HUMAN PROVENANCING – COMBINED ISOTOPIC AND GENETIC PROFILING OF LIMITED BONE AND TEETH MATERIAL OF ANCIENT HUMAN REMAINS

By

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Abstract

The ability to identify and to trace the origin and movement of individuals is of major interest in forensics, archaeology and anthropology. To date, DNA profiling is still the most effective approach for human identification. Despite the success story of DNA profiling, there is a constant search for additional methods to aid in the identification of human remains. In recent years increasingly strontium and lead isotopes analysis have proved particularly useful as tracers for individual residency and migration.

The re-occurring problem of limited sample material in archaeological and forensic investigations led to the second objective to investigate where sample material could be saved. The development of a new method for the simultaneous extraction of both strontium and lead from bone and teeth on a single Pb-specific resin proved to be successful. Both elements could be purified in sufficient amounts for successive isotope analysis. This work also investigated for the first time the feasibility of using the remaining bone residues after DNA extraction for further Sr and Pb isotope analysis. The first isotope results were promising and did not show any significant differences between fresh bone and bone residues. However, a more extensive trial is required to validate these exciting preliminary findings.

To maximize the evidence for individual identification an interdisciplinary approach was chosen for this study. DNA profiling and strontium/lead analysis were employed in two case studies on human remains from a) the Spanish Civil War (1936-1939) and b) from a Late Anglo-Saxon burial in Norfolk, UK. DNA analysis proved not to be achievable due to the degraded nature of the skeletal tissues in both case studies. Strontium and lead isotope analysis could identify two possible migrants among the Spanish burial population. In the Norfolk case, isotopic evidence implied that the group was unlikely to be of local (Norfolk) origin.

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Chapter 1

1 Introduction

1.1 Human Provenancing

The ability to identify and to trace the origin and movement of individuals is of major interest in forensics, archaeology and anthropology. To date DNA profiling is still the most important approach used for human identification in forensics and archaeology. While DNA is indispensible where biological relatedness is of interest it says very little about where an individual has lived or migrated from or to. To know the geographic whereabouts, however, can be equally important both in the forensic and archaeological context. Isotope analysis in human provenancing has been applied for more than a decade in archaeology to study migration patterns of people and is now becoming increasingly popular in forensics. In cases where DNA profiling cannot provide sufficient information for identification, stable isotope analysis has the potential to restrict the residency to a geographic area the individual has lived in and in some instances can provide a lead for further investigations. Strontium (Sr) isotopes in particular have proved to be strong indicators of geographic origin (Knudson and Buikstra, 2007, Price et al., 1994a, Price et al., 1994b, Bentley et al., 2002). Sr in combination with other elements such as oxygen (Evans et al., 2006a, Eckardt et al., 2009), or lead (Budd et al., 2001, Montgomery et al., 2005a) could provide even stronger evidence in favour of a specific geographic origin.

1.2 DNA Profiling

The human genome consists of approximately 3 billion base pairs (bp) of DNA harbouring around 30,000 genes. Despite the huge size of DNA only about 5 % encode genes. The vast majority of about 95 % contains non-coding DNA also referred to as "junk" DNA that is not directly involved in protein synthesis (Carracedo et al., 2008). Forensic identification is based on the great genetic variability that exists between individuals. Most of these variations, or DNA polymorphisms, are located in the non-coding DNA. Therefore the majority of forensic DNA markers used in human identification target primarily the non-coding region not only because it is most informative but also because it reveals no information that could disadvantage an individual. Ancient DNA studies, however, mostly rely on mitochondrial DNA analysis as nuclear DNA is often compromised and heavily degraded in historic human samples. The high copy number of mitochondrial DNA (mtDNA) present in hundreds to thousands of copies in a cell is much more abundant than nuclear DNA with only two copies. Hence the likelihood of retrieving mtDNA from ancient or degraded samples is much higher.

Relatively recently also archaeologists and anthropologists have started to embrace DNA profiling as a complementary method for identifying skeletal remains. Their primary tools to determine age, gender and disease are traditionally based on visual examination, odontology and metric analysis of skeletal parts. However, these techniques have limitations and cannot always lead to conclusive answers. Often DNA analysis delivers more precise details on the individual and his ethnic background and is nowadays frequently incorporated into archaeological research.

1.3 Isotopic profiling

Despite the success story of DNA profiling, there is a constant search for additional methods to aid in the identification of human remains. In recent years increasingly isotope analysis has emerged as a new tool in the identification of unknown skeletal remains both in the forensic and archaeological context.

$^{87}Sr/^{86}Sr$ composition in bone and tooth enamel has proved particularly useful for individual residency and migration studies. This technique is based on strontium variations in the geological environment and the bioavailable Sr fraction that is incorporated into bone and teeth (Bentley, 2006) through the intake of diet and water. The emphasis on using Sr isotopes for provenancing is based on the fact that Sr is passed on through the food chain without any significant fractionation thus reflecting the original isotopic signature of the Sr source. Similar to Sr, lead retains its isotopic signature once deposited in skeletal material. Lead isotopes have mainly been employed to trace the source of pollution in the environment and its effect on humans (Gulson, 2008, Gulson et al., 1999). The variability of isotopic compositions of lead ores, however, has also been exploited in forensics (Gulson et al., 2002b), and in numerous anthropological and archaeological studies (Budd et al., 2004b, Carlson, 1996).

Another important feature exploited in isotope analysis is the physiological difference that exists between bone and teeth. Each tooth records the isotopic signature incorporated during growth at different times of development, which is usually completed by the age of twelve and remains unchanged thereafter. Bone tissue on the other hand is constantly renewed thus the Sr composition is only representative for a specific time period. Hence the comparison of Sr ratios between tooth enamel and bone can be used to identify a change of residency between childhood and adulthood.

1.4 Objectives of the Norfolk case study

The cooperation between the Norfolk Archaeological Unit (NAU) in Norwich, UK and the Centre of Forensic Provenancing at the University of East Anglia (UEA) was established to investigate the origin of skeletal remains of fourteen individuals buried on the grounds of Snarehill Hall in Brettenham/Norfolk/UK. The foremost question posed was to identify local from non-local individuals that might have immigrated from continental Europe, in particular Anglo-Saxons and Vikings from Denmark or Norway.

a) Genetic analysis

The aim of the genetic analysis was to identify the ethnic background of the human remains. Considering that the skeletal material is an estimated 1,000 years old, it was expected that nuclear DNA would be highly degraded. Hence mitochondrial DNA markers were chosen for this study because both of the age of the sample material and the purpose of determining the ethnic background of the burial group.

b) Isotope analysis

The intention of this study was a) to analyse the elemental content and isotope composition of strontium and lead in bone and teeth and b) to investigate the geographic origin of the individuals at Snarehill Hall.

In order to identify residence and reconstruct mobility it requires that bones and teeth are well preserved so that they reflect the authentic geochemical record rather than a diagenetically altered elemental composition. To assess the preservation state of the sample material Rare Earth Elements (REE) analysis was included. These elements have no biological function and are only present in minute concentrations (~100 ppb) in modern bone (Trueman et al., 2006).

Under adverse chemical burial conditions, however, REE concentrations can increase up to 3- to 5-fold in bone (Trueman et al., 2006). Hence an increase in REE concentrations of this magnitude in skeletal tissues is an indication for diagenesis and soil contamination.

1.5 Objectives of the Spanish case study

In 2008 scientists from the Autonomous University of Barcelona (UAB) (Spain) and Binghamton University (USA) were involved in the exhumation of a mass grave in Villanueva del Rosario in the province of Malaga in southern Spain, which produced human remains of 16 victims executed during the Spanish Civil War (1936-1939). The cooperation between these two universities and the UEA was established to provide genetic and isotope analysis of the human remains in order to investigate their identity and kinship to the living relatives.

a) Genetic analysis

The goal of the Spanish case study was to retrieve and analyse nuclear DNA from the victims found in a mass grave in Villanueva del Rosario and to match the genetic information to those of the living relatives of the victims who provided reference samples (buccal swabs and blood) for this study. In modern samples usually nuclear DNA markers (Short Tandem Repeats – "STRs") are used to establish kinship. In cases were the DNA is highly degraded, "mini-STRs are routinely used in forensics because of their shorter amplicon length. As it could be expected that the human remains would be compromised to a certain extent, mini-STRs were chosen for DNA analysis in this case and also because of their specificity to establish kinship.

b) Isotope analysis

The principle aim of this study was similar to the Norfolk study, namely to a) investigate whether strontium and lead isotope analysis could identify two distinct populations ("locals" vs. "non-locals") and b) if these individuals had spent their childhood/adulthood in this particular area where the mass grave was located or have migrated between places. Such information could be verified by the relatives and hence provide further proof of identity.

1.6 Objectives of Method development

The title of this thesis implies that genetic and isotopic analysis is undertaken on "limited" bone and teeth sample material. This implication is based on the fact that forensic and archaeological investigations alike are often confronted with restrictive amounts of sample material for analytical procedures. Trace amounts of evidence material from the crime scene, or restrictions imposed by museums on the use of archaeological specimens have encouraged the aim to develop methods that make better use of the limited sample material available.

The following two new methods were investigated:

a) The development of an extraction protocol that allows for sequential separation of both strontium and lead on a single Pb-specific column instead of using separate columns. This will simplify the extraction procedure and save both sample material and processing time.

b) The assessment of whether or not the bone residue left over after DNA extraction can be used for further isotope analysis. The advantage of using the same bone material for both DNA and subsequent isotope analysis will again simplify the procedure, as often both analytical techniques are required to attain comprehensive information on a case. Additionally, it would not only save valuable sample material but also

time-intensive labour for sample preparation for different analytical techniques.

1.7 Thesis outline

This thesis is structured into eight chapters. The following two chapters (chapters 2 and 3) will provide the relevant background information on "Ancient DNA" and "Isotope Geochemistry" as the two complementary methodologies applied in this study.

Chapter 4 gives an overview of the structural and chemical composition of bone and teeth followed by a section on bone diagenesis.

Chapter 5 describes the "Materials and "Methods" used and tested for DNA and isotope analysis. It also includes a section on the development of the new method for the sequential extraction of strontium and lead. Further, also the feasibility of using bone residues from DNA extraction for subsequent isotope analysis will be investigated.

The first case study on the human remains from the Spanish Civil War is presented in Chapter 6 and the second case study on the human remains recovered from the Anglo-Saxon cemetery in Brettenham in Norfolk, UK, is discussed in Chapter 7.

The last chapter 8 of the thesis will give a final conclusion on the work relevant to the two case studies, methods used and future directions.

Chapter 2

2 Ancient DNA

2.1 Introduction

Ancient DNA (aDNA) studies started in the mid-1980's with Allen Wilson and his scholars Russ Higuchi (Higuchi et al., 1984) and Svante Pääbo (Paabo, 1985) pioneering the field of "Molecular archaeology". The first aDNA sequencing success of the extinct member of the horse family, the quagga, by Higuchi and a 2400-year-old Egyptian mummy by Pääbo initiated a wave of aDNA research. With the availability of the Polymerase Chain Reaction (PCR) in the late 80's the efficiency of aDNA analysis improved dramatically. PCR allowed the amplification of even single surviving molecules (Mullis et al., 1986, Willerslev and Cooper, 2005) giving rise to a thriving scientific discipline within the fields of population genetics, archaeology, anthropology and paleopathology to address questions in systematics, molecular evolution and geographic distribution of species.

Ancient DNA research has significantly contributed to the reconstruction of the hominin evolution (Krings et al., 1997, Krause et al., 2010, Green et al., 2006, Reich et al., 2010) or to the reconstruction of phylogenetic relationships between present and extinct species, such as New Zealand moas (Cooper, 2001, Bunce et al., 2005), the wooly mammoth (Capelli et al., 2006, Poinar et al., 2006), ground sloths (Greenwood et al., 2001) or cave bears (Stiller, 2012). Genetic data also provided evidence for human colonization spreading from Africa (Mellars, 2006, Campbell and Tishkoff, 2008, Reed and Tishkoff, 2006) to Asia and

Europe (Richards et al., 2000) or Oceania (Reich et al., 2011) and the Americas (Goebel et al., 2008, Raghavan et al., 2014, Rasmussen et al., 2014).

The most outstanding achievements in aDNA research to date have been the sequencing of the entire mitochondrial genome of a 30,000-year-old early modern human from a site in Russia (Krause et al., 2010) or the large-scale sequencing of eighty Neanderthal bones (Green et al., 2006). These achievements went hand in hand with the availability of new sequence technologies known as "Next Generation Sequencing" (NGS). The 454 pyrosequencing platform (454 Life Sciences/Roche) or Illumina sequencing technology (Illumina Inc.) are only two examples of the various possibilities available today. There are several advantages brought about by these new technologies for aDNA studies including the possibility to analyse fragments as short as 50 nucleotides at a high-throughput and accuracy, and importantly for research, at low cost.

Although technical developments in aDNA research have progressed significantly in the last two decades, this field is still confronted with a number of challenges, such as degradation of DNA, PCR inhibition by environmental factors and in particular the risk of contamination from modern DNA.

This chapter will start with a few basics on the nature of DNA and the major challenges in aDNA research will be discussed later as well as the precautions required to minimize contamination. Subsequently the analytical procedures in DNA analysis are presented in a brief overview. At the end of this chapter the major differences between ancient and forensic DNA procedures are highlighted.

2.2 DNA structure

Swiss chemist Johann Friedrich Miescher was the first to identify nucleic acids in 1869, and around 1919 Phoebus Aaron Levene determined its basic

polymeric structure (Guttman, 2013). Finally, in 1953 Francis Crick and James Watson succeeded in discovering the double-helical structure of DNA (Watson and Crick, 1953), which earned them the Nobel Prize.

The double-stranded DNA molecule consists of a chain of nucleotides on both strands. Each of the nucleotides comprises a 5-carbon sugar covalently bonded to a purine (adenine or guanine) or pyrimidine (cytosine or thymine) base, and the pentose sugar bonded to a phosphate group. Nucleotides are joined to each other by phosphodiester bonds that connect the 5′-phosphate group of one nucleotide to the 3′-hydroxyl (OH) group of the adjacent nucleotide forming a long chain. The nitrogenous bases on one strand pair in a particular manner – A(denosine) with T(hymine) and C(ytosine) with G(uanine) on the opposite strand by hydrogen bonds (Figure 1).

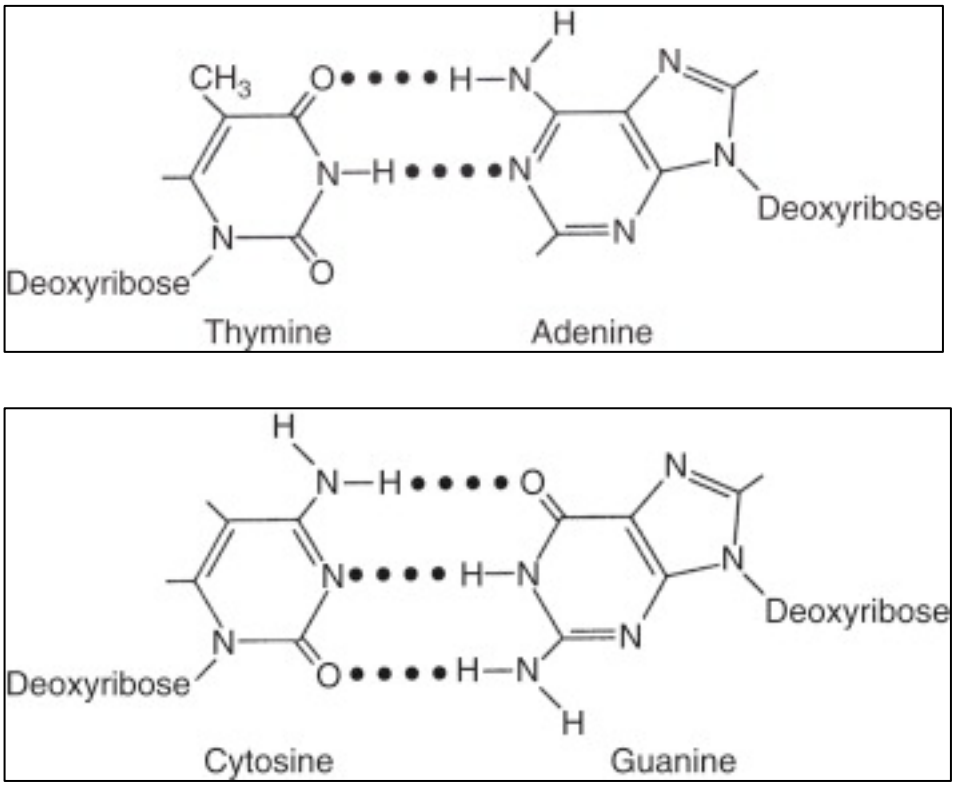

Figure 1: Hydrogen bonding between complementary base pairs (A to T, C to G) (Guttman, 2013)

2.3 DNA degradation in bone and teeth

In living organisms the integrity of DNA is constantly maintained by DNA repair mechanisms. After death these repair mechanisms cease to function, which consequently leads to the rapid degradation of DNA (Paabo et al., 2004). DNA decay is caused by enzymatic activity of cellular nucleases followed by microbial attack (bacterial, fungal and insect), which leads to increased porosity of the crystalline structure of bone and teeth causing further destruction (Poinar, 2003, Hedges, 2002).

Under favourable environmental conditions, such as rapid desiccation, constant low temperatures or high salt concentrations, when the enzymatic activity of nucleases is inactivated (Hofreiter et al., 2001), DNA is able to survive for thousands of years. In one earlier study it has been argued that that under physiological salt concentrations, neutral pH and a temperature of 15 °C, it would take about 100,000 years for hydrolytic damage to destroy all DNA that could reasonably be retrieved (Lindahl, 1993).

Even if conditions are favourable, any endogenous DNA that does survive over time survives only in small fragments that usually range between 100-500 base pairs (Handt et al., 1994, Hoss et al., 1994).

2.3.1 Types of aDNA damage

DNA is under permanent assault from both reactive products within a cell and environmental agents, such as UV-light or ionizing radiation. In a living organism there are several DNA repair mechanisms in place to maintain the integrity of DNA, including base excision repair, nucleotide excision repair, double-strand break repair, and cross-link repair (Sancar et al., 2004). If these repair mechanisms fail erroneous DNA replication can result in genetic malfunction and disease. In deceased organisms all these cellular repair

mechanisms come to a halt leading to an accumulation of DNA damage over time and fragmentation.

Ancient DNA from archaeological and historical material is generally characterised by a) low concentrations of DNA compared to modern samples, b) very short DNA fragments, and c) oxidative and hydrolytic damage (Hoss et al., 1996, Handt et al., 1996, Paabo, 1989).

In the following section the most common types of DNA damage will be discussed.

2.3.2 Molecular damage

The main types of damage in degraded DNA derive from hydrolysis and oxidative stress, which cause changes in nitrous bases. The most common changes in aDNA include the oxidation of the purines adenine (A) and guanine (G), and the formation of hydantoin derivatives of the pyrimidines cytosine (C) and thymine (T). Hydantoins (oxidised pyrimidines) are considered the most damaging modification as they distort the double helical DNA structure (O'Rourke et al., 2000) and thus effectively block PCR amplification (Hoss et al., 1996). Hydrolytic damage leads to the deamination of cytosine and thymine nucleotides to form uracil and hypoxanthine, respectively (Hofreiter et al., 2001). As these nucleotide changes can occur on both DNA strands the modifications appear as A to G and T to C (type 1), or C to T and G to A (type 2) transitions during subsequent PCR amplification (Gilbert et al., 2003a). Several studies on the DNA degradation process and resulting modifications have shown that C to T transitions are more frequently observed in ancient or degraded DNA samples than G to A transitions (Hofreiter et al., 2001, Gilbert et al., 2003b, Lamers et al., 2009).

Type of damage	Process	Effect on DNA
Hydrolytic damage	a) deamination of cytosine (C) to uracil b) deamination of adenine (A) to hypoxanthine	- Miscoding lesions: leading to C→T or A→G transitions. - DNA fragmentation
Oxidative damage	modifications of sugar residues and the deamination of the pyrimidines cytosine (C) and thymine (T)	- Miscoding lesions: C and T modified to hydantoins - Blocking PCR amplification

Table 1: Most common DNA modifications that occur post-mortem.

The major characteristics of DNA damage are single and double stranded breaks and internal and external cross-links. The most noticeable type of degradation is the reduction of DNA into small fragments caused by hydrolytic cleavage of phosphodiester-bonds in the phosphate backbone and the cleavage of glycosidic bonds between nitrous bases and the sugar backbone of the DNA double-helix generating single-stranded breaks (Paabo et al., 2004, Willerslev and Cooper, 2005).

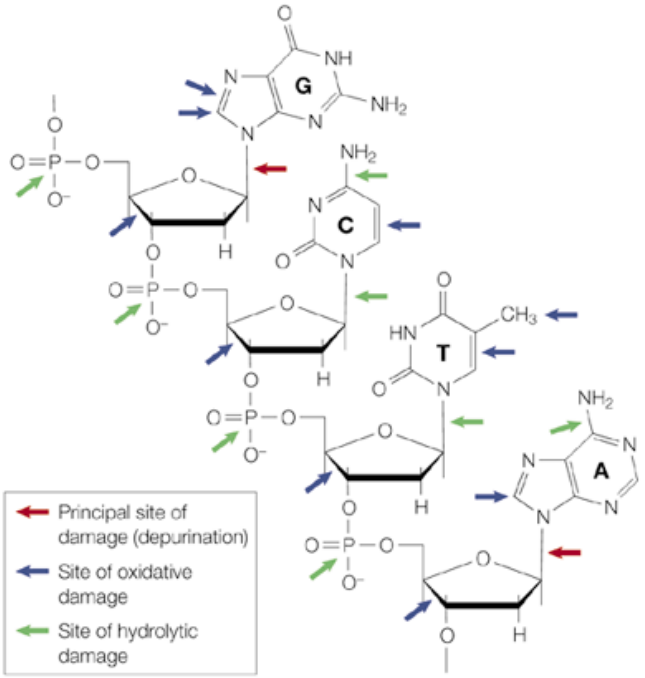

Figure 2: "*Single stranded DNA showing the four common bases G, C, T and A. The principal sites of damage are indicated by red arrows. Sites susceptible to hydrolytic attack are indicated by green arrows and sites prone to oxidative attack are shown in blue*" (Hofreiter et al., 2001).

2.3.3 Environmental damage

The quality of DNA surviving post-mortem depends to a high degree on the environmental conditions at the burial site. The main factors with a strong impact on the preservation of skeletal remains are environmental salt concentrations, exposure to radiation, pH, as well as the availability of oxygen and water (Lindahl, 1993). It has been widely acknowledged, however, that temperature seems to play the key role in the survival of ancient biomolecules (Lindahl, 1993, Hedges, 2002, Smith et al., 2001) as low temperatures effectively prevent damage from microbial attack (Hedges, 2002). The recovery of DNA and PCR amplification success for ancient samples increases with decreasing temperatures at the burial site (Lindahl and Andersson, 1972, Hoss et al., 1996, Paabo, 1989, Poinar et al., 1996). Hoss et al. (Hoss et al., 1996)

determined that a 20 °C decrease in temperature reduces base degradation 10- to 25-fold. It is therefore not unexpected that the best-preserved DNA has been found either in permafrost or dry environments protected from UV exposure, and in remains less than 100,000 years old (Hoss et al., 1994, Hagelberg et al., 1994). More recent examples of authenticated ancient DNA results have been reported from permafrost or cave environments that survived even longer than the previously predicted maximal DNA survival estimation of 100,000 years. For instance plants and insects recovered from ice cores from Greenland with an estimated age between 450,000 and 800,000 years (Willerslev et al., 2007), or 400,000-year-old bear fossils found in the Atapuerca cave system in Spain (Valdiosera et al., 2007) yielded authentic DNA.

Only recently a significant recovery of bones from a cave located in Sima de los Huesos ("pit of bones") in Northern Spain documented that DNA can survive not only in dry environments but also in humid conditions close to saturation and a constant temperature of 10.6 °C (Meyer et al., 2014). The fossil DNA from a femur found at the site was estimated to be approximately 400,000 years old and belongs to the early hominin form *Homo heidelbergensis*.

Although it is known that caves provide a favourable environment for the survival of DNA for two major reasons: 1) constant low mean annual temperatures (Smith et al., 2001) and 2) slightly alkaline pH in karstic caves (Collins et al., 2002), humid conditions, as previously mentioned, are generally considered less beneficial for DNA preservation (Tuross, 1994). In fact, DNA degradation is accelerated in the presence of water and by fluctuations in humidity (Nielsen-Marsh and Hedges, 2000).

These findings just underline that the factors responsible for the long-term DNA survival are much more complex and seem to depend on additional factors that are less well-defined (Nielsen-Marsh et al., 2007).

2.4 DNA survival and preservation in skeletal tissue

Numerous studies provided indisputable evidence that DNA can survive deep into time provided that the environmental conditions are favourable. However, apart from the obvious parameters such as temperature and pH that impact the longevity of DNA there are less well-characterised microenvironments with different biological and physiochemical properties that form within bone during fossilization, so-called molecular niches (Geigl, 2002) that seem equally important. One of the most proposed survival niches is the binding of DNA to mineralised collagen of bone and teeth, which is believed to delay the degradation process because of adsorption of DNA to hydroxyapatite (Paabo et al., 2004, Lindahl, 1993). The adsorption of DNA onto the hydroxyapatite is most likely based on the affinity of negatively charged phosphate groups of the DNA backbone to the calcium ions on the bioapatite surface (Grunenwald et al., 2014). The binding of DNA to the bioapatite matrix is assumed to protect the DNA from chemical deterioration and microbial degradation (Lindahl, 1993, Collins et al., 2002, Tuross, 1994) leading to a prolonged survival rate. If, however, the bioapatite matrix is exposed to water or acids and dissolution or reprecipitation can occur the protective shield disintegrates and the DNA becomes vulnerable to accelerated degradation (Götherström et al., 2002). Similarly, if bioapatite or collagen present in this matrix are exposed to repeated wetting and drying cycles the persistence of any captured DNA in these matrices is also likely to deteriorate (Nielsen-Marsh and Hedges, 2000).

Some studies sought to find an answer to where DNA is actually most likely to survive within bone. For instance, Salamon et al. (Salamon et al., 2005) have published a study where they successfully retrieved authentic DNA sequences from DNA extracts of crystal aggregates of fossilized animal and human bone. They also argued that the quality of DNA in the crystal aggregates was better preserved compared to the DNA of the total bone. Others have detected the presence of DNA within the osteocytic lacunae of ancient bones from Pompeii (47 AD) (Guarino et al., 2000). Lacunae are spaces in the bone that are occupied

by star shaped mature bone cells (osteocytes) (Campos et al., 2010) which seem to provide a sheltered location where DNA is able to persist.

Another critical factor in regard to DNA preservation is the way ancient specimens are treated at excavation sites or during subsequent storage. In a study published by Pruvost et al. (Pruvost et al., 2007) they compared DNA yields and PCR amplification success from archaeological bone based on differential excavation procedures. The results indicated that treating bones with standard excavations procedures (e.g. brushing, washing and treatment with chemicals) is detrimental to DNA preservation and that freshly excavated and non-treated unwashed bones produced six times more DNA and twice as many authentic DNA sequences as opposed to bones treated with standard procedures. Furthermore, they argued that the survival of DNA in archaeological specimens is very closely linked to the prevailing environmental conditions rather than a consequence of any differences in the fossilization process. Thus, once skeletal tissue is removed from its protective surroundings even minor changes in temperature, pH or moisture can lead rapidly to DNA degradation (Pruvost et al., 2007, Dobberstein et al., 2008).

2.5 Screening methods for DNA preservation

Several approaches have been proposed to assess DNA preservation in skeletal tissue, such as crystallinity index analysis, amino acid racemization (Poinar et al., 1996, Schwarz et al., 2009), collagen concentration, deamination patterns of cytosine to uracil, or thermal age calculations (Smith et al., 2001, Smith et al., 2003) with the aim to reduce unnecessary destruction of sample material and to increase the amplification success of ancient DNA.

Götherström and colleagues (Götherström et al., 2002) demonstrated that the preservation of DNA was strongly correlated with the crystallinity (crystal order) in the hydroxyapatite of ancient bone. Hence an increase in crystallinity

of hydroxyapatite would suggest an increase in diagenesis resulting in the loss or damage of DNA.

Amino acid racemization has been commonly adopted as a means to determine the preservation of DNA in ancient specimens. This method measures the extent of racemization of aspartic acid present in collagen. Poinar et al. (Poinar et al., 1996) suggested that if the D/L ratio of aspartic acid in ancient tissue exceeds 0.08 it is unlikely that DNA can be retrieved. However, more recently a larger study was undertaken and this questioned this technique as the results of this study could not determine any correlation between amino acid racemization and DNA preservation (Collins et al., 2009).

In addition, the total concentrations of amino acids and the amino acid composition in ancient specimens have also been suggested to be useful indicators of DNA preservation when present above certain levels (Poinar et al., 1996, Hofreiter et al., 2001).

Smith et al. (Smith et al., 2001) have introduced the concept of "thermal age" as a proxy for DNA preservation. The calculations are based on the chronological age of a specimen standardized to the temperature of 10 °C. Based on this model DNA in bone could survive approximately 20,000 years. However, due to the small sample size derived from a protective cave environment investigated in this study the results are probably not easily transferable to other less favourable depositional conditions.

2.6 Types of DNA: Nuclear and mitochondrial DNA

Eukaryotes have two distinct genomes – the nuclear and the mitochondrial genome (Table 2). Ancient DNA studies or forensic tests where often only a low amount of nuclear DNA is available, focus mainly on mitochondrial DNA (mtDNA) as it is more abundant than genomic or nuclear DNA (nDNA). While each cell contains just two copies of nDNA, mtDNA is present in thousands of

copies. Hence the probability of recovering mtDNA in ancient or degraded samples is much higher.

Nuclear DNA	mtDNA
Linear molecule	Closed, circular molecule
~ 3 billion base pairs	16,569 base pairs
95 % non-coding ("junk" DNA)	~ 1100 base pairs non-coding region
Inherited from both parents	Maternally inherited
Recombination	No recombination
2 copies per cell	50 to several thousand copies per cell

Table 2: Comparison between nuclear and mitochondrial genome

Mitochondria are cellular organelles that contain a double-stranded circular DNA molecule composed of 16,569 base pairs whereas the human nuclear genome has a size of 3.3×10^9 base pairs. The D-loop, also known as the control region or hypervariable regions 1 and 2 (HV1 and HV2), shows the most variation between individuals. The high variability in the mitochondrial genome is generated by a higher mutation rate due to the low fidelity of mtDNA polymerase and the lack of mtDNA repair mechanisms compared to those present in the nuclear genome (Budowle et al., 2003).

Mitochondrial DNA is only maternally inherited and therefore siblings and all maternal relatives share an identical mtDNA sequence. This feature makes it a very useful tool in kinship analysis and population migration studies.

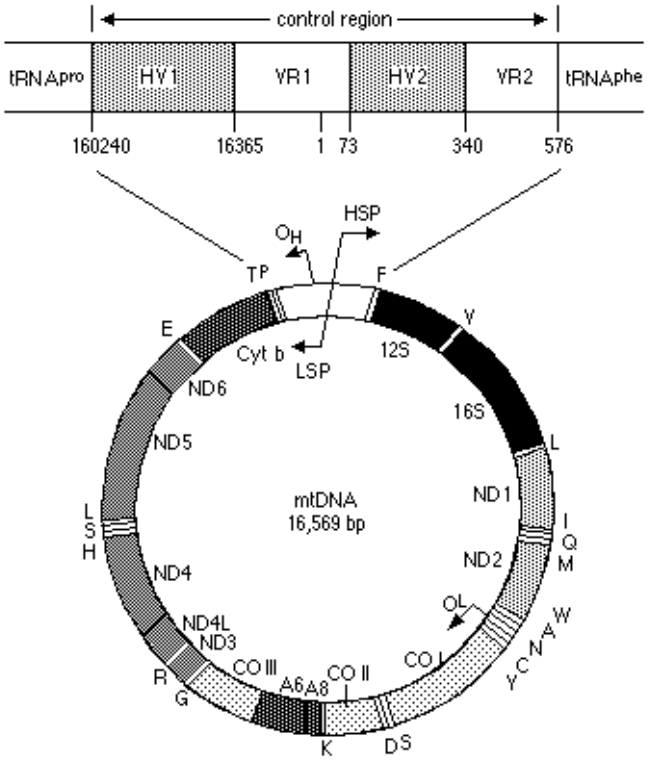

Figure 3: Mitochondrial DNA map with insert of control region HV1 and HV2 (Holland and Huffine, 2001).

Issues with mtDNA

Not so long ago individuals were considered to be homoplasmic, i.e. that only a single type of mtDNA existed within an individual. About a decade ago, however, it was observed that more than one mtDNA type (heteroplasmy) could be present within an individual leading to differences in the mitochondrial sequence that could affect one or more nucleotides. These variations can be found even within a single tissue or may vary among different tissue types (Budowle et al., 2003). Heteroplasmy occurs quite frequently in humans, especially in hair shafts, and hence has the potential to complicate the interpretation of forensic mtDNA results. Therefore mtDNA analysis should always include multiple samples to be able to exclude heteroplasmy.

Although mtDNA analysis is most commonly used where only small amounts of DNA is present or degraded such as in forensic samples like hair shafts or old

bone and teeth, it has only little power for individual discrimination (Melton and Nelson, 2001). Due to its maternal inheritance pattern it is not possible to exclude anybody along the maternal line in a specific case as each individual along this line shares the same mtDNA profile. In criminal cases for instance, where it is crucial to pinpoint the results to one possible individual, this method provides weaker evidence in court compared with STR (short tandem repeats) profiling evidence (Butler, 2005). However, the mtDNA characteristic of maternal inheritance is most valuable in forensic cases when reference samples of maternal relatives can be used to identify unknown human remains, an advantage that apart from the non-recombinant portion of the Y-chromosome, nuclear DNA markers are not able to provide (Budowle et al., 2003).

Issues with nDNA

Most archaeological and many forensic samples lack any retrievable nDNA due to degradation and inhibition. The DNA can be highly fragmented and contaminated from various sources, such as bacteria, fungi and very often by modern human DNA. In fact, contamination from modern human DNA is the number one obstacle in degraded or ancient DNA research. Numerous papers on authentication criteria have therefore been published (Cooper and Poinar, 2000, Hofreiter et al., 2001, Paabo et al., 2004, Willerslev and Cooper, 2005) to highlight the problems with aDNA and to emphasize the importance of strict criteria in order to produce reliable data for the work to be acknowledged by the relevant scientific community.

A brief summary of the "Top 10" criteria to be used in DNA laboratories to ensure authenticity of analytical results as suggested by Poinar et al. (Poinar, 2003) are listed below:

1) *Physically isolated work area*
   The minimum requirement for ancient/forensic DNA work is the spatial separation of pre- and post-PCR rooms to avoid contamination.

Professional DNA labs have additional facilities for sample storage and sample preparation where the dust generated can be contained.

2) *Extraction blanks and multiple negative PCR control amplifications* that contain no DNA should always be included alongside ancient DNA samples to detect any potential contamination introduced either during the extraction or amplification process.

3) *Test the molecular behaviour*
   Due to the fragmented nature of ancient or degraded DNA samples PCR products are usually short (<1000 bp). Thus any larger PCR products >1000 bp in length are generally a good indication of modern DNA contamination.

4) *Quantitation*
   The amount of DNA is best quantified with Real-time PCR. If the copy number of the template DNA at the start of the amplification is low (<1000 bp) then contamination can be largely excluded.

5) *Reproducibility*
   In order to verify the authenticity of a sample the results from the same as well as from different DNA extracts of the same sample should be reproducible.

6) Multiple clones
   PCR products should be cloned and sequenced ten times to detect potential differences between endogenous and exogenous sequences and damaged induced errors.

7) *Independent replication*
   In cases where new or unexpected results are obtained it is recommended that the analytical procedure of extraction, amplification and sequencing of separate samples of the same specimen are replicated in independent laboratories.
   NB: Although this measure could detect potential sources of

contamination from chemical reagents or lab personnel from one lab it would not be able detect any contamination that might have occurred already before arrival in the DNA lab.

8) *Biochemical preservation*
   It has been suggested that an assessment based on a combination of total amount of amino acids, the composition of amino acids, and their extent of racemization (structural change of amino acids) is the most reliable method to verify preservation in a specimen (Hofreiter et al., 2001, Paabo et al., 2004).

9) *Associated remains*
   The amplification of DNA from associated animal or plant material can provide supportive evidence for DNA survival of associated ancient human remains.

10) *Phylogenetic sense*
    Once reproducible DNA results from ancient specimens have been achieved they should match the correct taxonomic order in the phylogenetic tree (animal, human, bacteria, etc.).

It has to be noted, however, that despite all established authentication guidelines and painstaking efforts to achieve authentic DNA results the risk of errors still remains. For example, in one instance reproducible human DNA could be obtained from an approximately 30,000-year-old tooth from a cave bear in China despite adherence to authentication criteria (Hofreiter et al., 2001). This demonstrates that authentication criteria alone are not a warranty for authenticity and that results have always to be judged with a critical scientific mind.

