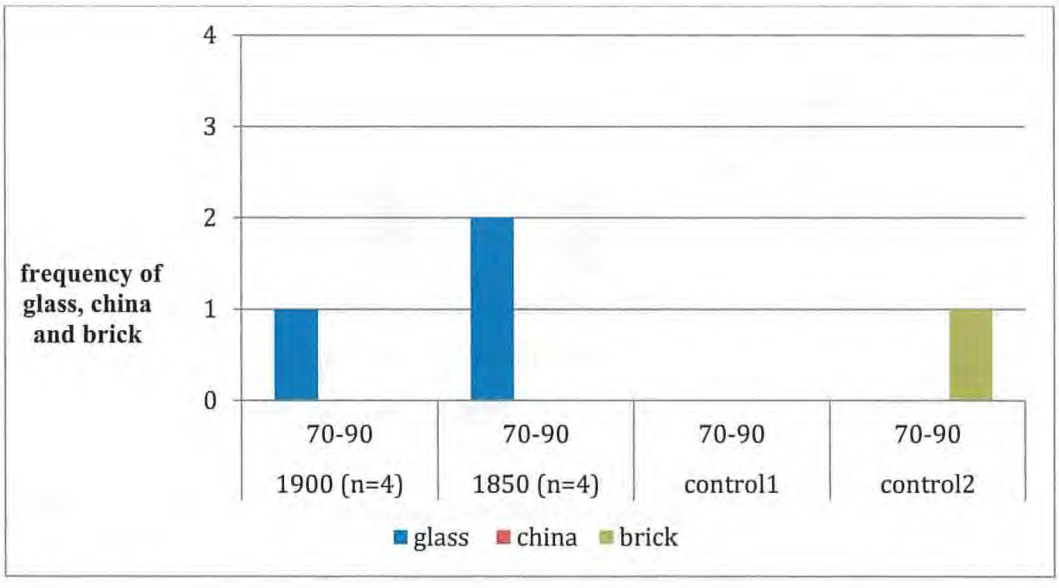


Figure 40: Frequency of domestic waste at 70-90 cm from Toxteth cemetery.

90-110 cm:

Domestic waste was no longer represented in the soil samples from West Allerton. Domestic waste including china was still being represented in the samples from Toxteth (Figure 41).

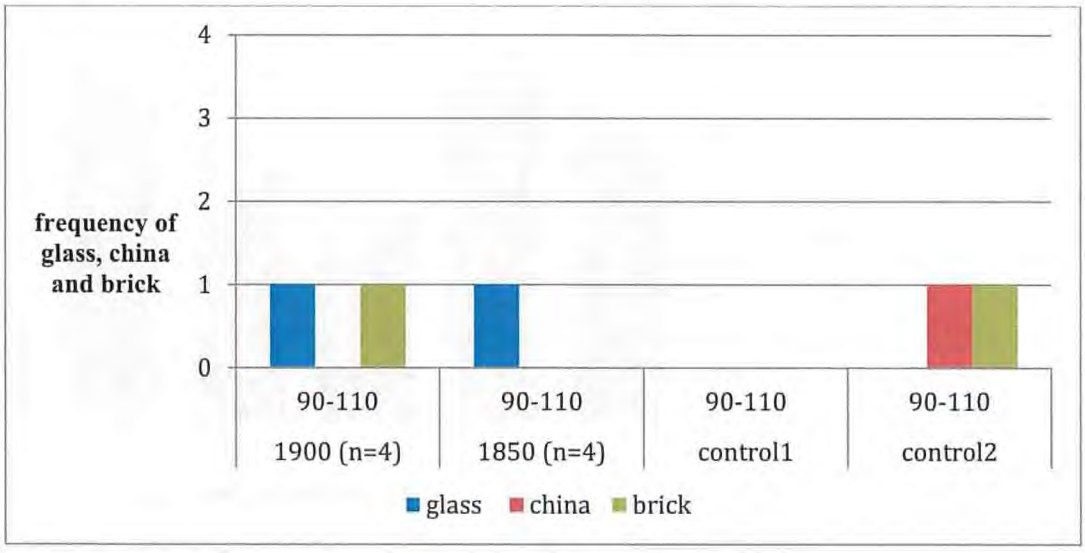


Figure 41: Frequency of domestic waste at 90-110 cm from Toxteth cemetery.

5.3.1. (ii) Soil X-ray Fluorescence Data Statistical Analysis

In order for the results to be statistically analysed it was important to ensure that there was no bias in the analysis. Initial tests of normality demonstrated that the data violated the assumptions and transformation of the data failed to convert to normality. The analysis was then moved to non-parametric tests.

Statistical analysis of left and right side of graves:

A Wilcoxon sign rank test demonstrated no significant difference between the elements of left and right sides of the grave soil samples, which allowed the data to be safely pooled. The grave year results were combined to obtain a mean with standard error displayed.

Statistical analysis of the different elements at each depth in relation to time:

A Kruscall- Wallis test was applied. The results for calcium (Figures 50 & 51) and potassium (Figures 44 & 45) were significant $p=0.017$, $p=0.041$ respectively. The differences in the proportion of iron (Figures 42 & 43), magnesium (Figures 48 & 49)

and phosphorus (Figures 46 & 47) were not significantly different between the four different depths across all time periods p=0.879, p=0.343, p=0.136 respectively.

Iron:

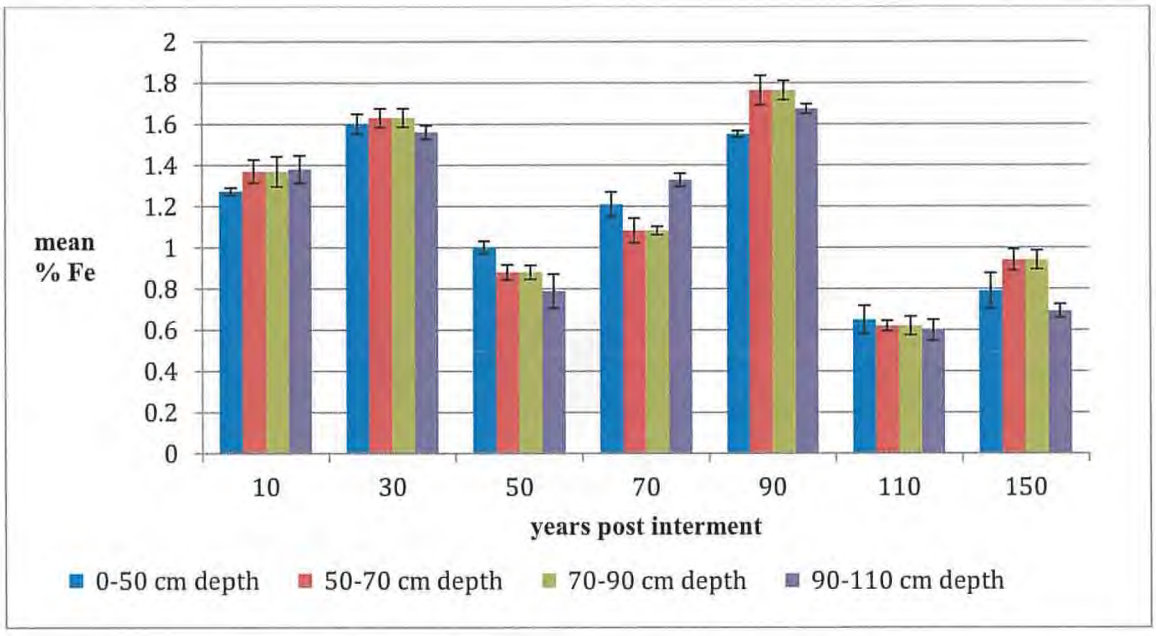


Figure 42: Mean percent iron from both cemeteries at the four depths (cm).

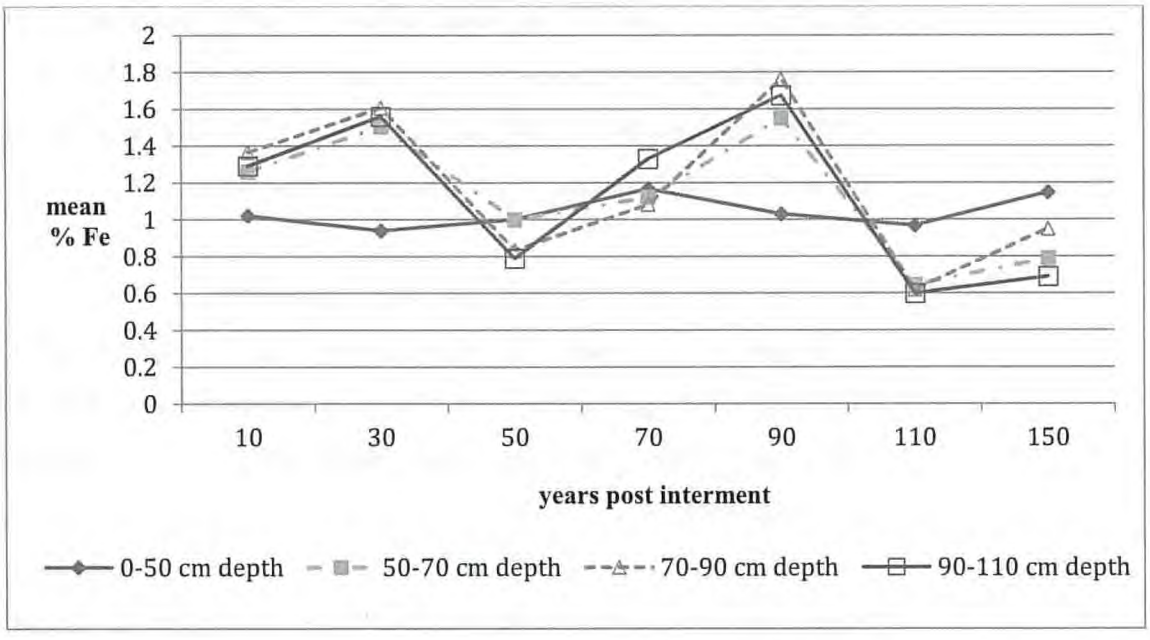


Figure 43: Mean percent iron from both cemeteries at the four depths (cm).

Potassium:

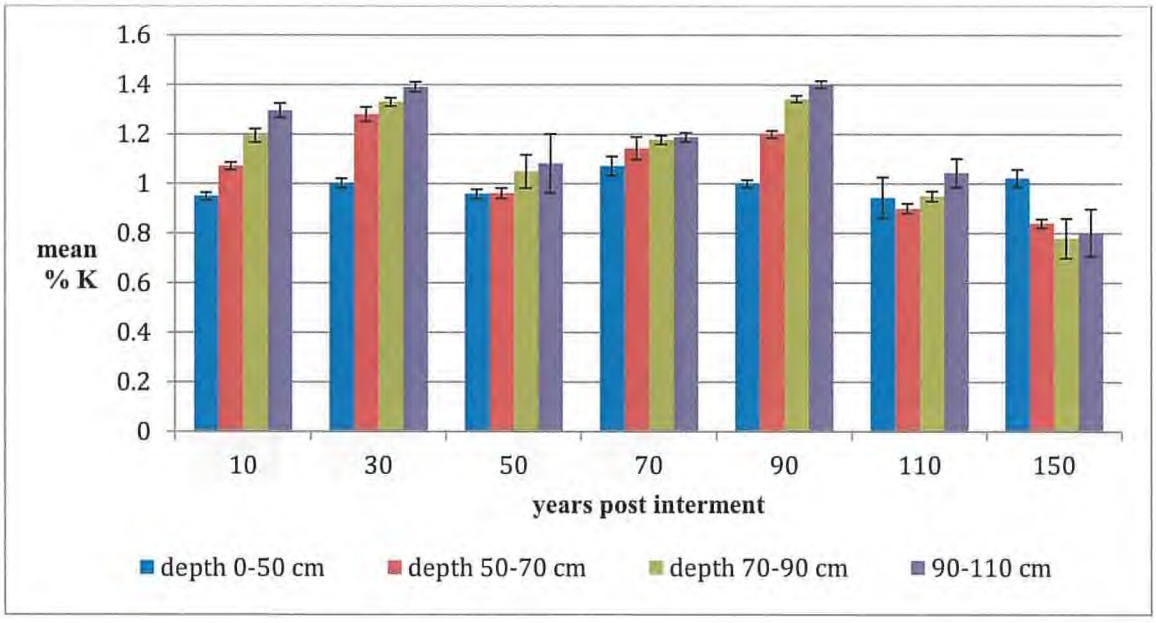


Figure 44: Mean percent potassium from both cemeteries at the four depths (cm).