2.6.1.1 Sources of DNA

DNA for forensic analysis can be obtained from various sources such as blood, semen, saliva, urine, hair roots, teeth, bone and tissue (Butler, 2005). Ancient DNA is mostly obtained from calcified skeletal material such as bone teeth, or hair. Analysis of ancient animal material also utilizes coprolites (Poinar et al., 2003, Hofreiter et al., 2001) or intestinal contents (Rollo et al., 2002) for additional insights on ancient diets. Sediments are now an additional source of aDNA where the presence of various animal, plant and microbial species can be reconstructed even in the absence of fossil material (Hofreiter et al., 2012).

2.7 Precautions when working with ancient/degraded DNA samples

Ancient DNA samples are by nature degraded and therefore prone to contamination with modern DNA that can override the genuine DNA signature and impede subsequent DNA analysis. It is therefore crucial to take all precautions possible to preserve authentic DNA and avoid any contamination from modern/exogenous sources during excavation and handling of samples in the lab. In the early days of aDNA research such precautions were not considered as they are today. Critical voices therefore have been questioning some of the early findings in aDNA research in respect to the authenticity of results. For instance, the recovery of alleged dinosaur DNA (Woodward et al., 1994) later turned out to be from human origin, representing a mitochondrial insertion into the nuclear human genome (Knapp et al., 2012).

Since the first authentication criteria published in the early 90's (Paabo, 1989) numerous authentication criteria have been added (Cooper and Poinar, 2000, Poinar, 2003, Gilbert et al., 2005, Willerslev and Cooper, 2005) and implemented and are now standard procedure in this field.

2.7.1 Precautions to prevent contamination of ancient DNA samples

Contaminants can be introduced during various stages of the sampling and analytical procedures of archaeological/forensic human tissue. The most commonly encountered source of contamination is modern human DNA present in old bone itself or introduced into equipment and chemical reagents in the lab later on. Measures to minimize contamination therefore need to be taken early on starting already at the sampling stage on excavation sites and should be subsequently carried through to the DNA lab and analytical stages.

2.7.1.1 Good practice procedures at the sampling stage

Field archaeologists are usually the first people who come into contact with ancient material at excavation sites. It was shown that contamination and degradation of ancient samples can be drastically reduced when special excavation protocols are followed and that the success rate of PCR for fossils using particular precautions (e.g. using gloves and clean tools) was significantly higher than that for fossils using standard excavations procedures (Pruvost et al., 2008).

To prevent contamination from modern DNA it is vital that precautions are already in place at this initial sampling stage. To minimize the chance of contamination, archaeologists should wear protective suits and masks and follow some general rules when handling ancient DNA specimens (Yang and Watt, 2005, Pruvost et al., 2008):

1) Samples for DNA analysis should not be cleaned on site as the layer of dirt may provide a protective shield against contaminants entering the skeletal material.

2) Bone tissue should not be washed with water as contaminating DNA might enter deeply into the tissue and could potentially also cause hydrolytic damage to ancient DNA.

3) Addition of any preservatives to samples should be avoided as such chemicals may inhibit PCR amplifications and may also introduce potential contaminants.

4) Samples should be stored in cool and dry conditions to prevent further degradation of ancient DNA.

5) Samples should always be stored separate from any modern reference samples to prevent cross-contamination.

6) Ideally, each sample should be handled with a new set of gloves and/or tools and individually stored in bags or tubes when dry.

7) Control samples should always be included to detect contamination from excavators and/or laboratory personnel.

2.7.1.2 Contamination controls in the DNA laboratory

Both ancient DNA and forensic DNA laboratories require a specific set-up to minimize the effects of contamination and ensure the authenticity of their results. A basic rule is that the workflow should be organized in a uni-directional manner so that personnel proceed always from a low concentration DNA area (i.e. extraction room, PCR preparation area) to a high concentration DNA lab such as the PCR amplification area (Willerslev and Cooper, 2005) in order to prevent contamination.

Generally, ancient and forensic DNA laboratories follow the same strict contamination prevention controls because of the sensitivity of the sample material. Sensitivity can be due to degradation or low amounts of DNA present in the sample itself but can also refer to the nature of the work. In forensic casework, for instance where DNA from suspects, victims or offenders are analysed in the same laboratory do not allow for any risk of contamination. Any cross-contamination between DNA samples could lead to false conclusions and in the worst case to the unjustified conviction of an individual. In principal, all DNA facilities need to follow basic rules to control contamination although they will be less rigorous in standard DNA laboratories that handle only modern samples with a high DNA content. For example, personnel in standard DNA facilities are not required to wear full cover, or decontaminate each item (equipment and consumables) prior to entering the DNA laboratory while for aDNA or forensic laboratories such measures are daily routine.

Before ancient/forensic DNA samples can be prepared for analysis several pre-requisites are required to ensure effective contamination controls (Willerslev and Cooper, 2005, Cooper and Poinar, 2000, Hofreiter et al., 2001):

1) A dedicated ancient/forensic DNA laboratory space. Furthermore, ancient DNA facilities should ideally be entirely isolated from any molecular DNA laboratory handling modern samples.

2) Physically isolated pre- and post PCR amplification areas

3) Isolated High-efficiency particulate air (HEPA)-filtered and positively pressured ventilation

4) Rigorous daily cleaning procedure with bleach and overnight UV irradiation of all work surfaces

5) Disposable protective suits, masks and gloves should be worn to prevent DNA shedding from laboratory personnel and resulting contamination of samples.

6) Disposable lab ware and filtertips

7) To prevent cross-contamination between samples, just one sample at a time must be handled and work areas decontaminated between samples.

8) Restricted access to all facilities for authorised personnel only

9) Fumigation (decontamination) of all incoming items such as equipment and consumables before entering laboratory facilities.

2.8 Genetic Markers

2.8.1 Short Tandem Repeats (STRs)

The human genome contains a high number of repetitive DNA sequences, which vary in length between individuals. These microsatellites or "Short Tandem Repeats" (STRs) have a length of two up to six nucleotide core repeat units typically repeated 5 to 30 times within a repeat region that is generally less than 350 base pairs long (Rapley and Whitehouse, 2007). STRs and are scattered along the whole genome making up around 3 % of the entire human genome (Lander et al., 2001). Microsatellites have a high mutation rate and hence variability among individuals which makes them highly suitable for forensic purposes (Carracedo and Sanchez-Diz, 2005, Butler, 2005). The most frequently used STR markers in forensics have four base pair core repeat units (Butler, 2005).

Forensic institutes have validated thirteen of the most polymorphic DNA markers for human identification purposes and established a standardised database known as CODIS (Combined DNA Index System), which is now used worldwide for DNA typing. The core set of 13 STR loci used in the CODIS are CSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, FGA, TH01, TPOX, and vWA. More recently Mini-STRs markers, shortened variants of STRs, have been developed and successfully applied to highly fragmented or degraded DNA samples (Butler et al., 2003, Coble et al., 2006). There have been attempts, however, to extend the core set of STR core loci (Guo et al., 2014).

2.8.2 Single Nucleotide Polymorphisms (SNPs)

Single Nucleotide Polymorphisms (SNPs) are DNA sequences that vary by a single nucleotide. These genetic markers are most popular in disease-association studies and to a lesser extent in forensic investigations. To achieve a useful

forensic result, it would take about 50-100 SNPs to match the discrimination power of 10-16 STR markers (Gill et al., 2004).

With the identification and mapping of 1.4 million polymorphisms the International SNP Map Working Group has created a powerful database (Sachidanandam et al., 2001) bringing immense benefit to medical research. Variations in single nucleotides influence both susceptibility and resistance to a specific disease. For instance, predisposition to Alzheimer's disease is associated with single base differences in the *APOE* gene (Bertram and Tanzi, 2004).

Another major field of SNPs application is in physical trait identification. In recent years an increasing number of publications on genes involved in phenotypic variations such as skin pigmentation (Myles et al., 2007), hair and eye colour (Sturm and Frudakis, 2004) have been published. Progress in this field, however, is still slow due to the complexity of factors that influence phenotypic appearance. Physical traits are not only under the control of multiple genes but are also determined by environmental factors as well as age. A major determinant for hair and skin colour is the Melanocortin 1 Receptor gene (MC1R) that regulates pigmentation in humans. A study published in 2007 suggested that Neanderthals had pale skin colour and red hair due to a SNP in MC1R (Lalueza-Fox et al., 2007).

SNPs have also become increasingly popular in ecology and conservation studies where they can be used as biological markers to investigate genetic diversity and population structure within a species (Morin et al., 2004, Aitken et al., 2004, Seddon et al., 2005, Roden et al., 2009, Karlsson et al., 2011, Mesnick et al., 2011, Olsen et al., 2011, Ogden et al., 2013). SNPs are highly informative in monitoring behavioural patterns, abundance, functions and processes in different environments. For example, projects such as FishPoptrace (https://fishpoptrace.jrc.ec.europa.eu/) and DEVOTES (http://www.devotes-project.eu) used SNPs to analyse fish population in conservation management studies or fish products for the purpose of traceability whereas DEVOTES aims

to understand the impact of human activities as well as variations due to climate change on marine biodiversity with applying SNP technologies.

2.8.3 Mitochondrial DNA and Y-chromosome markers (Lineage markers)

The genetic diversity among populations is generated by mutations in the nuclear and the mitochondrial genome alike and the resulting gene flow between populations. These variations are subject to evolutionary selection processes and the geographic distribution of a population. Ancient DNA studies mainly focus on the haploid mitochondrial and Y-chromosome genetic systems that are inherited by a single parent. Genetic variants of the maternally inherited mitochondrial genome and the paternally inherited Y-chromosome are not subject to genetic recombination and are therefore passed on from generation to generation without any major changes. By comparing lineage markers from modern populations with ancient populations they can reveal the ancestral history and migration of people (Rosser et al., 2001, Cavalli-Sforza and Feldman, 2003).

2.8.3.1 Mitochondrial markers

The most polymorphic regions in the mitochondrial genome are found within the hypervariable regions I and II (HVI and HVII). HVI stretches between positions 16024 to 16365 (342bp) and HVII spans the region between 73 and 340 (268 bp). Usually primers that cover the entire stretches of both control regions are used to determine the sequence. To increase the chances of DNA recovery from degraded DNA samples, overlapping mini-primers that generate short PCR products are used. The sequences are then compared to the revised Cambridge Reference Sequence (rCRS or Anderson sequence) to detect variations.

2.8.3.2 Y-Chromosome markers

Y-Chromosome markers are classified into bi-allelic loci with two possible alleles, and multi-allelic loci.

Bi-allelic markers include Y-SNPs (Y-chromosome single nucleotide polymorphisms) as well as insertion/deletion polymorphisms. Y-SNPs are most commonly used for phylogenetic analysis (van Oven et al., 2014) due to their specific distribution among populations. Y-SNPs, also known as unique event polymorphisms (UEPs), are associated with a specific haplogroup and as such are used as a tool to infer the origin, evolution and migration of human populations.

The multi-allelic markers of the Y chromosome include Y-STRs (short tandem repeats), are usually employed in forensic applications and paternity testing (Butler, 2005) and to a lesser extent in historical and genealogical studies.

2.8.3.3 Haplogroups

Individuals who share the same genetic lineage markers (SNPs) are defined as a haplogroup. Each mitochondrial DNA and Y-DNA haplogroup has a unique set of haplotypes that define their common ancestry (Budowle et al., 2003). The three basic mitochondrial haplogroups are L, M and N. Group L defines the common maternal ancestor from which M and N and all other descendants evolved. Mitochondrial DNA haplogroups in Europe are classed into ten major phylogenetic clusters, H, J, K, N1, T, U4, U5, V, X, and W (Torroni et al., 2000). Type H is the most frequent haplogroup found in the European genetic pool at about 50 % (Figure 4).

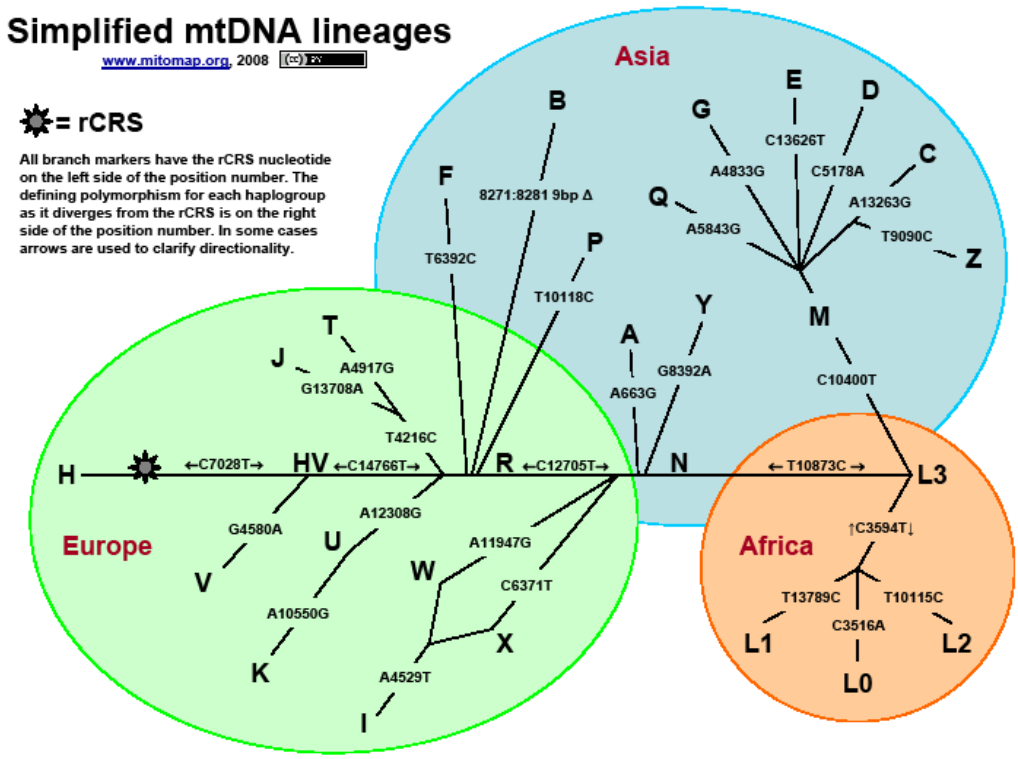

Figure 4: A simplified world map of mtDNA lineages (Mitomap)

Y-DNA is clustered into 20 major haplogroups (named chronologically with the letters A to T) and many more subclusters worldwide (not all subclusters are shown in Figure 5). The most common Y-SNPs in Caucasians are R1a and R1b, which are subclusters of haplogroup R.

NB: Y-DNA haplogroups are designated with capital letters and subclusters branching off major haplogroups with numbers and lower case letters.

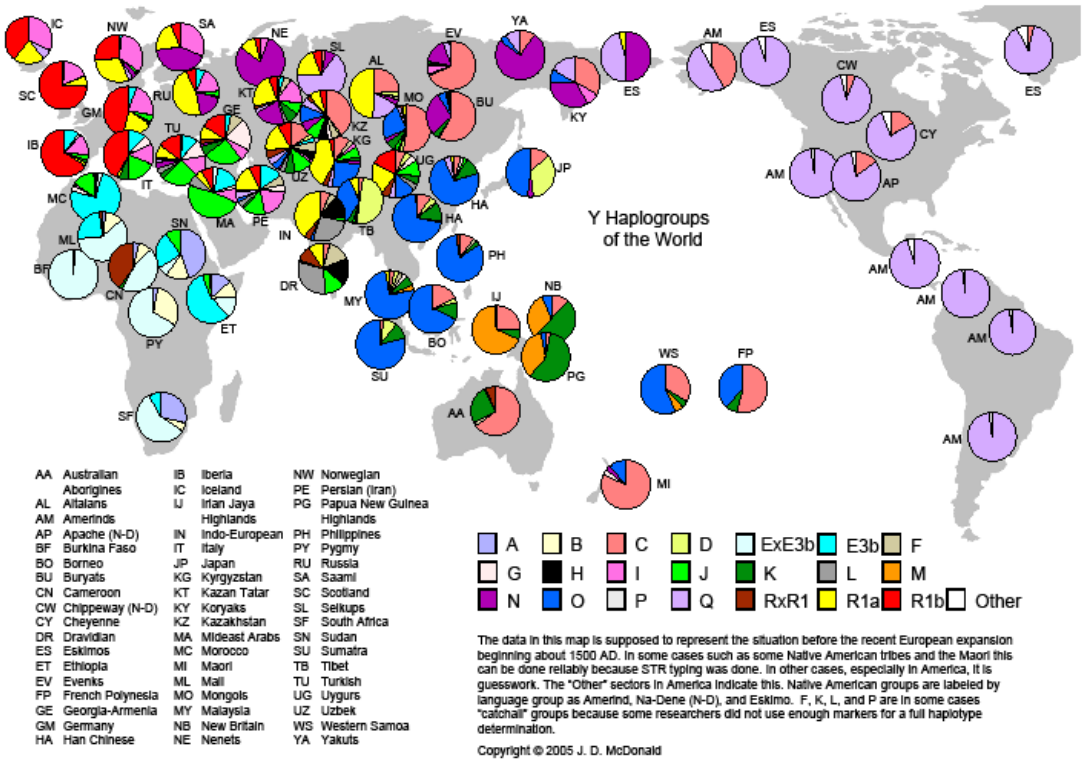

Figure 5: Y-haplogroups of the World (McDonald, 2005)

2.8.4 Databases

Worldwide a great effort has been put into the setting-up of databases to create an effective tool for forensic identification purposes, disease studies and population studies. Databases are available for genetic information on STRs, mtDNA and Y-chromosome markers, and SNPs. Most databases are publicly accessible and a crucial tool for all genetic research.

Forensic STR-Databases:

The rapidly evolving technologies in DNA analysis have led to a high reliability and acceptance of forensic genetics in court and to the establishment of extensive databases with DNA profiles from both suspects and convicted

offenders. The DNA databases are built on standardised genetic typing systems that have been recommended and accepted by scientific institutions such as the National Research Council (NRC) of the Academy of Sciences of the United States, and the International Society for Forensic Genetics (ISFG) (Schneider, 2007).

The Forensic Science Service (FSS) in the UK was the first and the most successful to launch a National DNA data bank in 1995. The United States followed in 1998 setting up the CODIS (Combined DNA Index System) linking all 50 US states in a national database based on the information of 13 core STR markers.

United States:	-	Center for Science Teaching and Learning, National Institute of Standards and Technology (CSTL, NIST) (http://www.cstl.nist.gov/strbase/)
UK	-	National DNA Database (NDNAD) (not publicly accessible)
Europe:	-	European Network of Forensic Science Institutes (ENFSI) database in progress (http://strbase.org)
International:	-	Interpol (http://www.interpol.int/INTERPOL-expertise/Databases)

International nucleotide sequence databases:

- National Center for Biotechnology Information (NCBI), USA (http://www.ncbi.nlm.nih.gov)
- European Molecular Biology Laboratory (EMBL) database (http://www.ebi.ac.uk/embl/
- DNA Data Bank of Japan (DDBL) database (http://www.ddbj.nig.ac.jp)

Y-STR haplotype reference databases:

- National Center for Forensic Science (for USA and Europe) (http://ncfs.ucf.edu/databases/)
- Centre for Science Teaching and Learning, National Institute of Standards and Technology (CSTL, NIST) (http://www.cstl.nist.gov/strbase/y_strs.htm

mtDNA databases:

- "Mitomap" - a human mitochondrial genome database (https://www.mitomap.org/MITOMAP)
- Institute of Legal Medicine Innsbruck, Austria (Empop) (http://empop.org)
- Federal Bureau of Investigation (FBI) (http://www.fbi.gov/)

SNPs databases:

- National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/snp)
- Centre for Science Teaching and Learning, National Institute of Standards and Technology (CSTL, NIST) (http://www.cstl.nist.gov/strbase/SNP.htm)

The databases listed here are only a small selection of the most important databases available on the World Wide Web.

2.9 Molecular techniques in ancient and forensic DNA Analysis

Analytical methods for DNA extraction, quantification, amplification and detection overlap greatly although forensic laboratories are increasingly using automated procedures for a high throughput of samples which is less common in aDNA research.

The following sections will give a general overview of the consecutive steps of DNA analysis.

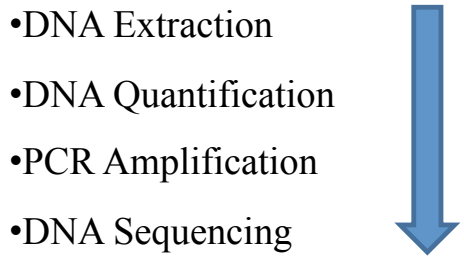

Figure 6: Overview of the consecutive steps of DNA analysis

2.9.1 DNA Extraction

The aim of extraction methods is to isolate purified DNA from cells and to remove any substances that could interfere with subsequent analysis.

Since the beginnings of aDNA analysis in the 80's a myriad of extraction protocols have been tested and modified. Currently there are typically two extraction methods that are successfully used for aDNA recovery in bone and teeth: a) phenol/chloroform extraction and b) a silica-based extraction method (Kaestle and Horsburgh, 2002). Also forensic geneticists use basically these two methods most frequently but in some instances some additional extraction techniques are employed such as Chelex extraction, solid phase extraction or extraction from FTA paper (Butler, 2005). The focus here will be on the two

most important extraction procedures and will only briefly touch on the latter ones.

Demineralization of bone and teeth tissue

Before DNA can be extracted from bone or teeth the mineral matrix needs to be dissolved to make the organic matrix accessible. Typically, skeletal tissue is first cleaned to remove any contaminants on the surface and then pulverized in a mill, grinder or by drilling. The resulting powder is incubated in a lysis buffer containing EDTA (ethylene diamine tetra-acetic acid), which both demineralizes the bone or teeth and inactivates DNAses by chelating them with Ca^{2+} and Mg^{2+} ions (Loreille et al., 2007). Most standard protocols discard the supernatant after demineralization and thus loose valuable DNA that remains in the mineral matrix/EDTA mix. To increase the recovery of DNA present in the bone and teeth tissue some modified demineralization protocols have emerged. Loreille et al. (Loreille et al., 2007) for instance use a total demineralization protocol where all the bone material is entirely dissolved. This method provides a significant improvement in terms DNA recovery and it also requires less sample material. Using less sample material also decreases the relative amount of co-extracted PCR inhibitors, which leads to improved amplification success. Some other studies have shown similar results of enhanced efficiency of DNA recovery when the EDTA supernatant containing the hydroxyapatite fraction is retained (Schwarz et al., 2009, Campos et al., 2012).

Organic (Phenol-chloroform) extraction

The original standard phenol-chloroform extraction protocol has been first described in Sambrook et al. (Sambrook et al., 1989) where samples are first digested in a lysis buffer containing Proteinase K and a detergent (sodium dodecyl sulphate (SDS) or Triton X-100) to break down the cells and make DNA accessible. The solution is then centrifuged which leaves the precipitated

protein and cellular debris in the organic phenol-chloroform phase while the DNA remains in the aqueous phase. The supernatant containing the DNA is transferred to a new tube and a mixture of phenol-chloroform-isoamyl alcohol mixture in the ratio of 25:24:1 is added and subsequently agitated and centrifuged to ensure that all proteins are removed. The resulting supernatant is then mixed with chloroform-isoamyl alcohol in a ratio of 24:1, agitated and centrifuged to remove the phenol. The supernatant is then transferred to a new tube and the DNA precipitated by either using ammonium acetate or cold 100 % ethanol, or concentrated by filter centrifugation (Kaestle and Horsburgh, 2002, Butler, 2005).

Although this method is very efficient in recovering aDNA/degraded DNA, it has some great disadvantages as it is a very time consuming process, involves the use of potentially very harmful chemicals and also requires multiple transfers between tubes which increases the risk of contamination.

Silica based extraction

Another more efficient approach for extracting DNA from ancient or forensic samples is the silica-based method (Hoss and Paabo, 1993, Boom et al., 1990). It takes advantage of the tendency of DNA to bind to silica or “glass milk” particles in the presence of high salt concentrations. This technique uses a lysis buffer that contains Proteinase K and a detergent (e.g. sodium dodecyl sulphate (SDS), Tween 20 or Triton X). The DNA binds to silica beads in the presence of high concentrations of a salt such as guanidium thiocyanate (GuSCN) or sodium chloride, which disrupt the protein structure by interfering with hydrogen bonding, Van der Waals interactions, and the hydrophobic interactions (Goodwin et al., 2007). After incubation the cellular proteins are removed by centrifugation and the supernatant containing the DNA is purified (Goodwin et al., 2007).

The silica-based extraction has the advantage that it does not require any toxic chemicals, produces a high yield of DNA and is less likely to co-extract PCR inhibitors (O'Rourke et al., 2000, Cattaneo et al., 1997). Silica-based extraction kits are available from a range of biotechnology companies and are routinely used for various aDNA and forensic applications (Tuross, 1994, Yang et al., 1998, Cattaneo et al., 1997, Castella et al., 2006, Baker et al., 2001, Phillips et al., 2012).

A modified silica based extraction kit has been developed by Promega, which involves the use of magnetic beads. Their DNA IQ™ system is very similar to the Qiagen kits except that the resin here is made of silica-coated magnetic beads. A great advantage of this method is that all DNA extraction steps can be carried out in a single tube by simply adding or removing solutions. Cells are first disrupted in a lysis and binding buffer (pH <7.5) in the presence of high salt concentrations (eg. GuSCN). Next, the silica-coated magnet beads are added to allow the binding of DNA. A magnet is then used to draw the magnetic beads with the immobilized DNA to the bottom and sides of the tube leaving the cellular debris in the solution. After the solution has been removed the magnetic beads are washed repeatedly to purify the DNA. Finally, the DNA is released from the magnetic beads using either water or low-salt buffer (Butler, 2005, Goodwin et al., 2007).

The advantage of this method is that it can be easily automated for high throughput DNA analysis (Greenspoon et al., 2004).

There have been studies that compared the performance of different extractions methods (Yang et al., 1998, Rohland and Hofreiter, 2007b) of mineralised tissues such as bone and teeth. Rohland and Hofreiter (Rohland and Hofreiter, 2007b, Rohland and Hofreiter, 2007a) tested systematically a variety of published protocols on ancient bone material including commercial kits. They found that a modified silica-based method which uses a buffer consisting solely of EDTA (EDTA chelates, or binds up, all of the free magnesium and thus prevents the nucleases from destroying the DNA; as nucleases need Mg to

degrade DNA) and proteinase K for bone digestion and subsequent purification using silica in combination with guanidium thiocyanate performed best.

Silica-based extraction commercial kits (e.g. Qiagen kits) are commonly used in both ancient DNA and forensic applications today. However, forensic laboratories also use additional techniques such as Chelex extractions for challenging samples such as semen, hair shafts or bloodstains (Butler, 2005).

Chelex Extraction

The Chelex extraction method was first introduced by Walsh et al. (Walsh et al., 1991) for the use on forensic samples such as blood, bloodstains, seminal stains, buccal swabs and hair. As the extraction procedure is simple and rapid it has become a favoured extraction technique among forensic geneticists. Chelex® 100 is a resin composed of styrene divinylbenzene copolymers containing paired iminodiacetate ions that chelate metal ions such as $Mg^{2+.}$ The resin is made into a 5 % solution using distilled water. The samples are added directly to the solution and boiled to disrupt the cells and destroy the cell proteins (Butler, 2005). Followed by a quick centrifugation spin the resin including all cell debris is deposited on the bottom of the tube. The supernatant containing the DNA can then be used straight away for PCR amplification.

The Chelex extraction procedure is a very fast method (takes ~1 hour) and does not require multiple tube transfers thereby reducing considerably the risk of cross-contamination. It also removes PCR inhibitors efficiently and has the advantage that the cost is very low (Goodwin et al., 2002).

2.9.2 Quantification

The determination of the amount of DNA extracted is a crucial step in DNA analysis. Most protocols require ~1 to 2.5 ng of purified DNA for the PCR

reaction to work successfully. Too much or too little DNA can cause the PCR reactions to fail or produces incorrect results when sequenced.

DNA quantification methods include UV spectrometry, gel-based techniques, blotting techniques as well as “real time” or quantitative PCR methods. Early techniques simply measured the total DNA present without differentiating the source of DNA (human, animal, bacterial or fungal). New approaches such as quantitative real-time PCR (q-PCR) are species-specific and more accurate in determining the amount of DNA present in a sample.

In the following section only a brief description of gel electrophoresis and the qPCR method are discussed as the relevant techniques used in this study.

2.9.2.1 Gel Electrophoresis

Agarose or polyacrylamide gels are still the most common and cost effective method for DNA quantification. PCR products stained with a fluorescent dye (eg. ethidium bromide) and a size standard are loaded into precast wells in the gel. As a current is applied (~120 V), the negatively charged DNA migrates towards the positively charged anode separating DNA fragments according to their size The rate at which DNA migrates through the gel is inversely proportional to its length, i.e. short fragments migrate faster than longer fragments. After the separation, the gel is visualized under UV-light. The fragment size can be estimated by comparing the size of the DNA fragment to size standards (“DNA ladder”).

2.9.2.2 Real-time Quantitative PCR (qPCR)

qPCR is very similar to conventional PCR amplification where specific DNA sequences are amplified exponentially over ~30 to 40 cycles but with the difference that this method uses fluorescently labelled DNA and that each amplification cycle can be directly monitored in “real time” as a fluorescent

signal. The qPCR process has three distinct phases: 1) exponential or geometric phase, 2) linear phase and 3) plateau region (Figure 7).

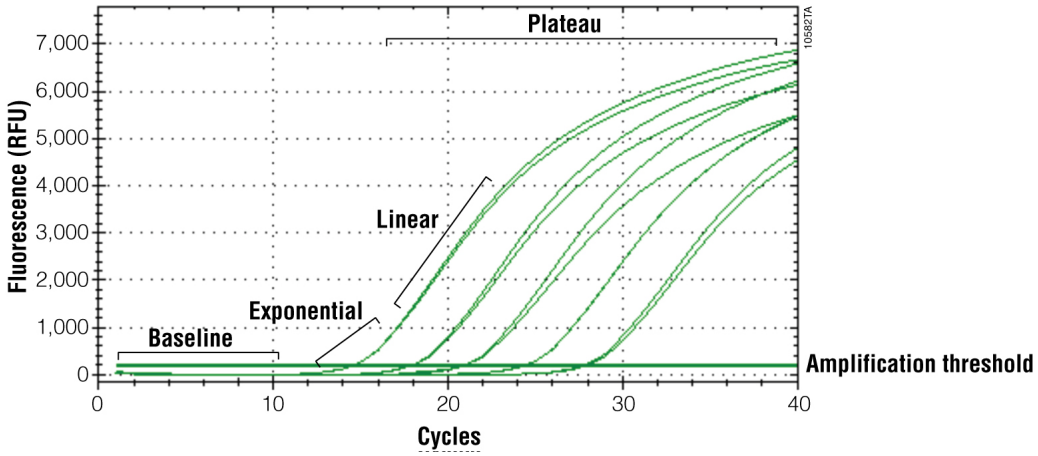

Figure 7: PCR phases (Source: (Promega))

The exponential phase starts when DNA is detected and an increase in fluorescence is observed. The point where fluorescence reaches a threshold level is called the Ct (threshold cycle). The amount of fluorescence released during amplification is directly proportional to the amount of amplified DNA, i.e. a high initial DNA content will show an earlier and faster increase in fluorescence. In the linear phase amplification continues with decreasing efficiency until it reaches the plateau phase where amplification stops.

The qPCR software calculates the unknown starting concentration of DNA in a sample by generating a standard curve of known concentrations against the Ct values of the sample. Alternatively, it can generate a relative quantification by comparing it to another sample.

2.9.3 PCR Amplification

The polymerase chain reaction (PCR) was developed by Kary Mullis in 1985 (Mullis et al., 1986) for which he and his colleagues earned the Nobel Prize in 1993. This significant event has revolutionized DNA research and forensic biology alike as it suddenly became possible to generate millions of copies of a specified DNA region for analysis in a matter of a few hours.

The PCR reaction requires a target DNA sequence, two primers that are designed to complement the 3' ends of each DNA strand, dNTPs (dideoxynucleotides: A, C, G, T), *Taq* DNA polymerase and magnesium chloride.

The DNA target sequence is the region of interest that needs to be amplified or copied to achieve a more concentrated sample suitable for further analyses. Usually approximately between 0.5 and 2.5 ng of extracted DNA are required for successful DNA profiling.

The two primers are a crucial component of the PCR reaction. They are short synthetically produced DNA fragments that are designed to identify a specific DNA region (Butler, 2005). They are usually about 18 to 30 nucleotides long and are complementary to the flanking regions of the target DNA sequence that is to be copied.

The most common DNA polymerase used today derive from the thermophilic bacteria *Thermus aquaticus* which was discovered in the 1960's in the hot springs of the Yellowstone National Park, USA (Goodwin et al., 2007). It is a very stable DNA polymerase with high sensitivity, specificity and yield of the reaction and as such has significantly contributed to the success of PCR (Saiki et al., 1988).

Another important component of the PCR reaction is magnesium chloride ($MgCl_2$), which is added to the mix to stabilize the reaction process. It increases the stability between DNA template and primer but is also required for the DNA polymerase to function (Goodwin et al., 2007).

The PCR cycling process

PCR is a repeated heating and cooling cycling procedure (~30x to 40x) that is driven by enzymatic processes of DNA replication (Goodwin et al., 2007). Each replication cycle consists of three stages: 1) denaturation 2) annealing and 3) extension. At the first stage of the PCR cycle the reaction is heated to 94 °C to break up the hydrogen bonds that hold the double stranded DNA together and to separate them. Then the denatured or "melted" DNA consisting of single strands is cooled down to about 50 °C to 60 °C to allow the forward and reverse primers to bind (anneal) to the 3' end of the template strand. In the final stage the temperature is increased to the optimal temperature of about 72 °C to start the enzymatic activity of the DNA polymerase which binds free dNTPs to the template strand creating a newly synthesised strand. Both the "old" and newly synthesised strand now serve as templates for the next cycling round which at the end produces four strands etc. thus increasing the number of DNA exponentially over repeated cycles (Butler, 2005).

Multiplexing PCR

Standard PCR amplification techniques use a single primer pair consisting of a forward and reverse primer, which amplifies one specific DNA target. In order to analyse larger regions commonly several short and overlapping target fragments (60-200 bp long) have to be amplified with multiple primer pairs in separate PCR reactions. This procedure is usually very time consuming and requires also plenty of sample material. The introduction of multiplex PCR technologies where multiple primer sets can be used in a single reaction has hugely increased the efficiency of DNA analysis as different DNA sequences of a genome can be amplified simultaneously (Krause et al., 2006, Endicott et al., 2006, Gilbert et al., 2007, Grubwieser et al., 2006, Rompler et al., 2006), which reduces significantly the time, cost and amount of sample material necessary.

2.9.4 Sequencing

Sequencing is the step where the order of nucleotides is determined. There is a wide range of new or “next generation” sequencing (NGS) methods available today. These techniques allow a high-throughput of large genomes in a short time and at low cost. Classical sequencing techniques such as the Sanger method, however, are still very commonly used for small scale DNA analysis. Frederick Sanger and colleagues developed the first sequencing method in 1977, which was based on the use of normal dNTPs (A, C, G, T bases) and modified nucleotides called dideoxynucleotide triphosphates (ddNTPs). ddNTPs contain a hydrogen group on the 3’ carbon instead of a hydroxyl group, which prevents chain elongation. Hence when ddNTPs are incorporated in place of normal dNTPs the reaction terminates (“chain termination method”). The method has been later improved using a nucleotide-specific fluorescent dye so that all four ddNTPs could be used in a single reaction. The distinct fluorescent signal of each ddNTP is detected by automated capillary electrophoresis.

2.10 Ancient DNA – Forensic DNA

Ancient DNA (aDNA) analysis and forensic DNA profiling are in many respects very similar methodologies. They use the same molecular techniques, follow the same authentication criteria, have to deal with a high risk of contamination, and often share the same problems of DNA degradation or inhibition. In some instances archaeologists and forensic geneticists have even participated in joint projects to investigate historical cases. One famous example is the "Romanov Case" where the fate of the Romanov family, the last Russian monarchy, was solved (Coble et al., 2009, Gill and Hagelberg, 2004).

2.10.1 Forensic DNA laboratory quality assurance guidelines

Although the analytical procedures in aDNA and forensic genetics show a great deal of similarities there are fundamental differences in the way forensic DNA work is organised and carried out. The acceptance and success of forensic DNA analysis in court cases is largely due to common standards that have been achieved by international forensic DNA working groups over the years.