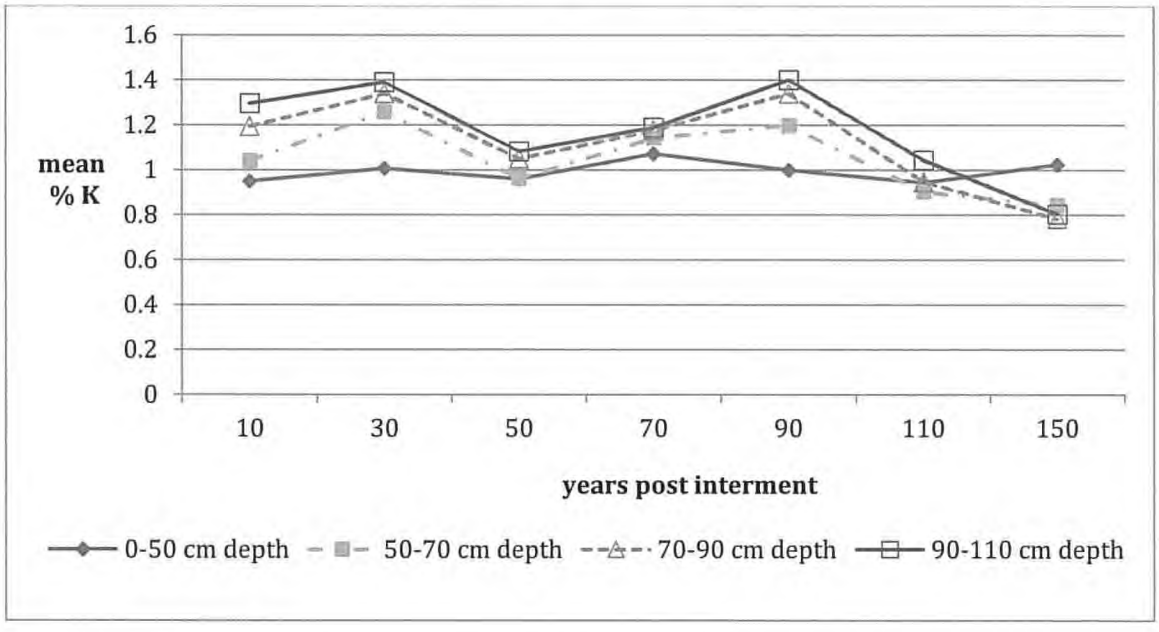


Figure 45: Mean percent potassium from both cemeteries at the four depths (cm).

Phosphorus:

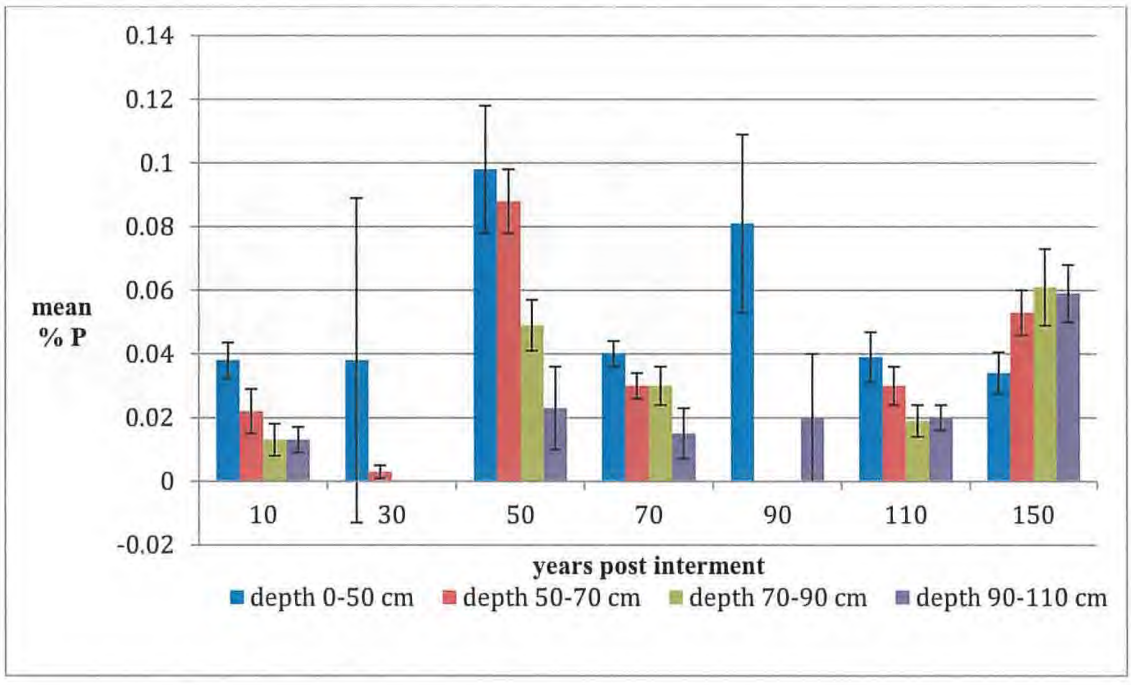


Figure 46: Mean percent phosphorus from both cemeteries at the four depths (cm).

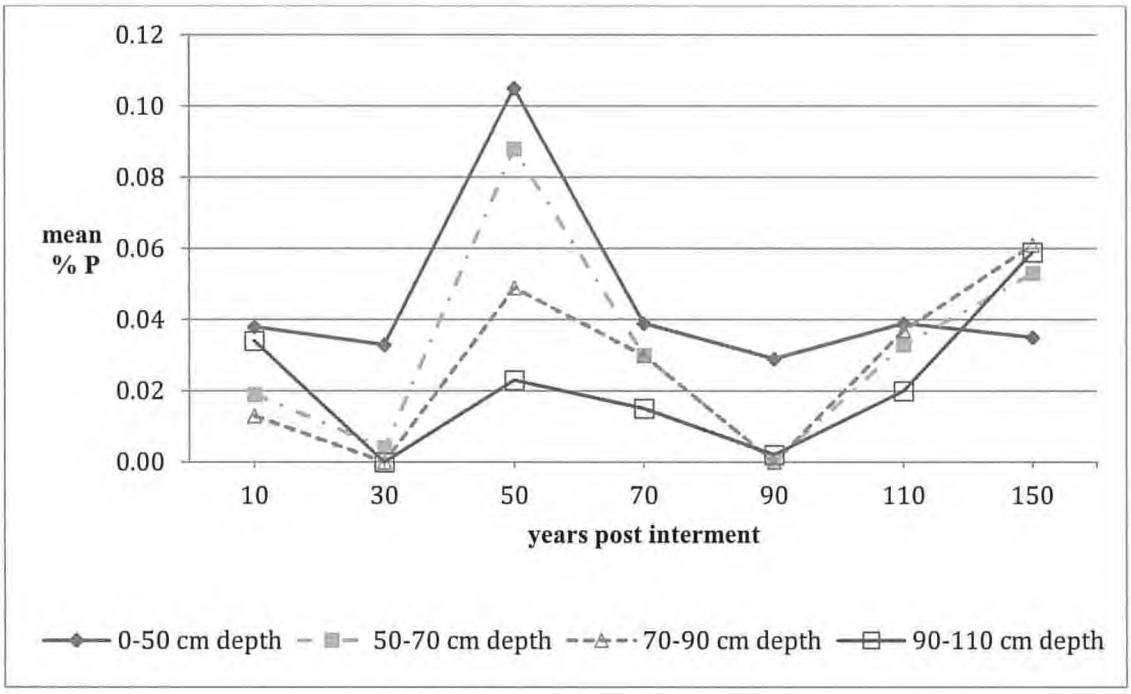


Figure 47: Mean percent phosphorus from both cemeteries at the four depths (cm).

Magnesium:

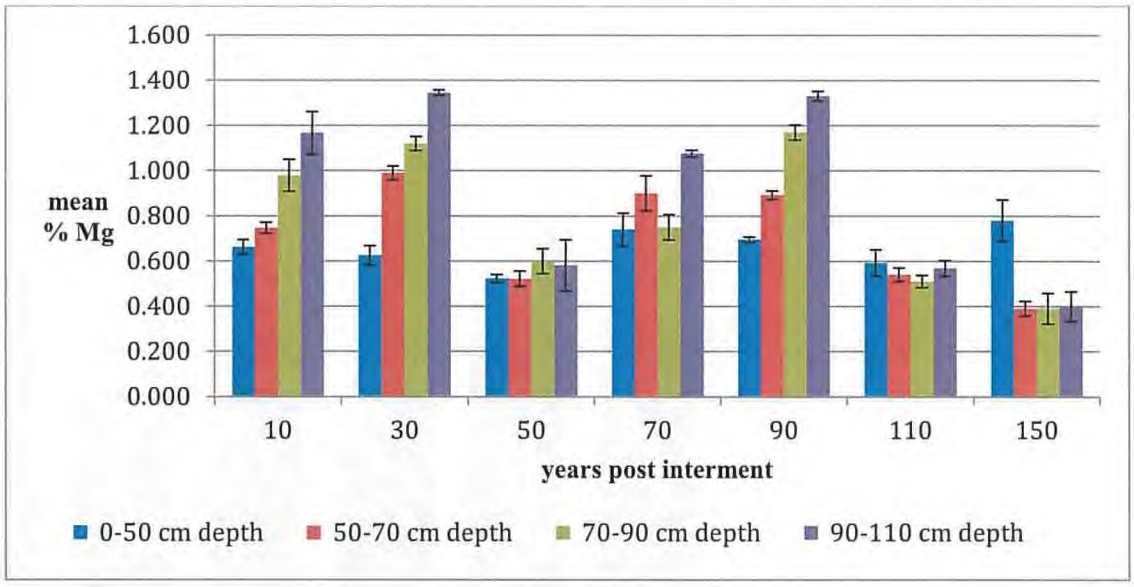


Figure 48: Mean percent magnesium from both cemeteries at the four depths (cm).

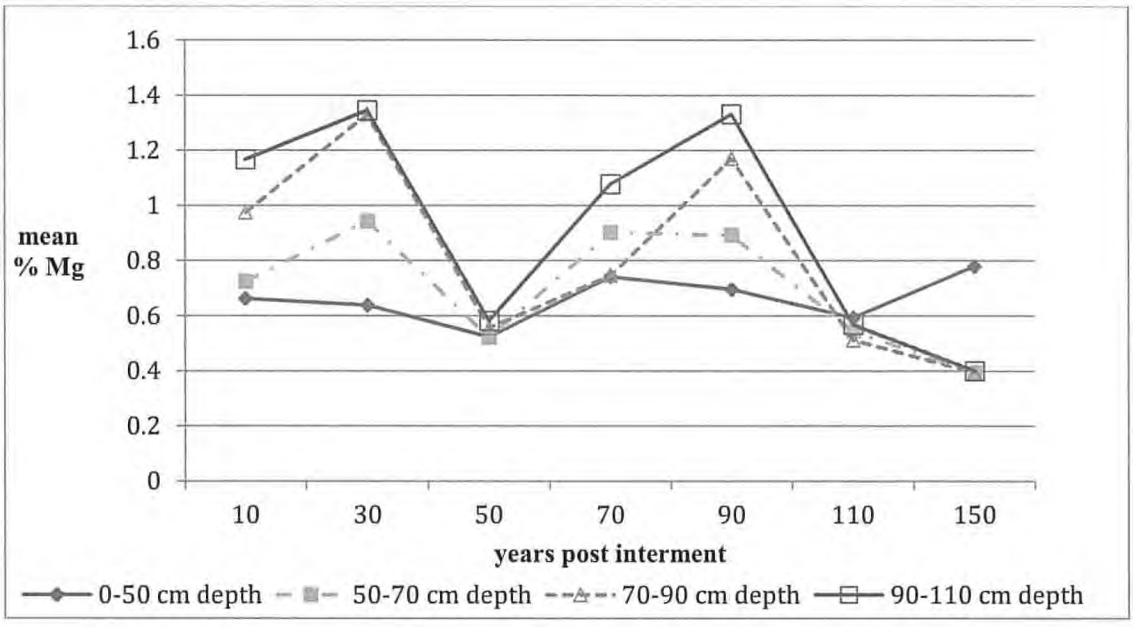


Figure 49: Mean percent magnesium from both cemeteries at the four depths (cm).