In the United States the first operational forensic standards were implemented in the late 1980's to assure the integrity of DNA evidence in criminal court cases. The two major bodies that were initially responsible for developing guidelines and standards for forensic DNA laboratories were the former Technical Working Group on DNA Analysis Methods (TWGDAM), now known as the Scientific Working Group on DNA Analysis Methods (SWGDAM) (www.swgdam.org), and the DNA Advisory Board (DAB) (www.cstl.nist.gov/strbase/dabqas.htm) which was established by the Director of the Federal Bureau of Investigation (FBI) (www.fbi.gov) under the DNA Identification Act of 1994 (Butler, 2005). In Europe the International Society for Forensic Genetics (ISFG) (www.isfg.org) with its national and international working groups and ENFSI (European Network of Forensic Science Institutes)

(www.enfsi.eu) have developed equivalent standards (Schneider, 2007). These guidelines and quality assurance standards for forensic DNA testing laboratories and are laid out in comprehensive handbooks covering each aspect of the process such as organization and management, personnel, facilities, evidence control, validation, analytical procedures, equipment calibration and maintenance, reports, review, proficiency testing, corrective action, audits, safety, and use of subcontractor laboratories (Cormier et al., 2005). Non-governmental DNA companies need to go through an accreditation process and comply with the same strict standards as governmental forensic laboratories (Schneider, 2007, Butler, 2005) before they can undertake forensic DNA casework.

Besides operational standards for forensic laboratories there are also technical standards in place that regulate DNA typing methodologies. Commercially available kits for DNA profiling have greatly contributed to the success of forensic DNA analysis as they provide validated markers that are utilized in forensic labs all around the world to facilitate individual identification on criminal databases.

2.10.2 Ancient and Forensic DNA targets

While aDNA is mainly focussing on mitochondrial DNA (mtDNA) as archaeological skeletal material often lacks endogenous nuclear DNA, forensic DNA analysis deals mostly with modern DNA samples, which allows both for nuclear and mitochondrial DNA to be investigated.

In forensic identification preferably nuclear DNA analysis based on autosomal STRs is used to compare individual genetic profiles to forensic DNA databases or other reference profiles available. STRs within the nuclear genome are unique and therefore provide a greater discrimination power than mtDNA markers for individual identification (Butler, 2005).

Ancient DNA research on the other hand is not so much concerned with individual identification as with elucidating the historic and evolutionary picture of humans and to investigate the origin and migration of human populations from the past. Lineage markers, such as mtDNA or Y-chromosomes markers, are therefore most suitable for the historic purpose.

For the reasons explained earlier in this chapter, the forensic DNA approach using highly discriminatory nuclear STR markers was chosen in the context of the Spanish study in order to establish a potential kinship between the relatives and the human remains from Villanueva del Rosario.

The Norfolk case study sought to find an answer to whether the remains found within the grounds of Snarehill Hall were of local (British) or non-local (Anglo-Saxon or Scandinavian) origin. In this instance the aDNA approach using mitochondrial lineage markers seemed the most appropriate and promising method to recover DNA from degraded skeletal material, and to determine ethnicity.

The analytical parts of the study will be presented in Chapter 5 (“Materials and Methods” – DNA analysis).

Chapter 3

3 Isotope Geochemistry

3.1 Introduction

In 1931 H.C. Urey made the ground-breaking discovery of deuterium (^2H) that had identical chemical properties as hydrogen but with a mass twice as large. At that time little was known about the existence of isotope fractionation. Yet, this event marks the beginnings of stable isotope geochemistry that has developed into an essential part of geochemistry.

Most naturally occurring chemical elements on earth have more than one isotope. Isotopes are variations of an atom of the same element but differ in the number of neutrons in the nucleus. These natural variations have been the foundation for scientific research in a wide field of applications in geological, environmental and dietary studies. In the last two decades isotope techniques have found a wide application as provenancing tool in archaeology and more recently in forensics such as human identification (Meier-Augenstein and Fraser, 2008), drugs (West et al., 2009), explosives (Benson et al., 2009), animal migration (Hobson, 2008) or food authentication (Gonzalvez et al., 2009).

The use of isotopes as natural tracers of the geographical origin of biological material is based on the potential differences in isotope signatures between distinctive geological, ecological or climatic backgrounds. The intake of nutrients and water from a particular geographic region leaves a certain isotopic fingerprint in plants, animals and eventually in humans, which are exploited for provenancing and authentication purposes in ancient population studies and modern forensic applications.

This chapter will introduce isotopes and their use in archaeology and forensics followed by a concise introduction to the fundamentals of isotope geochemistry focusing on strontium and lead isotopes as the relevant systems in this study. Next, the chemical properties of bone and teeth will be discussed as the relevant skeletal sample material used in this study including a section on bone diagenesis. Finally, the use of rare earth element analysis as a proxy for diagenetic processes will be briefly discussed.

3.2 Variation in isotope abundances

Isotopes of any given element contain an equal number of protons and electrons but differ in the number of neutrons. The majority of naturally occurring elements have more than one isotope. Variations in the number of neutrons do not affect the chemical properties of the element *per se* but they do change the atomic weight and hence their behaviour in chemical and physical processes also known as "isotope effects". According to the atomic mass isotopes can be divided into so-called light (e.g. C, N, O, H, S,) and heavy isotopes (e.g. Sr, Nd and Pb).

Apart from manmade enrichment of specific isotopes, there are two main natural processes generating isotopic abundance differences that are exploited in provenancing studies; 1) isotope fractionation due to chemical and physical processes being affected by subtle mass differences between the isotopes of an element and 2) radioactive decay (Aggarwal et al., 2008).

3.2.1 Isotopic Fractionation

Almost all isotopic fractionation processes are mass dependent. A change in the relative isotopic abundance is generally the result of two fractionation

mechanisms, 1) equilibrium reactions (also called thermodynamic or exchange reactions), and 2) kinetic reactions (Sulzman, 2007).

3.2.1.1 Equilibrium isotopic fractionation

Equilibrium isotope fractionation occurs in closed systems where the system will distribute the translational, rotational and vibrational energy between the isotopes in such a way that the overall energy of the system is at its lowest possible point (White 2013). The vibrational energy is the strongest contributor to the isotope fractionation effects and is related to temperature. Interestingly, more fractionation occurs at lower temperatures as the effect of the small mass difference between the isotopes on the total energy state of the system is more pronounced at lower temperatures.

3.2.1.2 Kinetic isotopic fractionation

Kinetic isotope fractionation occurs in open systems where the reaction rate for individual isotopes is affected by the differences in bond strengths for the different isotopes. The lighter isotope (e.g. ^{12}C versus ^{13}C, and ^{16}O versus ^{18}O) forms weaker bonds than its heavy counterpart and therefore separates easier and thus faster resulting in the light isotope being enriched in the reaction product. If the reaction product is removed, for example in the subsequent steps of an enzymatic metabolic pathway, then the isotope fractionation can be much larger than in an equilibrium reaction.

Kinetic fractionations are also associated with evaporation, condensation and diffusion. As with equilibrium fractionation these processes also depend on temperature and thus more pronounced fractionation will happen at lower temperatures.

3.2.1.3 Radioactive decay

A number of elements have naturally radioactive isotopes which undergo radioactive decay to form a more stable nucleus through either, alpha (α) decay, beta (β) decay and gamma (γ) decay. The heavier nuclides usually decay by α-decay whereby the parent nucleus emits α-particle (helium nucleus) to form a stable daughter nucleus. Beta-decay occurs when the nucleus emits an electron or positron, as well as a neutrino, e.g. the decay of ^{87}Rb to ^{87}Sr (Faure and Powell, 1972).

3.3 Oxygen Isotope Geochemistry

3.3.1 Introduction

For many years light stable isotopes have been preferably used to investigate fractionation processes in natural systems due to their high abundance in nature. In archaeological studies the light stable isotopes of carbon and nitrogen have greatly contributed to the better understanding of paleodiets. Carbon and nitrogen stable isotope ratios ($\delta^{13}C$, $\delta^{15}N$) vary between terrestrial and marine derived diets (Sponheimer and Cerling, 2014, Mays and Beavan, 2012) and are often used to characterize the dietary patterns in different cultural groups and the status of individuals within a certain group, e.g. rich versus poor (Privat et al., 2002). Oxygen isotopes ($^{18}O/^{16}O$) are commonly applied in (paleo)climatic research but have also proved useful in provenancing studies of historic people (Evans et al., 2006b, Eckardt et al., 2009) as the oxygen isotopic composition in human tissue is closely linked to that of local drinking water and that in turn is related to the local precipitation (Podlesak et al., 2008, Longinelli, 1984, Bowen et al., 2009a).

Isotopic data for the light stable elements is determined as a ratio of the heavy isotope abundance divided by the light isotope abundance relative to the isotopic ratio of a standard reference material. The ratios are noted as delta value (δ) per mil (‰) units. The standard reference material for oxygen isotopes in water is the Vienna Standard Mean Ocean Water (VSMOW). Isotope ratios are regarded as “heavy” or “enriched” when they consist of heavier isotopes in comparison to the VSMOW, and “light” or “depleted” if they contain lighter isotopes.

The oxygen isotope ratio is reported in δ notation relative to the standard VSMOW as defined by the following equation:

$$\delta^{18}O = \left[\frac{\left({}^{18}O/{}^{16}O \right)_{sample} - \left({}^{18}O/{}^{16}O \right)_{VSMOW}}{\left({}^{18}O/{}^{16}O \right)_{VSMOW}} \right] \times 10^{3}$$

If the $\delta^{18}O$ results in a positive value it means that the sample contains more of the heavy isotope than the VSMOW standard. Conversely, a negative δ value means that the sample contains less of the heavy isotope than the standard.

Although oxygen isotopes have not been part of this study they serve as a good model for the use of isotope processes in provenancing. The dynamics of the process is illustrated in Figure 8, which shows the cycling process between oceans and the atmosphere on land resulting from evaporation and condensation and the consequential changes in isotopic compositions of hydrogen. The isotopically lighter water molecules preferentially evaporate to generate an air-mass with water vapour and during condensation of that air-mass the heavier molecules preferentially leave the air-mass. The overall effect is that the air mass gets isotopically lighter when moving inland. (Bowen, 2010b). In concordance the isotope ratio of hydrogen and oxygen in precipitation decreases (more negative) with increasing distance from the evaporative source region. The effect is exacerbated by increased fractionation due to the decrease in temperature when an air mass moves inland and upland.

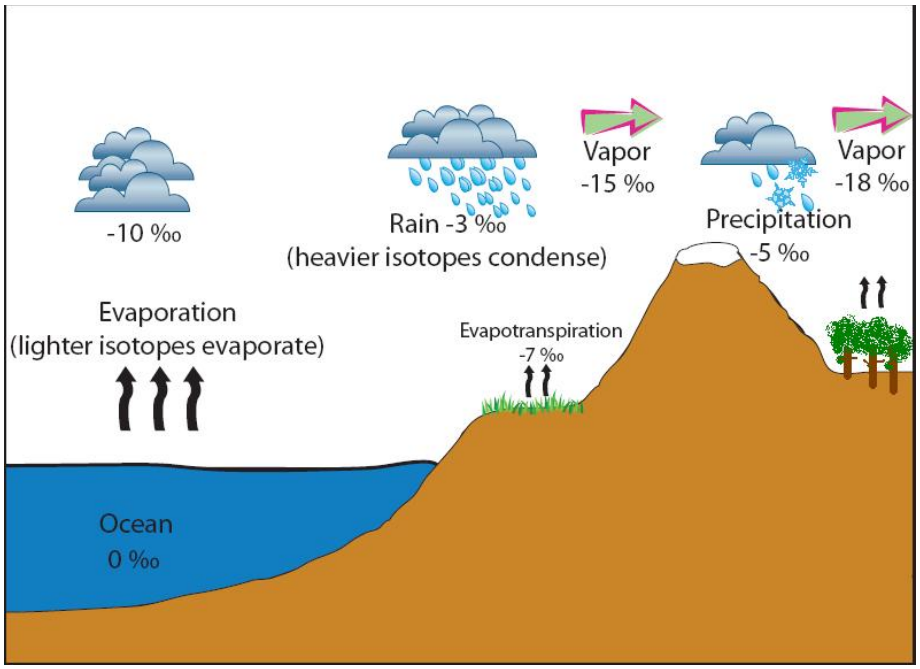

Figure 8: *A schematic diagram of the isotope fractionation process via evaporation, condensation, and evapotranspiration (combination of evaporation and transpiration). Notice that waters are lighter when they evaporate and are relatively heavier when condensed in the form of precipitation*. (Source: http://serc.carleton.edu/)

$\delta^{18}O$ depends on climatic conditions and changes systematically in the way that the relative heavy isotope abundance decreases with lower temperatures, high altitude and latitude. Hence the $\delta^{18}O$ of ingested water incorporated in human tissue is associated with a specific geographic origin which can also be valuable as a tracing tool for the movement of individuals (Bowen et al., 2005). Oxygen isotopes are not only fractionated through condensation and evaporation but also through biological processes (e.g. metabolism).

Antonio Longinelli (Longinelli, 1984) investigated the oxygen isotopic composition in body water and the phosphate in bones and his results delivered the first evidence that $\delta^{18}O$ in mammals is closely linked to the mean $\delta^{18}O$ of local meteoric water. Once it was revealed that $\delta^{18}O$ (and $\delta^{2}H$) in animals, humans and plants could be used to identify the isotopic composition of precipitation (Hobson, 2008, Bowen et al., 2005, Wassenaar et al., 2009) it rapidly found a wide area of provenancing applications in tracing the origin and

movement of animals (Bowen et al., 2005, Hobson et al., 2010), humans (Meier-Augenstein and Fraser, 2008) and food (Chesson et al., 2010, Heaton et al., 2008). Ehleringer et al. (Ehleringer et al., 2008) was the first to establish a close relationship between oxygen and hydrogen isotope composition of drinking water and keratin in hair. They analysed the spatial distribution of the isotope ratios of tap waters from across the US and compared them to the isotopic composition of hair keratin. Based on their findings they developed a prediction model for the expected average $\delta^{18}O$ and $\delta^{2}H$ isotope ratios in human hair as a function of the spatial distribution of the isotope ratios of tap waters and under the assumption of a "continental supermarket" dietary input, which was able to explain >85 % of the variation.

There has been a continuous progress in improving the predictability of the spatial distribution of $\delta^{18}O$ (and $\delta^{2}H$) of isotopic signatures on a global scale. Bowen and co-workers contributed significantly to the development of maps that represented the geographic distribution of hydrogen and oxygen isotopes ("Isoscapes"). They used fixed variables (latitude, altitude) to achieve a predictive $\delta^{2}H/\delta^{18}O$ in precipitation that could be used for a high-resolution global map of $\delta^{18}O$ (Bowen and Wilkinson, 2002). This model was later improved to decrease the error rate by 10-15 % relative to other tests (Bowen and Revenaugh, 2003). Other models have included more climatic parameters, such as temperature (van der Veer et al., 2009, Bowen, 2010a), or a combination of both meteorological (temperature, relative humidity, amount of snowfall, etc.) and geospatial variables to achieve an improved prediction of precipitation isotope composition (Terzer et al., 2013). Only recently a new "Regionalized Cluster-based Water Isotope Prediction (RCWIP) model was developed (Terzer et al., 2013) that uses subsets of climatic $\delta^{2}H/\delta^{18}O$ GNIP (Global Network for Isotopes in Precipitation) data, a network that has been developed by the IAEA (International Atomic Energy Agency) to provide monthly and annual isotope data for over 500 collection stations around the globe. This latest approach provides a more accurate prediction tool as it uses data that is specific for a defined climatic region in contrast to global isoscape models that do not account

for small regional variations. According to the authors, the RCWIP model has increased the predictive power >67 % of the time compared to previous approaches (Terzer et al., 2013). However, despite continuous progress in developing more precise isoscapes there is still a lack of research into the errors and uncertainties associated with these prediction models.

Some of the larger isotopic data maps for hydrogen are also publicly accessible via the following weblinks: Isoscapes (www.wateriso.utah.edu/waterisotopes/), IAEA (www-naweb.iaea.org/napc/ih/IHS_resources_isohis.html), and the Global Seawater Oxygen-18 Database (http://data.giss.nasa.gov/o18data/) created by Schmidt (Schmidt, 1999) and Bigg and Rohling (Bigg and Rohling, 2000).

While light stable isotopes are undeniably informative for studies that exploit the ability of natural fractionation processes, provenancing research with heavy isotopes like Sr and Pb exactly tries to circumvent this problem and looks for the stability of isotopic signals that are transferred without fractionation (or at least insignificantly) from the geosphere to the atmosphere and biosphere. But Sr and Pb have also other spatial distribution patterns, which add extra spatial resolving power for the use in provenancing applications.

3.4 Rubidium/Strontium Isotope Geochemistry

3.4.1 Introduction

Rubidium (Rb – atomic number 37) is an alkali metal found in group IA of the periodic table together with lithium, sodium, potassium, caesium and francium. Due to its similar ionic radius (1.48 Å) to potassium (1.33 Å) it substitutes in a number of potassium-bearing minerals such as igneous micas (muscovite, biotite, lepidolite) and potassium feldspar minerals (orthoclase and microline), certain clay minerals, and evaporites (sylvite, carnallite) (Faure, 1986). Although rubidium is abundantly present in common K-bearing minerals it is never concentrated enough to form its own mineral (Faure, 1986).

Rubidium has two naturally occurring isotopes, ^{85}Rb and ^{87}Rb with natural abundances of 72.17 % and 27.83 %, respectively.

Strontium (Sr – atomic number 38) is a naturally occurring alkaline earth metal with four stable isotopes, ^{84}Sr, ^{86}Sr, ^{87}Sr and ^{88}Sr. The most abundant is ^{88}Sr (82.53 %), followed by ^{86}Sr (9.87 %), ^{87}Sr (7.04 %) and ^{84}Sr (0.56 %). ^{87}Sr is the product of the radioactive decay of rubidium-87 (^{87}Rb) with a half-life of 48.8 x 10^9 years (Faure, 1986).

Sr is found in group IIA of the periodic table amongst beryllium, magnesium, calcium, barium and radium. Strontium ions are similar in size (1.13 Å) to those of Ca (0.99 Å), which allows Sr to substitute for Ca in calcium-bearing minerals, predominantly in igneous rock such as calcic plagioclase, apatite and calcium carbonate (Faure and Powell, 1972).

The average concentrations of Rb and Sr in igneous and sedimentary rocks are shown in Table 3.

Rock type	Rb (ppm)	Sr (ppm)	K (ppm)	Ca (ppm)
Ultrabasic	0.2	40	1	25,000
Basaltic	30	8,300	465	76,000
High Ca granite	110	25,200	440	25,300
Low Ca granite	170	42,000	100	5,100
Shale	140	26,600	300	22,100
Sandstone	60	10,700	20	39,100
Carbonate	3	2,700	610	302,300
Deep sea carbonate	10	2,900	2,000	312,400
Deep sea clay	110	25,000	180	29,000

Table 3: Average concentrations in igneous and sedimentary rocks (Turekian and Wedepohl, 1961, Faure, 1986)

It is noteworthy that these values only represent average concentrations that can vary considerably in rocks and that the Sr isotopic composition will depend on the original Rb/Sr ratio of the rock during crystallization and its age. These values, however, illustrate effectively the general correlation between Rb and K and Sr and Ca. Rubidium concentrations range from less than 1 ppm in ultrabasic rocks to 170 ppm in low-Ca granitic rocks. Strontium concentrations correlate with that of Ca and range between 1 ppm in ultramafic rocks to up to 2000 ppm in carbonate rocks. The Rb/Sr ratios of common igneous rocks show a wide range from 0.06 (basaltic rocks) up to 1.7 or more in highly differentiated granitic rocks with low calcium concentrations (Faure, 1986).

3.4.2 The Rb-Sr dating method

The radioactive decay of ^{87}Rb to ^{87}Sr is the foundation for the well-established Rb-Sr dating method that has been extensively employed to geochronological

studies since the early ‘50s and as such has also been used to determine the geological evolution of the continental crust and mantle of the Earth (Faure and Powell, 1972).

The radioactive isotope ^{87}Rb (half-life ~ 48.8 x 10^9 years) undergoes β-decay to form stable ^{87}Sr, which is expressed in the following equation.

Equation 1:

$^{87}Rb \rightarrow {}^{87}Sr + \beta^{-} + \upsilon + Q$ (0.275 MeV) (Faure and Powell, 1972)

Where β^{-} is the negative beta particle, υ is a neutrino and Q is the decay energy (in megaelectron volts).

The next equation describes how the $^{87}Sr/^{86}Sr$ in samples is determined:

$$\left(\frac{^{87}Sr}{^{86}Sr}\right)_t = \left(\frac{^{87}Sr}{^{86}Sr}\right)_0 + \frac{^{87}Rb}{^{86}Sr}(e^{\lambda_{87Rb}t} - 1)$$

$^{87}Sr/^{86}Sr_t$ is the ratio at the time the sample is measured, $^{87}Sr/^{86}Sr_0$ is the ratio at the time of mineral formation, $^{87}Rb/^{87}Sr$ is the ratio at the time the sample is analysed, λ is the decay constant (1.39 *10^{-11} yrs^{-1}), and t is the time elapsed since the last crystallization or homogenization of the sample (Faure and Powell, 1972).

3.4.3 The strontium cycle

As strontium is released from bedrock by weathering it enters soils and water starting the cycling process through plants and animals before being transferred to seawater by rivers. Oceans are the greatest reservoir of strontium and only a small part of that strontium is transferred back to the continents via precipitation (Capo et al., 1998) and most of it will over longer time become part of marine sediments.

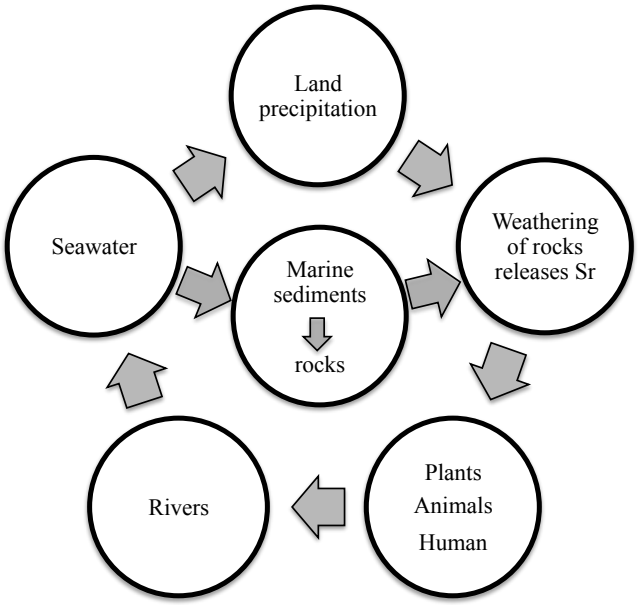

Figure 9: The strontium cycle (Source: author)

The average concentration of Sr in the different systems varies widely; in crustal rock 370 ppm, in soil 240 ppm, in seawater ~8 ppm and river water and precipitation have generally ~0.1 ppm Sr (Capo et al., 1998).

Because of its ubiquitous presence in nature Sr has become an important geochemical tracer for processes in ecosystems and biological systems alike. However, the Sr isotopic composition and dynamics in each of the systems within the strontium cycle is subject to complex inter-related geochemical (e.g. geology, soil), environmental (e.g. precipitation, dust, fertilizers) as well as biological factors (eg. diet, type of tissue), and requires a broad understanding on all these levels for a sound interpretation of Sr data.

3.4.4 $^{87}Sr/^{86}Sr$ values in rocks, soils and oceans

In the geologic history of the earth the Sr isotope composition has evolved due to tectonic events and the mixing of various geochemical reservoirs. Approximately 4.5 billion years ago the primordial Sr isotope ratio is estimated

to have been 0.699 and ever since has increased depending on the type of rock, its Rb content and the time passed since the formation of earth Figure 10.

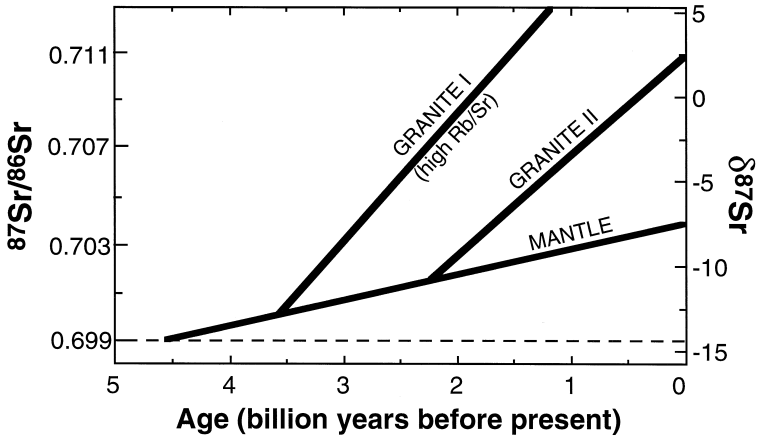

Figure 10: Sr isotopic evolution over geologic time (Capo et al., 1998).

The Sr isotopic composition in geologic systems ultimately depends on the geologic composition and the age of rocks and minerals. Rocks with a high K content (igneous, metamorphic, clay rich) that are naturally rich in Rb, will result in a more radiogenic $^{87}Sr/^{86}Sr$ ratio than that derived from Rb poor rocks. Generally, the more Rb is present and the more time has elapsed since the formation of the rocks, the more ^{87}Rb will have decayed to the stable isotope ^{87}Sr over time. Older rocks with an initially high $^{87}Rb/^{87}Sr$ ratio will now show a high strontium ratio above 0.710 while in young rocks with an initially low Rb/Sr ratio, $^{87}Sr/^{86}Sr$ will have values less than 0.706 (Price et al., 2002, Slovak and Paytan, 2011).

Values for $^{87}Sr/^{86}Sr$ ratios across different geological make-ups on earth range between 0.702 and 0.780. Igneous and metamorphic rocks (granites) from the continental crust have a Rb/Sr ratio approximately ten times higher to that in the rocks (basalts) of the upper mantle (Faure and Powell, 1972). Therefore high Sr

isotope ratios are typically found in the geology of the old continental crust (0.720 to 0.750) and lower values in younger basalts (0.702 to 0.704).

Sedimentary rock as the breakdown product of igneous and metamorphic rock can have any intermediate composition depending on the hinterland highlands that supply the source material (Faure et al., 1965). The strontium isotopic composition of marine carbonates reflects the ambient strontium isotopic composition of seawater at the time of deposition. The Sr composition of seawater over geological time is a function of the input from continental erosion. During different geological periods different geological hinterlands were the dominant sources of the eroded material leading to significantly different Sr isotopic composition over the eon. The "evolution" of Sr isotope ratios in marine sediments has been captured in the Sr curve by Veizer et al. (Veizer, 1989) and other authors (McArthur et al., 2012).

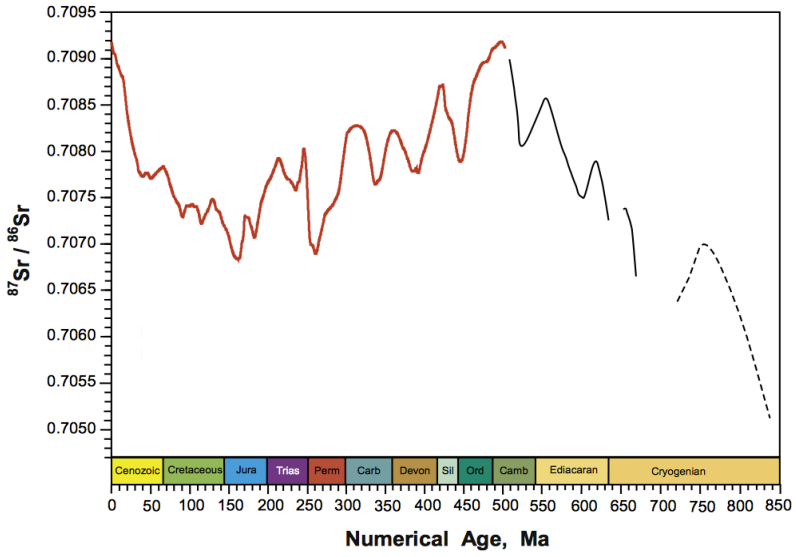

Figure 11: The evolution of $^{87}Sr/^{86}Sr$ ratios in seawater over geological time (Source: (McArthur et al., 2012))

Modern-day seawater and modern marine carbonate have a homogenous Sr ratio of ~0.7092, which is regarded as stable (Elderfield, 1986) because of the long residence time of Sr in seawater (approximately 2.5 million yrs) relative to the,

in geological time-scales, short mixing time (~1000 yrs) of oceans (Hodell et al., 1990). However, the Sr isotopic composition of seawater has changed considerably over geologic time due to the influx of isotopically different geological sources that were released and ultimately mixed in the oceans. The three main sources determining the Sr ratios in seawater derive from 1) hydrothermal circulation from mid-ocean ridges (low $^{87}Sr/^{86}Sr$ of ~0.703), 2) chemical weathering of old Rb-rich continental silicate rocks (high $^{87}Sr/^{86}Sr$ of ~0.711), and 3) marine carbonate rocks (intermediate $^{87}Sr/^{86}Sr$ of ~0.708) (Faure and Powell, 1972). Major tectonic events such as the uplifting of the Himalayas have contributed to changes in the Sr isotopic composition of the oceans (Capo et al., 1998, Hodell et al., 1990).

The isotopic composition in soils shows great variability due to the variability of contributing geologic sources on earth. The $^{87}Sr/^{86}Sr$ ratio in soils is generally defined by weathering processes of the underlying rock material. However, soils are also exposed to atmospheric input such as dust, precipitation and anthropogenic sources, which have shown to contribute and modify the Sr ratio derived from the underlying geology (Miller et al., 1993, Capo et al., 1998, Chadwick et al., 2009). For instance, the source of Sr contributing to the local vegetation in the Sangre de Christo Mountains/Mexico was investigated by Graustein *et al.* (Graustein and Armstrong, 1983) and it was found that more than 75 % of Sr derived from atmospheric deposition and less than 25 % from weathering of the underlying bedrock.

Natural locally-derived dust reflects similar Sr ratios to that of local or distant soil, sediments and bedrock (Capo et al., 1998) but Sr from anthropogenic sources (eg. fertilizers, coal combustion etc.) and dust transported by wind and storms can introduce isotopically distinct Sr signatures into the soil. So dust over the Atlantic Ocean and the Caribbean, respectively, could be traced back thousands of miles to its origin in the Sahara-Sahel zone (Kumar et al., 2014, Aarons et al., 2013).

Precipitation in coastal region has a similar isotopic composition of that of seawater but differs in Sr concentration, which is about three times lower in magnitude (<1 ppm) (Åberg et al., 1989). A study by Andersson et al. (Andersson et al., 1990) examined the isotopic composition of snow across central Scandinavia that lead to the conclusion that seawater contributes 90 % of the Sr to the isotopic composition in snow in the western coastal region, decreasing 10 to 30 % with increasing distance from the ocean. $^{87}Sr/^{86}Sr$ values ranged from 0.7098 at the Atlantic coast and 0.7194 about 300 km inland.

3.5 Sr isotopes as human provenancing tool - principles and considerations

Provenancing studies with heavy isotopes such as Sr and Pb take advantage of the lack of any significant fractionation of these elements when they are transferred from the geosphere to the biosphere. The use of Sr is based on the principle that the characteristic Sr isotopic composition found in the bioavailable fraction of the soil is directly transported to the food chain of plants, animals and humans (Aberg et al., 1998, Capo et al., 1998, Ericson, 1985, Blum et al., 2000, Bentley, 2006) is without any significant alteration of the Sr ratio. Since Sr readily substitutes for Ca the locally available Sr intake through food and water should therefore also be reflected in the Ca-rich tissues of bone and teeth.

The successful application of Sr in provenancing, however, depends on several factors. The most basic requirement is it that the geologic differences between the areas under investigation have to be sufficiently distinct that variations in $^{87}Sr/^{86}Sr$ can be detected. At the same time geologic variations within each area should be small enough, or ideally homogeneous, to display a characteristic Sr isotope composition. In numerical analytical terms, the actual observed variation in the Sr isotopic composition of a population is two or three orders in magnitude larger than the analytical uncertainty for an individual measurement, which is typically in the fifth or sixth decimal of the measured Sr isotope ratio.

Thus to confidently assume that differences between populations, or between outliers and a population, are significant one needs to observe differences in the order of 0.001 or larger in the Sr isotope ratio in general. This is due to the fact that humans are at the top of the food chain and each individual will consume his own variety from a number of different foodstuffs. At present no systematic studies have been performed to assess the exact relation between intake, metabolic pathways and final deposition in bone for strontium isotopes. Only recently one group achieved such a study for the composition of hydrogen in hair (Ehleringer et al., 2008). Realistically, ideal discriminatory isotopic conditions are rarely the case and therefore it has been suggested that incorporating a variety of biological samples (plants, animals) should give a better indication of the local bioavailable Sr range (Price et al., 2002).

Another critical factor to consider is that using Sr isotope analysis for discriminating between people living in coastal areas might not always be effective particularly if the main food source derives from the sea and thus reflects the isotopic composition of that of seawater ($^{87}Sr/^{86}Sr$ 0.7092). This argument is particularly significant for ancient populations from coastal areas as the dependency on marine food was much greater then than it is now in modern people.

The source of consumed food is one important issue to consider in provenancing studies using Sr isotopes. Ancient populations sourced their food and water mostly locally. However, exceptions can occur in populations that utilized sea salt, either obtained from local coastal production or acquired via salt trade. The isotopic signature of sea salt might differ significantly from terrestrial sources and can impede the interpretation of "local" versus "non-local". For instance the Sr isotope data from most human remains found in the ancient Maya city of Tikal differed from the local bioavailable Sr signatures (Wright, 2005). Wright suggested that imported sea salt could be responsible for the mismatch and used a model to demonstrate that dietary $^{87}Sr/^{86}Sr$ may be raised to the level found at Tikal by a daily intake of only 6 g of sea salt.

While the Sr method is generally very effective in ancient population studies it could be problematic to apply it to modern day humans without taking into account the change in the way food is sourced today. The majority of people in the developed world lives on mixed diets sourced from imported and locally grown food. The ease of access to supermarkets, globalisation and mass production of processed food from ingredients sourced internationally has reduced the effectiveness of Sr isotope analysis in modern human provenancing studies. It has been suggested that isotope values will be homogenized globally if the trend of the global supermarket culture continues to grow (Hawkes, 2006, Phillips, 2006). Especially Ca- and Sr-rich imported foods (dairy products, leafy greens, legumes) and sea salt will have a significant effect on the Sr isotopic signature in human tissue, even when consumed in small amounts (Slovak and Paytan, 2011, Wright, 2005). Consequently, whether Sr isotope analysis can be used successfully for provenancing purposes in humans ultimately depends if individuals have consumed food from local production or not.

Provenancing studies increasingly include multiple isotopes such as lead and oxygen as complementary tracers for distinguishing “local” from “non-local” (Budd, 2003, Aberg et al., 1998, Montgomery et al., 2005a, Eckardt et al., 2009, Chenery et al., 2010, Price et al., 2012a). For instance, oxygen isotopes in bioapatite are known to correlate to the values of ingested water and as the isotopic composition of water depends on precipitation (Bowen et al., 2005, Longinelli, 1984) it can define the climatic environment the individuals lived in.

A number of studies investigated the isotopic impact of local drinking water vs. food intake in individuals using oxygen and hydrogen isotopes (Ehleringer et al., 2008, Bowen et al., 2009a, Thompson et al., 2010, O'Brien and Wooller, 2007). Ehleringer et al. (Ehleringer et al., 2008) for instance looked into the isotopic composition of these elements in human hair keratin taken from individuals from 65 cities in the United States. This study showed that 35 % of oxygen and 27 % of hydrogen atoms in hair were directly linked to drinking water. They developed a model to predict the isotopic composition of oxygen and hydrogen as a function of drinking water, bulk diet and dietary protein intake. They found

that drinking water had a significant impact on the isotopic composition on hair keratin contributing >85 % to the variation in the population while diet was responsible for the remainder. Thompson et al. (Thompson et al., 2010) collected isotope data (H, O, C, N, S) from across Asia and used Ehleringer's prediction model to test the relationship between local drinking water and hair keratin. The results suggested the 42 % and 39 % of H and O isotopes, respectively, originated from drinking water. O'Brien et al. (O'Brien and Wooller, 2007) determined somewhat lower values and found that drinking water was responsible for 27 % oxygen (and 36 % hydrogen) in human facial hair. Based on the analysis of hydrogen, Sharp et al. (Sharp et al., 2003) determined that 31 % of hydrogen in human hair derived from ingested water and the remainder from food.

Furthermore, lead isotopes can complement Sr isotopes as geochemical tracers due to the high variability and characteristic isotopic composition of Pb deposits (Reimann et al., 2012). Lead isotope analysis therefore has the potential to further restrict potential geographical areas of origin. Hence, the best or most promising approach is probably to use a combination of different parameters to gain supportive information and thus increase the significance of Sr data in the provenancing process.

Comparing isotopic signatures from different tissues from the same individual (e.g. tooth and bone) have also been suggested as a means to differentiate between migrants and locals (Slovak and Paytan, 2011). As discussed later on in this chapter, tooth enamel is mineralized during childhood and the isotopic composition captured at the time of formation remains unchanged throughout life. Bone on the other hand is constantly remodelled and only reflects the isotopic composition of the last 5 to 10 years (Grupe et al., 1997, Price et al., 1994a) in the life of an individual. In migration studies the differences in isotopic composition between tooth enamel and bone in the same individual hence can provide evidence that a person has changed residency between childhood and adulthood.

3.5.1 Defining the local bioavailability of $^{87}Sr/^{86}Sr$

As a starting point, the local bedrock geology can provide an estimate of the $^{87}Sr/^{86}Sr$ range in a particular area (Capo et al., 1998, Beard and Johnson, 2000) but as some studies have shown, the Sr values of the underlying geology can differ significantly from the bioavailable $^{87}Sr/^{86}Sr$ (Price et al., 2002, Poszwa et al., 2004, Hedman et al., 2009). To establish the Sr isotopic value it has been recommended to analyse a variety of small animals living locally that are less likely to roam great distances (Price et al., 2002). This approach decreases environmental variability as animals obtain an averaged local signal which have been shown to be very close to the range in human tissue (Bentley, 2006).

A more recent study (Maurer et al., 2012) measured Sr ratios of different sample types (water, soil leachate, snail shells, deer enamel, ground vegetation, tree leaves) to determine the best proxy for local bioavailable Sr in archaeological studies. Comparing their data to $^{87}Sr/^{86}Sr$ from bones and teeth of two 5–6th century A.D. cemeteries, they concluded that modern water and vegetation (tree leaves in particular) most closely reflect the Sr isotopic composition of the local biosphere. A similar outcome has been reported in a previous study (Poszwa et al., 2004) where the isotopic composition in leaves of tropical rainforest trees in French Guyana was less variable (0.714-0.716) compared to that in bulk soils (0.720-0.770) and thus represented a more consistent proxy of the local bioavailable Sr than soils.

3.6 Lead Isotope Geochemistry

3.6.1 Introduction

Lead has four naturally occurring stable isotopes, ^{204}Pb (1.4 %), ^{206}Pb (24.1 %), ^{207}Pb (22.1 %) and ^{208}Pb (52.4 %). The latter three isotopes ^{206}Pb, ^{207}Pb and ^{208}Pb are the end products of radioactive decay series of ^{238}U (uranium series), ^{235}U (actinium series) and ^{232}U (thorium series), respectively. ^{204}Pb is a so-called primordial nuclide that has already existed before the creation of earth. It is the rarest lead isotope and due to its non-radiogenic nature it is used as a reference isotope for presenting lead isotope ratio data. The abundance of Pb isotopes depends entirely on the concentrations of primordial Pb, U and Th, and the decay rates of both parent and intermediate isotopes.

Pb isotope data can be expressed in several ways ($^{206}Pb/^{204}Pb$, $^{206}Pb/^{207}Pb$, $^{208}Pb/^{206}Pb$) but the most effective way to discriminate between different ore deposits is achieved by normalization to ^{204}Pb ($^{206}Pb/^{204}Pb$, $^{207}Pb/^{204}Pb$ and $^{208}Pb/^{204}Pb$) (Komarek et al., 2008). In early human studies the data were reported without ^{204}Pb ratios because of the difficulty in analysing the low abundance ^{204}Pb isotope (Gulson, 2008) with anything but a TIMS (Thermal Ionization Mass Spectrometry). Modern state-of-the-art MC-ICP-MS technology, however, can now measure low abundances with high precision and larger numbers.

Lead is distributed over a wide range of U- and Th-bearing mineralogies either as a major constituent or as a trace element. Lead, however, can also form its own mineral, galena (PbS), which is the main Pb ore mineral consisting of 86.6 % Pb with extremely low concentrations of U and Th (Kamenov and Gulson, 2014).

Similar to Sr, Pb^{2+} is found in K-feldspar where it is known to substitute for K^+ but is otherwise not restricted to Ca-rich minerals (Elias et al., 1982). Lead accumulates with the transition metals zinc (Zn), cadmium (Cd) and trace

amounts of silver (Ag) in ore deposits. The presence of Pb in different low U/Pb and Th/Pb bearing minerals, such as galena, plagioclase and K-feldspar, as well as in highly radiogenic Pb sources such as zircon, apatite or in U- and Th- rich granites, contributes to the great isotopic variations of lead (Faure, 1986). The isotopic composition of any given ore deposit has been described as a function of four parameters: 1) the decay rate of parent isotopes, 2) the initial ratio of the abundance of the parent to the abundance of Pb ($^{238}Pb/^{204}Pb$, $^{232}Th/^{204}Pb$) in the source reservoir (e.g. mantle or continental crust), 3) the initial isotopic composition of reservoir Pb and 4) the duration of reservoir evolution prior to separation of Pb by geological processes (Sangster et al., 2000).

As Pb is a high atomic mass element no significant fractionation of the original Pb signature occurs hence the characteristic Pb source of a particular rock or location is retained and transferred unaltered to biological systems. The isotopic variety can derive from spatial differences within one ore field or from weathering processes of heterogeneous rock phases that are mixed within soil and groundwater ultimately resulting in a homogenous source representative of the local underlying geology (Erel et al., 1994).

Very old ore deposits like those in Broken Hill and Mt. Isa in Australia that formed about 1700-1800 million years ago have typically a low isotope ratio whereas younger ore deposits formed in the Palaeozoic era, eg. the Mississippi Valley Type (MVT) in the USA, are more radiogenic (Gulson, 2008, Kamenov and Gulson, 2014).

3.6.2 Anthropogenic lead

Lead is not naturally present, or in any way essential, in human tissue. In fact it is highly carcinogenic and environmental health studies have used Pb isotope analysis to identify the source of lead deposited in human tissue (Gulson, 2008, Rabinowitz, 1995, Lanphear et al., 1998, Rabinowitz, 1991, Ettinger et al., 2004, Mushak, 1998, van Wijngaarden et al., 2009, Weisskopf et al., 2009).

Lead has a long history of industrial and commercial use. People have been exposed to lead since the introduction of mining and smelting ~5000 years ago in Asia (Komarek et al., 2008). In historical populations chronic lead intoxication was a very common health problem resulting from the use of leaded kitchen and household goods. In some early cultures (Romans and Greek) lead production thrived accompanied by increasing exposure to dangerous emissions and extensive use of metals containing lead such as silver, copper and bronze to produce household utensils, such as lead glazed pottery (Aberg et al., 1998), pipes, cisterns, pewter ware, coffins etc., even in jewellery (Montgomery, 2010b). Ancient Greeks, for example, used to store wine in lead vessels which was reported to cause sterility, abortion, headache and sleeplessness (Drasch, 1982). Interestingly, another major source of lead poisoning in humans is soft drinking water, or acidic water from lead pipes, cisterns and vessels. Hard water on the other hand, which is high in Ca, is known to largely reduce the uptake of lead by the gut (Montgomery, 2010b).

Since the introduction of industrial and commercial use the anthropogenic lead pollution in the environment has steadily increased. The changes of Pb isotopic signals in Europe and the US induced by anthropogenic lead sources over time are illustrated in Figure 12 (Source: (Kamenov and Gulson, 2014)). When Australian Pb was introduced in Europe as additive in gasoline regional Pb isotopic compositions fell dramatically with the effect that local natural background values became entirely obscured (Kamenov and Gulson, 2014). Similarly, when the more radiogenic Pb from the MVT substituted the low radiogenic Pb from an ore deposit in Idaho, the Pb isotope ratios in the US increased accordingly.

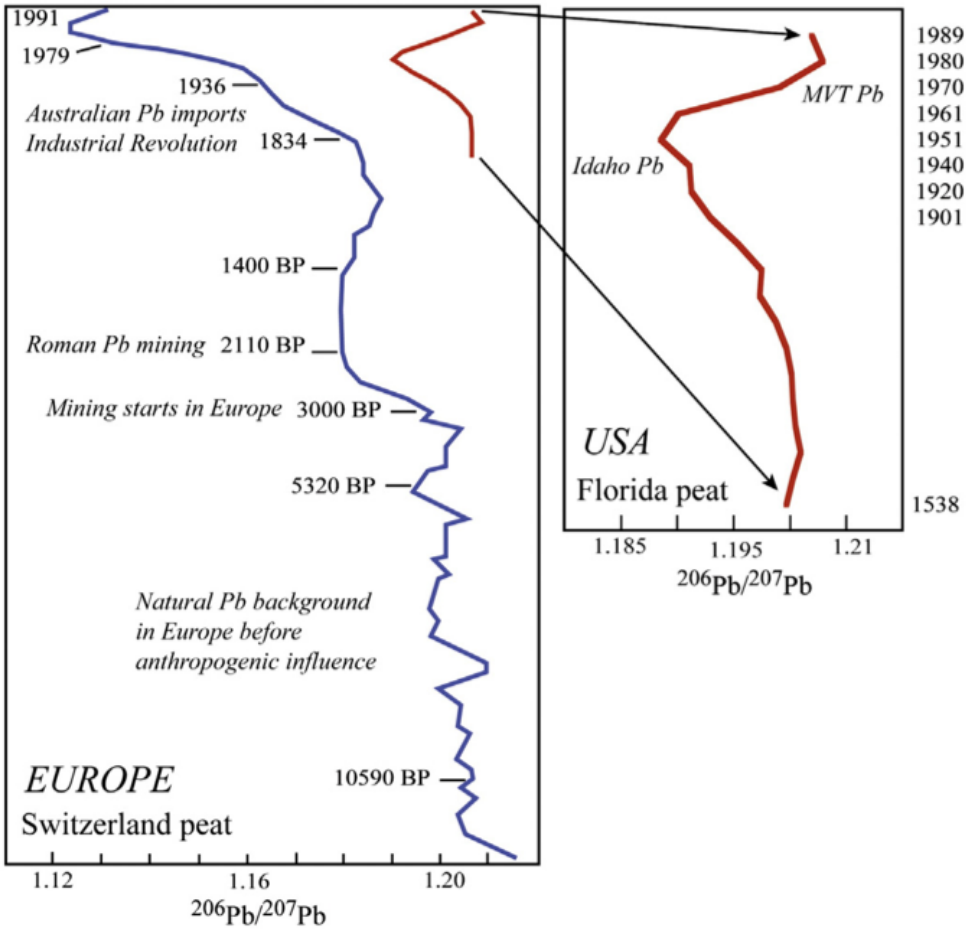

Figure 12: "*Pb isotopic background through time in Europe. The two major shifts in the Pb isotopic background in Europe correspond to the onset of mining during the Romano-British period and the introduction of Australian Pb. The USA data comprise approximately the last 500 years, providing record from the discovery of the New World to present. The Pb isotopic shifts identified in the USA during the last 100 years correspond to the use of different Pb ores*" (Source: (Kamenov and Gulson, 2014)).

During the 60's and 70's gasoline additives containing lead was becoming the major source of lead contamination until leaded gasoline was successively banned globally by 2012.

3.7 Pb isotopes in human provenancing

Isotopic studies utilizing Pb have mainly focussed on tracing changes in pollution and the source of lead in environmental studies (Miller et al., 2004, Tyszka et al., 2012), health-related research (Gulson et al., 2006, Gulson, 2008) or forensics (Zeichner et al., 2006, Henderson et al., 2005). In particular Gulson et al. published a series of papers on the impact of man-made lead burdens in modern humans (Gulson et al., 2002a, Gulson et al., 1999, Gulson et al., 2003, Gulson, 2008). For example, in a study by Gulson's group they proved that Pb isotopes could be successfully applied to distinguish first generation European immigrants from individuals born in Australia based on specific isotope signatures of different Pb sources they were exposed to (Gulson and Gillings, 1997).

The great variability of lead isotopic signatures also makes it a valuable instrument in human provenancing (Valentine et al., 2008). As lead is not measurably fractionated by human metabolic turnover (Carlson, 1996) or other chemical processes, the isotope ratio in human tissue reflects the original isotope composition from the lead source. However, exposure to various environmental lead contaminants such as combustion of coal, mining and smelter can alter or even override the lithogenic isotopic signal (Faure, 1986). Hence Pb isotope ratios are often used as a proxy for differentiating between pre-historic and modern populations. Generally, Pb isotope ratios derived from anthropogenic sources tend to exhibit higher Pb values than geologically derived isotope ratios (Bird, 2011), and hence can provide evidence if people were exposed to a natural Pb source or to lead from anthropogenic pollutants in provenancing studies.

Some archaeological studies have compared modern and pre-industrial bone lead and it emerged that modern bones contained more elevated lead levels than archaeological human bone (Budd et al., 1998) due to increased anthropogenic lead exposure. A precise quantification, however, proved difficult because of the

very low trace amounts found in pre-industrial human tissue and also because of post-mortem environmental lead contamination (Budd et al., 1998). In a later study Budd et al. (Budd et al., 2004b) published lead concentrations for tooth enamel from 77 individuals buried in England covering approximately 5000 years from the Neolithic until the 16th century A.D. Pb concentrations in prehistoric individuals showed a range between 0.04 to ~0.4 ppm and increased dramatically during the Romano-British and medieval periods to approximately 40 ppm, which would be equivalent to industrial Pb levels (Budd et al., 2004b).

Kamenov et al. (Kamenov and Gulson, 2014) also investigated the correlation between Pb isotopic signatures from historic and modern human teeth derived from Europe and the Americas and their usefulness in provenancing applications. The majority of Pb isotope results for historic and modern European and South American teeth in the same region showed significantly different Pb isotope ratios. Samples from the Iron Age to late Medieval times (ca. 1000 B.C. to 1500 A.D.) in Europe had $^{206}Pb/^{204}Pb$ of <18.6 and in modern samples <18.5 due to the introduction of less radiogenic Australian ore. Historical teeth samples from South America on the other hand showed average $^{206}Pb/^{204}Pb$ of 18.73 +/-0.20 compared to modern values of >18.50. The general outcome suggests that while it may be possible to discriminate individuals between Europe and the US based on the Pb isotopic signatures, the differences are less well pronounced between individuals within Europe. Only Australian individuals can generally be identified anywhere in the world based on their distinctly low Pb isotopic composition in teeth.

Apart from measuring isotope ratios from biological material, anthropogenic Pb isotope ratios can also be obtained from the analysis of archaeological artefacts, (eg. coins, coffins etc.). Comparing lead isotopes between historical metal objects recovered from archaeological sites and lead ores can sometimes also prove valuable in provenancing applications of individuals. Lead extracted from a particular ore retains its original isotope and is not significantly fractionated during ancient metallurgical processes in smelting, refining, working, casting or corrosion processes (Stos-Gale, 2009). Lead isotope analysis from cultural

artefacts and burial goods cannot only provide valuable information on the cultural context, e.g. status, trade relationships or manufacturing skills but can also help shed light on the geographic background of the individual itself.

3.8 Combined oxygen, strontium and lead provenancing

Provenancing studies have mainly focused on the use of oxygen isotopes (White et al., 1998) and the heavy isotopes of strontium (Price et al., 2000, Price et al., 2002, Andrushko et al., 2009) and lead (Carlson, 1996) to reconstruct origin and mobility of people and animals. However, as light stable isotopes are subject to fractionation as they move through geological and biological systems the isotopic fingerprint can be altered. Light stable isotopes are therefore frequently applied in combination with heavy isotopic systems such as Sr and Pb (Budd, 2003, Eckardt et al., 2009, Evans et al., 2006a, Hughes et al., 2014). Strontium in particular has also the advantage that the small-scale fractionation that occurs can be corrected for during ICP-MS analysis by normalization to a fixed value of the stable isotope $^{86}Sr/^{88}Sr$ ratio (Steiger and Jäger, 1977).

3.9 Reference databases and Isoscapes

The provenancing of people, food or materials involves the comparison of samples to reference materials in order to establish or predict with variable probability if samples and reference material have a common source, or if they are unlikely to have derived from the same source.

Due to the lack of comprehensive reference databases and/or spatial isotope data, researchers presently mainly rely on geological maps and individually chosen reference samples for subsequent isotope analysis of human bone and teeth. To compile databases that accurately define isotopic signatures would require systematic sampling of a variety of sample material of a specified region. An attempt has been made to produce prediction models based on the Sr isotopic variations in bedrock in the USA (Beard and Johnson, 2000, Bataille and Bowen, 2012) as a basis for large-scale provenancing studies. Although this

map of geologic Sr variations provides a good base for further research it lacks information on bioavailable Sr values, which would be equally important in the context of provenancing studies of biological material (Hoogewerff, 2012).

In another large-scale study Sr isotope ratios from 650 different European natural mineral waters surface waters and soils were analysed (Voerkelius et al., 2010) as one major part of the European wide "TRACE" project previously mentioned. This part is particularly significant as water and soil $^{87}Sr/^{86}Sr$ are important proxies for bioavailable Sr values in bone and teeth. Apart from these two large studies the availability of isotopic reference data for Sr and Pb alike is very limited. The few spatial databases that exist are mainly the product of individual research groups. For example, Evans et al. (Evans et al., 2010) investigated the strontium composition in plant and water for major lithologies and geographical areas in Britain and produced a first map for bioavailable $^{87}Sr/^{86}Sr$ in the UK. Also Sr data from surface waters and soils in Denmark has been published (Frei and Frei, 2011, Frei, 2013). Maps of bioavailable Sr ratios have also been produced for the Sterkfontein Valley/South Africa (Sillen et al., 1998), for Mesoamerica (Hodell et al., 2004) and for the Eastern Mediterranean (Hartman and Richards, 2014). A summary of smaller Sr datasets from numerous archaeological studies is reviewed in Slovak and Paytan (Slovak and Paytan, 2011).

Reimann et al. (Reimann et al., 2012) argued that it was important to know the Pb background concentrations and isotopic composition on a global or least continental scale if Pb isotopes were to be used in forensic and environmental studies. They produced the first and most comprehensive lead isotope data sets from continental European agricultural soils, which constitutes a valuable source for provenance research. However, for other parts of the world no comparable large-scale compilation of lead isotope data is available. An interesting find is the collection of archaeological lead isotope databases (OXALID) for ore deposits and archaeological artefacts that were analysed in the years 1978-2001 in the Isotrace Laboratory at the University of Oxford. It includes datasets from parts of Bulgaria, Spain, Italy, Cyprus, Greece, Turkey and Britain and provides

a useful but restricted resource for lead isotope provenancing studies as ore lead will be representative for environmental or bedrock Pb.

In recent years spatial mapping of stable isotope variations ("Isoscapes") has become an increasingly popular tool. The term isoscapes ("Isotope Landscapes") was first created by J. West and G. Bowen (Bowen et al., 2009b) and describes a method where isotopic variations are presented in a geographic information system (GIS). Particularly oxygen and hydrogen isoscapes have been well developed and are also publicly accessible (see previous section 3.3 for details). The data used for isoscapes can be based on a single or on a multi-isotope approach, which could be particularly advantageous for narrowing down geographic origins. Isoscapes have been created in a wide range of applications such as ecology (Wassenaar et al., 2009), animal migration (Hobson et al., 2010) and forensics (Ehleringer et al., 2010) and will most likely become available for many more areas in future.

3.10 Application of isotopes in archaeology and forensics

The wealth of isotopic information recorded in skeletal material such as bone and teeth over time provides valuable insight into past events. A variety of factors such as geology, climate and diet leave a specific isotopic signal in these tissues that allows addressing different questions in paleodietary, paleo-ecological and ancient population studies and is now increasingly applied in modern day forensics.

3.10.1 Archaeological applications

Ecologists were the first to demonstrate that Sr isotopes could be used to map geographical movement of animals such as elephants (Koch et al., 1995) or

migratory birds (Chamberlain et al., 1997), and for tracing environmental materials (Gosz et al., 1983, Hurst and Davis, 1981, Åberg, 1995, Ericson, 1985). In a pioneering paper published by Ericson et al. ((Ericson, 1985), the great potential of Sr isotopes for investigating different aspects of prehistoric humans such as residency, migration or as a tool to estimate terrestrial versus marine derived diet was first recognized. In the following years archaeologists adopted isotope analysis successfully to study dietary patterns (Sealy and Sillen, 1988, Sealy et al., 1991, Sillen et al., 1998, Sillen and Sealy, 1995), and to reconstruct residential mobility and colonization of ancient populations (Grupe et al., 1997, Price et al., 1994a).

In one of the earlier studies strontium isotopes in bone and tooth enamel were used to identify migration in the late prehistoric (14^{th} century) period in the mountain province of east-central Arizona/USA (Price et al., 1994b) or the late Neolithic Bell Beaker people in Bavaria (Grupe et al., 1997) followed by a number of migration studies in the ancient city of Teotihuacan in Mexico (Price et al., 2000) or the African diaspora in colonial Campeche/Mexico (Price et al., 2006). Knudson et al. investigated residential mobility of cave mummies in Bolivia (Knudson et al., 2005) and of people in Chiribaya Polity/Peru (Knudson and Buikstra, 2007).

Isotope analysis has been used for considerable time in archaeology to reconstruct paleodiets but most previous studies have focused on the light isotopes carbon and nitrogen to determine the food source or strontium based on $^{87}Sr/^{86}Sr$ ratios. Fairly recently, Knudson et al. (Knudson et al., 2010), reasoned that despite the fact the stable strontium isotopic fractionation (reported as $\delta^{88}/^{86}Sr$) is only small, it could still result in different isotopic values along the food chain as organisms preferentially incorporate the lighter isotope, i.e. Sr ratios should decrease with higher trophic level. They compared $\delta^{88/86}Sr$ to isotopic data ($^{87}Sr/^{86}Sr$) and light stable isotope data ($\delta^{15}N$ and $\delta^{13}C$) from tooth enamel and bone of the same individuals buried in Chiribaya/Andes to determine both the geologic and dietary Sr source (marine vs. terrestrial Sr

intake) and concluded that mass-dependent fractionation of Sr ($\delta^{88/86}$Sr) could identify the trophic level of marine and/or terrestrial food sources.

Strontium isotope analysis has also been employed to trace the origin of cultural artifacts such as pottery, textiles and building material made from natural materials (Benson et al., 2006, Frei et al., 2009, Reynolds et al., 2005, Minniti et al., 2014).

Apart from Sr, also Pb isotopes have become increasingly applied in archaeological provenancing studies of ancient metals and historical artefacts from the Bronze Age (Cattin et al., 2011, Ling et al., 2013), or to reconstruct historical developments in metallurgy (Baron et al., 2009).

3.10.2 Forensic applications

More than a century ago the first to recognize the great potential of geochemistry to aid in forensic investigations were Sir Arthur Conan Doyle, a physician and the writer of the renowned Sherlock Holmes detective series (1887 to 1893), and the Austrian criminologist Professor Hans Grosz (Murray, 2004). It was not until the mid seventies, however, that forensic geology was recognized as a potential analytical forensic tool to aid in solving criminal cases. Particularly in the last decade an increasing demand for additional forensic tools to routinely used DNA techniques has emerged and various isotopic elemental systems (H, O, C, N, Sr, Pb) have been employed in the pursuit to provide supplementary evidence in court. The application of isotopes in the forensic context has been used to trace eg. unidentified human remains (Meier-Augenstein and Fraser, 2008, Mutzel Rauch et al., 2009), illicit drugs such as heroin and marijuana (Casale et al., 2006, West et al., 2009), or to determine the source of forensic material such as gun powder (Gulson et al., 2002b).

The use of isotopes in forensics is not yet as well established as DNA analysis, which is mainly due to the lack of extensive reference databases available. However, as reference material becomes more available and accessible isotope

analysis holds great potential to become a more acknowledged and legitimate tool in delivering supporting evidence to solve criminal cases. The usefulness of the method in forensics was first demonstrated in a case where a mutilated murder victim could be identified by carbon and nitrogen isotope analysis (Meier-Augenstein and Fraser, 2008).

3.10.3 Authentication of foodstuffs

The growing concern of producers as well as consumers that high value produce can be modified or even entirely replaced with cheaper substitutes has led to a whole new field of research using stable isotopes for traceability and authentication purposes. This method has been employed in many studies to verify the origin of animal products such as meat and milk (Heaton et al., 2008, Piasentier et al., 2003, Crittenden et al., 2007)), plant products like wine (Martin et al., 1999, R.D. Di Paola-Naranjo, 2011), olive oil (Spangenberg and Ogrinc, 2001), crops (Ariyama et al., 2011) and many other produce.

The EU-funded TRACE project (www.trace.eu.org) initiated a large-scale study to collect stable isotope data for a variety of food commodities, such as honey cereals, lamb, beef, olive oil, and mineral water. Also surface water and soil samples were integrated for stable isotope analysis. The data collected in this study is not only suitable for food authentication purposes but provides also an invaluable pool of isotopic information for the provenancing of other biological material (Voerkelius et al., 2010).

Chapter 4

4 Bone and teeth

4.1 Introduction

Bone and teeth are the main sample material available for isotope/DNA analysis of human skeletal material. The isotopic signature preserved in bone and teeth reflects distinct periods of a lifetime (eg. childhood vs. adulthood) due to physiological differences of the tissues (i.e. dental formation and development, bone turnover rate). It is therefore essential to understand the biological and chemical composition of these tissues and the processes involved in bioapatite diagenesis. The following sections will give a brief overview of the organic and inorganic building blocks of bone and teeth followed by a description of diagenetic effects on bone and teeth.

4.1.1 Chemical composition of bone

Bone is a composite of approximately 25 % organic and 75 % inorganic matter. The inorganic constituent is mostly carbonated calcium phosphate ($Ca_{10}(CO_3PO_4)_6(OH)_2$), which is embedded in a protein matrix predominantly composed of collagen fibres (Figure 13). Collagen is a large protein molecule, which constitutes the major organic content of bone (90 %) (Horvath, 2006). Collagen is the main source for organic carbon, nitrogen, oxygen and sulphur in bone. While all elemental concentrations are ultimately controlled by metabolic pathways the isotopic composition of carbon ($^{13}C/^{12}C$), nitrogen ($^{15}N/^{14}N$) and, to a lesser extent, oxygen ($^{18}O/^{16}O$), sulphur ($^{34}S/^{32}S$) and hydrogen (^{2}H) are determined by the diet (Price et al., 1985).

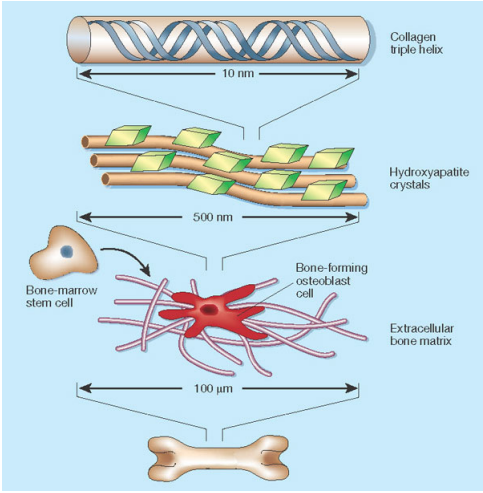

Figure 13: Organic and inorganic components of bone (Taton, 2001)

Bone occurs as cortical (compact) and trabecular (spongy) bone. Trabecular bone consists of a honeycomb like structure within the bone matrix while cortical bone forms the outer sheath of all bones, and is the densest type of bone.

Bone apatite consists of very small plate shaped crystals with an average dimension of 50 x 25 x 2-3 nm (Figure 14) and has a very large surface area of about 240 m^2/g (Berna et al., 2004). Its large surface and crystal strain makes it chemically and physically unstable, which has major consequences in the diagenetic process described later on.

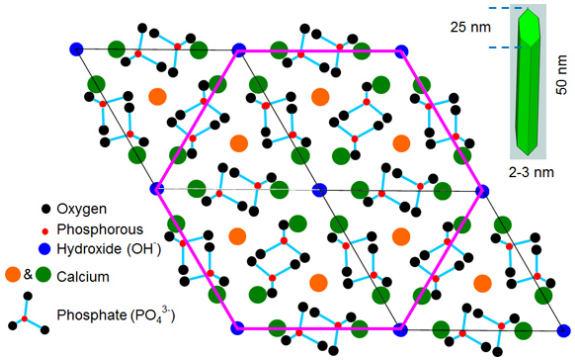

Figure 14: Hydroxyapatite crystal lattice
(Source: www.iupui.edu/bbml/Figures/HA.jpg).

Bone with its large organic content is a dynamic tissue, which is constantly remodelled throughout life (Beard and Johnson, 2000). The turnover rate of bone varies between individuals, sex and age (Schwarcz, 2014), and depends on the nature of bone. The turnover rate of bone is difficult to determine but it has been estimated to be between 5 to 10 years (Ericson, 1985, Grupe et al., 1997) depending on the type of bone. The dense structure of cortical bones remodels at a slower rate than trabecular bone and should be taken into account when selecting bones for migration studies (Price 2002). In a study on the Tyrolean Iceman ("Ötzi") Hoogewerff *et al.* (Hoogewerff et al., 2001) analysed both rib (trabecular bone) and femur (compact bone) and the $^{87}Sr/^{86}Sr$ results indicated that Ötzi had travelled between different geographical regions based on the differences between the two different tissues. However, since compact bone (eg. diaphysis of the femur) is much less susceptible to diagenetic factors (Schwarcz 2014) than trabecular bone it is the preferred bone material in archaeological studies.

4.1.2 Chemical composition of teeth

The tooth structure comprises four major parts, the enamel, dentin, pulp and cementum (Figure 15). Enamel is the most mineralised and in fact the hardest tissue in the human body. It consists of approximately 96 % of inorganic material, mostly hydroxyapatite (crystalline calcium phosphate), and 4 % organic matter (mostly collagen) and water (Schroeder, 1991). The dense crystalline structure of the enamel makes its surface non-porous.

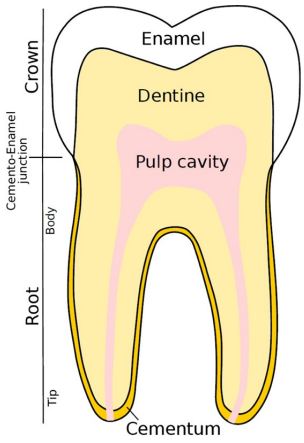

Figure 15: Diagram of the basic structure of a human molar tooth (Adler et al., 2011)

Dentin is less mineralized than enamel (approximately 70 %) with a 20 % organic component, which is mainly made of collagen, and 10 % water. The cementum is the least mineralized part of the tooth with approximately 45 % hydroxyapatite, 33 % collagen and 22 % water (Schroeder, 1991). The pulp is located between dentin and the cementum and consists of soft connective tissue that contains the blood vessels and nerves.

Dental development proceeds in two stages with the deciduous teeth starting to form already *in utero* or shortly after birth and is completed at the age of around 2 to 3 years of age. The permanent set of teeth develops usually between 7 and the age of 12 years and remains essentially unchanged for life (apart from "wear and tear"). Since tooth enamel is mineralised at different stages the isotopic signal captured at these stages is retained throughout life (Budd et al., 2000).

Isotopic studies have shown that compared to other skeletal material enamel preserves the isotopic composition best (Gulson and Gillings, 1997). Its dense inorganic structure is highly resistant to diagenetic effects and therefore reflects the geochemistry for the childhood residency during the formation and mineralization phase of teeth (Aggarwal et al., 2008).

4.1.3 The uptake of Sr and Pb into bone and teeth

In many respects Pb and Sr systematics in human tissue are very similar. Both Sr and Pb^{2+} can substitute for Ca^{2+} in the hydroxyapatite crystal lattice of bone and teeth, and are both incorporated into human tissue through ingested water and food, but also through inhalation of airborne particles (dust, smoking). The most striking difference, however, is the toxicity of lead in humans and especially in children whereas strontium is considered relatively non-toxic within the range of a normal dietary intake.

4.1.3.1 Sr uptake in humans

Food, drinking water and inhalation are the principal pathways in which Sr is incorporated into the human body. Strontium has a great similarity to Ca and follows the same metabolic pathway in the human body where it readily substitutes for Ca^{2+} in the mineral matrix. Most of the insights into the metabolic pathway of Sr in the human body have predominantly derived from clinical studies with radioactive Sr isotopes because of their similar kinetic behaviour with Ca in human tissues (Pors Nielsen, 2004). The biological pathway of Sr was intensely investigated in the 1950's because of the nuclear fall-out that was produced from nuclear fission and the resulting deposition of radioactive ^{90}Sr in the human body (Bryant et al., 1959, Bryant et al., 1958, Comar et al., 1957b, Rivera, 1964).

Reported Sr concentrations in soil and drinking water vary between 0.001 and 39 mg/l, and the average modern diet contains 2-4 mg Sr/day, which derives mostly from vegetables and cereals (Pors Nielsen, 2004). In a different study by the Agency for Toxic Substances and Disease Registry in the US (ATSDR, 2004) it was suggested that humans ingest approximately 3.3 mg of strontium per day where on average up to 2 mg originate from drinking water.

In bone and teeth Sr concentrations depend to a great extent on the dietary composition and are regarded as "dose-dependent" unlike Ca, which is strictly

under homeostatic control (Pors Nielsen, 2004). Vegetables, dairy products and fish have a naturally high Sr content whereas meat is a very poor Sr nutrient. These differences in Sr and Ca content, respectively, have been exploited in numerous paleodietary studies to discriminate between trophic positions in food webs (herbivores vs. carnivores) (Sealy and Sillen, 1988, Sillen and Kavanagh, 1982). Research has shown that the Sr/Ca ratio in skeletal tissue decreases up the food chain, in a process known as "biopurification", and that only 20 % to 40 % of ingested Sr is absorbed compared to 40 % to 80 % of dietary Ca (Comar et al., 1957a, Sillen and Kavanagh, 1982). This method, however, seems not always to be straightforward as individual parameters such as age, sex or health status influence Sr/Ca as well and might change Sr/Ca ratios (Sillen and Kavanagh, 1982).

Maybe an important aspect that needs to be mentioned here is that while the diet has a great impact on Sr concentrations in human skeletal tissue, Sr isotope ratios are independent on ingested Sr concentrations and therefore should represent the average strontium isotope ratio of ingested Sr.

4.1.3.2 Pb uptake in humans

The primary route into human tissue is via ingestion or inhalation of soil and dust (Mielke and Reagan, 1998, Gulson, 2008, Kamenov, 2008). It was estimated that humans ingest and/or inhale on average between 23 and 625 mg (Davis and Mirick, 2006) on a daily basis, which in extreme cases can increase to several grams or more (Simon, 1998). Once lead has entered the body it is initially rapidly transferred from plasma to red blood cells and subsequently at a slower rate into liver and soft tissues, bone, and to excreta (Chamberlain, 1985). Most of the lead intake (~90-95 %) is deposited in skeletal tissue (Rabinowitz, 1991, Drasch, 1982) where it accumulates over a lifetime. Pb concentrations vary between different bone types but are typically ~3-60 ppm in modern adult bone (Drasch, 1982).

As bone is constantly remodelled, the turnover rate of lead varies depending on the type of bone but is generally very slow. In dense cortical bone the lead turnover has been estimated at about 1 % per year (Rabinowitz, 1991) and in trabecular bone somewhat faster (Montgomery et al., 2000). As the permanent set of teeth is mineralised at different stages during early childhood to 12-16 years of age, the Pb isotopic signature captured during these different phases of dental development is locked in for life.

Interestingly, in vivo human experiments revealed that the amount of ingested lead depends whether the lead has been ingested or inhaled during a fasting period or immediately after a meal (Maddaloni et al., 1998). They observed that during fasting conditions on average 26 % (+/− 8 %) of the soil Pb would be absorbed whereas a much lower amount, on average 2.5 % (+/−1.7 %), is absorbed if soil is ingested immediately after a standardized breakfast.

Typical Sr and Pb concentrations and isotope ratios from modern and historic human bone and enamel have been reported in the literature (Aberg et al., 1998, Budd et al., 2004b, Evans et al., 2006b, Montgomery, 2010b, Mays, 2003, Bentley, 2006). However, these values represent only average values drawn from different studies and can exceed these values depending on the type of tissue as well as on the geological background and environmental exposure.

Tissue	**Sr (ppm)**	**Pb (ppm)**
Bone modern	100-300	3-60
Bone historic	50-500	0.04- 300
Enamel modern	50-150	0.5-1
Enamel historic	30-150	0.02~40

Table 4: Typical Sr and Pb concentrations for modern and historic bone and enamel reported in the literature. (NB: The data shown here is drawn from different studies and represent only average values). Sources: (Aberg et al., 1998, Budd et al., 2004b, Evans et al., 2006b, Montgomery, 2010b, Mays, 2003, Bentley, 2006).

The extent to which these concentrations of archaeological bone and teeth might have been subject to the uptake of diagenetic Pb or Sr will depend on the conditions in the burial context, such as temperature, soil pH or groundwater composition.

4.2 Bone diagenesis

The analysis of Sr isotopes from bone and teeth has proved to be valuable in inferring geographic origin and residential mobility. This method, however, depends to a great extent on the preservation of the sample material and its authentic isotope signature, which is often compromised by post-burial alterations.

While in living bone mineral and protein (collagen) form a very strong bond this association starts to weaken post-mortem leading to diagenetic changes of the organic and inorganic bone matrix over time. The most pronounced alterations in bone are molecular damage and loss of the biological component (mostly collagen) as previously discussed in chapter 2 ("Ancient DNA"). The loss of the collagen, which constitutes approximately 40 % in living bone, (Trueman et al., 2006) leads simultaneously to structural changes of the bone mineral such as porosity and microstructural changes which are correlated with the simultaneous increase in crystallinity of the bone mineral (Hedges, 2002). "Crystallinity" defines the structural order within crystals (Trueman et al., 2004), which differs between biogenic and diagenetic bone mineral (Shemesh, 1990). Generally, crystallinity increases in diagenetically altered bone due to extensive substitutions of carbonate for phosphate in the lattice, which leads to disorder (Berna et al., 2004).