Calcium:

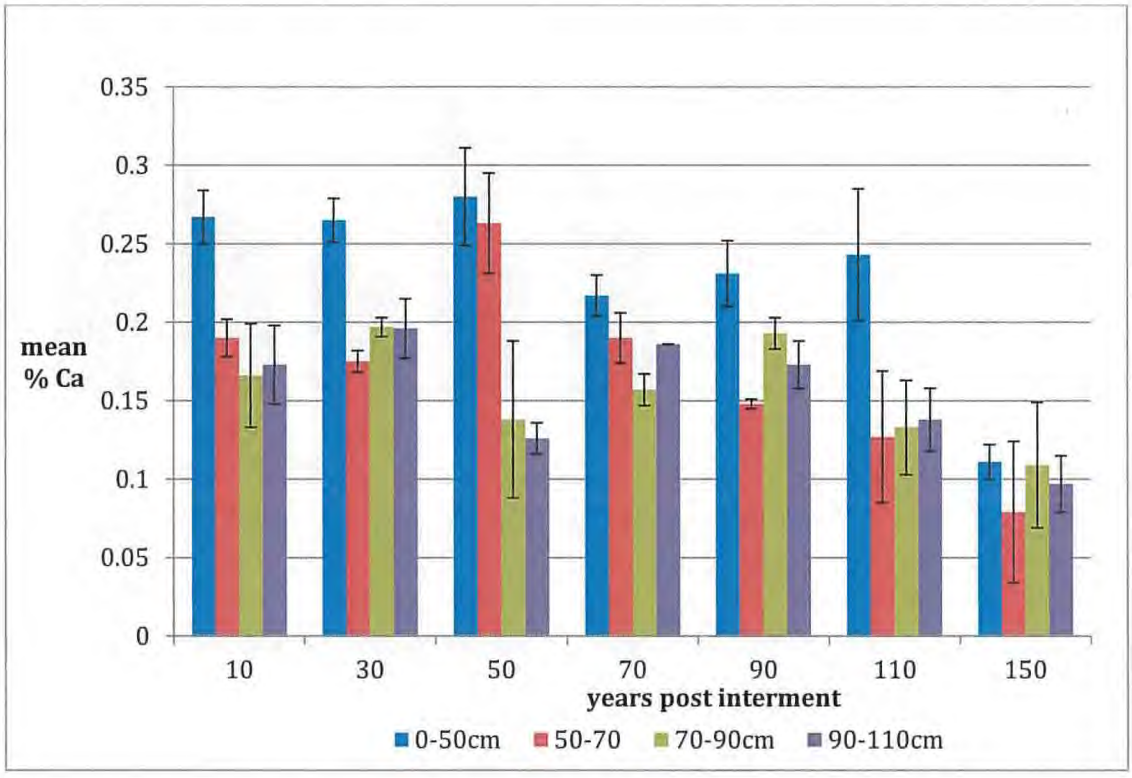


Figure 50: Mean percent calcium from both cemeteries at the four depths (cm).

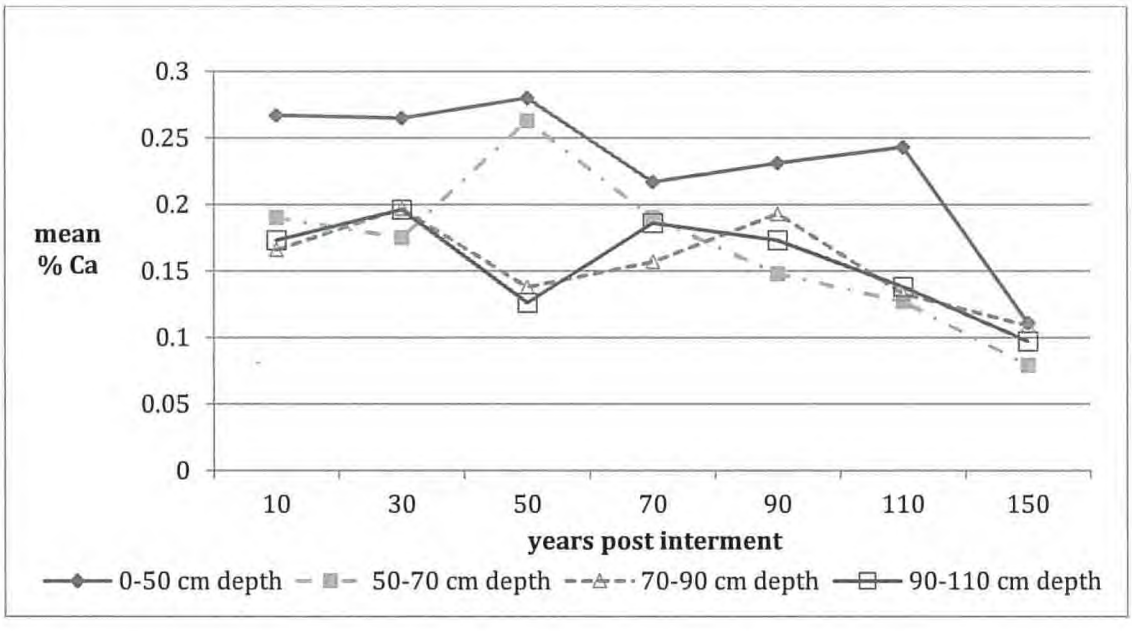


Figure 51: Mean percent calcium from both cemeteries at the four depths (cm).

Correlation Co-efficient of elements at each depth in relation to time:

Calcium was significant at all depths, but was most prominent at 70-90cm p=0.05, 90-110cm, p=0.01, this showed a strong negative correlation between the levels of Calcium and time r= -0.802, r= -0.832, respectively. This was demonstrated by the close clumping in the values (Figures 52 & 53). Iron and potassium were significantly different at 50-70cm p=0.05, r= -0.543, r= -0.574 respectively. Magnesium proved significantly different at 70-90 and 90-110cm, showing a correlation between the levels of Magnesium and time, r= -0.547, r= -0.615, there is less clumping of the values than for calcium (Figures 54 & 55).

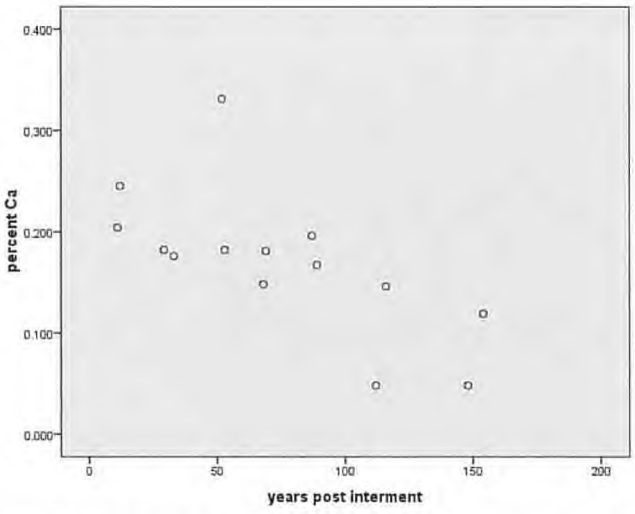


Figure 52: Correlation of calcium levels and time 70-90cm both cemeteries combined r=0.802.

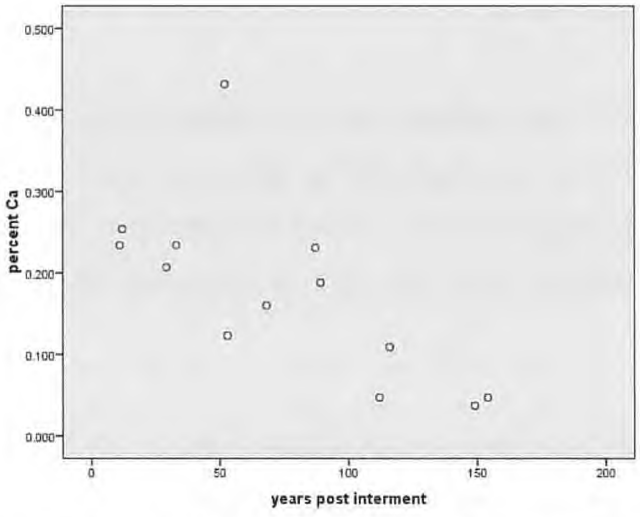


Figure 53: Correlation of calcium levels and time 90-110cm both cemeteries combined r=0.832.

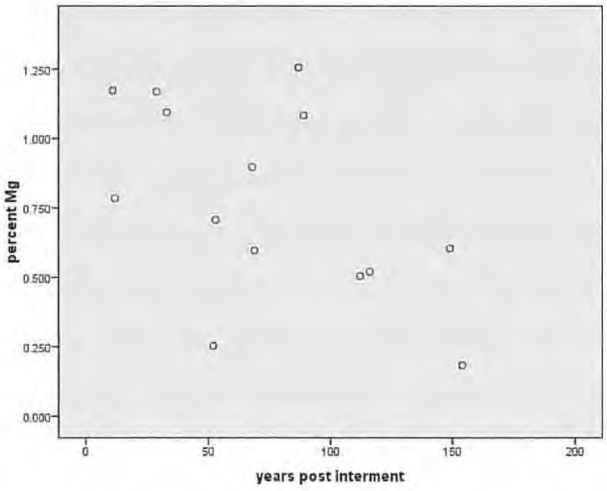


Figure 54: Correlation of magnesium levels and time 70-90cm both cemeteries combined r=-0.547.

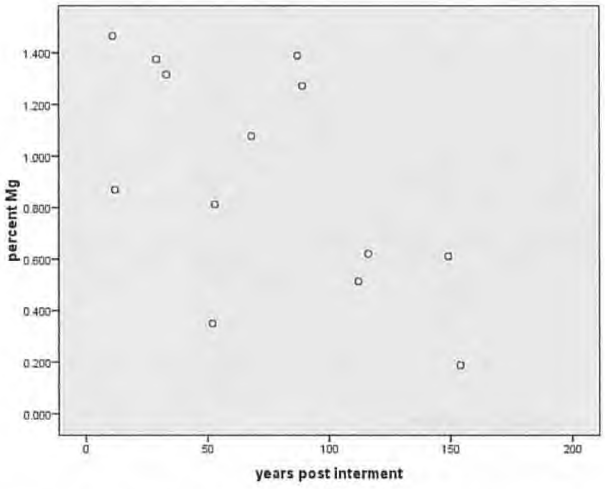


Figure 55: Correlation magnesium levels and time 90-110cm both cemeteries combined r=0.615.

Statistical analysis of the different elements from the graves and control samples: A Mann Whitney U test was applied to the elemental results of each grave soil sample at all depths and compared to the control samples. Iron was significantly different from the control samples across all depths, across nearly all the time periods (Figures 56 & 57).

In the year 1940 there were inconsistencies across all the elements apart from Calcium. This discrepancy could be explained by the fact that the sampling for one of these graves was incomplete (90-110cm) due the impenetrable hard sandstone parent material, which could have been responsible for skewing the results. Potassium was

only significant in 2/7 time periods (Figures 58 & 59), Magnesium 3/7 (Figures 62 & 63), phosphorus 3/7 (Figures 60 & 61), iron 4/7 (Figures 56 & 57), Calcium 7/7 (Figures 64 & 65). By comparing the results of the graves to the controls at each depth, the differences become more evident the deeper the soil sample.

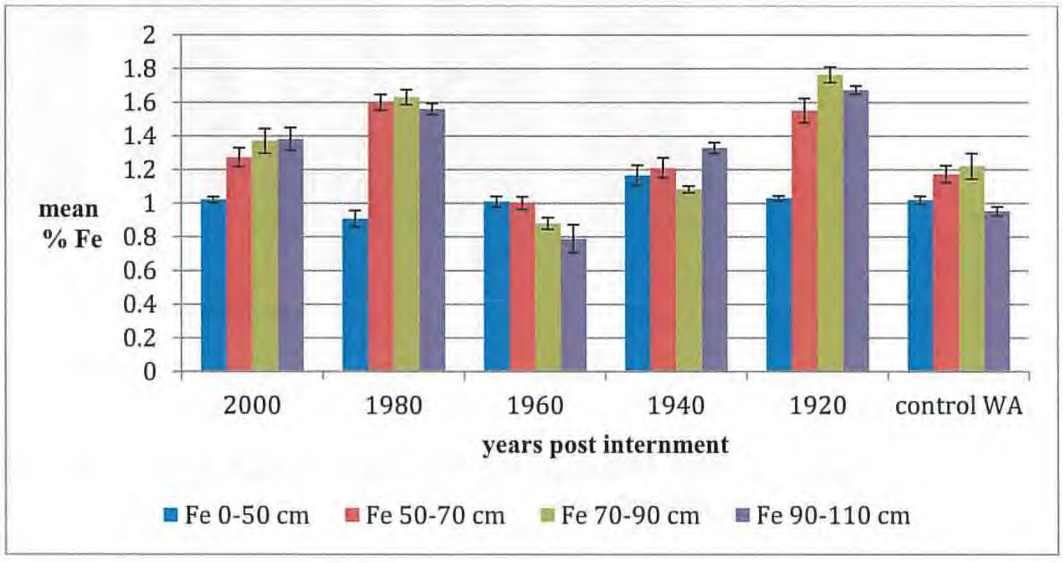


Figure 56: West Allerton cemetery mean iron levels at the four depths compared to controls.