Bone crystals are very small in size but at the same time have a high surface area/mass ratio, which puts considerable strain on the crystal lattice (Berna et al., 2004). Strain is further increased if ions of a different ionic radius are replaced in the lattice or by addition of a solute (Brice, 1975) as it may occur post-mortem when the crystal surface of bone is exposed to pore water. The effects of strain can be quantified with the lattice strain partitioning model originally developed by Brice (Brice, 1975) and which was later modified by Blundy and Wood (Blundy and Wood, 1994) to predict the equilibrium

partitioning behaviour of cations on crystal lattice sites for different parameters such as pressure, temperature and material properties (eg. elasticity).

Because of their small size bone crystals are highly reactive and thus either dissolve or recrystallize when exposed to the environment (Berna et al., 2004). Several environmental factors such as soil acidity, water content and temperature determine the rate of bone diagenesis. The pH in soil has the most impact on bioapatite preservation (Gordon and Buikstra, 1981). The long-term survival of hydroxyapatite is therefore strongly influenced by neutral pore water acidity of around pH 8 (Sonke, 2006), which favours the growth of secondary apatite. If the pH of the groundwater solution falls below 7, fresh or recrystallized bone mineral will rapidly dissolve (Berna et al., 2004). Under favourable conditions in the burial environment, the unstable hydroxyapatite can be transformed into the thermodynamically more stable fluorapatite (francolite) (Pasteris and Ding, 2009), and is often found in fossilized bone.

The diagenetic recrystallization process is estimated to take <100 ka years (Herwartz et al., 2011, Trueman and Martill, 2002, Trueman and Tuross, 2002) and ends when all open pore spaces originally occupied by collagen are filled (Trueman and Tuross, 2002). By that time the biogenic isotopic signature in ancient bone can be entirely concealed by the surrounding pore water signal. This has been shown to be the case with terrestrial animals recovered from marine sediments (Trueman et al., 2003).

Although both bone and teeth consist of hydroxyapatite there are differences in the crystalline structure and this has implications for the survival of the intrinsic isotope composition. Unlike in enamel, the porous matrix and large surface area in bone apatite favours the adsorption of trace elements (eg. Sr, Pb, REE), and post-burial recrystallization (Hedges, 2002, Kohn, 2008). Tooth enamel is much more resistant and less effected by diagenesis mainly because phosphate crystals are relatively large (>1 mm) forming a dense and rigid structure (Kohn et al., 1999). Enamel is therefore the preferred sample choice over bone for isotope analysis.

Diagenetic effects on bioapatite can cause major problems in isotope analysis and various methods to remove diagenetic contaminants have been suggested including mechanical and chemical cleaning procedures (Price et al., 1992) although these measures proved not always effective (Kohn and Cerling, 2002).

In order to ascertain whether skeletal material has been subject to diagenetic contamination also the soil isotope composition of the burial environment should be routinely analysed and incorporated into isotope studies. If the isotopic signature between skeletal tissue and soil differs this could arise for two reasons, a) due to a "true" isotopic variation or b) is the outcome of diagenetic alteration. Proving which of these two scenarios might be responsible for the prevalent isotopic composition in skeletal tissue is often very difficult or even impossible. In such a case it will be virtually impossible to differentiate between "local" or "non-local" populations.

A theoretical mixing model is shown in Figure 16. It illustrates the possible variations in isotope ratios of bone caused by the uptake of increasing fractions of groundwater. The assumption here is that the biogenic Sr concentration in bone is 100 ppm and that of groundwater 10 ppm, and the initial Sr isotope ratio for bone is 0.7091 and that for water 0.7100, respectively. Depending on the fraction of groundwater taken up by the bone from the burial deposit it incrementally changes the Sr isotope ratio of the bone. Up to ~70 % of added groundwater the isotope ratio of bone increases relatively slow whereas more than 80 % of diagenetic uptake of water has a very significant effect on the original isotopic signature, which essentially can entirely obscure the original Sr isotopic signature of bone.

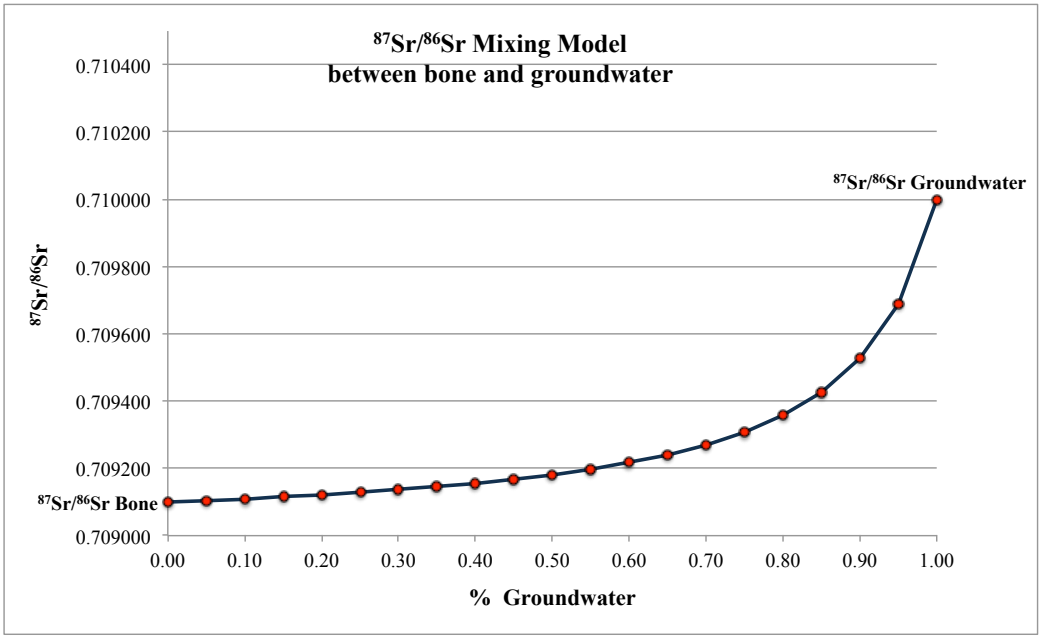

Figure 16: A hypothetical model illustrating the change of Sr isotope ratios of bone with increasing mixing with groundwater.

4.2.1 Screening methods for bone diagenesis

A significant effort has been put into understanding the diagenetic process in bioapatite and to find suitable techniques to assess the preservation status in order to ensure the authenticity of analytical data. The four most commonly applied parameters are histological preservation, protein content, crystallinity, and porosity described below. The analysis of Rare Earth Elements (REE) in bone provides another powerful tool to determine the extent of diagenesis, and will be briefly discussed at the end of this chapter.

4.2.1.1 Visual examination

The first approach to examine signs of diagenetic changes is usually done by visual examination of skeletal material. Destructive traces also referred to as "bioerosion" or "microscopical focal destructions" (MFD) first described by

Hackett (Hackett, 1981) are caused by microbial attack and can be observed as tunnels or borings. The dimension of Wedl-type tunnels range from 5-10 μm in diameter, which correlates with the size of microbial infiltration (Bell, 1990). Wedl tunnels are visible as an interconnected branched network of tunnels under the electron microscope (Jans et al., 2004, Trueman and Martill, 2002). MFD appears in three forms, 1) linear longitudinal, 2) budded and 3) lamellate (Hackett, 1981) and are generally assumed to stem from bacteria and fungi. In order to cause MFD, it has been suggested that these microorganisms must have the ability to initially dissolve the mineral matrix as well as to metabolize collagen (Trueman and Martill, 2002).

4.2.1.2 Histology index

During the first stage in diagenesis bone is subject to microbial attack from bacteria, fungi and protozoa resulting in the histological destruction of the matrix as described in the previous section. Hedges et al. (Hedges, 1995) introduced a histological destruction index (the Oxford Histology Index, OHI) as a means to assess histological preservation. It consists of a simple graded system of 0 (no original histological features) to 5 (<5 % affected, which is equal to that of fresh bone).

4.2.1.3 Protein content

Collagen is the most abundant protein in bone and about 95 % of the nitrogen in bone derives from collagen (Collins et al., 2002). Post-mortem the collagen content in bone deteriorates with time under normal burial conditions, which correlates with the loss of nitrogen. A method to assess the preservation of bone accurately is to measure the nitrogen content in powdered whole bone provided that more than 10 % of collagen is still present (Nielsen-Marsh and Hedges, 2000).

4.2.1.4 Crystallinity

Crystallinity is regularly applied as a method to screen for diagenetic changes in the bone mineral. It can be measured using either X-Ray Diffraction (XRD) or Fourier Transform Infra-red Spectroscopy (FTIR). The extent of crystallinity can be determined by calculating the crystallinity index (CI), which can derive from either the infrared splitting factor (IRSF), or from X-ray diffractometry (Shemesh, 1990). The CI reflects a combination of the relative sizes of the crystals and the extent of the crystal order in the lattice (Weiner and Bar-Yosef, 1990). Samples exhibiting a CI above that for fresh bone (2.8-3.1) (Weiner and Bar-Yosef, 1990) are likely to have experienced diagenetic alterations. Evidence for the validity of the FTIR and XRD screening method for diagenetic modifications has come from numerous studies (Hedges, 1995, Lee-Thorp and Sponheimer, 2003, Grunenwald et al., 2014, Schwarz et al., 2009, Nielsen-Marsh and Hedges, 2000, Trueman et al., 2004). Trueman and colleagues (Trueman et al., 2008b), however, tested the validity of this conventional method and noticed that in older bones where recrystallization has been completed, i.e. ion exchange between bone mineral and pore waters no longer persists, the CI of bone is not a reliable measure any longer. Once the bone becomes a closed system, the CI between altered and pristine mineral is virtually indistinguishable. In other words, the CI is only informative as long crystal growth and recrystallization in bone is incomplete.

4.2.1.5 Rare Earth Element (REE) analysis in bone diagenesis

Rare earth elements (REE), also known as rare earth metals, are a group of seventeen elements in the periodic table; fifteen of these elements comprise the lanthanide series, which includes atomic numbers 57 through 71 (La, Ce, Pr, Nd, Pm, Sm, Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu). Despite their name, REE are abundant in the earths crust (Table 5) but not so much in biological tissue such as bone and teeth.

Rare Earth Element	Symbol	Atomic number	Abundance (ppm)
Lanthanum	La	57	30
Cerium	Ce	58	64
Praseodymium	Pr	59	7.1
Neodymium	Nd	60	26
Promethium	Pm	61	n/a
Samarium	Sm	62	4.5
Europium	Eu	63	0.88
Gadolinium	Gd	64	3.8
Terbium	Tb	65	0.64
Dysprosium	Dy	66	3.5
Holmium	Ho	67	0.8
Erbium	Er	68	2.3
Thulium	Tm	69	0.33
Ytterbium	Yb	70	2.2
Lutetium	Lu	71	0.32

Table 5: Relative abundances of REE in the earth's crust (Taylor and McClennan, 1985)

The total REE content in living bioapatite is usually very low with approximately ~100 ppb (Trueman and Tuross, 2002). Post-mortem, however, concentrations can increase by 3–5 orders in magnitude over time (Trueman and Tuross, 2002, Kocsis et al., 2010) due to the influx of REE from the surrounding environment into the bone/enamel matrix. During the fossilization process the growth of secondary apatite (i.e. recrystallization) is driven by the negatively charged apatite surface, which attracts and incorporates positively charged REE and other elements from pore water into the Ca sites in the apatite lattice.

It has been widely acknowledged that the uptake of REE into skeletal material occurs during early diagenesis and that the REE composition captured at that time reflects that of the burial environment. Hence the characteristics of different REE signatures reflected in bone have been exploited as a tracing tool for origin in numerous paleoenvironmental (Kemp and Trueman, 2003, Kocsis et al., 2009, Elderfield and Pagett, 1986), taphonomic (Trueman, 1999, Trueman et al., 2003, Suarez et al., 2010) and provenance studies (Trueman et al., 2006, MacFadden and Hulbert Jr, 2009, Staron et al., 2001, Tütken et al., 2011).

Generally, fossil bone is highly enriched with elements such as Yttrium, Rare Earth Elements (REE), Uranium and Fluor (Trueman and Tuross, 2002) and measuring these elements in bioapatite provides information on the degree of diagenesis present in a sample (Kohn et al., 1999). Some other trace elements in fossils (Na, Mg, Cl, K, Zn, Sr) are less informative as they have shown similar concentrations in modern bone (Trueman and Tuross, 2002).

Recent studies applied high spatial resolution LA-ICP-MS methods to investigate the intra-bone REE variability of fossil bones from a wide range of diagenetic settings and geological time periods (Hinz and Kohn, 2010, Suarez et al., 2007, Herwartz et al., 2011, Herwartz et al., 2013, Trueman et al., 2011). Herwartz *et al.* (Herwartz et al., 2013) analysed fossil bones (n=54) from 25 locations ranging from Early Triassic to early medieval time periods and noted a high variability in terms of REE fractionation not only between individual sites but also within a single bone. The highest REE concentrations in all samples analysed were always located at the outer rim of the cortex while the gradient towards the central cortex varied greatly between samples and resulted in the depletion of light and middle REE in the inner cortex.

REE fractionation is dependent both on the physiochemical properties of pore waters and on the structural characteristics of bone. The complexity of the process itself, however, is still poorly understood. Nonetheless, the REE composition and concentration deposited in bone is most useful as a proxy for post-mortem bone crystal-pore water interaction and as such often valuable in

determining the extent of diagenetic changes, which is most important in isotopic provenancing studies.

Chapter 5

5 Materials and Methods

5.1 Introduction

This chapter describes "Materials and Methods" for both isotope and DNA analysis.

In the first part the procedure for strontium and lead isotope analysis will be discussed including any sample preparation that was largely identical for both isotope and DNA work. The aims of the isotope study were a) to investigate the feasibility to use bone/teeth powder residues from DNA extraction for subsequent isotope analysis of strontium and lead, b) the development of a new protocol which allows for sequential separation of both strontium and lead from bone/enamel material using the Eichrom™ lead resin, and c) to investigate the isotope results in context of the group of human remains found in the mass grave in Villanueva del Rosario in Southern Spain.

The second part is dedicated to the analytical steps of DNA analysis. It will briefly introduce the general methodology followed by the specific techniques applied in this study. Different extraction protocols for ancient/degraded DNA are investigated for their effectiveness on the Spanish and Norfolk bone and teeth samples and the resulting outcomes presented.

5.2 Strontium and lead isotope analysis

5.2.1 Introduction

Sr and Pb isotope systematics have been extensively applied in geochronological studies (Faure, 1986) but often also used in combination in archaeological traceability and provenance studies (Aberg et al., 1998, Budd et al., 2001, Montgomery et al., 2000, Montgomery et al., 2005a, Font et al., 2012), or ancient artefacts (Degryse and Shortland, 2009). The combination of both Sr and Pb isotopic analysis in such studies provides a means for associating samples both with the geological and environmental (eg. lead pollution) background found in biological matrices.

The quality of Sr and Pb isotope data analysis by multiple collector mass spectrometry (MC-ICP-MS) depends to a high degree on the purity of Sr and Pb. Since MC-ICP-MS instruments separate ions by their mass-to-charge ratio, analysing elements of the same mass will result in isobaric interferences. As ^{87}Sr is the product of beta-decay of ^{87}Rb, both rubidium and strontium are present, which can lead to isobaric interference of ^{87}Rb on the ^{87}Sr signal. Isobaric interference can also result from Ca dimer ions ($^{40}Ca^{44}Ca^{+}$, $^{42}Ca^{2+}$, $^{40}Ca^{46}Ca^{+}$, $^{42}Ca^{44}Ca+$, $^{43}Ca2^{+}$, $^{40}Ca^{48}Ca^{+}$, $^{42}Ca^{46}Ca^{+}$, $^{44}Ca^{2+}$) and $ArCa^{+}$ molecular ions ($^{36}Ar^{48}Ca^{+}$, $^{38}Ar^{46}Ca^{+}$, $^{40}Ar^{44}Ca^{+}$, $^{38}Ar^{48}Ca^{+}$, $^{40}Ar^{46}Ca^{+}$, $^{40}Ar^{48}Ca^{+}$) on Sr ($^{84}Sr^{+}$, $^{86}Sr^{+}$ and $^{88}Sr^{+}$) signals (De Muynck et al., 2009). To prevent isobaric interference of Rb and Ca ions on Sr an efficient separation method for Sr is therefore essential for MC-ICP-MS analysis.

The most common separation method for Sr is cation exchange chromatography on resin AG 50W-X8 using HCl as eluent. While this resin is efficiently retaining Sr, the removal of the interfering Ca can be compromised particularly if present in higher concentrations (Slovak and Paytan, 2011).

The commercially available crown ether-based Sr-specific and Pb-specific resins (Eichrom™), respectively, are also frequently used for separating Sr and Pb for isotope analysis by mass spectrometry. Several methods have been

reported where Sr and Pb were sequentially extracted from geological and environmental samples using just the Sr specific resin (Deniel and Pin, 2001), (Makishima et al., 2008, Thériault and Davis, 2000, Smet et al., 2010) but to the best of the author's knowledge, no one has attempted the use of the Pb-specific resin for the sequential purification of both Sr and Pb so far.

Although the Sr-spec resin is very similar to the Pb spec resin the latter has the advantage of having a very low retention factor for Ca, which is abundantly present in the bone/teeth matrix (Horwitz et al., 1994). Ca as well as Na have hardly any effect on the retention of Pb at molarities <1 M HNO_3. Hence it was speculated that the Pb-spec resin would be better suited for the specific application in this study.

In combination with the Pb resin different mobile phases at various concentrations have been proposed for the separation of Sr and Pb from interfering ions (eg. Rb, K and Ca) such as nitric and hydrochloric acid amongst other acidic media. The most useful eluent for Pb is ammonium oxalate as it strips Pb off more efficiently and is also easily removed via sublimation once eluted samples are evaporated to dryness (Horwitz et al., 1994).

Sr has a maximum capacity factor (k') of approximately 90 at a nitric acid concentrations range of 5 M to 8 M HNO_3 on the Pb resin while Rb shows very low affinity (k'<1) and peaks in the low concentration range around 0.5 to 1.5 M HNO_3. Lead has the highest retention (k'>100) on the Pb resin across a very wide range of nitric acid concentrations (0.1 M to 10 M) and other acidic media. Considering the different elution behaviours of Sr, Rb and Pb on the Pb-specific resin it was speculated that an efficient separation from the same column should be achievable by adjusting HNO_3 concentrations accordingly for each element.

5.3 Sample preparation for isotope and DNA analysis

Ancient sample material is usually contaminated from the burial site and/or the individuals handling the material during and/or after excavation. Before bone or teeth can be used for extraction the specimens have to be decontaminated either by mechanical (sanding) or chemical cleaning (bleach) or the combination of both and by additional UV-irradiation.

5.3.1 Precautions to prevent contamination of samples for isotopic studies

Due to the subtle differences in natural isotopic ratios, which can be as small as 1 part per 10,000, isotope work needs to be undertaken in clean labs with similar precautions as required for ancient and forensic DNA work in order to preserve the original geological fingerprint.

The greatest concern in isotopic studies is the contamination by environmental metal elements. Clean laboratory conditions and suitable equipment are required to achieve accurate results. The most important guidelines are listed below:

a) Protective suits, masks and gloves should be worn when handling samples

b) Only metal-free labware, tools and containers should be used

c) All labware, tools and containers used for isotope work need to be acid cleaned

d) Sample preparation and extractions should be carried out in Class 100 laminar flow hoods

For this study, all bone and teeth samples were prepared in a dedicated lab space within the facilities of the Centre for Forensic Provenancing at the School of

Chemical Sciences at UEA. The DNA analysis procedure itself was undertaken in isolated Class III clean lab spaces within the School of Biological Sciences at the University of East Anglia (UEA) and is discussed in detail in this chapter (Ancient DNA).

At any time during the preparation process only a single specimen of either bone or teeth was prepared for subsequent DNA/isotope analysis within a Dead Air Box (AirClean™). After each use all the working areas were intensely cleaned with bleach and UV-irradiated overnight before the next sample material was handled. Due to the time-consuming preparation process to ensure that cross-contamination or extraneous contamination could be excluded it took about 5 months before the samples from Norfolk (n=32) and Spain (n=21) were ready for DNA/isotope analysis.

5.3.2 Bone preparation

Bone (femur) samples were first cleaned with a disinfectant (5 % Trigene) to remove extraneous surface materials. This was followed by a thorough rinse with MilliQ water and 70 % ethanol (Sigma-Aldrich, Ethanol *BioUltra* for molecular biology). The bone was then placed in the Dead Air Box (AirClean™), UV irradiated for 15 minutes on each side and left to air-dry overnight.

The dry bone was sanded about approximately 2 mm deep using a single-use Dremel® coarse sanding band to remove surface contamination as far as possible. Subsequently, for further DNA analysis bone powder was collected from the interior part of the femur by drilling at low speed using a Dremel® drill bit. A diamond cutting wheel was used to attain a bone fragment for subsequent isotope analysis.

5.3.3 Teeth preparation

The entire tooth was sanded with a Dremel® coarse sanding band to remove extraneous surface contamination. The tooth was sonicated twice in DNA free water (Fisher Bioreagents) and once in absolute ethanol (Sigma-Aldrich, Ethanol *BioUltra* for molecular biology) for 15 minutes each time, placed in a hood under UV-light for 10 min and then left to air-dry overnight.

The following day the crown was cut circumferentially to a depth of approximately 1 mm and enamel edged off using a diamond-cutting wheel. Where necessary, the adhering dentin was removed from the enamel using a Dremel® Tungsten carbide cutter.

For subsequent isotope analysis, the enamel was placed into an agate container together with agate grinding balls and powdered in a Retsch® mixer mill (MM2000). The enamel was milled for approximately 20 to 30 minutes at a frequency of 90 Hz depending on the size of the enamel piece.

For DNA analysis, the remaining dentin was drilled using a low-speed dental drill (Osseo Scientific™, NJ, USA) to prevent DNA damage by overheating, and the powder collected. All subsequent steps of DNA analysis are discussed separately later on ("DNA analysis").

5.4 Extraction and Analysis of Sr and Pb from bone/enamel:

5.4.1 Extraction from bone and enamel

Approximately 100 mg of either the resulting bone or the enamel powder were placed in an acid cleaned Savillex™ Teflon 15 ml vial and digested in 1 ml distilled HNO_3 (16.0 M) and 0.5 ml Merck ultrapure hydrogen peroxide (H_2O_2) for approximately 12 hrs. The acid was evaporated on a hotplate for about 3 to 4 hrs and the residue re-dissolved in 0.5 ml 8.0 M HNO_3. As control, standard

reference material for bone meal (NIST 1486) was included along with all bone/teeth samples as well as triple blanks.

5.4.2 Extraction from soil

Approximately 20 g of soil were extracted by adding 50 ml of 1 mol/L ultrapure ammonium nitrate into a 250 ml PE bottle. The mixture was shaken for about 2 hours at normal laboratory temperatures (~22 °C) until all solid components were well suspended. The closed bottles were left to stand for one hour to allow the solids to settle. The supernatant was then filtered using a 0.45 µm membrane filter. The first part of the filtrate (~1 ml) was discarded and the remainder collected in a fresh bottle for storage. To stabilize the filtrate, ~10 µl high purity nitric acid was added per 1 ml filtrate.

5.5 Method development for sequential extraction of Sr and Pb on a Pb-specific Eichrom™ resin

Strontium (Sr) and lead (Pb) Eichrom™ resins are commonly used to extract Sr and Pb from various matrices such as soil, food and water for isotope ratio measurements by mass spectrometry. The extraction chromatographic material of both the Pb-resin and the Sr-resin consists of 4,4'(5')-di-t-butylcyclohexano-18-crown-6 ether (crown ether) but differ in concentrations and the diluents used. The Sr resin comprises 1.0 M crown ether in n-octanol while the Pb resin is made of 0.75 M crown ether in isodecanol that facilitates lead elution from the resin (Horwitz et al., 1994).

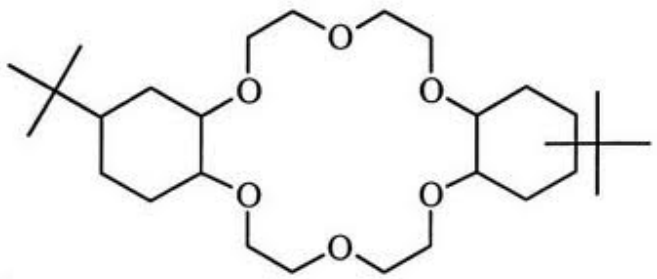

Figure 17: 4,4'(5')-di-t-butylcyclohexano-18-crown-6 (crown ether) used in the Sr and Pb resins.

A comprehensive study into the behaviour of different ions separated both on the Eichrom Sr and Pb resin at increasing concentrations of HNO_3 was published by Horwitz et al. (Horwitz, 1998, Horwitz et al., 1994) as shown in Figure 18 and Figure 19. It is shown that the Sr-resin has a strong selectivity not only for Sr itself but an even greater selectivity for Pb (Figure 18), which can prevent effective stripping of Pb from the Sr resin (Horwitz et al., 1994).

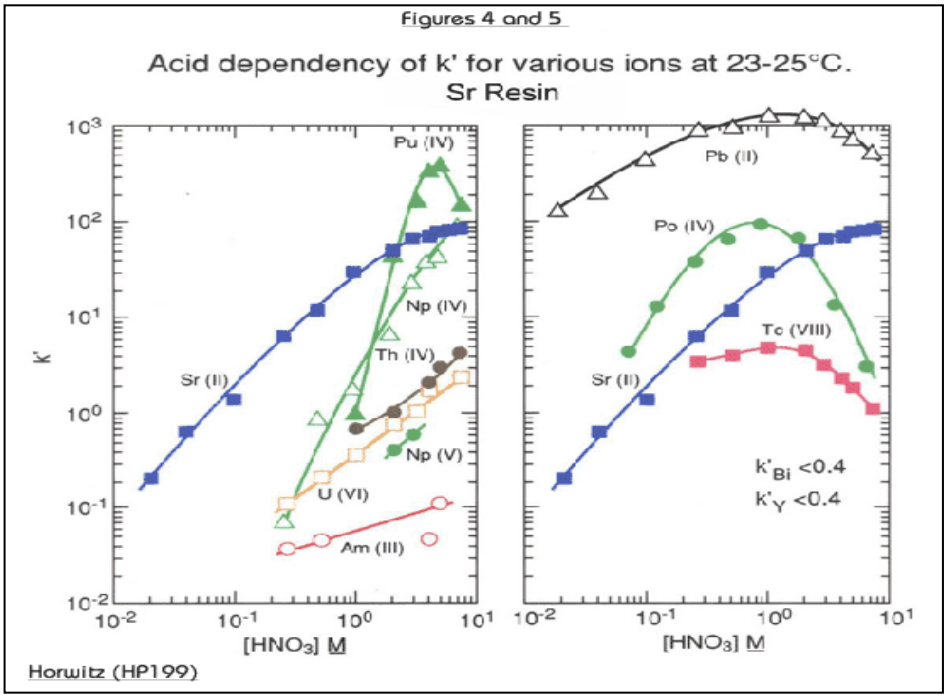

Figure 18: Elution behaviour for selected divalent metal ions on the Eichrom specific Sr resin (Source: (Horwitz, 1998)).

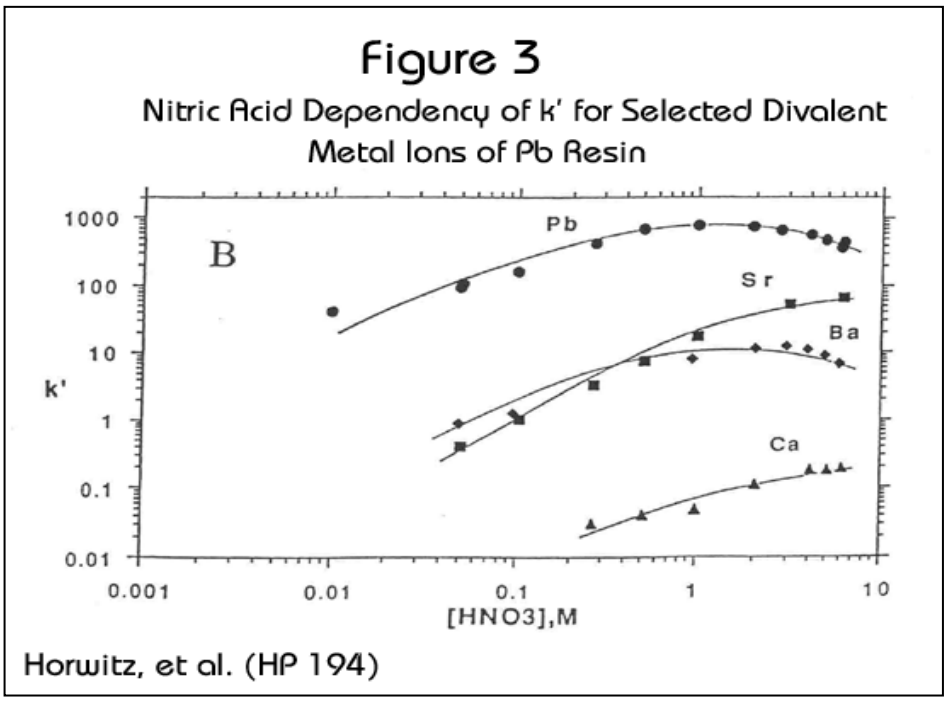

Figure 19: Elution behaviour for selected divalent metal ions on the Eichrom Pb-specific resin (Source: (Horwitz et al., 1994)).

Biological samples such as bone and teeth usually contain very low levels of Pb whereas Sr is much more abundant. Hence emphasis has been put on the optimal recovery of Pb by using the Pb-specific resin for this study. The Pb-resin is most selective for Pb at a concentration of approximately 1 M HNO_3 whereas Sr selectivity peaks at around 8 M HNO_3 (Figure 19). Based on the different elution behaviours of Sr and Pb with the Pb-resin and the more favourable conditions for recovering Pb the aim was to develop a method where both ions could be extracted from the Pb-resin in a sequential manner instead of using separate resins for extracting either Sr or Pb. For stripping Pb off the columns ammonium oxalate was chosen instead of using HNO_3. Ammonium oxalate emerged as the best eluent out of three tested eluents including HNO_3 and HCl in a study carried out by Horwitz et al. (Horwitz et al., 1994).

The following section describes the development of a sequential extraction method of Sr and Pb on the Pb-specific resin. To validate and optimise the extraction of Sr and Pb on the same column two protocols (A and B) were tested for this purpose and the results discussed at the end of this section.

A) 1st Protocol:

5.5.1 Sample preparation for validation of sequential extraction of Sr and Pb

To validate the sequential extraction procedure, standard reference material of bone meal (NIST SRM 1486) was selected for its similarity to bone and dental tissue along with reference materials for the isotopic composition of Sr (NIST SRM 987), Pb (NIST SRM 981) and Rb standard (CPI International's Peak Performance Rubidium standard).

All samples were handled in a vertical laminar flow hood (AirClean™ 4000) during the extraction procedure to eliminate contamination from extraneous sources.

5.5.2 NIST Sr/Pb/Rb and NIST bone meal sample preparation

2x samples, each made up from mixing 1.0 ml NIST SRM 987 Sr carbonate isotopic standard (37.7 ppm), 1.0 ml NIST SRM 981 $Pb(NO_3)_2$ isotopic standard (9.984 ppm) and 0.5 ml CPI International's Peak Performance Rubidium (Rb) standard which was diluted (0.1 ml Rb standard solution (1000 ppm) plus 9.9 ml 2% ultrapure HNO_3) before being added to the mix of Sr and Pb standards.

a) 2x NIST SRM 1486 bone meal standards (~100 mg)

   The samples were digested for approximately 12 hrs by adding 1.0 ml laboratory distilled HNO_3 and 0.5 ml ultrapure hydrogen peroxide (H_2O_2) to each sample/blank in a Savillex™ vial. The digest was then dried on a hotplate and the residue re-suspended in 5.0 M HNO_3 (0.5 ml).

5.5.3 Sequential extraction method for Sr and Pb on Pb-specific Eichrom™ resin:

Acid cleaned columns (5 mm diameter) were layered with glass wool followed by 450 µl Pb-specific Eichrom™ resin and another layer of glass wool on top.

The column was first washed with 5 ml 0.1 M HNO_3 to remove any contamination and then conditioned with 5 ml 5 M HNO_3. After loading the sample (0.5 ml sample in 5 M HNO_3) on the column, Rb was eluted twice using 1 ml 5.0 M HNO_3 each time and collected in two separate fractions (F1 and F2). Sr was eluted in a single fraction (F3) from column using 5 ml 1.0 M HNO_3. In the last two elution steps Pb was stripped off the column with 5 ml 0.05 M ($(NH_4)_2C_2O_4$) in each elution step (F4 and F5).

Eluted Sr and Pb solutions were then dried on a hotplate and the residue re-dissolved in 3 ml 2 % HNO_3.

All fractions were quantified on the Agilent 7500ce Q-ICP-MS to evaluate the purity of Sr and Pb stripped off the column.

5.5.4 Results for first protocol

The results of the measurements in the different fractions are summarized in Table 6. It must be noted upfront that the Rb concentration in NIST 1486 was close to detection limit resulting in deviations of >100 % (NIST 1486/1 of ~107 % and NIST 1486/2 of ~106 %).

The bulk of the total Rb content in all samples could be eliminated in the first two elution steps using 2x 1 ml 5.0 M HNO_3. In the other two reference samples (SrPbRb/1 and Sr/Pb/Rb/2) approximately 80 % and 83 %, respectively, of the Rb could be removed. Most of Rb was washed off in the second elution step indicating that it takes at least 2 ml of the eluent to strip off most of the Rb from the column.

In the third fraction between ~70 % and ~96 % of the Sr content could be recovered from all four samples analysed. Although most of the Sr was eluted off the column with 5 ml 1.0 M HNO_3 in fraction 3 there was still a considerable amount (~7 % and 15 %) left in the fourth eluent. In the last elution step the amount of Sr tailed off leaving between 0.08 % and 0.13 % in the Pb fraction. Although the recovery of Sr in fraction 3 is generally very high these results show an average loss of ~12 % of Sr to fractions 2 and 4.

In the case of Pb, most of the content was stripped off the column in step 4 (~92 % to 99 %) with the first 5 ml 0.05 M $(NH_4)_2C_2O_4$ leaving a remainder of <0.6 % in the final fraction.

Values for procedural blank duplicates were below detection limit for Rb and Pb, and for Sr 0.03 ng/ml.

Sample Name	Rb	Sr	Pb	Rb Recovery	Sr Recovery	Pb Recovery	Rb Recovery	Sr Recovery	Pb Recovery
	weighed in	weighed in	weighed in	ug	ug	ug	%	%	%
	ug	ug	ug						
NIST1486/1 - F1	0.03	26.87	0.14	0.00	0.01	0.00	0.00%	0.02%	0.06%
NIST1486/1 - F2	0.03	26.87	0.14	0.04	1.11	0.00	**107.08%**	4.12%	0.15%
NIST1486/1 - F3	0.03	26.87	0.14	0.00	25.74	0.00	0.00%	**95.80%**	0.18%
NIST1486/1 - F4	0.03	26.87	0.14	0.00	2.10	0.13	0.00%	7.81%	**99.07%**
NIST1486/1 - F5	0.03	26.87	0.14	0.00	0.02	0.00	0.00%	0.09%	0.48%
NIST1486/2 - F1	0.03	26.76	0.14	0.00	0.00	0.00	0.00%	0.01%	0.00%
NIST1486/2 - F2	0.03	26.76	0.14	0.04	0.68	0.00	**106.86%**	2.55%	0.16%
NIST1486/2 - F3	0.03	26.76	0.14	0.00	18.80	0.00	0.00%	**70.25%**	0.05%
NIST1486/2 - F4	0.03	26.76	0.14	0.00	1.96	0.12	0.00%	7.33%	**92.16%**
NIST1486/2 - F5	0.03	26.76	0.14	0.00	0.02	0.00	0.00%	0.08%	0.56%
SrPbRb/1 - F1	506.25	19.05	10.24	42.11	0.00	0.00	8.32%	1.80%	0.00%
SrPbRb/1 - F2	506.25	19.05	10.24	421.88	0.17	0.00	**83.34%**	0.89%	0.00%
SrPbRb/1 - F3	506.25	19.05	10.24	0.02	15.33	0.00	0.00%	**80.46%**	0.00%
SrPbRb/1 - F4	506.25	19.05	10.24	0.00	2.78	10.14	0.00%	14.60%	**98.96%**
SrPbRb/1 - F5	506.25	19.05	10.24	0.00	0.02	0.05	0.00%	0.13%	0.52%
SrPbRb/2 - F1	503.48	19.08	10.21	68.70	0.00	0.00	13.65%	0.01%	0.02%
SrPbRb/2 - F2	503.48	19.08	10.21	404.85	0.34	0.00	**80.41%**	1.77%	0.00%
SrPbRb/2 - F3	503.48	19.08	10.21	0.00	17.69	0.00	0.00%	**92.74%**	0.01%
SrPbRb/2 - F4	503.48	19.08	10.21	0.00	1.54	10.12	0.00%	8.05%	**99.12%**
SrPbRb/2 - F5	503.48	19.08	10.21	0.00	0.02	0.05	0.00%	0.11%	0.53%

Table 6: Recovered Rb, Sr and Pb content (ug and %) in each fraction of the two bone meal standards (NIST 1486) and Sr/Pb/Rb reference standards (NIST 987, NIST 981, Rb standard).