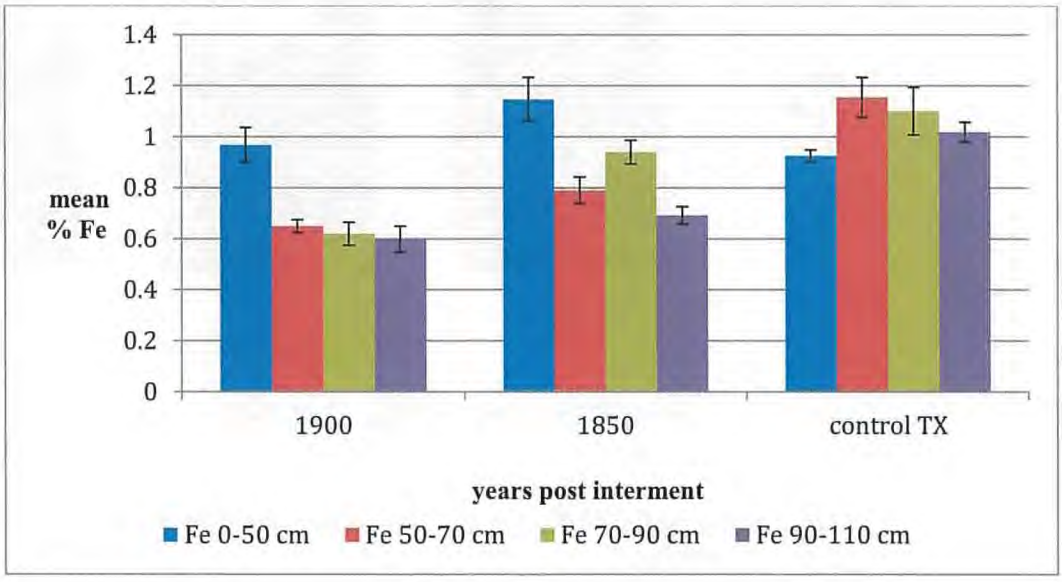


Figure 57: Toxteth cemetery mean iron levels at the four depths compared to controls.

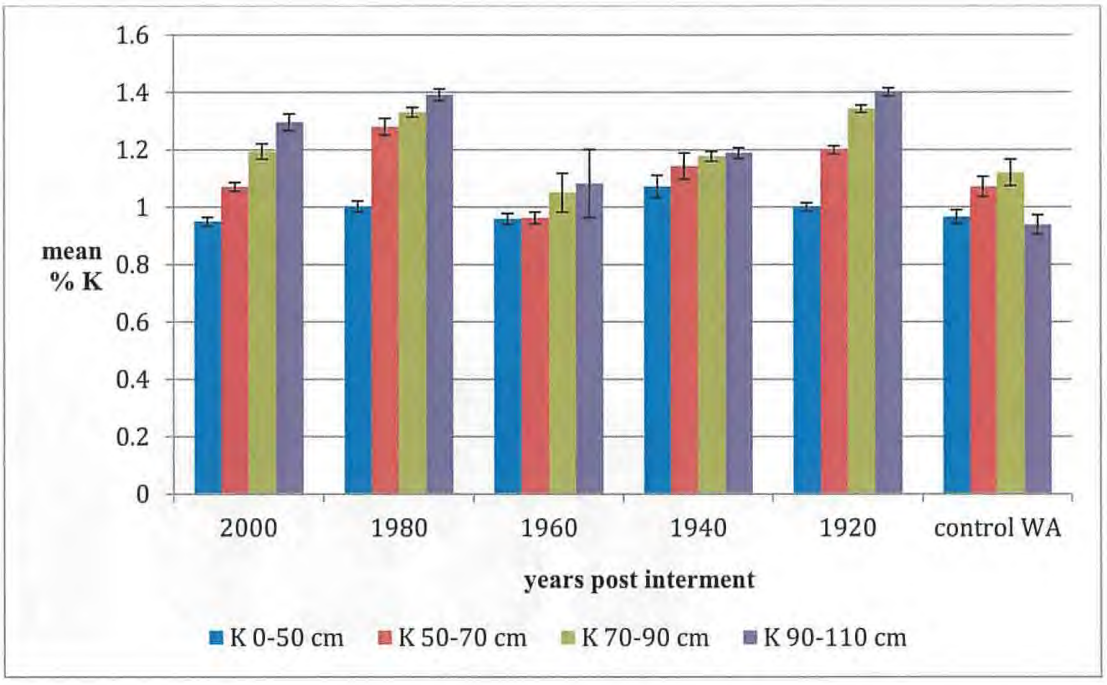


Figure 58: West Allerton cemetery mean potassium levels at the four depths compared to the controls.

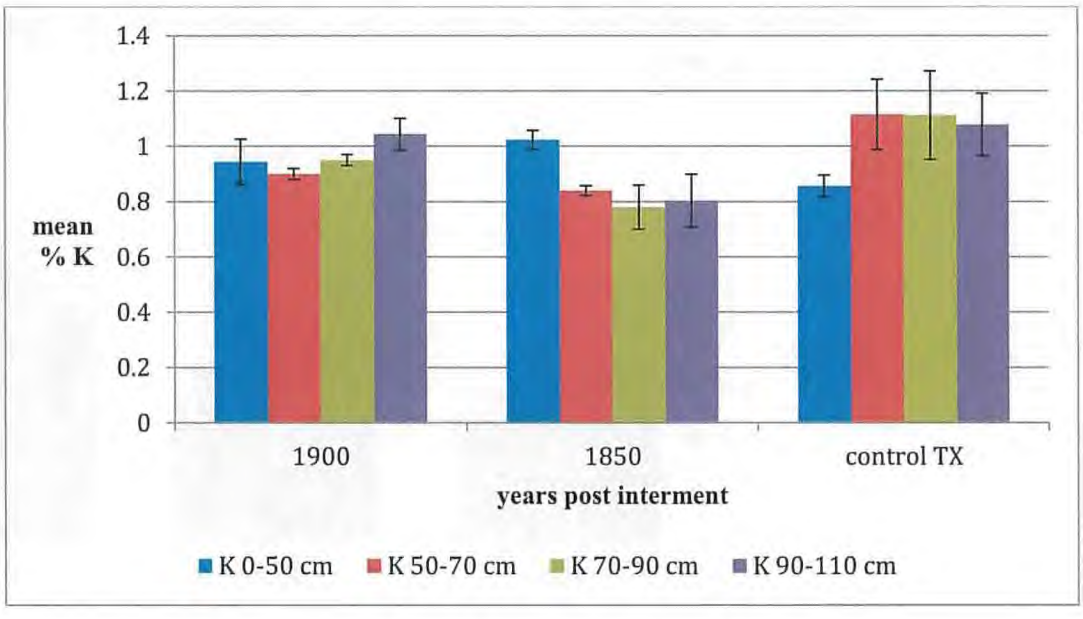


Figure 59: Toxteth cemetery mean potassium levels at the four depths compared to the controls.

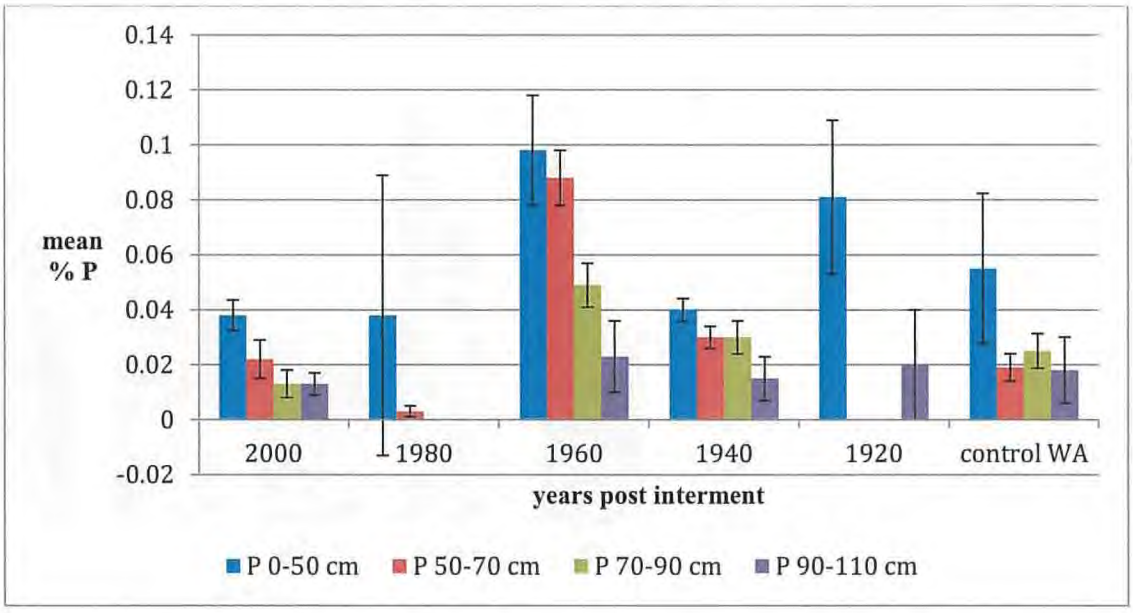


Figure 60: West Allerton cemetery mean phosphorus levels at the four depths compared to the controls.

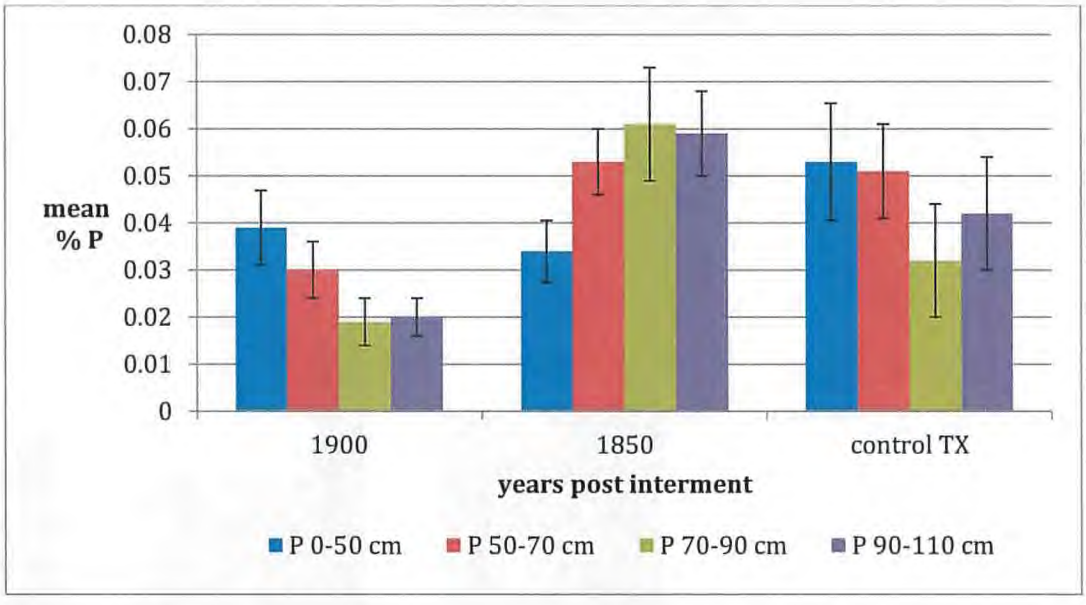


Figure 61: Toxteth cemetery mean phosphorus levels at the four depths compared to the controls.

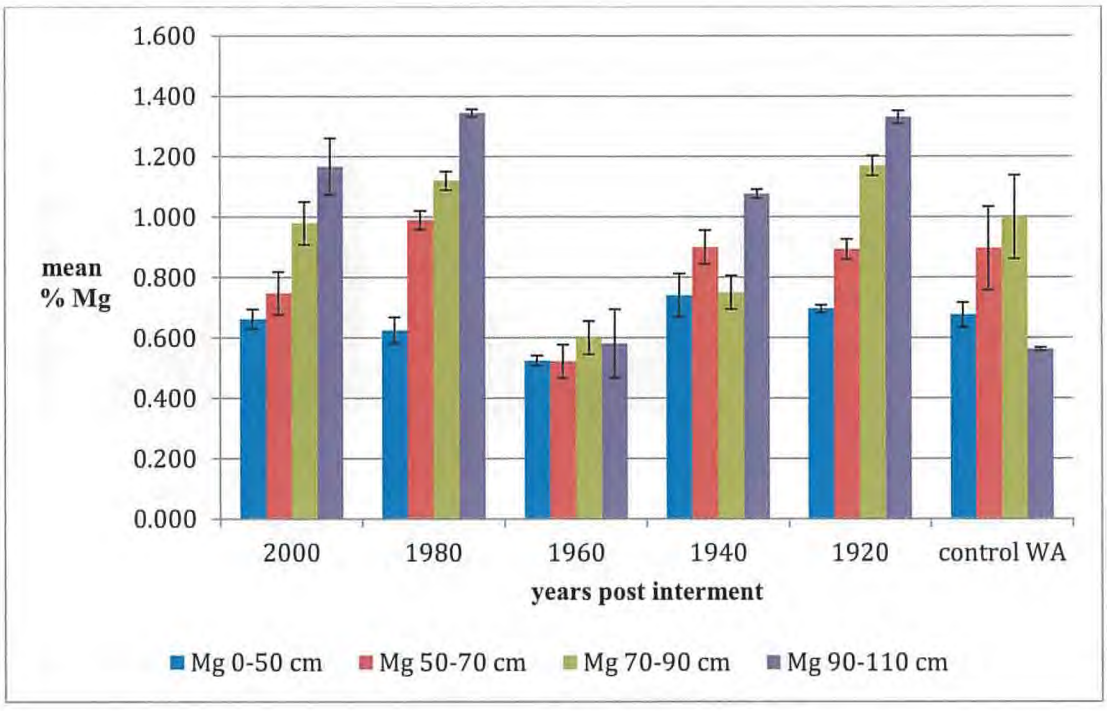


Figure 62: West Allerton cemetery mean magnesium levels at the four depths compared to the controls.

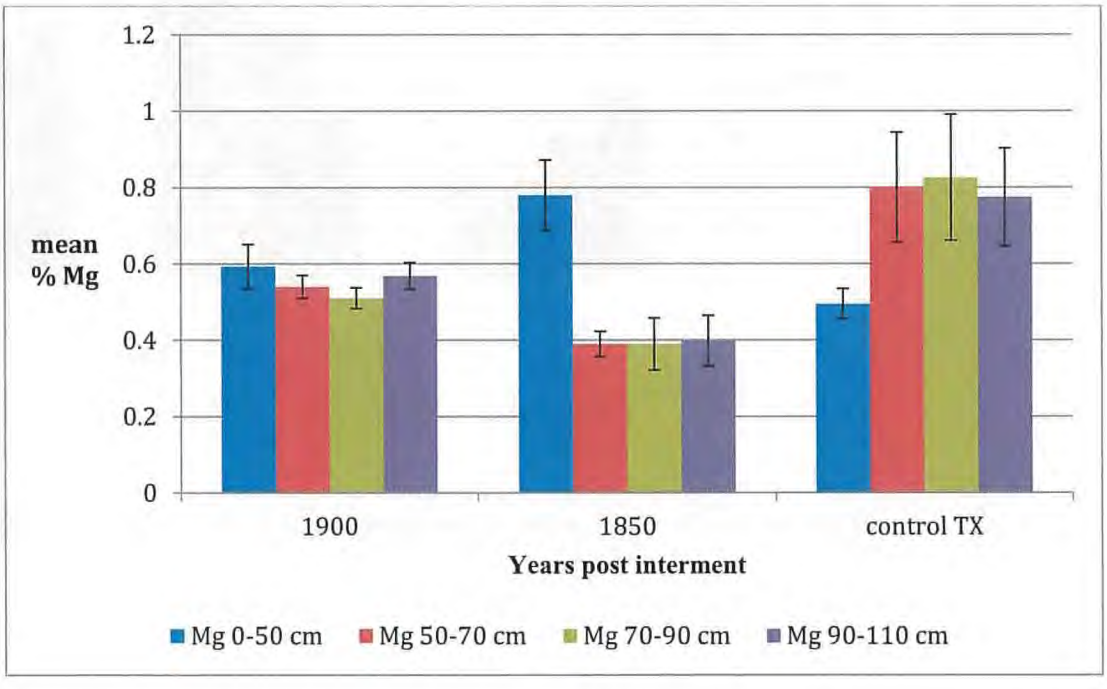


Figure 63: Toxteth cemetery mean magnesium levels at the four depths compared to the controls.

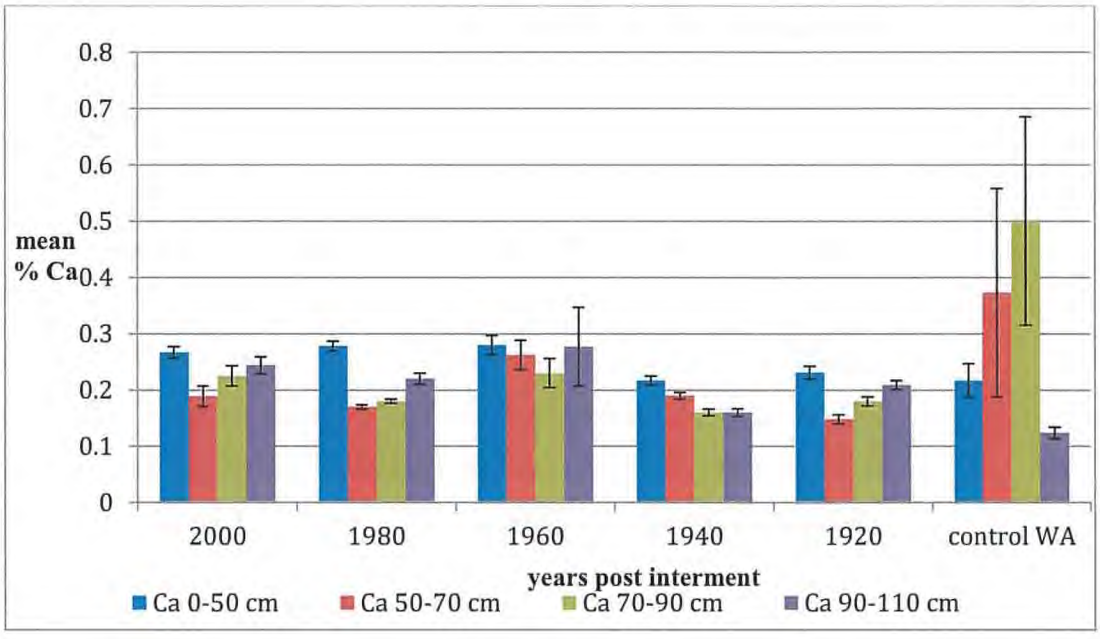


Figure 64: West Allerton cemetery mean calcium levels at the four depths compared to the controls.

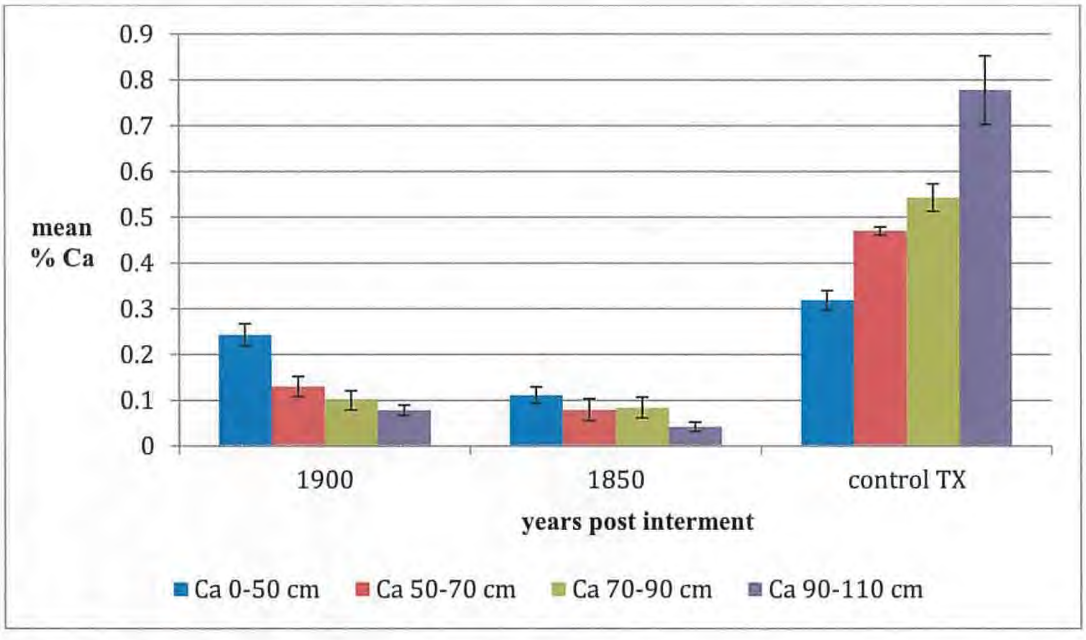


Figure 65: Toxteth cemetery mean calcium levels at the four depths compared to the controls.

5.3.1. (iii) Cemetery Soil PH Results

The results for the pH of the cemeteries were not normally distributed and proved resistant to transformation, therefore non-parametric tests were applied to the data.

Statistical analysis of the left and right side of the graves:

A Mann Whitney U test was applied to the data from West Allerton and Toxteth separately. There was a significant difference between the left and right side of the graves for the data from West Allerton cemetery, p= 0.025, Z= -2.236, but not for Toxteth p=0.665, Z= -0.432. It was then decided to test whether there was a significant difference between the four depths in order to be able to pool the data for further analysis.

Statistical analysis between the four depths:

A kruscall-Wallis test was performed on the data from both cemeteries on the left and right separately. There was a significant difference between the four depths at West Allerton cemetery, left p=0,001 3df, right P=0.001. There was a significant difference between the four depths at Toxteth from the left side of the grave p= 0.021 3df, but no significant difference between the four depths on the right p= 0.796 3df. The left and right were combined at the four depths and found to be significantly different p=0.041. It was then decided to pool the data for further analysis (Figure 70).

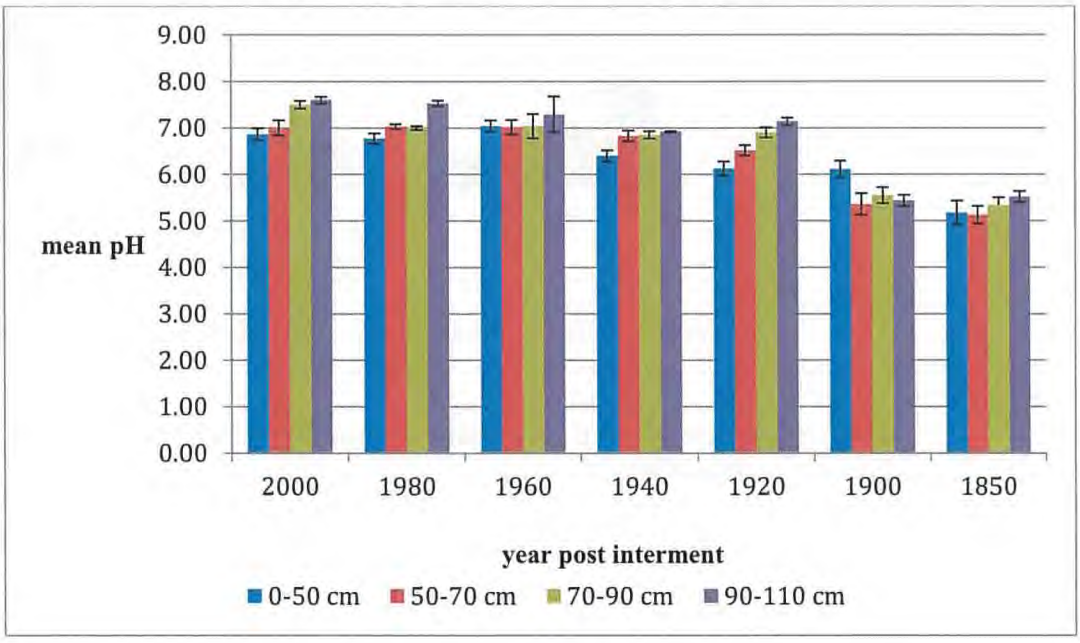


Figure 66: Mean pH of the cemetery soils from West Allerton and Toxteth cemeteries at the four depths.