The percentage recovery of Rb, Sr and Pb from each of the five fractions is illustrated in Figure 20 below.

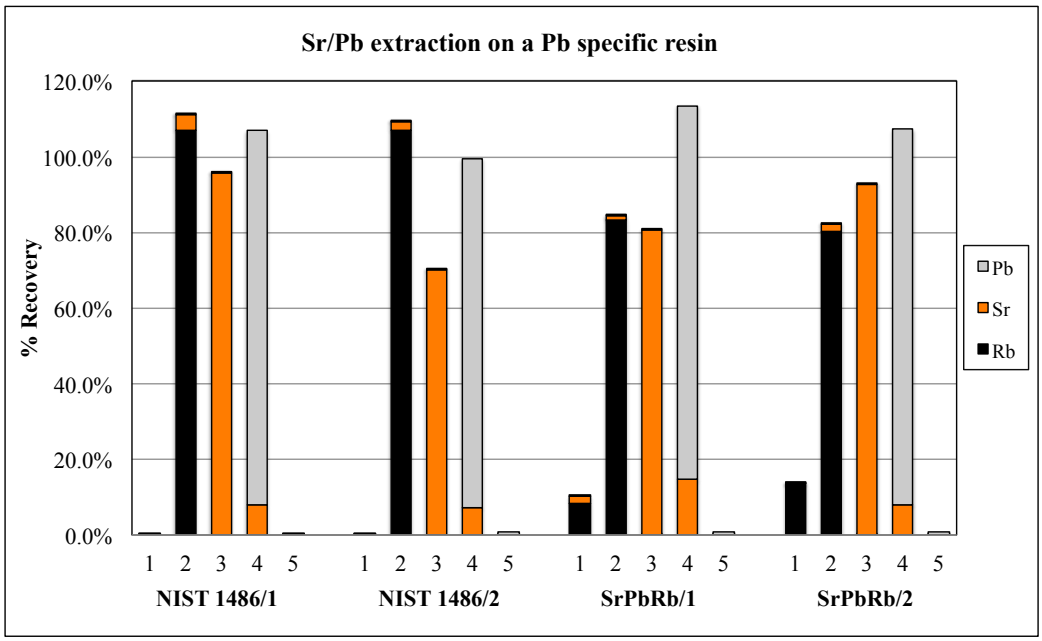

Figure 20: % recovery of Rb, Sr and Pb in each of the five fractions (F1=Rb, F2=Rb, F3=Sr, F4=Pb, F5=Pb) from the four reference samples (NIST 1486/1), NIST 1486/2, SrPbRb/1 and SrPbRb/2).

B) Modified 2nd Protocol:

Instead of using bone meal reference material and Sr/Pb/Rb standards as in the first protocol, here bone and enamel from three individuals (VR-01, VR-02, VR-03) from the Spanish Civil War collection were analysed for comparison.

Based on the first results the protocol was optimised in regard to elution volume, nitric acid concentration of elution medium and fractions collected. Firstly, the elution volume was increased to 8 ml for each elution step to ensure that most, if not all, of each relevant ion could be stripped off and collected as one fraction. Also, the nitric acid concentration was changed from 5.0 M to 8.0 M HNO_3 to verify if Rb could also be removed effectively at a higher nitric acid concentration.

5.5.5 Sample preparation

The samples (100 mg bone powder) were digested for approximately 12 hrs by adding 1.0 ml laboratory distilled HNO_3 and 0.5 ml ultrapure hydrogen peroxide (H_2O_2) to each sample/blank in a Savillex™ vial. The digest was then dried on a hotplate and the residue re-suspended in 8.0 M HNO_3 (0.5 ml).

5.5.6 Modified sequential extraction method

The column was first washed with 5 ml 0.1 M HNO_3 to remove any contamination and then conditioned with 5 ml 8 M HNO_3. After loading the sample (0.5 ml sample in 8.0 M HNO_3) on the column the first fraction (F1) was eluted with 8 ml 8.0 M HNO_3 to collect Rb. Sr was then eluted with 8 ml 1.0 M HNO_3 (fraction 2) and in the last fraction (F3) Pb was stripped off the column using 8 ml 0.05 M ammonium oxalate ($(NH_4)_2C_2O_4$).

After extraction, the Sr and Pb solutions were evaporated on a hotplate and the residues re-dissolved in 3 ml 2 % HNO_3.

All fractions were quantified on the Agilent 7500ce Q-ICP-MS to evaluate the purity of Sr and Pb stripped off the column.

5.5.7 Results for modified protocol

Most of the Rb content was removed with the first elution step using 8 ml 8.0 M HNO_3. The results for Rb displayed in Table 7 show that across all samples taken from three individuals (VR-01, VR-02 and VR-03) the Rb percentage removed in the first fraction was the highest (74.6 % to 92.9 %), however, values vary significantly between bone and teeth. The recovery of Rb from enamel is generally higher than from bone with values ranging from 87.1 % to 94.5 % for enamel across all individuals while for bone values span the region from 74.6 % to 78.5 % in fraction 1.

In contrast, the bulk Sr content recovered in the second elution step was generally higher for bone than enamel. Between 83.2 % and 96.5 % Sr could be recovered for bone and for enamel the values varied between 82.4 % and 91.0 % eluted in fraction 2.

In the final elution step 98.9 % to 99.8 % of Pb was collected using 8 ml 0.05 M $(NH_4)_2C_2O_4$. The recovery of Pb in fraction 3 does not display any significant variations looking across both sample types and all three individuals analysed.

The Rb content in the Sr fraction (F2) lies between <0.1 % and 7.0 % over all individuals and both sample types. Analogous to the Sr fraction, values in bone samples are higher (5.3 % and 7.0 %) than in enamel (1.9 % and 2.5 %) in two individuals (VR-01 and VR-02). For individual VR-03 the separation between Rb and Sr could be efficiently completed with values below detection limit for both bone and teeth in this fraction (F2).

Values for procedural blank duplicates were below detection limit for Rb and Pb and the mean Sr blank 0.22 ng/ml (n=2).

Sample	Fraction	Rb (ng)	Rb	Sr (ng)	Sr	Pb (ng)	Pb
VR-01-Bone F1	1	2.65	**78.5%**	191	2.4%	1.03	0.1%
VR-01-Bone F2	2	0.24	7%	7716	**96.5%**	0.47	0.1%
VR-01-Bone F3	3	0.49	14.5%	91	1.1%	846	**99.8%**
VR-01-Enamel F1	1	5.15	**91.3%**	843	12.9%	1.06	0.3%
VR-01-Enamel F2	2	0.10	1.9%	5622	**86.3%**	0.61	0.2%
VR-01-Enamel F3	3	0.38	6.8%	52	0.8%	323	**99.5%**
VR-02-Bone F1	1	1.58	**74.6%**	332	5.6%	1.05	0.1%
VR-02-Bone F2	2	0.11	5.3%	5531	**93.1%**	0.44	0.1%
VR-02-Bone F3	3	0.43	20.2%	80	1.3%	745	**99.8%**
VR-02-Enamel F1	1	4.33	**87.1%**	469	7.8%	0.97	0.3%
VR-02-Enamel F2	2	0.13	2.5%	5484	**91%**	0.67	0.2%
VR-02-Enamel F3	3	0.51	10.3%	76	1.3%	315	**99.5%**
VR-03-Bone F1	1	1.28	**73.6%**	943	15.4%	0.93	0.3%
VR-03-Bone F2	2	<DL	< 0.1%	5087	**83.2%**	0.31	0.1%
VR-03-Bone F3	3	0.46	26.4%	86	1.4%	266	**99.5%**
VR-03-Enamel F1	1	5.74	**92.9%**	641	15.9%	0.89	0.7%
VR-03-Enamel F2	2	<DL	<0.1 %	3314	**82.4%**	0.47	0.4%
VR-03-Enamel F3	3	0.44	7.1%	66	1.6%	122	**98.9%**

Table 7: Recovered Rb, Sr and Pb content (ng) in each fraction of the Spanish bone/teeth samples analysed (F1=Rb, F2=Sr, F3=Pb)

The points of separation during the elution steps and the percentage recovery for Rb, Sr and Pb are illustrated in Figure 21.

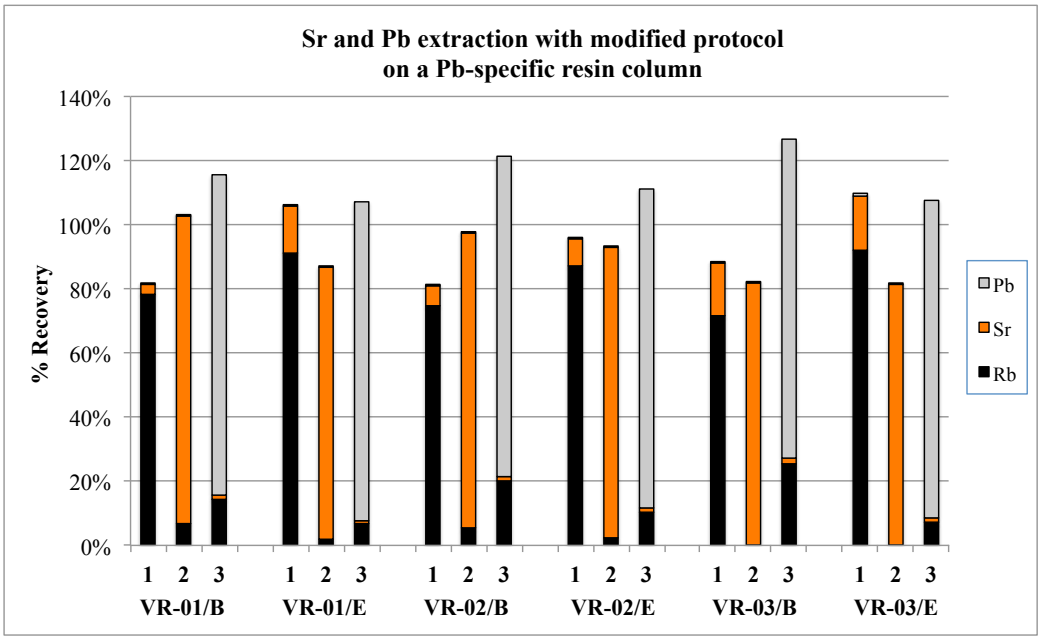

Figure 21: % recovery of Rb, Sr and Pb in each of the three fractions of Spanish bone/teeth samples (VR-01/B, VR-01/E, VR-02/B, VR-02/E,VR-03/B, VR-03/E) using modified protocol. “B”=Bone, “E”=Enamel

The table below shows the complete elemental data set from the analysis of these three Spanish samples.

Sample Name	Na	Mg	Al	K	Ca	Sc	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	Ga	As	Se	Rb	Sr	Y	Zr	Mo	Ag	Cd
VR-01-Bone F1	9.E+04	4.E+04	233	1347	1.E+07	2.6	6234	123	125	747	979	17	57	176	3714	9.4	7.4	13.3	0.88	64	0.78	2.68	8.76	0.35	0.21
VR-01-Bone F2	2.E+02	5.E+01	54	-19	1.E+04	0.3	49	0.1	0.2	5.2	7.3	0.2	2.2	0.2	28	0.10	0.6	0.9	0.08	2572	0.03	0.04	0.11	0.11	-0.03
VR-01-Bone F3	3.E+01	3.E+01	7	-19	9.E+02	0.6	2.3	0.1	0.6	0.9	10.8	-0.02	1.2	0.4	6	0.03	0.5	-0.2	0.16	30	0.01	0.15	0.11	-0.06	-0.03
VR-01-Enamel F1	2.E+05	1.E+05	84	5038	1.E+07	2.8	7904	1.6	33	64	688	18	35	31	3815	12.4	1.2	5.9	1.72	281	0.46	0.51	4.88	0.47	0.13
VR-01-Enamel F2	7.E+01	2.E+01	18	-19	5.E+03	0.2	3.8	0.1	0.05	1.2	2.0	-0.02	1.9	0.1	11	0.01	0.6	0.5	0.03	1874	0.04	-0.02	0.05	0.12	-0.03
VR-01-Enamel F3	2.E+01	2.E+01	8	-19	8.E+02	0.6	1.8	0.1	0.5	0.7	13.6	0.03	1.4	0.4	6	0.02	0.5	-0.2	0.13	17	0.02	0.06	0.08	-0.06	-0.03
VR-02-Bone F1	1.E+05	4.E+04	245	1560	1.E+07	3.1	6782	6.4	85	59	1240	17	71	258	3571	10.0	5.3	8.6	0.53	110	2.01	14.6	6.36	0.36	0.22
VR-02-Bone F2	6.E+01	1.E+01	21	-19	2.E+03	0.1	3.3	0.0	0.3	0.9	1.7	-0.02	2.0	0.1	5	0.01	0.6	-0.2	0.04	1844	0.02	0.04	0.05	-0.06	-0.03
VR-02-Bone F3	2.E+01	3.E+01	9	-19	1.E+03	0.7	2.4	0.0	0.6	1.0	9.4	-0.02	1.1	0.5	6	0.03	0.7	0.3	0.14	27	0.02	0.12	0.07	-0.06	-0.03
VR-02-Enamel F1	2.E+05	9.E+04	82	5623	1.E+07	3.0	7915	1.3	14	202	595	18	32	18	4302	12.4	1.5	4.9	1.44	156	0.32	0.37	5.50	0.44	0.35
VR-02-Enamel F2	1.E+02	2.E+01	20	-19	4.E+03	0.2	3.1	0.1	0.1	1.1	2.4	-0.02	1.8	0.1	7	0.02	0.5	-0.2	0.04	1828	0.03	0.03	0.31	0.09	-0.03
VR-02-Enamel F3	3.E+01	3.E+01	11	-19	1.E+03	0.6	2.2	0.1	0.3	1.0	6.0	-0.02	1.2	0.4	7	0.03	0.8	0.8	0.17	25	0.01	0.06	0.04	-0.06	-0.03
VR-03-Bone F1	9.E+04	4.E+04	148	1528	1.E+07	2.8	7248	15	71	44	747	18	54	74	2578	11.3	4.7	8.1	0.43	314	0.11	0.44	6.42	0.45	0.16
VR-03-Bone F2	4.E+01	1.E+01	13	-19	2.E+03	0.1	2.5	0.05	0.1	0.7	2.1	-0.02	1.6	0.1	4	0.01	0.5	-0.2	-0.03	1696	0.02	0.02	0.06	-0.06	-0.03
VR-03-Bone F3	2.E+01	3.E+01	8	-19	2.E+03	0.8	2.1	0.1	0.8	1.1	7.2	-0.02	1.2	0.4	6	0.02	0.9	0.8	0.15	29	0.02	0.05	0.11	-0.06	-0.03
VR-03-Enamel F1	2.E+05	8.E+04	253	5164	1.E+07	3.0	7970	1.8	7.5	113.3	707	19	26	11	3802	12.8	1.4	5.4	1.91	214	0.33	0.26	3.03	0.23	0.15
VR-03-Enamel F2	4.E+01	1.E+01	13	-19	2.E+03	0.2	2.4	0.1	0.6	1.0	2.4	-0.02	1.9	0.05	6	0.01	0.5	-0.2	-0.03	1105	0.02	-0.02	0.08	-0.06	-0.03
VR-03-Enamel F3	2.E+01	2.E+01	7	-19	1.E+03	0.7	1.8	0.1	0.5	0.9	8.7	-0.02	1.1	0.4	8	0.02	1.1	1.0	0.15	22	0.01	0.05	0.06	-0.06	-0.03

Sample Name	Sn	Sb	Te	Cs	Ba	La	Ce	Pr	Nd	Sm	Eu	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu	Hg	Tl	Pb	Bi	Th	U
VR-01-Bone F1	12.6	9.98	0.9	0.04	2818	0.39	1.42	0.10	0.41	0.13	0.664	0.15	0.033	0.16	0.032	0.099	0.016	0.10	0.021	47	0.08	0.34	0.161	0.141	1.272
VR-01-Bone F2	1.01	0.15	-0.3	0.01	15	0.06	0.61	0.01	0.04	0.01	0.004	-0.02	-0.001	-0.01	0.001	-0.002	-0.001	-0.01	-0.004	23	0.01	0.16	-0.003	0.008	0.020
VR-01-Bone F3	0.27	-0.01	-0.3	0.04	0.7	0.55	4.69	0.05	0.16	0.01	0.003	0.05	-0.001	-0.01	-0.001	0.004	-0.001	-0.01	-0.004	18	0.02	282	-0.003	0.004	-0.005
VR-01-Enamel F1	6.91	0.21	-0.3	0.02	79	0.48	2.08	0.10	0.43	0.09	0.039	0.11	0.026	0.08	0.014	0.045	0.007	0.03	0.011	60	0.06	0.35	0.122	0.038	0.184
VR-01-Enamel F2	1.16	0.01	-0.3	0.01	0.8	0.05	0.41	0.01	0.03	0.02	0.002	-0.02	-0.001	-0.01	0.001	-0.002	-0.001	0.01	-0.004	-15	-0.01	0.20	-0.003	0.006	0.008
VR-01-Enamel F3	0.25	-0.01	-0.3	0.03	0.4	0.38	2.86	0.04	0.08	0.01	0.002	0.02	0.002	-0.01	-0.001	0.002	0.002	-0.01	-0.004	-15	0.01	108	-0.003	-0.003	-0.005
VR-02-Bone F1	27.8	3.85	-0.3	0.02	670	2.93	6.97	0.66	2.41	0.45	0.261	0.44	0.073	0.38	0.076	0.225	0.034	0.22	0.033	58	0.03	0.35	0.025	2.286	0.537
VR-02-Bone F2	0.95	-0.01	-0.3	0.00	2	0.05	0.46	0.01	0.02	0.01	0.003	-0.02	0.002	-0.01	-0.001	0.004	-0.001	-0.01	-0.004	18	-0.01	0.15	-0.003	0.008	0.011
VR-02-Bone F3	0.27	-0.01	-0.3	0.03	1	0.49	3.80	0.04	0.12	0.01	0.003	0.03	-0.001	-0.01	0.001	0.003	-0.001	-0.01	-0.004	-15	0.02	248	-0.003	0.007	-0.005
VR-02-Enamel F1	6.01	0.30	-0.3	0.02	112	0.41	1.83	0.10	0.36	0.07	0.045	0.08	0.022	0.07	0.014	0.037	0.006	0.03	0.011	32	0.05	0.32	0.024	0.040	0.152
VR-02-Enamel F2	1.22	0.01	-0.3	0.00	1	0.06	0.56	0.01	0.03	-0.01	0.003	-0.02	-0.001	-0.01	0.001	0.002	-0.001	-0.01	-0.004	-15	-0.01	0.22	-0.003	0.008	0.010
VR-02-Enamel F3	0.27	-0.01	-0.3	0.03	0.5	0.56	4.51	0.05	0.14	0.02	0.003	0.04	0.003	-0.01	0.001	-0.002	-0.001	-0.01	-0.004	-15	0.01	105	-0.003	0.003	-0.005
VR-03-Bone F1	8.74	2.91	0.3	0.02	1141	0.20	0.84	0.04	0.16	0.04	0.288	0.05	0.015	0.03	0.006	0.015	0.004	0.02	0.008	80	0.02	0.31	0.007	0.023	0.075
VR-03-Bone F2	0.96	0.01	-0.3	0.00	4	0.04	0.38	0.01	0.03	-0.01	0.003	-0.02	-0.001	-0.01	-0.001	0.002	-0.001	-0.01	-0.004	-15	-0.01	0.10	-0.003	0.005	0.005
VR-03-Bone F3	0.33	-0.01	-0.3	0.03	0.8	0.45	3.26	0.04	0.10	0.02	0.002	0.04	-0.001	-0.01	-0.001	-0.002	-0.001	-0.01	-0.004	-15	0.01	89	-0.003	0.003	-0.005
VR-03-Enamel F1	2.98	0.68	0.3	0.04	72	0.35	1.38	0.09	0.34	0.09	0.040	0.09	0.024	0.06	0.011	0.041	0.005	0.02	0.010	43	0.04	0.30	0.017	0.057	0.233
VR-03-Enamel F2	0.91	-0.01	-0.3	0.00	1	0.03	0.34	0.01	0.02	-0.01	0.003	-0.02	-0.001	-0.01	0.002	-0.002	-0.001	-0.01	-0.004	-15	-0.01	0.16	-0.003	0.004	0.007
VR-03-Enamel F3	0.19	-0.01	-0.3	0.03	0.3	0.42	2.81	0.03	0.10	-0.01	0.004	0.02	-0.001	-0.01	0.001	-0.002	-0.001	-0.01	-0.004	-15	0.01	41	-0.003	0.004	-0.005

Table 8: Measured multi-elemental values in ng/ml in fractions 1, 2 and 3 for the Spanish bone/enamel samples VR-01, VR-02 and VR-03. Negative values indicate detection limits.

5.6 Conclusion

In the first protocol the procedure was divided into five fractions to validate the points of separation of each element at the different elution steps. From the results shown in Figure 20 it is apparent that the first and fifth elution step contained only minimal elemental fractions. Based on the outcome of the first protocol the aim of the second protocol was to optimise the elution steps and collect the bulk of each element into one fraction. Changes to the first protocol included a) the increase of the elution volume to 8 ml in each step, b) increase of the molarity of HNO_3 from 5.0 M to 8.0 M in order to verify if Rb could also be removed at a higher molarity, and c) to reduce the collected fractions from five to three.

Although the results from the two protocols cannot be directly compared to each other as standard reference material was evaluated versus actual ancient biological samples, it is still evident that an efficient sequential separation of Sr and Pb on the Pb specific resin can be achieved. The purity of Sr obtained with the new method was generally high (82.4 % to 96.5 %) across both bone and enamel samples with Sr contents between 3314 ng and 7716 ng per 100 mg sample material (i.e. 33.14 ppm to 77.16 ppm in original sample). The bone Sr values are within a range of 50.88 ppm and 77.15 ppm which corresponds to normal Sr concentrations in human bone of 50 ppm to 500 ppm (Hoogewerff et al., 2001). In two enamel samples (VR-01 and VR-02) Sr concentrations were measured with 56.22 ppm and 54.83 ppm, respectively, and 33.14 ppm in individual VR-03. The first two samples are within the normal range while the latter was below the expected value of approximately 50 ppm.

In two samples (VR-03/Bone and VR-03/Enamel) Rb was below detection limit in the Sr fraction. In the remaining four samples an insignificant fraction of interfering Rb (< 0.24 ng Rb) overlapped with the Sr fraction. ^{87}Rb is a concern with Sr isotope analysis on the ICP-MS because of its identical mass to ^{87}Sr, which leads to isobaric interference during measurement. In this case, however,

the content of Rb in the Sr fraction was not significant compared with the consistent high yield of Sr in these samples (approximately 5,500 ng to 7,700 ng Sr per 100 mg sample) in the samples analysed. The high elemental Sr/Rb ratio between 28624 and 131084 of these samples indicates that the Sr fraction recovered from the column was very pure and insignificantly contaminated with Rb.

The Pb content in bone ranged between 266 ng to 846 ng and in enamel 122 ng to 323 ng per ~100 mg sample material could be recovered. The new method produced also a consistently high recovery of Pb with values between ~ 98 % to 99 % in bone as well as in enamel. A crossover of Rb between 6.8 % and 26.4 % of the total Rb content could be observed in the Pb fraction. However, because of the great differences in mass between these two elements mass dependent interference is not of concern when analysed by mass spectrometry ($^{85}Rb^{2+}$ = 170, $^{87}Rb^{2+}$ = 174. $^{85}Rb^{3+}$ = 255, $^{87}Rb^{3+}$ = 261 vs. ^{204}Pb, ^{206}Pb, ^{207}Pb, ^{208}Pb).

The sequential separation of multiple elements on a single column, as attempted in this study, involves a balancing act to achieve the best possible recovery for each element with the least effort. The result might not match single element extractions in terms of quantity and quality. Nevertheless, the purity and high content for both Sr and Pb achieved with this new method are well above expectation and sufficient for the purpose of isotope analysis by mass spectrometry, especially when considering that just a few ng of purified Sr are necessary for isotope analysis, and that an amount of just 0.4 to 1 µg of Sr can produce several high precision measurements (Capo et al., 1998).

In retrospect, however, it would have been important to measure also the Sr isotope ratio of NIST 1486 in each fraction in order to determine the amount of Sr fractionation of the method. In a future experiment the Sr isotope composition of the non-Sr fraction could provide the ultimate evidence of Sr isotope fractionation between the different fractions.

Extraction procedures are lengthy, time and cost consuming especially when a high sample number needs to be prepared. If different elements of interest have to be extracted separate columns need to be washed and conditioned followed by multiple elution steps to recover just a single purified element for subsequent isotope analysis. This newly developed protocol could be an efficient alternative to the traditionally used methods and suggests the suitability of the Pb-resin to recover efficiently both Sr and Pb in a sequential elution process halving the time and cost effectively.

5.7 Analytical methods for strontium and lead isotope ratio analysis

5.7.1 Determination of Rb, Sr and Pb concentration in the extracted eluent on the Agilent 7500ce ICP-MS

The extracted Sr and Pb samples were dried on a hotplate and re-dissolved in 3 ml 2 % HNO_3 as previously described.

For further analysis on the ICP-MS the samples (0.5 ml) were diluted with 2 % HNO_3 containing a 10 ppb internal standard (Rh, Ge and Pt) to a total volume of 5.0 ml.

Calibration standards were prepared using Merck (Darmstadt, Germany) multi element standard solution VI (batch 110580) in six dilutions (x100 – x20000 dilutions in 2 % HNO_3 plus internal standard). 3 aliquots of NIST SRM 1640 standard reference water (NIST, Gaithersburg, MD, USA) were analysed alongside as control material. Triple blanks were included in all measurements.

5.7.2 Isotope ratio analysis on the Isoprobe MC-ICP-MS

Strontium and lead isotope ratios were measured on a GVI instruments Isoprobe Multi Collector - Inductively Coupled Plasma - Mass Spectrometer (MC-ICP-MS). Isotopic Sr ratios were corrected for mass fractionation by normalisation to the stable $^{88}Sr/^{86}Sr$ ratio 0.1194. Pb was corrected for mass bias using a bracketing standard calibration method and the geometric mean of the bracketing standards and NIST 981 values. Instrumental precision was determined by repeated NIST SRM 987 (Sr standard) and NIST SRM 981 (Pb standard) analysis, respectively.

Samples with strontium concentration of >200 ppb were adjusted to 200 ppb by adding 2 % HNO_3 to a total volume of 2.5 ml. The results were corrected using a 200 ppb NIST SRM 987 $SrCO_3$ standard (NIST, Gaithersburg, MD, USA).

Samples for lead analysis were diluted with 2 % HNO_3 to Pb concentrations below ≤200 ppb (100 ppb, 50 ppb, 20 ppb, 10 ppb) depending on the amount quantified for each sample. Corresponding concentrations of NIST SRM 981 lead standard (NIST, Gaithersburg, MD, USA) were used to correct for instrumental mass fractionation.

5.8 Investigating the feasibility of using DNA extraction residues from bone for subsequent Sr/Pb isotope analysis

Isotopic profiling from human remains aims to determine geographical origin and/or migration of individuals while DNA profiling provides information on ethnicity and kinship. Using isotopes in combination with DNA analysis can complement each other and provide further valuable information on individuals. To facilitate the simultaneous retrieval of both isotopic and genetic information this part of the project investigated the feasibility of using the bone powder residue resulting from DNA extraction for subsequent Sr/Pb isotope analysis.

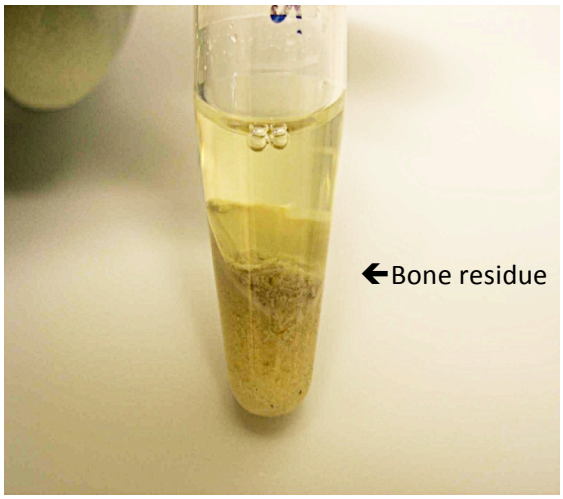

Figure 22: Bone residue after DNA extraction

There were several reasons for developing a more efficient method for using the same sample material for isotopic and genetic profiling: first, when creating reference databases from irreplaceable museum material it is desirable or even mandatory to remove as little authentic material as possible to preserve valuable archaeological collections. Second, in forensic or archaeological casework often only minute fragments are found or are free from diagenetic effects. Splitting the

limited material available reduces the accuracy of the final results. Finally, in order to be able to retrieve both genetic and isotopic information from an archaeological/forensic specimen a partially merged sample preparation would save considerable sample preparation time and cost.

5.8.1 Preliminary analysis of DNA extraction reagents

DNA extraction buffers routinely used contain EDTA, Proteinase K and other additives depending on the quality of the sample material extracted. In a preliminary analysis Sr and Pb concentrations of the reagents used in the extractions of this study (EDTA, Proteinase K and Sarkosyl) were determined to establish to which extent the inherent Sr/Pb concentrations of these components influence/contaminate the final concentrations of the extracted bone/teeth residues.

5.8.1.1 Sr/Pb Analysis of DNA extraction buffer components:

Sample preparation of DNA extraction reagents (EDTA, Proteinase K, Sarkosyl):

EDTA (100 µl) (Ambion®), Proteinase K (100 µl) (Invitrogen™) and Sarkosyl (100 µl) (Sigma Aldrich®) were placed in an acid cleaned Savillex™ Teflon vial and digested in 1 ml ultrapure HNO_3 (16.0 M) and 0.5 ml Ultrapure H_2O_2 for ~12 hrs. The acid was evaporated on a hotplate for ~3-4 hrs and the residue re-dissolved in 2.0 ml 1.0 M HNO_3.

Dilutions (x1000 and x10000 including internal standard) of all 3 reagents (EDTA, Proteinase K, Sarkosyl) were prepared and analysed for their Sr/Pb concentration on the Agilent 7500ce ICP-MS. NIST SRM 1640 water reference standards and triple blanks were also included in the measurements.

Sample preparation for ICP-MS quantification:

EDTA, Proteinase K and Sarkosyl samples were diluted (x1000 and x10000) with 2 % HNO_3 containing a 10 ppb internal standard (Rh, Ge and Pt) to a total volume of 10.0 ml.

Calibration standards were prepared using Merck (Darmstadt, Germany) multi element standard solution VI (110580) in six dilutions (x100 - x20000 dilutions in 2 % HNO_3 plus internal standard). Three aliquots of NIST SRM 1640 standard reference water (NIST, Gaithersburg, MD, USA) were analysed alongside as reference material. Triple blanks were included in all measurements.

5.8.2 Results

The extraction buffer consisting of 2.8 ml EDTA, 0.1 ml Proteinase K and 0.1 ml Sarkosyl contains a total of 0.41 µg/3ml (0.136 ppm) Sr and 0.01 µg/3ml Pb (0.002 ppm) (Table 9).

DNA Extraction solution	**[Sr] ppm**	**[Pb] ppm**
EDTA	0.124	0.002
Proteinase K	0.004	0.000
Sarkosyl	0.008	0.000
TOTAL in extraction buffer (3 ml)	**0.136**	**0.002**

Table 9: Sr and Pb concentrations in the respective volumes used for DNA extraction

Mean Sr levels in normal bone have been reported with values between 50 ppm and 500 ppm (Beard and Johnson, 2000, Hoogewerff et al., 2001, Mays, 2003)

and mean Pb levels of 0.65 ppm (Gulson, 2008, Shafer et al., 2008) in uncontaminated bone. If a low mean Sr level of 50 ppm and 1 ppm Pb is taken as a lower limit in normal bone, and considering that 200 mg bone powder was used for DNA extraction the Sr content of the DNA extraction buffer accounts for 4 % of the total Sr present in the bone residues measured. The Pb content of the reaction reagents results in 3 % of the total Pb content in the bone residues.

Although the Sr and Pb content in the three DNA extraction reagents is in a low range compared to normal Sr concentrations in bone and teeth and thus not very likely to interfere with isotope analysis, it would be worthwhile testing different manufacturers in the future and, if possible, use reagents containing even less Sr and Pb. Also, it might be useful to investigate more efficient methods to remove interfering ions from the reagents.

With the known Sr and Pb concentrations in the DNA extraction solution present the bone residues were analysed along with freshly ground bone and teeth for comparison.

5.8.3 Sr/Pb isotope analysis of fresh bone in comparison to bone residues resulting from DNA extraction

After DNA extraction bone powder residues are usually discarded and thus valuable sample material lost. The aim of this part of the study was to investigate the potential of these residues to be used for subsequent strontium and lead isotope analysis, respectively.

The flow-chart below (Figure 23) illustrates the process of sample preparation for subsequent DNA and isotope analysis in this study.

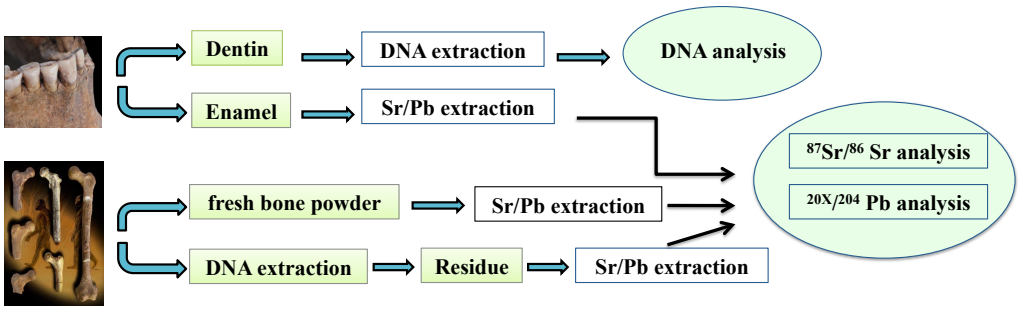

Figure 23: A flow chart showing the process of sample preparation for subsequent DNA/isotope analysis

Bone samples used for this study originate from five individuals excavated from a mass grave from the Spanish Civil War in Andalucía/Southern Spain.

Two sample types were prepared for isotope analysis: a) freshly pulverized bone and b) bone residue resulting from DNA extractions with EDTA, 20 % Sarkosyl and Proteinase K.

Approximately 100 mg of each sample type (except for bone residue from individual #1 where only 20 mg were available) were placed in an acid-cleaned Teflon vial and digested in ultrapure HNO_3 and H_2O_2 overnight. The dissolved sample material was then dried and subsequently suspended in 0.5 ml 8.0 M HNO_3.

Both Sr and Pb were separated on a single column using a Pb-specific Eichrom resin and analysed as described previously in section 5.5.

5.8.4 Results

The following Table 10 shows the accumulated Sr and Pb data for fresh bone versus bone residues from five individuals.

Sample ID	Sample Type	$^{87}Sr/^{86}Sr$	NIST 987 Mean	2σ	$^{206}Pb/^{204}Pb$	$^{207}Pb/^{204}Pb$	$^{208}Pb/^{204}Pb$	NIST 981 Mean	2σ
VR-02	Bone	0.70884	0.71026	0.00005	18.27	15.61	38.37	38.39	0.12
VR-02	Bone Residue	0.70888	0.71025	0.00012	18.69	15.68	38.63	38.49	0.18
VR-05	Bone	0.70928	0.71026	0.00005	18.30	15.62	38.42	38.39	0.12
VR-05	Bone Residue	0.70930	0.71025	0.00012	18.35	15.67	38.53	38.49	0.18
VR-08	Bone	0.70910	0.71026	0.00005	18.30	15.63	38.42	38.39	0.12
VR-08	Bone Residue	0.70901	0.71025	0.00012	18.30	15.64	38.46	38.49	0.18
VR-09	Bone	0.70901	0.71026	0.00005	18.32	15.63	38.46	38.39	0.12
VR-09	Bone Residue	0.70897	0.71025	0.00012	18.32	15.63	38.46	38.49	0.18

Table 10: Accumulated Sr and Pb data for fresh bone vs. bone residues measured from five individuals.