Spearman's Correlation analysis of depth and time:

The Spearman's coefficient result was statistically significant at all four depths p=0.001. The R^2 values increased in their strength descending through the soil horizon before finally stabilising: 0-50 cm R^2= 0.650, 50-70 cm R^2= 0.730, 70-90 cm R^2= 0.829, 90-110 cm R^2= 0.799 (Figures 67-70).

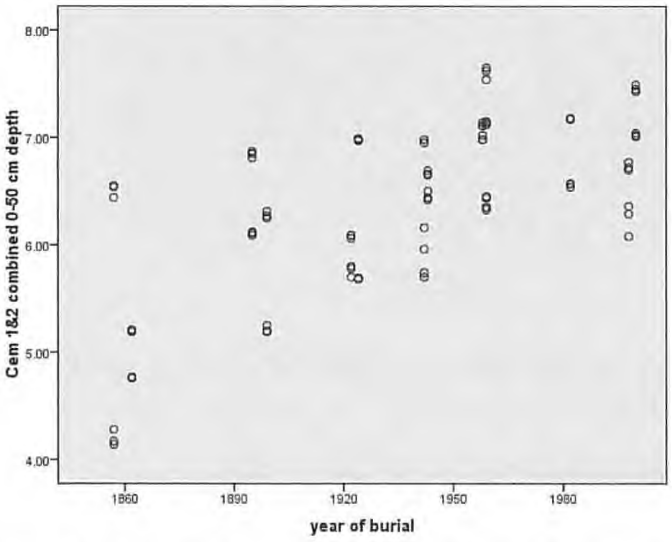


Figure 67: 0-50 cm correlation coefficient of pH with time both cemeteries combined R^2=0.650.

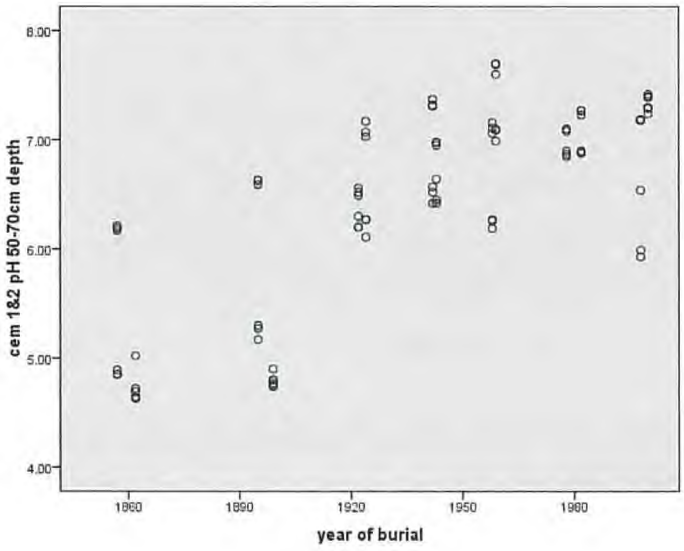


Figure 68: 50-70 cm correlation coefficient of pH with time both cemeteries combined R^2= 0.730.

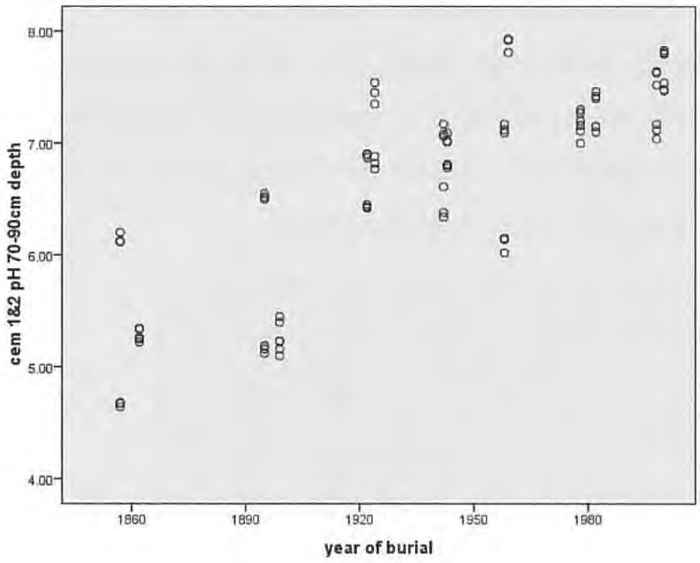


Figure 69: 70-90 cm correlation coefficient of pH with time both cemeteries combined R^2= 0.829.

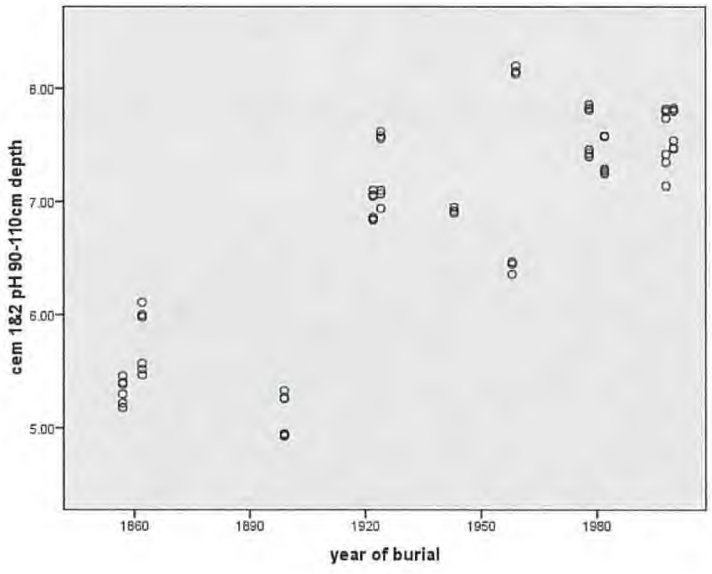


Figure 70: 90-110 cm correlation coefficient of pH with time both cemeteries combined R^2= 0.799.

Mann Whitney U test comparing cemetery and control samples pH:

The cemetery sample results were compared to the control samples at all four depths. The two cemeteries were analysed separately.

The pH results from the graves from West Allerton were statistically different from the control samples at all four depths (Figure 71):

0-50 cm p= 0.001, Z= -3.815 n= 60, 15

50-70 cm p= 0.007, Z= -2.705 n= 60, 12

70-90 cm p= 0.023, Z= -2.280 n= 57, 12

90-110 cm p= 0.001, Z= -3.948 n= 45, 6

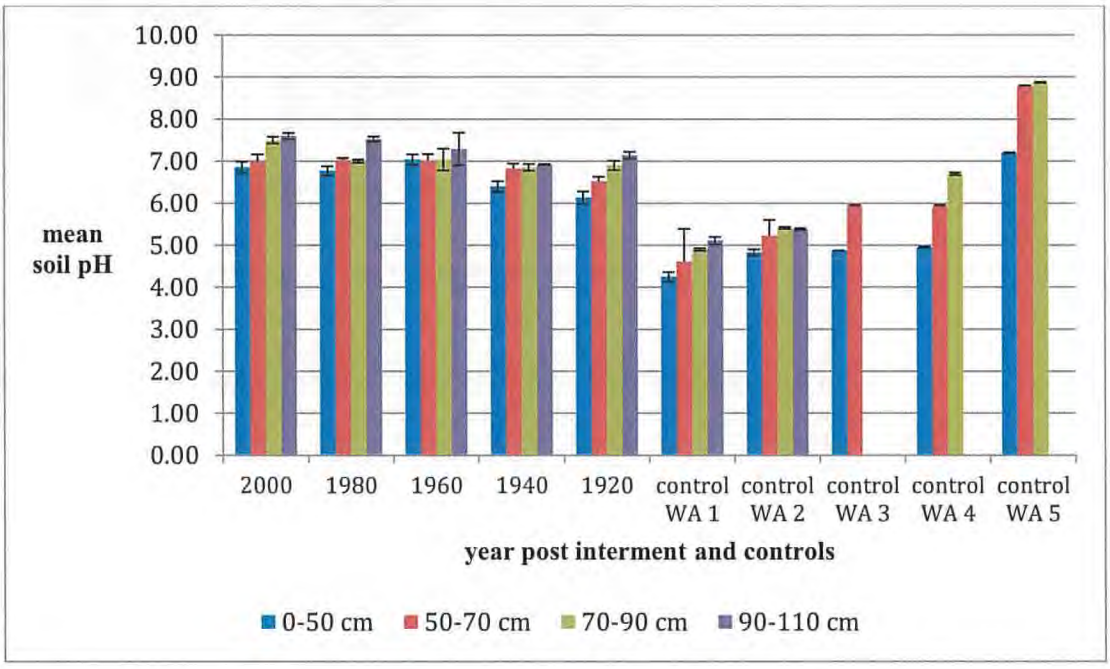


Figure 71: Mean pH of West Allerton cemetery soils compared to control samples.

The pH samples from the graves Toxteth were statistically different from the control samples at three out of four depths (Figure 72).

0-50 cm p= 0.104, Z= -1.660, n= 24,6

50-70 cm p=0.005, Z= -2.697, n= 24, 6

70-90 cm p= 0.003, Z= -2.802, n= 24, 6

90-110 cm p=0.001, Z= -3.734, n=24, 6

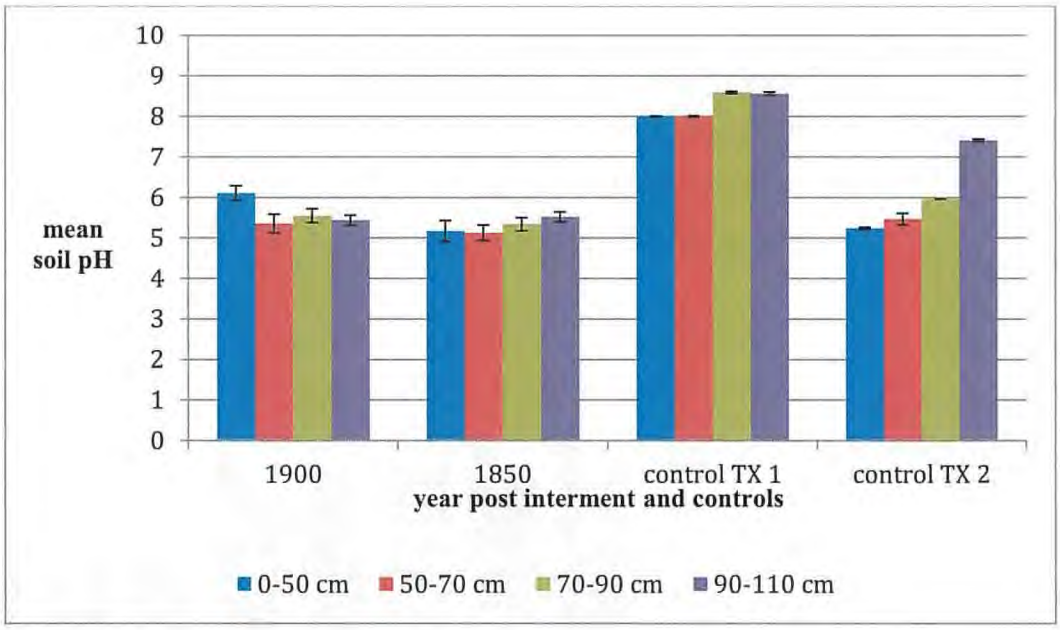


Figure 72: Mean pH of Toxteth cemetery soils compared to control samples.

5.4. Discussion

The remains from Liverpool were interred in an environment that was conducive to accelerated decomposition by being subjected to the effects of the temperate climate of the UK, frequent incursions of the water table, with the open textured nature of the parent material consisting mainly of quartz (Figures 12-19). Due to these mechanisms there was a significantly increased decomposition rate of biological material causing the bone components to be degraded until they were fully incorporated into the soil within a few decades.

The microscopy results indicate a previously contaminated soil horizon with coal and charcoal featuring in the West Allerton grave samples and the occasional control sample at all depths. The land at West Allerton had been used primarily as agricultural land in the past with coal and wood burning ash being dumped on the land from the available woodland and domestic dwellings (Figures 20, 22, 24, 26,). Toxteth, in contrast, developed as an urban area with coal featuring as the main fuel source, with charcoal featured in only one sample (21, 23, 25, 27). During the industrial and domestic development of Liverpool, large quantities of wood and coal would have been burnt and deposited on the land in order to dispose of it. Anthropogenic activities such as coal and charcoal burning can have both a temporary and lasting effect on the pH of the soil. Wood burning produced a highly alkaline ash containing mostly hydroxides (>90%) and small quantities of calcite (<10%), and metallic residues of calcium (Ca), potassium (K), magnesium (Mg), phosphorus (P) and silicon (Si), but the effect was generally temporary, with only insoluble compounds such as calcite remaining for any length of time (Etiegni and Campbell, 1990; Ohno and Erich, 1990; Ulery et al., 1993). In direct contrast to wood-burning, coal produced acidifying effects on the environment via the production of suphur dioxide, nitrogen oxides and chlorine gases. These gases undergo a series of chemical reactions with atmospheric water to produce weak acid solutions of sulphuric, nitric and hydrochloric acids; these enter the eco system through precipitation of water onto the land and water courses (Egeback et al., 1984; Flues et al., 2001; Menz and Siep, 2004; Newberry et al., 1990). The grave soil pH from West Allerton was generally higher which corresponded with the presence of charcoal (Figure 71), and Toxteth produced a more acidic pH associated with coal burning (Figure 72), but the pH increased downward through the soil horizon towards the coffin area in West Allerton, but remained about the same for

Toxteth. The control samples followed the increasing trend seen in the West Allerton grave samples, but to a lesser degree. As the deposition of coal and charcoal occurred over a hundred years ago it is questionable as to the present day effects, but acid rain from coal emissions continue to this day from power plants and domestic properties, and it reasonable to assume that some of this would percolate through the soil horizons, but its role in the decomposition of bone and teeth would be more difficult to determine.

The domestic waste found in the samples consisted mainly of broken red brick, glass and crumbling china (Figures 34-41). Brick and glass tend to be very hard substances that remain pH inert in the soil. The decomposing china was found in the second control sample from Toxteth (Figure 41), this had a lower pH than the first control, and about the same as the cemetery samples. The first control sample did not contain any artefacts that would explain the rise in the pH. There was a vast range of pH readings from the Toxteth and West Allerton control samples which were not explained by any artefacts present, but could have resulted from components that might not have been detected using microscopy such as dissolved cement from past building works or demolition.

The presence of humic acids (HA) from the breakdown of biological plant matter can result in chelation with metals, and may be the reason that the metals from bone decomposition remained in the soil (Baker, 1973; Brown et al., 2000; Kerndorff and Schnitzer, 1980; Petrovic et al., 1998) HA exist in all soils that contain plant matter, and contribute partly in the acidification of the soil, and are an important part of the soil's fertility. They can be found in all soil horizons but in greater quantities in those horizons that contain the largest quantity of degraded plant matter (usually the topsoil). Each metal ion chelates at a different pH (usually low). The HA can also intrude and chelate with buried bone metals causing inhibitory problems during the polymerase chain reaction (PCR), and their removal is necessary for DNA studies (Kalmar et al., 2000; Tuross, 1994). The presence of roots can also facilitate the retention of metals, iron in particular. The cemetery soil's contained root and plant matter in greater quantities in the topsoil in both cemeteries (Figures 28 & 29), decreasing in the lower horizons (Figures 28-34).

The levels of five elements: iron (Fe), calcium (Ca), magnesium (Mg), potassium (K) and phosphorus (P) were analysed at four different depths over seven different time periods to assess their suitability to be used for investigating the mobility of bone components in the burial environment as well as detecting the presence of decomposing bone. The graves from fifty years post interment demonstrated an interesting elemental status with low levels of iron and magnesium compared to phosphorus, calcium and potassium, indicating the mobility of these ions under certain conditions (Figures 42-51).

Difficulty using elemental analysis stems from the fact that these elements may come from sources other than human soft and hard tissue decomposition, especially given Liverpool's prolific industrial past. The spike in the levels of the elements during the war years (1918-1945) may have been due to the increased industrial output in Liverpool during these times. The values for iron were not significantly different ($P<0.05$) across the four depths, but were significantly different at all depths to the control samples (Figures 56 & 57). As can be seen from the graph (Figures 42), there is little variation in the iron content between the four depths at each time period. This shows that the levels of iron vary little throughout the soil horizon. This can be seen more clearly as the mean trend line in Figure 43, seen in the closeness of the trend lines, apart from 0-50 cm. The levels for iron appear to exhibit two main events over the time ranges, rising in the initial stages of decomposition (10 and 30 years post interment) and declining 50 years post interment. There is another rise between the war years (70-90 years post interment), which could be attributed to an increase in industrial output. The levels pre-war (110-150 years) demonstrate a marked overall decline compared to the other time periods. The presence of roots and plant material can sequester iron, and are routinely removed before analysis (Thomine and Lanquar, 2011). However during processing, the large root fragments were removed manually or by sieving, and the remaining tiny fragments do not appear to have had an adverse effect on the iron levels. The iron levels at 0-50 cm that included the turf roots were consistently lower than other depths across all time periods, which may indicate the sequestering of iron in the roots. There was a significant difference between the levels of K at the four depths ($p>0.05$), from both cemeteries, and across all time periods, this can be seen with greater clarity in Figure 44 and the mean trend line in Figures 45. Figure 44 shows the levels 10 – 110 years post interment demonstrating an increase

through the soil horizon, with 150 years showing a decrease. There are two events at 30 and 90 years that follow the iron trend. The potassium results from the control soil samples showed a significant difference in only two samples compared to the cemetery soil (58 & 59). Whilst the levels of phosphorus show no significant difference ($p<0.05$) through the four horizons, trends can be seen that indicate a decrease through the soil horizons (figure 46 & 47). Some of the deeper values were = 0, showing the possible high mobility of this element through the soil environment. Phosphorus forms a major part of the organic component of bone and its interaction with the soil matrix in regards to mobility is important; however phosphorus is a common element found in the enivironment from multiple sources including products of decomposition from plants and animals, fertilizers, industry, and domestic activities such as wood burning. The phosphorus levels at the coffin depth (110 cm) appear to be very low compared to the rest of the soil horizons except for 150 years post interment, this could be explained by multiple inhumations, however the levels for other graves that had muliple inhumations did not follow this trend. Elevated levels of phosphorus in the soil of decomposing skeletal material may occur earlier in the decomposition process due to its highly mobile characteristics, making it suitable for initial skeletal degradation detection work, but unsuitable for later detection studies in this type of soil where decomposition occurs quickly (< 50 years). The levels were highest at 50 years post interment compared to the other values, which could account for residual phosphorus in the soil once the bone dissolved away and there was a sudden release of phosphorus into the soil. This event follows later than the 30 year trend of iron and potassium. Phosphorus commonly exists as compounds, the most common being the apatites and only soluble under specific conditions. Phosphorus can form compounds with other elements such as iron, potassium, magnesium, aluminium, manganese, and sodium that form part of the parent material. Generally phosphorus is less soluble in low soil pH (<5), however when in a compound such as calcium phosphate the reverse is true and can become more soluble. One explanation for the decreasing levels through the horizons could be removal of P by the water table or microorganisms (Larsen, 1967). The P results demonstrated significance difference with the control soil samples in three time periods (Figures 60 & 61). Magnesium showed no significant difference ($P<0.05$) through the four depths at time periods. The results from Figure 48 demonstrate a similar pattern to potassium. Whilst the results were not significant together through time, some patterns could be seen in individual

time periods (10, 30, 90 years post interment) with the elevated levels of Magnesium. Figure 49 demonstrated these trends more clearly. The control samples (Figures 62 & 63) were significanly different at three time periods (1920, 1980, 2000). The results for calcium were significantly different p=0.017 at all depths, time periods (Figure 50), and controls (Figures 64 & 65). The results from Toxteth demonstrated higher calcium levels in the control soils, which may be due to the high levels of cements and building material contained in these samples (Figure 65). The trend in Figure 51 demonstrated a general decrease in the calcium levels through time, rather than event peaks seen in other elements.

The results from this study have demonstrated a clear negative correlation between the movement of calcium and magnesium across depth and time (Figures 52-55), despite the complex problem of differences between soil matrices. All the elements studied proved to be useful at lower depths, nearer to the coffin, making this a useful indicator for the presence of interred remains. The higher significant levels of the elements iron, potassium and phosphorus at 50-70 cm could have resulted from deposition by the water table incursions, and may have normally been found lower in the soil horizon.

The pH of the soil may have been affected by the interred human remains, parent material, hydrology, and previous use of the land. The control soil samples varied in their pH, but contrasted directly with the cemetery samples (Figures 71 & 72). The control samples tended towards either end of the pH scale, in contrast to the cemetery samples that tended towards neutral then increasing in pH down through the horizons. The frequent refreshing of the water table could have assisted in the acceleration of the dissolution of the mineral component of the bone matrix once the soft tissues had decomposed; the water not becoming fully saturated with ions that could buffer the water pH and provide some bone preservation. The solubility of bone ions can be pH dependant especially compounds of calcium and phosphorus (Larsen, 1967). The soil pH demonstrated a clear negative correlation with time across all depths and time periods, with 70-90 cm being the most prominent (Figures 67 – 70).

5.5. Conclusion and Further Work

The detection of older buried remains has tended to focus on the physical density of the soil to identify voids that may locate an interment, or use biological decomposition to detect remains, but the effectiveness significantly reduces after a couple of years as the corpse degrades. This new method using a multidisciplinary approach to bone leaching provides a soil and chemical analysis that is relatively quick, cheap and easy to use.

The taking of control samples to use for comparison may prove to be obsolete, with the development of "hotspot" areas of interest being more relevant and useful. The areas where the control samples were taken from were primarily tree-lined grass verges with copious amount of roosting bird guano, but the levels of phosphorus and potassium were consistent with the grave findings, and were not particularly high compared to the graves. The presence of plant roots in the soil samples that may skew the results as they can sequester iron, but given the consistency of the iron results from the Liverpool cemeteries this does not appear to be the case here. Visible roots were removed manually, and small ones can be removed by water flotation and skimming, but will extend the soil preparation time considerably.

The increase in soil pH demonstrated a clear significant correlation with time, the soil pH became more acidic through time as the bone components were mobilised out of the coffin area. This is useful for applying to decomposition of remains, but further study would be needed to clarify the consistency of these findings with different soil types. There was some difference between the cemeteries and controls, but this picture was not clear, the usefulness of pH analysis for the detection of remains would need further clarification.

Extending the soil preparation to include particle size would help to assess the soil in greater detail. Pelleting the samples may help to reduce the within sample variation. Further study of other cemeteries of different soil types will further the understanding of bone leaching and mobility under different conditions, parent material and environment.

General Conclusions and Recommendations

The DNA extraction methods used in this PhD study, using Medieval bones resulted in DNA being extracted but it was too degraded to amplify, as the bones used were from the worst rather than the best bones in the collection. Using highly fragmented, disarticulated bone fragments, interred with only a thin layer of soil to protect them, would leave them exposed to the worst extremes of environmental conditions and render the DNA in a poor condition. Future work would benefit from:

- Use whole, robust, well preserved, undamaged bones, or teeth if possible.
- Bones/teeth that have been excavated from deep (> 1m) graves.
- Freezing bones at -80°C once excavated would help to preserve any useable DNA, and prevent the destructive agents such as DNAases, bacteria or fungi, from degrading the DNA.
- Using a range of different polymerases that may be more tolerant (such as iron tolerant Klentaq®) and DNA technologies may help to over-ride metal or soil component PCR inhibition problems.
- Investigate the soil first before starting DNA work to identify contaminating factors first such as metal, humic acid, clay or biological contaminants, using XRF and FT-IR.
- Use FT-IR to assess the bone components, as this technology only requires 2mg of bone sample, and is capable of identifying the preservation of the organic and inorganic phases of bone, and the degree of soil component intrusion.
- Future DNA should involve the use of sequencing primers, as this would make understanding the degradation of the DNA sample easier, but also enable human genetic familial lineage/ disease lineage comparison software to be used.
- Further investigation into soil extraction techniques, such as raising/lowering the pH of the EDTA extraction buffer to investigate the precipitation and removal of the salts that may enable full analysis of the effects of soil contaminants of DNA preservation and PCR.

This study has shown that the preservation of the Medieval bone was directly linked to the interment soil environmental conditions. Future investigation of a burial cemetery site should include:

- Recording of the pH of the soil across the whole of the cemetery site. As the site is at various stages of excavation careful consideration would have to be given to the depth and distance of sampling.
- Full soil analysis including particle size, humic quantification, and moisture content (hydrology).
- Full analysis of the metal range and content.
- All excavations should refrain from storing soil around the perimeter of the cemetery, as this can result in contamination of the underlying soil.