$^{87}Sr/^{86}Sr$ Fresh Bone/Bone Residue

As shown in Figure 24, the Sr ratios for fresh test bones have values ranging from 0.70884 to 0.70928 (+/-0.00005, 2σ), and for bone residues resulting from DNA extraction from the same five individuals range between 0.70888 and 0.70930 (+/-0.00012, 2σ). By applying the t-test these isotope values show insignificant variation compared to the matching fresh bone ($p=0.33$). Four out of the five individuals (VR-02, VR-05, VR-09 and VR-11) have near identical Sr ratios for both bone and bone residue. For the one remaining individual VR-08 the Sr ratio between fresh bone and bone residue differs slightly more (by 0.00009) but is still within analytical error. All three procedural blanks were below detection limit (<0.18 ppb).

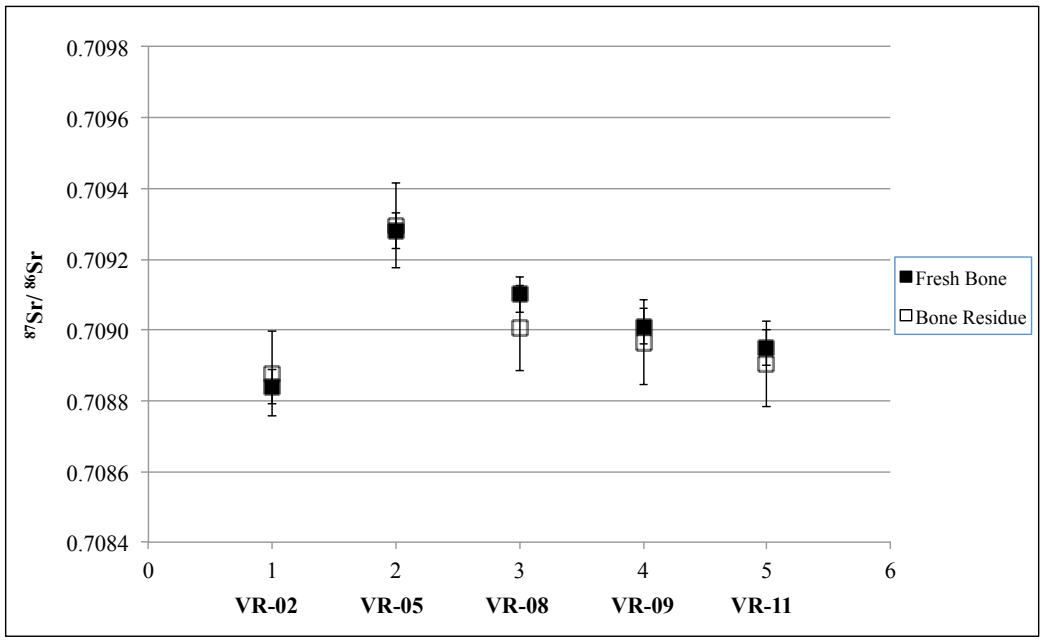

Figure 24: Comparison of $^{87}Sr/^{86}Sr$ isotope ratios between fresh bone and bone residues from DNA extraction of 5 individuals (VR-02, VR-05, VR-08, VR-09, VR-11) from Villanueva del Rosario/Spain. Error bars represent 2σ of standard reference material NIST 987.

$^{208}Pb/^{204}Pb$ Bone/Bone Residue

The $^{208}Pb/^{204}Pb$ values for the five fresh bone samples lie within a range of 38.37 and 38.46 and for bone residues 38.46 and 38.63 (Figure 25). All three procedural blanks for lead were below detection limit (<0.01 ppb).

With the exception of individual VR-09, where values for fresh bone and bone residue were identical, the data obtained from the other four samples vary between the two sample types but do still overlap within the analytical error margin. The Pb isotope ratios obtained for individual VR-02 shows the greatest deviation between fresh bone and bone residue. However, the Pb concentration in this sample was extremely low (0.01 ppm) which might have resulted in the greater variability of Pb isotope ratios than observed in the other four samples. Nevertheless, it seems that Pb isotope ratios from bone vs. bone residues show generally a greater variability compared to Sr isotope ratios.

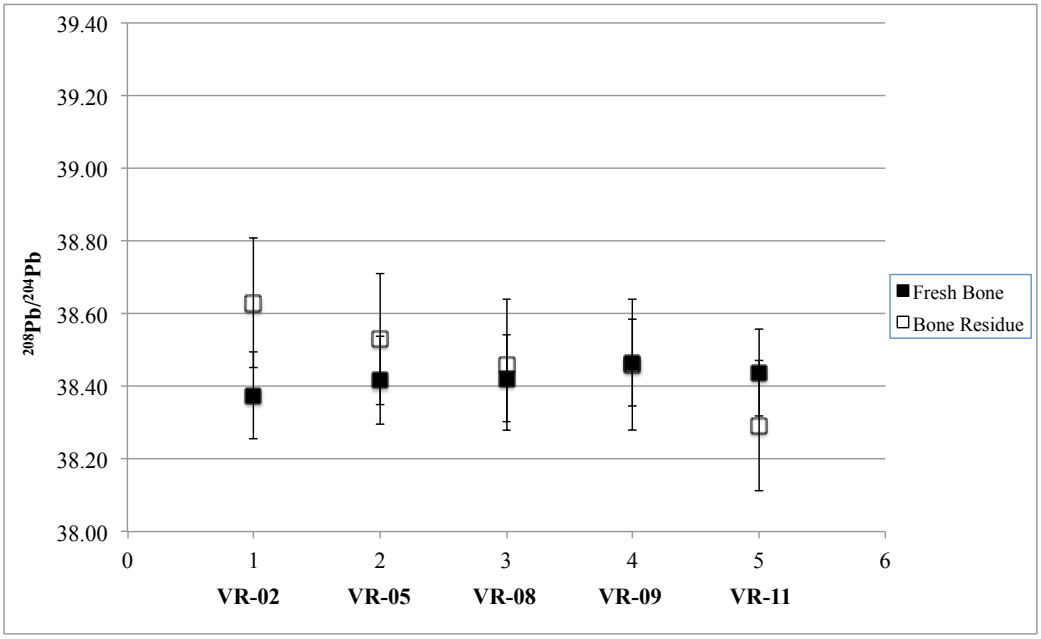

Figure 25: Comparison of $^{208}Pb/^{204}Pb$ isotope ratios between fresh bone and bone residues from DNA extraction from 5 individuals (VR-02, VR-05, VR-08, VR-09, VR-11) from Villanueva del Rosario/Spain. Error bars represent 2σ of $^{208}Pb/^{204}Pb$ ratios of standard reference material NIST 981.

5.9 Conclusion

Once the DNA has been extracted bone residues are treated as waste products and discarded. It was reasoned, however, that while the genetic information would be lost the bone residue could still retain the original trace elemental information that could be exploited for subsequent isotope analysis. The isotope data gained from this first study to investigate the suitability of bone residues from DNA extraction in comparison to fresh bone demonstrates that it can be used effectively for successive Sr and Pb isotope analysis. Generally, Pb isotope ratios observed in both samples types, however, are less consistent compared to the measured Sr isotope ratios which might be the consequence of the fundamental differences in the initial elemental concentration present in bone.

Thus it appears that very low Pb concentrations in these samples do show a higher variability resulting in a lower accuracy during measurement.

Although the preliminary results look very promising, the limited bone residue sample material available for this study and lack of relevant data to compare with, a greater study with a more representative sample number would be required to confirm the outcome of this experiment. Being able to use a single bone sample for both genetic and isotope analysis would be of great advantage in archaeological and forensic applications where sample material is very often limited.

5.10 Rare Earth Element (REE) sample preparation

For REE ICP-MS analysis the digested samples (0.1 ml) were diluted with 0.9 ml 2 % HNO_3 and 9.0 ml HNO_3 containing a 10 ppb internal standard (Rh, Ge and Pt) to a total volume of 10.0 ml.

REE and HFSE standards (Claritas™) were prepared in 4 dilutions (x1000, x2000, x10000, x20000-dilutions in 2 % HNO_3 plus internal standard). Three aliquots of NIST SRM 1640 standard reference water (NIST, Gaithersburg, MD, USA) were analysed alongside as control material. Triple blanks were included in all measurements.

REE analysis has been used on the Norfolk bone/teeth samples and the results will be discussed in detail in the relevant chapter (Chapter 7: ”Norfolk – Anglo-Saxon Case Study”).

5.11 DNA Analysis

This section describes the analysis of the Spanish and Norfolk bone and teeth samples. Because of the degraded nature of the sample material four different protocols were tested for comparison and the results thereof presented at the end of this section.

5.11.1 Introduction

Reading through "Material and Methods" sections of articles on aDNA it becomes apparent that there is no standard methodology used to extract, purify and amplify DNA from ancient material or degraded forensic samples. Instead many different procedures have been found to recover DNA from damaged and extremely degraded samples (O'Rourke et al., 2000, Pusch et al., 2003).

Extraction procedures used depend very much on the conditions present at the specific burial site where the remains are found. Extraction and amplification protocols have to be modified accordingly to remove possible PCR inhibitors. Inhibitors prevent cell lysis, which is vital to liberate the DNA for analysis. The majority of extracted PCR inhibitors are soil-derived degradation products such as tannins, humic acids, and fulvic acids (Hummel et al., 1992, Tuross, 1994) as well as Maillard products. The latter are by-products of sugar reduction, which cross-link macromolecules, including nucleic acids (Paabo, 1989). But also endogenous inhibitors such as metal ions (Ca^{2+}), haemoglobin and collagen can be responsible for the low recovery of undamaged DNA from archaeological and forensic samples. In the case of bone, Maillard products and collagen type 1 are the main inhibitors of PCR amplification (Lindahl, 1993).

5.12 Sample preparation for DNA analysis

One of the greatest challenges in ancient DNA research is to prevent contamination with modern DNA. The handling of ancient samples therefore requires utmost care and cleanliness. Isolated pre- and post-PCR working areas are only the most basic requirements and so is the full cover of the individuals dealing with such ancient specimen. A comprehensive list of precautions is described in chapter 2 ("Ancient DNA").

The principle preparation procedure of bone and teeth samples has already been described in the previous section 5.3 ("Sample preparation for isotope and DNA analysis"). A low-speed dental drill (Osseo Scientific™, NJ, USA) was used to collect bone powder from the innermost part of the femur. This method is easy to handle and the risk of contamination is lower, too, because of its protected location. Also dentin was drilled with the low-speed dental drill in order not to overheat and destroy any DNA present.

The pulverized bone and dentin were stored in sterile containers in a cool, dry, dark place until extraction on the following day.

Each bone or teeth sample was extracted in a Class II safety cabinet on separate days and after a thorough clean-up and sterilisation procedure of all tools between extractions.

5.13 DNA Extraction protocols tested on Spanish and Norfolk bone and teeth samples

As there is no standardised extraction protocol available for degraded samples, several different methods that have been previously successfully applied to degraded bone and teeth samples by renowned labs of either ancient DNA research or forensic human identification were tested:

a) "Nature protocol" published by Rohland & Hofreiter ("Modified silica-based extraction using GuSCN binding solution") (Rohland and Hofreiter, 2007a)

b) AFDIL (Armed Forces Identification Lab, USA) protocol ("Mystery Solved: The Identification of the Two Missing Romanov Children Using DNA Analysis"). (Coble et al., 2009)

c) Bone Extraction Protocol to be used with the DNA IQ System (Promega, 04/2008)

d) Qiagen DNA Investigator Kit (Qiagen, 2007)

a) Rohland & Hofreiter, 2007 (Leipzig/Germany)

This modified silica-GuSCN (guanidium thiocyanate) version by Rohland & Hofreiter (Rohland and Hofreiter, 2007a) uses a buffer consisting only of EDTA and proteinase K for bone digestion. In a comparative study Rohland and Hofreiter (Rohland and Hofreiter, 2007b) showed that only EDTA and proteinase K seem to have a positive effect on DNA yields. Other chemicals routinely used in ancient DNA protocols that were tested in this study did not improve DNA yields. DNA is purified by binding to silica in the presence of high concentrations of guanidium thiocyanate.

The downside of this method is the length of preparation time of the buffers, solutions and suspensions and the high contamination risk associated with it.

Modified silica method GuSCN binding solution (Rohland and Hofreiter, 2007b):

500 mg of powder was incubated in 10 ml extraction buffer (0.45 M EDTA and 0.25 mg/ml Proteinase K) at room temperature overnight. After centrifugation, the supernatant was added to 4 ml binding buffer (5 M GuSCN, 25 mM NaCl,

50 mM Tris (pH 4.0) and 90 µl silica suspension. This mixture was incubated for three hours at room temperature under rotation. After centrifugation the supernatant was discarded, the silica pellet was washed with 1 ml 5 M NaCl and then transferred into a 2 ml tube. After short centrifugation, the NaCl was removed with a pipette, the pellet resuspended in 1 ml wash solution (51.3 % Ethanol, 125 mM NaCl, 1 mM EDTA (pH 8.0), 10 mM Tris (pH 8.0), briefly centrifuged, and the supernatant removed with a pipette. The last washing step was repeated. Subsequently, the silica pellet was air dried at room temperature for 15 min, resuspended in 100 µl 1x TE and after 8 min incubation at room temperature, the silica was collected by centrifugation at maximum speed (16,000 g). The supernatant, representing the extract, was transferred into a fresh tube.

b) AFDIL protocol

The total demineralisation protocol developed by the AFDIL lab (Armed Forces DNA Identification Lab, USA) is now the preferred method for extracting DNA from severely degraded samples for both STR and mitochondrial DNA profiling (Coble et al., 2009, Loreille et al., 2007).

The novelty of this protocol is that the bone powder is fully dissolved at a high concentration of EDTA, which increases DNA yields considerably. The majority of other protocols lose significant quantities of undissolved bone or teeth material left in the lysis buffer and when disposed during the washing steps.

Loreille et al. (Loreille et al., 2007) have previously determined that for each gram of powder, 15 mL of EDTA 0.5 M was necessary to completely dissolve the bone powder. The optimal amount of bone or teeth material for extraction ranges between 170 mg and 200 mg. More powder usually increases simultaneously the co-extraction of inhibitors and less powder contains insufficient DNA leading to negative DNA profiles.

The extraction process of the AFDIL protocol consists of the following steps: Approximately 200 mg of powder is incubated at 56 ºC overnight in 0.5 M EDTA, 0.5 % Sarkosyl and Proteinase K. The supernatant is then removed and concentrated in a Centricon 30 Ultrafiltration unit to ~100 µl and subsequently purified with the MinElute Purification kit from Qiagen.

(NB: In this study the QIAquick PCR Purification kit for 10 µg PCR products of 100 bp to 10 kb was used instead of the MinElute Purification kit suitable for purification of up to 5 µg PCR products (70 bp to 4 kb) in low elution volumes.

c) Promega DNA IQ™ System

The DNA IQ System is a DNA isolation system designed specifically for forensic and paternity laboratories. This system makes use of paramagnetic particles to isolate DNA and remove PCR inhibitors frequently encountered in degraded samples.

Promega also provides a protocol with the system specifically for the extraction from bone. However, for DNA extraction Promega recommends the amount of 200 mg of bone powder, which is considerably more compared to other extraction methods (Promega, 04/2008).

DNA extraction following Promega protocol (Promega, 04/2008):

Approximately 2000 mg of pulverized bone were placed into 3 ml of freshly prepared Proteinase K Digestion Solution and incubated at 56 °C for 1 hour.

The remaining bone powder was removed by centrifugation at 5000 rpm for 5 minutes and the solution transferred to a new 15 ml tube. Two volumes of DNA IQTM Lysis Buffer and 15 µl DNA IQTM Resin were added to the solution and vortexed for 5 seconds at high speed.

The sample was incubated at room temperature for 10 minutes, mixing one to three times by inverting the tube. Then the sample was vortexed for 5 seconds at high speed, and placed in the Magnetic Stand. After the resin has collected at the side of the tube, the solution was carefully removed and discarded without disturbing the resin. Subsequently more sample/Lysis Buffer/resin mixture was added and repeated until all of the resin was captured and all of the solution has been removed.

Next, 100 µl of lysis buffer was added, vortexed for 2 seconds at high speed and the mixture carefully transferred to a 1.5 ml tube, making sure all of the resin has been transferred. The sample tube was then vortexed for 2 seconds at high speed, placed in the Magnetic Stand, and the solution carefully removed and discarded.

In the following step 100 µl of prepared 1x Wash Buffer was added and the tube removed from the Magnetic Stand. After being vortexed for 2 seconds at high speed the sample was returned back to the Magnetic stand and all of the Wash Buffer disposed of. The washing steps were repeated three times and all of the solution removed after the last wash.

With the lids open, the tubes were left in the Magnetic Stand for 5 minutes to air-dry the resin. Then 50 µl of Elution Buffer was added, the lids closed and the tube vortexed for 2 seconds at high speed. Then tube was placed into the Thermomixer (Eppendorf Thermomixer® comfort) at 65 °C for 5 minutes. Next, the tube was vortexed again for 2 seconds at high speed, and immediately placed in the Magnetic Stand.

The DNA solution was transferred to a fresh tube and stored at 4 °C.

d) QIAamp DNA Investigator Kit (Qiagen)

Qiagen DNA extraction kits are all based on the same principle, following the process of 4 steps:

1) Lyse: sample is lysed with proteinase K

2) Bind: DNA binds to the membrane in the spin column and contaminants flow through

3) Wash: residual contaminants are washed away

4) Elute: pure, concentrated DNA is eluted from the membrane

The QIAmp DNA Investigator Kit was specifically developed for forensic applications where only a low copy number of DNA is expected (Qiagen, 2007).

DNA Extraction was performed following the protocol for "Isolation of Total (genomic and mitochondrial) DNA from Bones and Teeth" (Qiagen, 2007):

100 mg of powdered bone were placed into a 1.5 ml microcentrifuge tube with 360 µl Buffer ATL and 20 µl proteinase K and incubated overnight at 56 °C.

After a brief spin of the tube to remove drops from the inside of the lid, 300 µl Buffer AL were added and mixed by pulse-vortexing for 10 s to yield a homogeneous solution.

The tube was then placed in the thermomixer and incubated at 70 °C with shaking at 900 rpm for 10 min. Next the tube was centrifuged at full speed (20,000 x g; 14,000 rpm) for 1 min, and the supernatant carefully transferred to a new 1.5 ml microcentrifuge tube. Subsequently, 150 µl ethanol (96–100 %) was added to the sample and mixed by pulse-vortexing for 15 s.

The entire lysate from the previous step was carefully transferred to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp MinElute column was placed in a clean 2 ml collection tube, and the collection tube containing the flow-through was discarded.

600 µl Buffer AW1 were then added and the tube centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp MinElute column was transferred in a new 2 ml collection tube, and the collection tube containing the flow-through was discarded.

In the next step 700 µl Buffer AW2 was added and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp MinElute column was again placed in a clean 2 ml collection tube, and the collection tube containing the flow-through discarded.

After the washing steps, 700 µl of ethanol (96–100 %) was added to the tube and centrifuged at 6000 x g (8000 rpm) for 1 min before placing the QIAamp in a clean 2 ml collection tube. The previous collection tube containing the flow-through was discarded.

The MinElute column was subsequently centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min to dry the membrane completely before being transferred in another clean 1.5 ml microcentrifuge tube. The QIAamp MinElute column was left with the lid open to incubate at room temperature (15–25 °C) for 10 min or at 56 °C for 3 min.

In the last step, 20–50 µl of Buffer ATE was applied to the centre of the membrane to ensure that the DNA bound to the membrane was completely eluted. The tube (with closed lid) was incubated at room temperature (15–25 °C) for 1 to 5 min and stored at 4 °C.

Protocol	Extraction buffer components	Bone powder (mg)	Purification
Rohland & Hofreiter	- 0.45 M EDTA - 0.25 mg/ml Proteinase K, pH 8.0	500	*By binding to silica suspension* *Binding buffer:* 5 M GuSCN, 25 mM NaCl, 50 mM Tris *Washing buffer:* 50 % Ethanol, 125 mM NaCl, 10 mM Tris, 1 mM EDTA (pH 8.0)
AFDIL	- 0.5 M EDTA - 20 mg/ml Proteinase K, pH 8.0 - 0.5 % laurylsarcosinate (Sarkosyl)	170-200	MinElute PCR Purification Kit (Qiagen)
QIAampDNA Investigator Kit	- Buffer ATL (contains EDTA and SDS (sodium dodecyl sulphate)) - Buffer AT (contains guanidinium salt) - Proteinase K	≤100	- Buffer AW1 (with Ethanol and guanidinium chloride) - Buffer AW2 (with Ethanol) - Buffer ATE (elution buffer)
Bone Extraction Protocol to be used with the DNA IQ™ System (Promega)	- Bone Incubation Buffer (contains guanidinium thiocyanate) - 18 mg/ml Proteinase K - DNA IQ™ Lysis Buffer (contains 1 M DTT (Dithiothreitol))	≥2000	2x Wash Buffer (contains ethanol and isopropyl alcohol)

Table 11: Overview of the components used in the four protocols tested

5.14 Amplification and quantification of Spanish samples on the ABI 7500 Real-time PCR using the ABI Quantifiler DUO Human Identification Kit

The Quantifiler Duo DNA Quantification Kit (Applied Biosystems, 2008) is designed to simultaneously quantify the total amount of amplifiable human female DNA and human male DNA in a sample. The markers target the human specific Ribonuclease P RNA Component H1 (RPPH1) with an amplicon length of 140 bp and the male specific sex-determining region Y (SRY) consisting of 130 bp in the nuclear genome. This assay also contains an internal PCR control (IPC) of 130 bp, which is a synthetic DNA sequence not found in nature.

Calculation of Male to Female DNA Ratio

The Quantifiler Duo kit provides the quantity of human (NB: "human" is used for "female" in this context as designated by ABI) and human male DNA in biological samples. From these values, one can calculate the ratio of male to female DNA using the following equation (quantities in ng/µL) (Applied Biosystems, 2008):

Male DNA : Female DNA Ratio =

$$\frac{\text{Male DNA}}{\text{Male DNA}} : \frac{\text{Human (Female)DNA} - \text{Male DNA}}{\text{Male DNA}}$$

This ratio determines the extent of the mixture, which is used to decide whether to proceed with autosomal STRs or Y-STRs (the latter for male DNA analysis only).

The Quantifiler Duo assay has several advantages when limited sample material is available as it is the case with the Spanish samples:

- It determines the ratio human male to female DNA, even with very little male and excess female DNA (1:1000 or greater)
- it provides information on the presence of inhibitors
- it is highly sensitive, detection limit 6 pg/µl of DNA which is equivalent to one human diploid cell
- specific to human DNA
- saves sample material due to dual quantification system

Sample preparation:

Samples were made up with 2 µl DNA in a 23 µl reaction mix (10.5 µl Quantifiler® Duo Primer Mix and 12.5 µl Quantifiler® Duo PCR Reaction Mix) to a total volume of 25 µl.

Amplification reactions were performed in a 7500 Real-Time PCR System (Applied Biosystems) following the manufacturer's instruction with conditions as follows: 50 ºC, 2 min; 95 ºC, 10 min; 40 cycles of 95 ºC, 15 sec and 60 ºC, 1.0 min. The data were analysed using 7500 System sds Software v1.2.3 (Applied Biosystems).

5.15 PCR Amplification and quantification of Norfolk bone and teeth samples

5.15.1 PCR amplification

The Norfolk samples were amplified with the standard PCR method.

PCR amplification was carried out using 4 µl of DNA template in a total reaction volume of 25 µl. The reaction mix was prepared with 2.5 µl 1x PCR

Gold buffer containing 1.5 mM $MgCl_2$ (Applied Biosystems, Warrington, UK), 0.5 µM of each primer (custom synthesized, Eurofins MWG Operon, Ebersberg, Germany), 0.25 µM of each dNTP, and 1U AmpliTaq Gold polymerase (Applied Biosystems, Warrrington, UK).

Positive (lab staff member) and negative PCR controls were used alongside the samples and reagent blanks.

A list of the mitochondrial primers of the HVI region used for PCR amplification of the Norfolk samples is shown below:

Primer names	**Sequence 5' to 3'**	**Length (bp)**
F15997	CACCATTAGCACCCAAAGCT	189
R16185	GGGTTTTGATGTGGATTGGG	
F16112	CACCATGAATATTGTACGGT	147
R16258	TGGCTTTGGAGTTGCAGTTG	
F16190	CCCCATGCTTACAAGCAAGT	221
R16410	GAGGATGGTGGTCAAGGGAC	

Table 12: Mitochondria primer pairs used for Norfolk samples

DNA extracts amplified in a total reaction volume of 25 µL on a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems, Warrington, UK) using the following cycling parameters: 95 °C for 10 min followed by 38 cycles of 30 s at 94 °C, 60 s at 54 °C, and 60 s at 72 °C; holding at 4 °C.

A second PCR amplification was performed separately with different PCR settings because of the poor quality of the first results, which showed multiple unspecified extra bands. In order to improve the quality, PCR cycles were

reduced to 35 and the annealing temperature increased to 55 °C, additionally the amount of DNA was decreased to 3 µL.

The PCR product of the Norfolk bone and teeth were quantified on a conventional 2 % agarose gel (200 ml 1x TBE Buffer and 4 g agarose powder). DNA (5 µl) and 6x loading dye (1 µl) were loaded on the gel alongside a 100 bp DNA ladder as size marker.

The changed parameters of the second amplification did not show any improvement of the results and except for the DNA ladder and positive control no bands were visible.

5.16 STR analysis on the ABI 310 Genetic Analyser using the ABI MiniFiler Kit

The AmpFlSTR MiniFiler PCR Amplification Kit is a reduced size short tandem repeat (STR) assay optimized for genotyping degraded and/or inhibited DNA samples. It amplifies eight autosomal STR loci (D13S317, D7S820, D2S1338, D21S11, D16S539, D18S51, CSF1PO and FGA) and the sex-determining marker, Amelogenin, in a single PCR reaction. The amplicon lengths range from 70 to 283 nucleotides.

All Spanish samples including all previously negatively quantified samples were amplified with the Minifiler Kit for subsequent sequencing. DNA samples were prepared according to the ABI Minifiler kit protocol (Applied Biosystems, 2007). According to the protocol ~0.5 to 0.75 ng of total DNA in a total of 10 µL are required but because of the poor DNA quality of the Spanish samples 10 µL pure DNA extracts were added to the reaction mix (10 µL AmpFlSTR® MiniFiler™ Master Mix plus 5.0 µL AmpFlSTR® MiniFiler™ Primer Set).

DNA extracts were amplified in a total reaction volume of 25 µL on a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) using the following

cycling parameters: 95 °C for 11 min; 30 cycles of 94 °C/20 s, 59 °C/2 min, 72 °C/1 min; 60 °C extension for 45 min; holding at 4 °C.

5.16.1 Capillary electrophoresis on the ABI 310 Genetic Analyser

1.5 µL of each PCR product and 25 µL of the formamide: size standard mixture (0.5 µL GeneScan™ 500 LIZ® Size Standard and 24.5 µL Hi-Di™ formamide (Applied Biosystems) was added to each well in a 48-well reaction plate. A well containing 1.5 µL AmpFlSTR MiniFiler allelic ladder was included on the plate. Samples were run on a capillary electrophoresis (CE) sequencer (ABI 310 Genetic Analyser) using Data Collection software Genescan 3.0 and analysis software GeneMapper ID version 3.2 for Human Identification (Applied Biosystems).

Chapter 6

6 Case Study I: Spanish Civil War mass grave

6.1 Historical background

The Spanish Civil War (1936-1939) was the result of a major political conflict between Nationalists and Republicans that caused a deep rift between the Spanish people. The conflict was further greatly influenced by international involvement. The ideological support for Nationalists came from Fascist Italy, Nazi Germany and Portugal while the Republicans received aid from volunteers of the Soviet Union, Mexico, the international Marxist movement and the International Brigades from countries like the UK, USA and others. The war ended in the defeat of the Republicans leading to the rule of the Nationalists under General Francisco Franco (1939-1975). During the war and the successive Franco dictatorship an estimated 150.000 people lost their lives. Individuals were executed and buried in unmarked mass graves often alongside roads or within wooded areas (Ferllini, 2010). The victims were referred to as "paseadeos" ("taken for a ride"), which was an expression commonly used for the practice of execution during the war years (Ferllini, 2010). Although these atrocities and human rights repressions were on-going, Franco imposed silence over the crimes committed until his death in 1975, as did all subsequent democratic governments.

Only a fairly recent social movement led to the foundation of the "Association for Recovery of Historical Memory of the Civil War" (ARMH), with the aim to collect written and oral testimonies of witnesses and relatives of the victims about locations of mass graves and individuals buried in them. The organisation

operates in collaboration with forensic scientists, archaeologists and anthropologists. Since 2000 many mass graves have been exhumed on initiation and request of relatives and the guidance of ARMH, so that human remains could be identified and reburied in dignity.

However, this chapter of Spain's history still remains a highly sensitive subject even for its governments today so that future investigations and exhumations are uncertain.

6.2 Archaeological background

In 2007 a team of archaeologists and geologists from the Autonomous University of Barcelona (UAB) excavated a mass grave from the Spanish Civil War in Villanueva del Rosario in the province of Malaga in Southern Spain. The exhumation was an initiative of the Spanish Federation of Civil War Memory Forums and the families of the victims. The mass grave contained the bodies of eleven civilians executed by gunshots in 1937 shortly after Franco's troops occupied the town. This grave is one of many in the region of Malaga where it is estimated that 4,500 bodies were buried in mass graves during the war.

The mass grave was located at the side of the road between the towns of Villanueva del Rosario and Villanueva del Trabuco, which is a typical location for the majority of mass graves from the Spanish Civil War.

6.3 Geological setting of Villanueva del Rosario/Malaga

Villanueva del Rosario is located about 40 km north of Malaga in the mountain range of the Alta Cadena in the south of Spain. The mountain range lies geologically within the Betic Cordillera and consists of Jurassic carbonate rocks.

The lithology is composed of mainly limestones, mixed with clays and marly limestones and marls on top (Mudarra and Andreo, 2011). A geologic map of the area around Villanueva del Rosario is illustrated below (Figure 26).

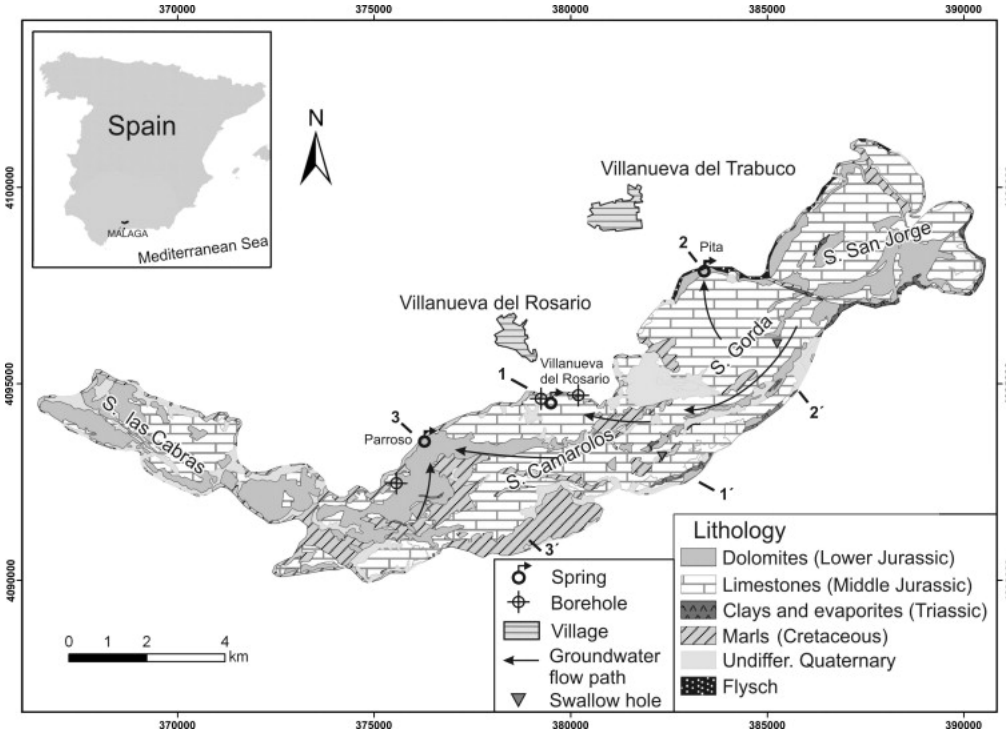

Figure 26: The lithology of Villanueva del Rosario (Mudarra and Andreo, 2011)

6.4 Bone and teeth samples from the mass grave

In 2008 our Spanish collaborators provided bone and teeth fragments from the eleven men exhumed from the mass grave in Villanueva del Rosario for DNA and isotope analysis.

The bones were provided as small open fragments taken from the femur and in different sizes (Table 13). As far as we were aware, no special precautions in regard to contamination prevention were followed at the time of excavation.

List of samples:

Skeletal number	Skeletal element	Size (cm)	Weight (g)	Skeletal number	Skeletal tooth	Location	Weight (g)
VR-01	R Femur	6.62 x 2	9.73	VR-01	M^3	Maxilla	1.66
VR-02	L Femur	2 x 2.5	3.96	VR-02	M^3	Mandible	2.09
VR-03	L Femur	3 x 1.5	3.40	VR-03	^2M	Maxilla	2.69
VR-04	R Femur	3.5 x 2.5	3.68	VR-04	no teeth	---	---
VR-05	R Femur	2.9 x 2.4	3.36	VR-05	M_3	Mandible	1.99
VR-06	L Femur	1.5 x 2.5	2.37	VR-06	^3M	Maxilla	1.83
VR-07	L Femur	6 x 2.3	8.23	VR-07	M^3	Maxilla	2.28
VR-08	R Femur	6.3 x 2.1	8.41	VR-08	^3M	Maxilla	1.74
VR-09	L Femur	2 x 1.7	2.68	VR-09	M^3	Maxilla	1.87
VR-10	R Femur	5.5 x 2.4	9	VR-10	M^2	Maxilla	2.87
VR-11	L Femur	6.7 x 2.4	9.9	VR-11	M_1	Mandible	3.09

Table 13: List of bone and teeth sample from Villanueva del Rosario/Spain

6.5 Aim of the Spanish case study

The identification of human remains from mass graves is on many levels a challenging and sensitive undertaking. Individuals were in most cases victims of human rights violations, executed and buried at anonymous locations. Therefore forensic investigations to locate a mass grave and collect all relevant

information about the individuals buried in it initially rely on testimonies of witnesses and relatives. The description of special physical features or the comparison of dental records (where available) can be important for positive identification in such cases. Commonly, standard forensic anthropological methods are applied first to estimate sex, age and physical condition. The successful identification based on this method, however, depends very much on the available ante-mortem information provided from relatives or other testimonies. If no ante-mortem evidence is available for identification or when human remains recovered from mass graves are in a putrefied and co-mingled state, anthropological methods alone are not sufficient. In such circumstances DNA analysis is usually more successful but in many instances still problematic due to DNA damage and the high risk of cross-contamination in mass graves.

6.6 DNA analysis

When DNA is degraded to much too short fragments, the commonly used forensic STR markers fail because of their relatively long amplicon length. An alternative profiling method for compromised samples involves the use of mtDNA markers as discussed earlier in chapter 2 (“Ancient DNA”). However, the major drawback of mtDNA is the lack of specificity compared to STRs, which in forensic cases is of course crucial. Mitochondrial DNA typing cannot discriminate between individuals along the maternal lineage as they carry identical mtDNA information. In recent years increasingly short amplicons of STRs (“mini STR“) are frequently used which offer the specificity required for kinship analysis and at the same time are suitable for the analysis of degraded sample material.

For the reasons explained above, “mini STRs” were chosen in the first approach to identify kinship between the human remains and the living relatives who provided buccal swabs and/or blood samples for this study. The reference samples of the relatives were analysed (partly by the author) in the “normal-

level" DNA laboratory of a local company specialised in kinship analysis (Anglia DNA, Norwich, UK) in order not to risk any contamination of the low-level aDNA laboratory at the University. While the DNA profiles of the relatives could be determined with mini-STRs, they failed with the samples of the war victims. In the second attempt mtDNA markers were used but failed equally to produce satisfactory results. The analytical procedures have already been discussed in chapter 5 ("Materials and Methods" -"DNA analysis") so that the following sections will only present the outcomes of the DNA analysis.

6.6.1 Quantification results for Spanish bone and teeth

DNA extracts from Spanish bone and teeth samples (including three test samples from Norfolk and one modern sample) were used to assess the efficiency of the different extraction protocols.

Procedural blanks (2x) and reaction negatives were included in the quantification and remained below detection limit.

Data is only shown for those runs where positive results could be achieved. (

Table 14):

a) Results from the first analysis:

Sample (Bone)	Bone powder (mg)	Extraction Method	DNA Quantification
VR-01	200	AFDIL	0.00078 ng/µl – Human female DNA
VR-02	200	AFDIL	0.0039 ng/µl – Human female DNA

Table 14: Sample list of two Spanish bone samples (VR-01 and VR-02) extracted with the AFDIL method.