The use of soil analysis techniques to assist in explaining the preservation of soil-interred bone, using the dissolved human remains from modern contemporary cemeteries has proved a useful tool in assessing how long chemical components from human remains can persist in the soil. This study would benefit from:

- Being extended further to include cemeteries with different soil types.
- Increase in the number of graves from 2 from each time period to 5 or more to clarify the results obtained, as the limited number of samples used in the study would not usually be enough for clear statistical analysis results.
- Comprehensive soil study including particle size, humus and biological content quantification, moisture content (hydrology), and metal ion quantification.

Appendix

Chapter 3.

Statistical results:

1: Levels of significance for the elements from recently excavated and stored crania

Element	P value	Z value	n
Calcium	0.003	-2.966	15,15
Phosphorus	0.015	-2.427	15,15
Iron	0.029	-2.178	15,15
Zinc	<0.001	-4.343	15,15
Lead	0.001	-3.452	15,14
Strontium	0.001	-3.592	15,15
Silicon	0.983	-0.021	15,15
Aluminium	0.852	-0.187	15,15
Magnesium	0.101	-1.639	15,15
Potassium	0.820	-0.228	15,15
Titanium	0.418	-0.81	10,11
Sulphur	0.281	-1.079	15,15
Manganese	0.071	-1.805	15,15

2: Levels of significance for the elements from recently excavated and stored long bones

Element	P value	Z value	n
Calcium	0.007	-2.717	15,15
Phosphorus	0.014	-2.458	15,15
Iron	0.033	-2.137	15,15
Zinc	<0.280	-1.079	15,15
Lead	0.118	-1.564	15,15
Strontium	0.042	-2.037	15,15
Silicon	0.575	-0.560	15,15
Aluminium	0.852	-0.187	15,15
Magnesium	0.852	-0.187	15,15
Potassium	0.983	-0.021	15,15
Titanium	0.137	-1.487	15,15
Sulphur	<0.001	-4.210	15,15
Manganese	0.743	-0.327	15,14

3: Levels of significance for the elements from stored cranium and long bones

Element	P value	Z value	n
Calcium	0.081	-1.763	15,15
Phosphorus	0.285	-1.099	15,15
Iron	0.001	-3.132	15,15
Zinc	0.001	-3.222	15,15
Lead	<0.001	-4.25	15,15
Strontium	0.006	-2.703	15,15
Silicon	0.161	-1.431	15,15
Aluminium	0.202	-1.307	15,15
Magnesium	0.021	-2.302	15,15
Potassium	0.098	-1.680	15,15
Titanium	0.004	-2.762	11,7
Sulphur	0.595	-0.560	15,15
Manganese	0.098	-1.682	15,15

4: Levels of significance for the elements from recently excavated crania and long bones

Element	P value	Z value	n
Calcium	<0.001	-4.584	15,15
Phosphorus	<0.001	-4.211	15,15
Iron	0.512	-0.685	15,15
Zinc	<0.001	-3.176	15,15
Lead	0.914	-0.133	14,15
Strontium	0.001	-3.109	15,15
Silicon	0.012	-2.510	15,15
Aluminium	0.041	-2.054	15,15
Magnesium	0.074	-1.805	15,15
Potassium	0.106	-1.639	15,15
Titanium	0.019	-2.331	11,7
Sulphur	<0.001	-4.606	15,15
Manganese	0.760	-0.306	15,14

5: The levels of significance for the stored cranium and long bones

Element	P value	Z value	n
Calcium	0.003	-2.928	15,12
Phosphorus	0.001	-3.074	15,12
Iron	<0.001	-4.396	12,15
Zinc			
Lead			
Strontium			
Silicon	<0.001	-4.392	15,12
Aluminium			
Magnesium	<0.001	-3.487	8,15
Potassium	0.036	-2.077	11,15
Titanium			
Sulphur	<0.001	-4.392	12,15
Manganese			

6: Levels of significance for stored long bone and pork bone control

Element	P value	Z value	n
Calcium	<0.001	-4.392	15,15
Phosphorus	<0.001	-3.873	15,15
Iron	<0.001	-4.396	15,15
Zinc			
Lead			
Strontium			
Silicon	<0.001	-4.392	15,15
Aluminium			
Magnesium			
Potassium	0.002	-3.036	15,15
Titanium			
Sulphur	<0.001	-4.392	15,15
Manganese			

7: Levels of significance for recently excavated cranium and pork control

Element	P value	Z value	n
Calcium	<0.001	-4.392	15,15
Phosphorus	<0.001	-4.392	15,15
Iron	<0.001	-4.396	15,15
Zinc			
Lead			
Strontium			
Silicon	<0.001	-4.392	15,15
Aluminium			
Magnesium	<0.001	-3.874	15,15
Potassium	<0.001	-4.282	15,15
Titanium			
Sulphur	<0.001	-4.393	15,15
Manganese			

8: Levels of significance for recently excavated long bone and pork control

Element	P value	Z value	n
Calcium	0.002	-2.977	15,15
Phosphorus	0.001	-3.075	15,15
Iron	<0.001	-4.397	15,15
Zinc			
Lead			
Strontium			
Silicon	<0.001	-4.392	15,15
Aluminium			
Magnesium	<0.001	-3.874	15,15
Potassium	<0.001	-4.101	15,15
Titanium			
Sulphur	<0.001	-4.392	15,15
Manganese			

Chapter 4

FTIR spectra

The FTIR spectra used for comparison of the humic acids, collagen, and soil intrusion of the archaeological bone:

1. Samples 1-20 archaeological samples.
2. Pork bone samples (acetates in separate file)
3. Poulton soil samples (acetates in separate file).
4. Bone standard 1486

Bone samples FTIR spectra identification:

1: 1a
2: 2a
3: 3a
4: 4a
5: 5a
6: 1b
7: 2b
8: 3b
9: 4b
10: 5b
11: 1c
12: 2c
13: 3c
14: 4c
15: 5c
16: 1d
17: 2d
18: 3d
19: 4d
20: 5d

Chapter 5

Proposal for cemetery soil sampling:

The need to conduct soil sampling is part of a larger body of work for a PhD, looking at soil components and their effects on human bone. Metals in the soil have been of particular interest, as their possible persistence in the soil could be of great scientific, archaeological and forensic significance. It is hoped that by sampling the soil from between known graves it may be possible to avoid the destructive and time-consuming work that detecting forensic and archaeological clandestine graves can bring, making this work easier by allowing a large area of ground to be surveyed in a much shorter period of time. The work proposed would involve:

- Taking cores of soil using a 5cm auger, usually 2-3 from the soil between graves, to a depth of about 1-2m. Direct access to the graves will be avoided as we only need the indicators of the body's presence.
- 3 control soil samples from outside each of the grave areas; this is to compare the grave soil components to.
- Minimum disturbance to the surface soil appearance, plugging with turf if necessary.
- Unsophisticated burials would be preferred, as embalming and robust coffins may not allow for detection of leached components from the remains.
- Graves from a wide range of time spans would be appreciated, from 10, 20, 30, 50 years etc. up to the oldest, according to the graves that are available to have access to. As experiments are done in triplicate, it would be preferable to have 2- 3 graves from each time period to sample from.

The greatest respect will be shown to these graves at all times. Any requirement as to reassurance to the quality of the work being done, including monitoring by cemetery staff, if required, will be adhered to. Work will be stopped if any problems arise and would not resume until any issues raised have been resolved.

Statistical results:

Cemetery 1: West Allerton, cemetery 2: Toxteth

1: Wilcoxon sign rank left and right p = 0.05

element	cemetery	Z value	P value	N
Fe	Cem1	-1.340	0.180	40
Ca	Cem1	-1.228	0.219	40
P	Cem1	-0.264	0.792	40
Mg	Cem1	-1.633	0.103	40
K	Cem1	-0.070	0.944	40
Fe	Cem2	-0.931	0.352	16
Ca	Cem2	-1.344	0.179	16
P	Cem2	-0.414	0.679	16
Mg	Cem2	-0.052	0.959	16
K	Cem2	-0.879	0.379	16

2: P=0.05 for significance.

element	Chi sq.	df	P value	N	sig
Fe	0.684	3	0.879	55	no
K	8.272	3	0.041	55	yes
Mg	3.330	3	0.343	55	no
Ca	10.148	3	0.017	55	yes
P	5.542	3	0.136	55	no

3: P=0.05 for significance

Cemetery 1 and Cemetery 2 0-50cm:

element	Spearman's rho	P value	sig	N
Fe	r=0 .002	0.994	no	14
K	r= 0.359	0.208	no	14
Mg	r= 0.059	0.840	no	14
Ca	r= -0.736	0.003	yes	14
P	r= -0.352	0.217	no	14

Cemetery 1 and Cemetery 2 50-70cm:

element	Spearman's rho	P value	sig	N
Fe	r= -0.543	0.045	yes	14
K	r= -0.574	0.032	yes	14
Mg	r= -0.437	0.118	no	14
Ca	r= -0.587	0.027	yes	14
P	r= 0.162	0.579	no	14

Cemetery 1 and Cemetery 2 70-90cm:

element	Spearman's rho	P value	sig	N
Fe	r= -0.393	0.164	no	14
K	r=-0.481	0.081	no	14
Mg	r= -0.547	0.045	yes	14
Ca	r= -0.802	0.001	yes	14
P	r= 0.335	0.241	no	14

Cemetery 1 and Cemetery 2 90-110cm:

element	Spearman's rho	P value	sig	N
Fe	r= -0.495	0.086	no	13
K	r= -0.484	0.094	no	13
Mg	r= -0.615	0.025	yes	13
Ca	r= -0.832	0.000*	yes	13
P	r= 0.365	0.220	no	13

*This was significant at 0.01 level

4: P=0.05, 2- tailed Mann Whitney U, cemetery/control samples

element	cemetery	date	Z value	P value	N	sig
Fe	Cemetery 1	2000, 1998	-1.564	0.119	32	no
Fe	Cemetery 1	1982, 1978	-3.026	0.002	32	yes
Fe	Cemetery 1	1959, 1958	-2.287	0.009	29	yes
Fe	Cemetery 1	1943, 1942	-0.614	0.559	29	no
Fe	Cemetery 1	1924, 1922	-3.185	0.001	32	yes
Fe	Cemetery 2	1899, 1895	-3.338	0.001	24	yes
Fe	Cemetery 2	1862, 1857	-1.838	0.066	24	no
K	Cemetery 1	2000, 1998	-1.564	0.118	32	no
K	Cemetery 1	1982, 1978	-3.356	0.001	32	yes
K	Cemetery 1	1959, 1958	-1.316	0.188	32	no
K	Cemetery 1	1943, 1942	-1.491	0.136	29	no
K	Cemetery 1	1924, 1922	-2.606	0.009	32	yes
K	Cemetery 2	1899, 1895	-0.184	0.854	24	no
K	Cemetery 2	1862, 1857	-0.735	0.462	24	no
Mg	Cemetery 1	2000, 1998	-1.131	0.258	32	no
Mg	Cemetery 1	1982, 1978	-2.073	0.038	32	yes
Mg	Cemetery 1	1959, 1958	-2.675	0.007	29	yes
Mg	Cemetery 1	1943, 1942	-0.175	0.861	29	no
Mg	Cemetery1	1924, 1922	-2.374	0.018	32	yes
Mg	Cemetery 2	1899, 1895	-0.705	0.481	24	no
Mg	Cemetery 2	1862, 1857	-1.347	0.178	24	no
Ca	Cemetery 1	2000, 1998	-2.940	0.003	32	yes
Ca	Cemetery 1	1982, 1978	-2.865	0.004	32	yes
Ca	Cemetery 1	1959, 1958	-2.456	0.014	29	yes
Ca	Cemetery 1	1943, 1942	-2.500	0.012	29	yes
Ca	Cemetery 1	1924, 1922	-2.544	0.011	32	yes

Ca	Cemetery 2	1899, 1895	-3.860	0.000(5)	24	yes
Ca	Cemetery 2	1862, 1857	-3.920	0.000(5)	24	yes
P	Cemetery 1	2000, 1998	-0.228	0.820	32	no
P	Cemetery 1	1982, 1978	-2.537	0.011	32	yes
P	Cemetery 1	1959, 1958	-3.161	0.002	29	yes
P	Cemetery 1	1943, 1942	-1.692	0.091	29	no
P	Cemetery 1	1924, 1922	-2.792	0.005	32	yes
P	Cemetery 2	1899, 1895	-1.594	0.111	24	no
P	Cemetery 2	1862, 1857	-1.041	0.298	24	no

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