The two Spanish bone samples VR-01 and VR-02 were extracted with the AFDIL protocol as previously described in Chapter 5 “Materials & Methods”. DNA concentrations were measured for VR-01 with 0.00078 ng/µl and for VR-02 0.0039 ng/µl could be detected (Table 14). According to the results, the DNA in both samples was of female origin.

A screenshot of the amplification curves of the two positive samples VR-01 and VR-02 is shown below (Figure 27).

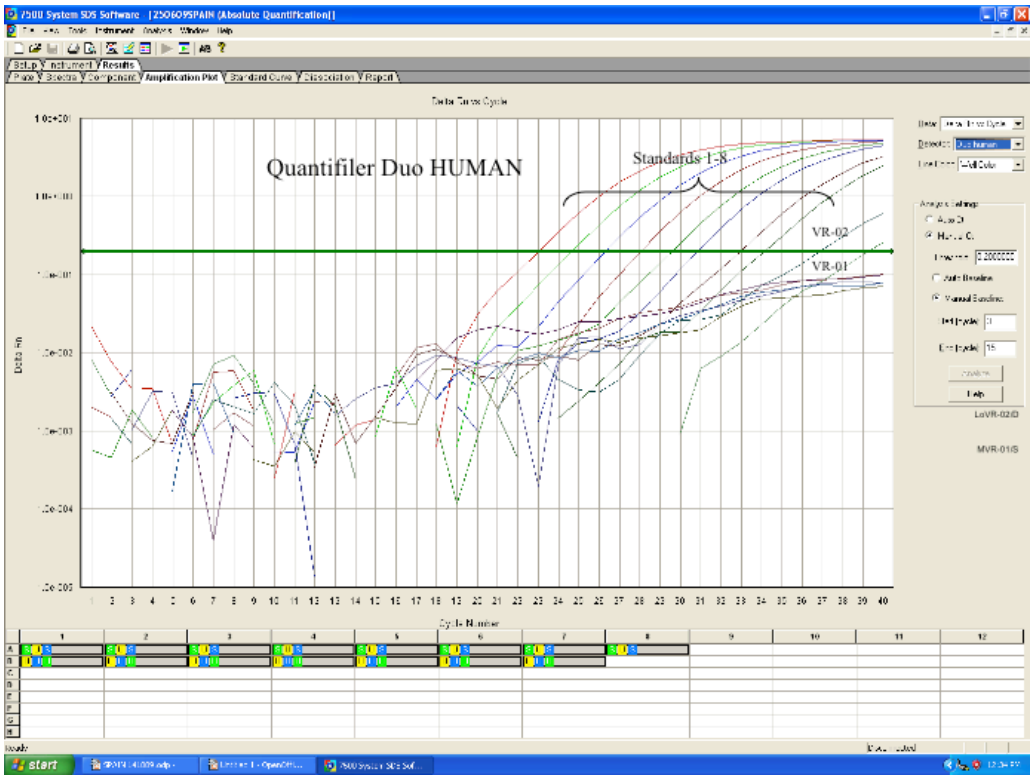

Figure 27: A screenshot of the human amplification curve for Quantifiler Duo Standards and samples. Samples VR-01 and VR-02 show positive results for female DNA of 0.00078 ng/µl and 0.0039 ng/µl, respectively.

b) Results from the second analysis:

Bone Sample	Bone powder (mg)	Extraction Method	DNA Quantification
VR-04	220	AFDIL	------
VR-10	290	AFDIL	0.00136 ng/µl / female DNA
SUSJ3_3 (modern sample)	400	AFDIL	1.16 ng/µl / male DNA 1.61ng/µl / female DNA

Table 15: Sample list containing two Spanish bone samples (VR-04 and VR-10) and one modern bone control sample (SUSJ3_3) extracted according to the AFDIL protocol.

Only the Spanish bone sample VR-10 contained a very low concentration of female DNA (0.00136 ng/µl). The modern control sample SUSJ3_3 was positive for both male and female DNA indicating a mixed DNA samples. To determine the ratio between the male and female content for SUSJ3_3 the DNA ratio was calculated as follows:

$$\frac{1.16\ \text{ng/µl}}{1.16\ \text{ng/µl}} : \frac{(1.61\text{ng/µl} - 1.16\ \text{ng/µl})}{1.16\ \text{ng/µl}} = 1 : 0.38$$

This resulting ratio indicates that this DNA sample is predominantly from a male individual.

The amplification curves of the two positive samples VR-10 and SUSJ3_3 are shown in the screenshot below (Figure 28).

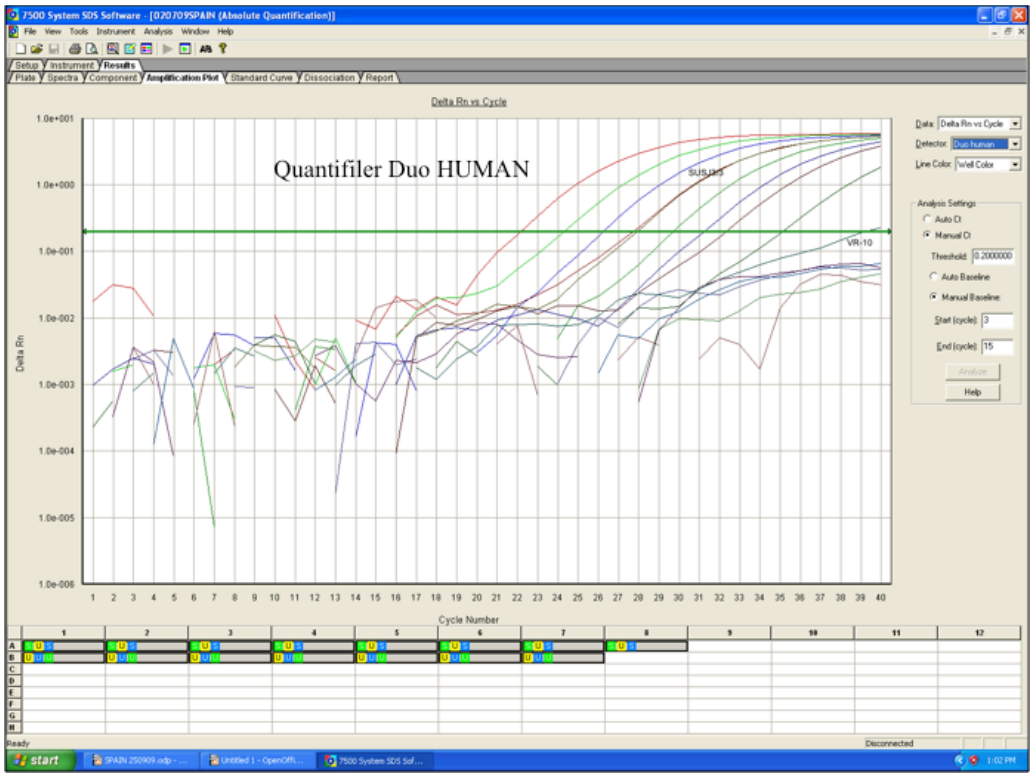

Figure 28: A screenshot of the human amplification curve for Quantifiler Duo Standards and samples (Table 15). In the Spanish sample VR-10 human female DNA of 0.00136 ng/µl was detected and in the modern sample SUSJ3_3 1.16 ng/µl male DNA and 1.61ng/µl female DNA.

A summary of the quantification results for the Spanish bone samples (VR-xx), and one modern sample (SUS_JR/3) resulting from the extractions with the AFDIL method are shown in the following table (Table 16).

Bone sample	**Weight (mg)**	**Quantification results for AFDIL extraction method**
VR-01	200	0.00078 ng/µl female DNA
VR-02	200	0.0039 ng/µl female DNA
VR-03	200	*negative*
VR-04	220	*negative*
VR-05 (4x)	200-280	*negative*
VR-06	270	*negative*
VR-07	200	*negative*
VR-08 (2x)	150/240	*negative*
VR-09	300	*negative*
VR-10	290	0.00136 ng/µl female DNA
VR-11 (2x)	240/360	*negative*
SUS_JR/3 (modern sample)	400	1.16 ng/µl male DNA / 1.61 ng/µl female DNA

Table 16: Summary of results from the Spanish bone samples extracted with the AFDIL method

Due to the limited amount of teeth material available from most Spanish teeth samples, only one method for extraction could be used. In this case the AFDIL method was chosen because it produced the only positive results for bone as previously shown

A summary of the quantification results for the Spanish teeth samples (VR-xx) and one modern tooth extracted according to the AFDIL method is shown on the following page (Table 17):

Method of extraction	Teeth sample	Weight (mg)	DNA Quantification with Quantifiler DUO
AFDIL	VR-01	~100	*negative*
	VR-02	~120	0.00178 ng/µl male DNA
	VR-03 (2x)	~200	*negative*
	VR-05 (2x)	~120	*negative*
	VR-06 (2x)	100	*negative*
	VR-07	110	*negative*
	modern tooth	~50	0.198 ng/µl human DNA

Table 17: Summary of results from Spanish teeth samples and one modern tooth extracted according to the AFDIL protocol.

6.6.2 Sequencing results

The MiniFiler kit was chosen because of the degraded state of the samples from Spain and because STRs are the preferred markers used for kinship analysis due their highly polymorphic nature. To match the Spanish family reference samples with the unknown bone or teeth samples from the excavation site in Villanueva del Rosario autosomal markers would provide a greater discrimination power than lineage markers. However, the analysis with mini-STRs failed for all Spanish samples so that it was necessary to resort to mitochondrial DNA for kinship analysis.

Due to time constraints, the Spanish samples were sent to a professional analytical DNA company, Cellmark (Abingdon/Oxfordshire/UK), for further mitochondrial analysis. However, none of the samples analysed at Cellmark produced a positive result that could be used for conclusive kinship analysis.

6.6.3 Conclusion

From the quantification results it is evident that none of the protocols tested (see Chapter 5.13) could retrieve sufficient DNA for further sequencing of the Spanish samples. Out of the four protocols tested only the AFDIL method succeeded to recover a low amount of DNA from three Spanish bone samples (VR-01: 0.00078 ng/µl human DNA, VR-02: 0.0039 ng/µl human DNA, VR-10: 0.00136 ng/µl human DNA) and one tooth sample (VR-02: 0.00178 ng/µl male DNA). However, these results could not be reproduced. Moreover, the quantification result for the bone of individual VR-02 indicates that the DNA was of female origin while the result for the tooth of the same individual points to male origin. The contradictory nature of the two results which might have resulted from cross-contamination in the mass grave or from contamination later in the field or lab, as well as the low recovery of DNA in general, are problematic and hence no reasonable conclusion can be drawn at this stage. It would require more replicates to verify whether the extraction, PCR methods or the samples themselves are the cause for failure. However, due to the limited sample material from Spain it was not possible to investigate the causes any further.

6.7 Strontium and lead isotope analysis

Strontium and lead isotope analysis was employed to investigate if two distinct populations ("locals" vs. "non-locals") could be discerned in the mass grave. If results revealed that individuals had migrated between places such information could be verified by the relatives and used to provide further proof of identity.

6.7.1 Sr and Pb isotope results

Spanish bone and teeth samples (n=21) were analysed for their Sr and Pb isotope composition by MC-ICP-MS (GVI Isoprobe). Instrumental precision was determined by repeated measurements of NIST 987 strontium and NIST 981 Pb reference standards.

The NIST 987 Sr standard showed a reproducibility of 0.71026 ± 0.00002, 1σ and the NIST 981 standard (n=21) 0.05891±0.00002 ($^{204}Pb/^{206}Pb$), 0.9154±0.0005 ($^{207}Pb/^{206}Pb$) and 2.1721±0.0006 ($^{207}Pb/^{206}Pb$) during measurement.

All Sr and Pb blanks were below the detection limit.

The isotope results for the Spanish samples are summarized in the following Table 18:

Sample ID	Sr (ppm)	87Sr/86Sr	Pb (ppm)	206Pb/204Pb	207Pb/204Pb	208Pb/204Pb	207Pb/206Pb	208Pb/206Pb	206Pb/207Pb
VR-01/Bone	57	0.7089	0.80	18.25	15.60	38.34	0.854	2.101	1.170
VR-01/Enamel	42	0.7085	0.30	18.24	15.61	38.34	0.855	2.102	1.169
VR-02/Bone	49	0.7088	0.62	18.27	15.61	38.37	0.855	2.101	1.170
VR-02/Enamel	42	0.7086	0.42	18.26	15.60	38.35	0.854	2.100	1.170
VR-03/Bone	44	0.7090	0.21	18.30	15.61	38.41	0.853	2.098	1.172
VR-03/Enamel	29	0.7088	0.09	18.29	15.62	38.41	0.854	2.100	1.171
VR0-4/Bone	68	0.7091	0.89	18.31	15.62	38.44	0.853	2.099	1.172
VR0-4/Enamel	---	---	---	---	---	---	---	---	---
VR-05/Bone	64	0.7093	1.06	18.30	15.62	38.42	0.854	2.100	1.171
VR-05/Enamel	81	0.7098	0.42	18.21	15.61	38.28	0.857	2.102	1.166
VR-06/Bone	71	0.7090	0.27	18.30	15.62	38.42	0.854	2.100	1.171
VR-06/Enamel	49	0.7087	0.12	18.29	15.62	38.40	0.854	2.099	1.171
VR-07/Bone	73	0.7091	0.65	18.31	15.63	38.44	0.853	2.099	1.172
VR-07/Benamel	41	0.7087	0.11	18.28	15.62	38.40	0.854	2.100	1.170
VR-08/Bone	64	0.7091	0.51	18.30	15.63	38.42	0.854	2.099	1.171
VR-08/Enamel	32	0.7088	0.09	18.31	15.63	38.43	0.854	2.099	1.171
VR-09/Bone	62	0.7090	0.58	18.32	15.63	38.46	0.853	2.099	1.172
VR-09/Enamel	48	0.7088	0.10	18.31	15.63	38.42	0.854	2.099	1.171
VR-10/Bone	72	0.7098	0.75	18.16	15.61	38.23	0.860	2.106	1.163
VR-10/Enamel	28	0.7088	0.05	18.28	15.61	38.38	0.854	2.099	1.172
VR-11/Bone	53	0.7089	0.31	18.30	15.63	38.44	0.855	2.101	1.170
VR-11/Enamel	31	0.7086	0.18	18.35	15.64	38.49	0.853	2.098	1.173

Table 18: The Sr and Pb isotope results of the Spanish bone/teeth samples

6.7.2 $^{87}Sr/^{86}Sr$ of Spanish bone and enamel samples

The $^{87}Sr/^{86}Sr$ ratios for the measured samples cover a range between 0.7088 and 0.7098 (0.7091±0.0005, 2σ) for bone and 0.7089-0.7093 (0.7088±0.0007, 2σ) for enamel. As illustrated in Figure 29, 19 out of the 21 values for bone and enamel are found within a narrow range most likely characteristic for the local strontium signature. Only two ratios differ significantly from the local strontium values. One is from individual VR-10, which has the highest ratio for bone (0.7098) implying that this person probably spent considerable time outside the local region during his last years before returning to the region where his remains were found. The other noticeable higher value of 0.7098 for tooth enamel belongs to individual VR-05. While the strontium ratio for bone of

individual VR-05 correlates with the local values, the strontium integrated in the tooth enamel during childhood derives from a distinct environment suggesting that the person must have migrated between childhood and adulthood.

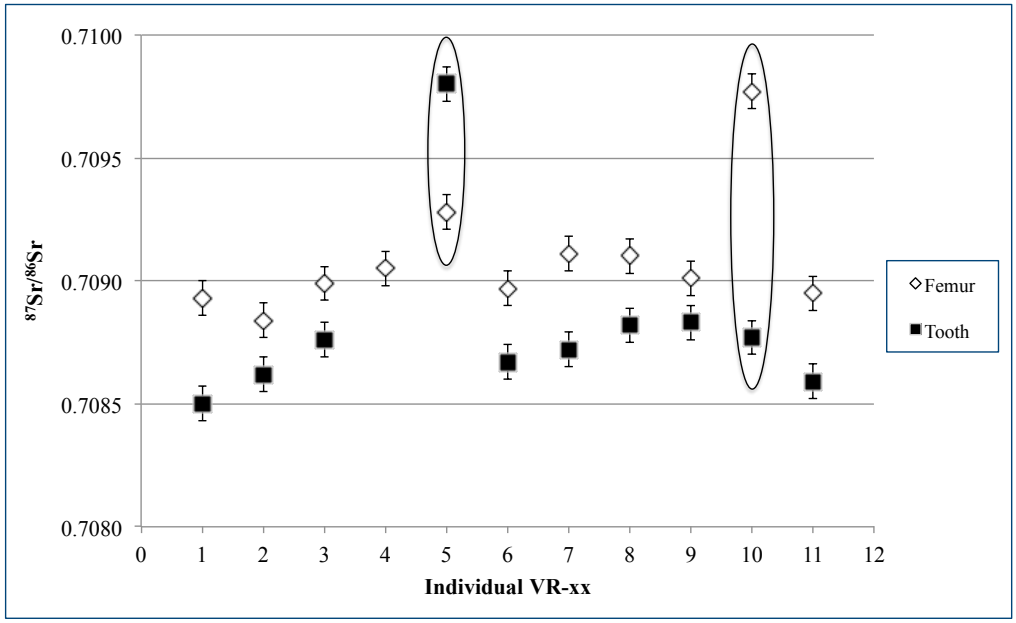

Figure 29: $^{87}Sr/^{86}Sr$ of Spanish bone/enamel samples (error bars represent 2σ of standard reference material NIST987, n=25). The ellipses show the two outliers VR-05 and VR-10.

Sr isotope literature data for this region in Southern Spain (Voerkelius et al., 2010) produced a range from 0.70701 to 0.70900, which coincides with the Sr isotopic ratios obtained from this study. A new addition to geospatial reference data has been introduced only recently and contains a comprehensive compilation of bioavailable $^{87}Sr/^{86}Sr$ data from European soils (Hoogewerff, 2012). The Sr isotope range documented for this particular area in Southern Spain also correlates with the data obtained from Villanueva del Rosario.

6.7.3 $^{208}Pb/^{204}Pb$ of Spanish bone and enamel samples

$^{208}Pb/^{204}Pb$ lead isotope ratios for bone cover the range from 38.23 to 38.44 (38.40±0.06, 1σ) and for enamel from 38.28 to 38.49 (38.39±0.06, 1σ) (Figure 30). Most Pb isotope ratios obtained from bone and enamel at Villanueva del Rosario form a homogenous group. Only the two same individuals (VR-05, VR-10) exhibit a significantly different pattern again as with the Sr isotope ratios. While the bone Pb ratio of bone for individual VR-05 is consistent with the majority of the group, the $^{208}Pb/^{204}Pb$ ratio is significantly higher in enamel compared to all other samples. In contrast, VR-10 shows an inverse analogy to VR-05, with the bone Pb ratio distinctly apart from the group while enamel correlates with all other enamel Pb ratios in the group.

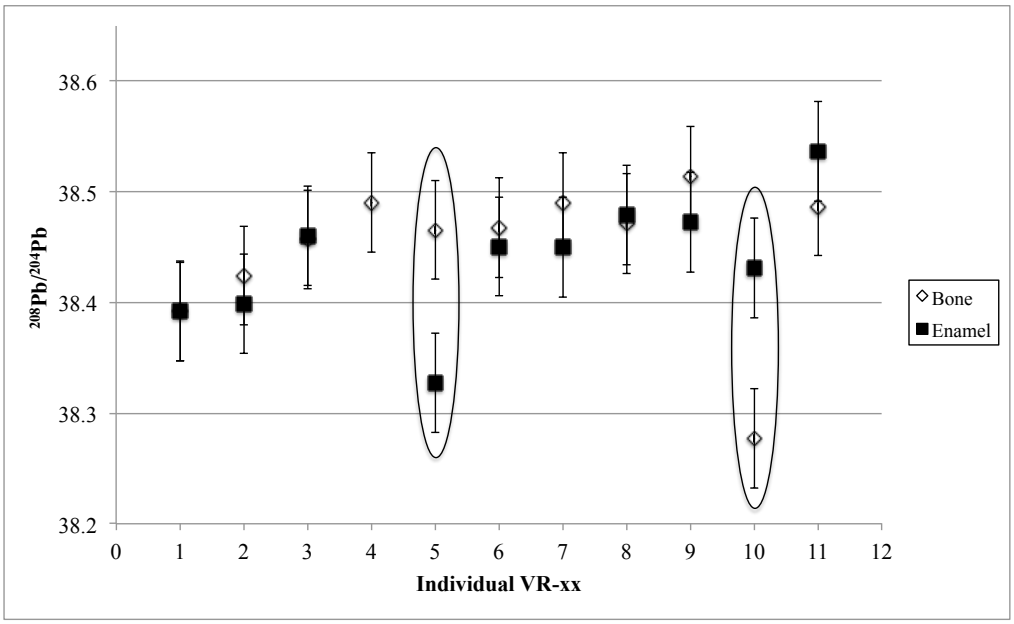

Figure 30: $^{208}Pb/^{204}Pb$ ratios for bone and enamel samples from Spain. Error bars represent 2σ of NIST981 reference standard (n=21). The ellipses show the two outliers VR-05 and VR-10.

Comparative Pb isotope data for this part of Spain is scarce and the only Pb reference data found, derives from a European wide assessment of agricultural

soils (Reimann et al., 2012), which was mentioned earlier in chapter 3 ("Isotope Geochemistry"). The majority of $^{206}Pb/^{207}Pb$ ratios obtained from this region ranged from 1.191 to 1.202. However, a small pocket in this part of Spain also exhibits slightly lower ratios from 1.174 to 1.191, which are somewhat closer to the results obtained from the Spanish bone and enamel samples ($^{206}Pb/^{207}Pb$ 1.163 to 1.170) but are still a magnitude higher than the sample data. Such low Pb ratios of <1.174 as seen in the sample material from Villanueva del Rosario are very rare for Spain and only present as tiny inclusions further north and in the north-west of Spain. Looking further afield, similar Pb ratios are more commonly found in northern Europe in countries such as the UK, the Netherlands and Northern Germany.

6.8 Discussion Sr and Pb combined

Based on the information provided by the relatives of the victims the human remains in this particular mass grave contained their fathers, uncles, nephews, cousins and grandfathers. This circumstances suggest that the bone and enamel Sr isotopes ratios observed in the majority of these individuals most likely represent the variation of the local Sr and Pb signature.

The results for Sr and Pb isotope ratios from Spain generally show a consistent group of individuals that most likely spent their childhood as well as at least the last years of their lives within the same location apart from individuals VR-05 and VR-10 whose results strongly indicate a change of residency between childhood and adulthood. Interestingly, these two "outliers" become evident from both Sr and Pb isotope analysis. While the bone Sr isotope ratio of VR-05 falls within the "local" range, the significantly higher Sr isotope ratio (0.7098) incorporated during enamel mineralisation reflects a different isotopic background to that of Villanueva del Rosario. Sr ratios of this magnitude can be found further inland towards central and northern parts of Spain and could therefore be a plausible location for childhood residency.

In contrast, the Sr isotope ratios observed for bone and enamel in VR-10 reveals an inverse life history to VR-05. The result for enamel is part of the homogenous cluster while the Sr and Pb isotope ratios in the bone are located outside the "local" group. Hence it can be reasoned that this individual had spent considerable time (possibly more than 10 years) either in a region further inland or in the north of the country itself or even abroad.

From a group of people who spent all their lives in the same location and lived from local food and water sources such homogeneity as seen in this burial population could be expected. However, slight variations in Sr as well as Pb isotope ratio within a population might still be evident as each individual has his food preferences and consumes varying amounts of a particular nutrient. If the nutrients ingested have different isotope ratios the isotope ratios within an individual will reflect the relative contributions of these different food sources. Isotope ratios will not only depend on the type of water or food (eg. carnivorous or herbivorous) intake but also on the metabolic condition or age of the individual. Yet, Pb isotope ratios in individuals seem to be generally less effected by the diet compared to Sr isotope ratios (Montgomery, 2002).

Despite the overall consistency among the majority of individuals, there is still a difference evident between the Sr isotope ratios of bone and enamel within each individual. Generally, enamel can be regarded as a more reliable proxy for the original isotope signal due to its inherently greater resistance to diagenetic factors than bone. An offset between bone and teeth Sr signal is usually observed if, 1) migration between distinctive Sr geological regions between childhood and adulthood has taken place, 2) the local bioavailable Sr isotope ratio has been altered due to foreign atmospheric or dietary input, or 3) post-mortem contamination from the burial ground has occurred. Before any conclusion can be drawn in respect to "local" or "non-local" individuals all these possibilities need to be considered and investigated.

Considering the proximity of Villanueva del Rosario to the coast (~40 km) the affect of rainwater or sea-spray on the locally grown crops could be considered

as a potential cause of increased Sr isotope values in the local biosphere and the consequential deposition in bone. Rainfall occurs mostly in winter and spring in Villanueva del Rosario associated with wet winds from the Atlantic, but also southern marine winds from the Mediterranean provide humidity to the region (Mudarra and Andreo, 2011). Hence it seems most likely that the Sr values in Villanueva del Rosario will be affected to a certain degree by marine Sr influx.

Alternatively, the higher Sr isotope ratios in bone might have also arisen from a salt-rich or marine derived diet during adulthood causing the offset observed between bone and enamel. Assuming this was the case, the Sr signature in bone would reflect an intermediate Sr isotope ratio between that of the local and seawater Sr isotope ratio. Given that modern day seawater has a $^{87}Sr/^{86}Sr$ of 0.7092 and that the observed mean (outlier excluded) in enamel was 0.7087±0.0002 (assuming enamel reflects the local value), the intermediate Sr isotope ratio for bone would result in ~0.7090. This is approximately the mean seen in the bone (0.7090±0.0002, 2σ) of the group, exclusive of the single outlier. This mixing effect in coastal or near-coastal regions has been discussed in a number of provenancing studies (Wright, 2012, Montgomery, 2010b, Frei and Frei, 2013, Price et al., 2012a) and hence would support the assumption that the elevated Sr isotope ratios observed in bone could have derived from marine Sr input. However, the consumption of marine seafood meat alone is probably not enough to cause the offset whereas sea salt or salt-preserved meat is more likely to have such an effect (Montgomery, 2010a).

Finally, the possibility of diagenetic Sr contamination has to be taken into account as explanation for the raised Sr isotope ratios in bone. In order to ascertain this possibility it would require the analysis of the mobile soil and water from the burial environment. However, the interpretation can still be very problematic if the intrinsic Sr isotope ratio of bone acquired from a distinct geological, environmental or dietary background is identical to that of the burial environment. If this occurs, it will not be possible to discriminate between the biogenic and diagenetic Sr isotopic signature.

The lead data provides a stronger evidence for the homogeneity of the burial group, which seems to reflect the local Pb signature. All Pb isotope ratios in bone and enamel are overlapping within the analytical standard deviation with the exception of VR-05 and VR-10. While the Pb isotope ratio of enamel from individual VR-10 compares to the "local" group, the result for bone implies that this person had spent his last years (possibly >7 years) in a distinct location with a less radiogenic Pb isotope ratio than that characteristic for Villanueva del Rosario. The opposite applies to VR-05 who seems to have migrated some time after completion of enamel mineralisation in childhood to Villanueva del Rosario and lived there during his last years (probably >7 years).

The relatively low Pb isotope ratios seen in the Spanish bone and enamel samples ($^{206}Pb/^{207}Pb$ 1.163 to 1.170) are not comparable to reported literature data (Reimann et al., 2012) from the area ($^{206}Pb/^{207}Pb$ 1.191 to 1.202). However, this study also inferred the presence of regional anomalies in terms of Pb isotopic signals. Such anomalies could be directly related to the geology rather than linked to a Pb deposit or contamination (Reimann et al., 2012). This might also explain the irregularity seen in Villanueva del Rosario since a low Pb isotopic composition of <1.174 has not been documented in the vicinity of the study location. Furthermore, the consistency of the burial group in terms of the Pb isotope ratios analysed hints towards a "local" but unknown Pb source.

It can also be speculated that the lower Pb ratios among the burial population were acquired from the main water supply of Villanueva del Rosario. The water supply derives from the local spring, which drains the Alta Cadena carbonate aquifer. The carbonate rocks of the Alda Cadena are composed mainly of Jurassic and Cretaceous limestones (Mudarra and Andreo, 2011). Hard water sources derived from such geologic background are known to offer some protection against Pb-uptake (Jaworowski, 1990) hence lowering the isotopic Pb ratio in human tissue. This is based on some studies that have shown that inhabitants of urban areas with hard water have lower Pb burdens than people living in rural areas that have soft water, even when hard water areas are considerably polluted (Jaworowski, 1990, Jaworowski et al., 1985).

Any unusual high source of environmental lead contamination (eg. from pollution or impact of lead pipes carrying drinking water) seems highly unlikely as the lead concentrations in bone are within a normal range of 0.21 to 0.80 ppm, and 0.05 to 0.42 ppm in enamel.

6.9 Conclusion and future work

Given the homogenous clustering of the majority of bone and enamel samples, the results from Sr and Pb isotope analysis favour the conclusion that most human remains from the mass grave in Villanueva de Rosario were of local origin, and that the offset between bone and enamel observed is more likely to reflect a possible impact of marine atmospheric input or variations of dietary Sr intake rather than the individuals themselves being of non-local origin. Particularly the Pb isotopic ratios seem to support this hypothesis, as they show no significant offset between the two sample types. The more significant isotopic differences between bone and enamel in the two individuals VR-05 and VR-10 suggests that they had migrated between childhood and adulthood. One individual (VR-05) seems to have joined the local population later in life (>12 years of age) while the other person (VR-10) left the local group after having spent his childhood there. These findings could potentially be very important information in the identification of these individuals. The remainder of the group did not exhibit any isotopic particularities that could potentially be useful to confirm their identities.

This case study has demonstrated that Sr and Pb analysis can generate valuable information in revealing a potential migratory history, which could contribute to a certain extent to human identification. At the same time it is also evident that the identification of individuals depends also greatly on additional information to make sense of the isotopic data, as isotopic signatures are seldom sufficiently "unique" that they could be attributed to one specific geographic origin.

In order to determine the possibility of migration it requires the knowledge and definition of the local geology and bioavailable Sr isotopic composition that can be related to the isotopic composition in human skeletal tissue. Without this knowledge any conclusions in terms of provenancing is a guess at best. It is therefore unfortunate, that no suitable sample material was available for isotope analysis that could have provided comparable biosphere data from this specific area. This could have delivered a clearer picture of the local isotopic composition and might have strengthened the findings of the isotopic results.

Concluding, it is presently only possible to say that the Sr and Pb results indicate the presence of two migrants among the presumably local burial group but that neither of the groups can be ascertained as "true" locals without further investigation and analysis of suitable reference sample material. Also, no conclusion on the potential migratory history of the two individuals, VR-05 and VR-10, can be drawn at this point as the findings of this study remain to be compared with the ante-mortem information and the testimonies of the relatives of the victims that were collected by the Spanish collaborators in this study.

If the Spanish case should be pursued in future, it is not only crucial to have the necessary access to environmental reference samples for establishing a baseline Sr isotopic signature for the location for comparison, but it might also be worthwhile to include supportive evidence from oxygen isotope analysis to shed further light on the geographic origin of the individuals. As previously discussed in chapter 3 ("Isotope Geochemistry"), the analysis of O isotopes has been commonly utilized in provenancing studies of humans. Oxygen isotopic systems reflect climatic conditions, which vary with geography and weather. The oxygen isotopic composition from rainwater is ultimately absorbed and retained in bone and teeth mainly through the uptake of local drinking water. Particularly human hair has been found to reflect the ingested water source very accurately (Ehleringer et al., 2008). For archaeological studies, however, enamel is the most interesting tissue as it preserves the original isotopic oxygen composition of the ingested drinking water during the time of enamel mineralization (Budd et

al., 2001) and thus could provide an additional link to the childhood place of origin.

The greatest shortfall of this case study is certainly the lack of genetic data that could have established kinship with their living relatives and thus their identities. Such data would have immensely facilitated the provenancing process and concluded the debate whether these individuals were locals or migrants. However, the methods and protocols, respectively, tested in this study were not successful in retrieving sufficient DNA for analysis. If more sensitive methods and additional sample material would become available it might be interesting to re-investigate the case but above all it would be most important for the living relatives to find the answer they were hoping for.

Chapter 7

7 Norfolk – Anglo/Saxon case study

7.1 Historic Background

After the fall of the Roman Empire around 410 A.D., the *Adventus Saxonum* marked the invasion of the British Isles by the Germanic tribes from Angeln and Saxony in what is now Northern Germany. The Anglo-Saxon period lasted for ~600 years (410 to 1066 A.D.) and ended with the Norman Conquest in 1066. During the early Anglo-Saxon rule in the 5th and 6th century the seven kingdoms of Northumbria, Mercia, East Anglia, Essex, Kent, Sussex and Wessex were established. During the late Saxon period Anglo-Saxon Britain increasingly suffered from Viking invaders arriving from Denmark and Norway who were drawn to the British Isles by the wealth of the Anglo-Saxons. Historic records about the Viking Age are written in the "Anglo-Saxon Chronicle" (Ingram, 1823 (translated 1912)). They document that the Viking (Danish) settlements in England were predominantly found in the northern and eastern part of England, an area called "Danelaw", which refers to the area where the Danish law was executed as a result of a treaty in 884 between the Danish Viking chieftain Guthrum and Alfred, the Great King of Wessex. The Scandinavian influx from the Viking Age is still apparent in some of the names of villages ending with -by, such as Hemsby, Ormsby, Whitby etc. The East of England was in most cases the first port of call for all invaders from the Continent be it Saxons or Vikings. Many of them settled in Norfolk where excavations of burial sites in North Elmham ("Spong Hill"), Sutton Hoo in Woodbridge/Suffolk and other places in East Anglia have revealed the great cultural influence of Anglo-Saxons on this region.

Traditionally, the Anglo-Saxon immigration from the continent has been described as mass migration, that led to the wipe-out of the British indigenous people (Thomas et al., 2006). Only in the late 1980's the view of historians and archaeologists changed to favour the "elite replacement" theory where only a small elite of immigrants from the continent achieved military, political and social power (Hughes et al., 2014). Nowadays it has been widely accepted among historians and archaeologists that immigrants arriving in Britain, from the Romans to the Normans, arrived in small groups in Britain rather than by mass invasion (Pattison, 2008).

A great research effort has gone into investigating the genetic past of the people in the British Isles. Y-chromosome markers were applied to trace the possible Anglo-Saxon origin of males in north Wales, central and eastern England, and using Friesland in Netherlands as a positive control as Friesian are considered close living relatives of the original Saxons (Weale et al., 2002). This study revealed that the English males were genetically different from those in Wales, and similar to those in Friesland. Weale et al. concluded that their findings indicated a massive migration of Frisian males into central England contributing 50 to 100 % to the gene pool at that time.

In a larger study on Y-chromosomes Capelli et al. 2003 (Capelli et al., 2003) followed up the previous findings of Weale and colleagues and investigated males from across Britain including sample sets from Norway, Denmark and Germany (Schleswig-Holstein). Comparing samples from Schleswig-Holstein (Northern Germany) and from Denmark with British data indicating that southern England was "predominantly indigenous" (Capelli et al, 2003, 982) while the results for eastern and central England still pointed to significant continental genetic influence. The highest degree of German and Danish contribution was found in York and Norfolk.

7.2 Archaeological Background

Anglo-Saxon cemeteries have been predominantly found in the eastern and southern parts of England, which were in use over centuries. The graves discovered within the grounds of Snarehill Hall in Brettenham, Norfolk, UK date back to Late Anglo-Saxon and medieval times.

The following information on the excavation has been retrieved from the Norfolk Archaeology Unit excavation report (Whitmore, 2001).

In June and July 2001 the Norfolk Archaeological Unit (NAU) carried out a building recording and excavation external to the east wall of a disused Late Saxon church within the grounds of Snarehill Hall, Brettenham, in southwest Norfolk. The work revealed the presence of eighty-nine intercutting graves of Late Saxon and medieval date (10^{th} to 16^{th} century).

The excavation undertaken within the grounds of Snarehill Hall produced a total of 157 contexts. Almost 50 % of the human skeletal remains excavated were well preserved with a completeness of 75 %.

7.2.1 Excavation site

Snarehill Hall lies approximately 2 km to the east of the centre of Thetford on a piece of land south of the River Thet and north of the River Little Ouse. The site is situated on the edge of a plateau of land with the ground sloping away northwards towards a slight bend in the River Thet. The church lies within the grounds of the Hall approximately 30 m to the north of the main Hall buildings (Whitmore, 2001). To the east of the church wall a total of eighty-nine partial or complete graves were identified, which were all oriented east to west. The graves contained evidence of coffins in the form of wood staining, and textile fragments. Some of the earliest burials appeared to have had small chalk blocks

placed at strategic positions around the body, possibly reflecting a Saxon tradition.

7.3 Geology of Norfolk/East Anglia

The geological foundation of most of Norfolk has been laid during the Cretaceous period 144 to 65 million years ago. The bedrock of marine limestone stretches all the way from the North Norfolk coast down to the south-western corner of Norfolk and beyond (Figure 31).

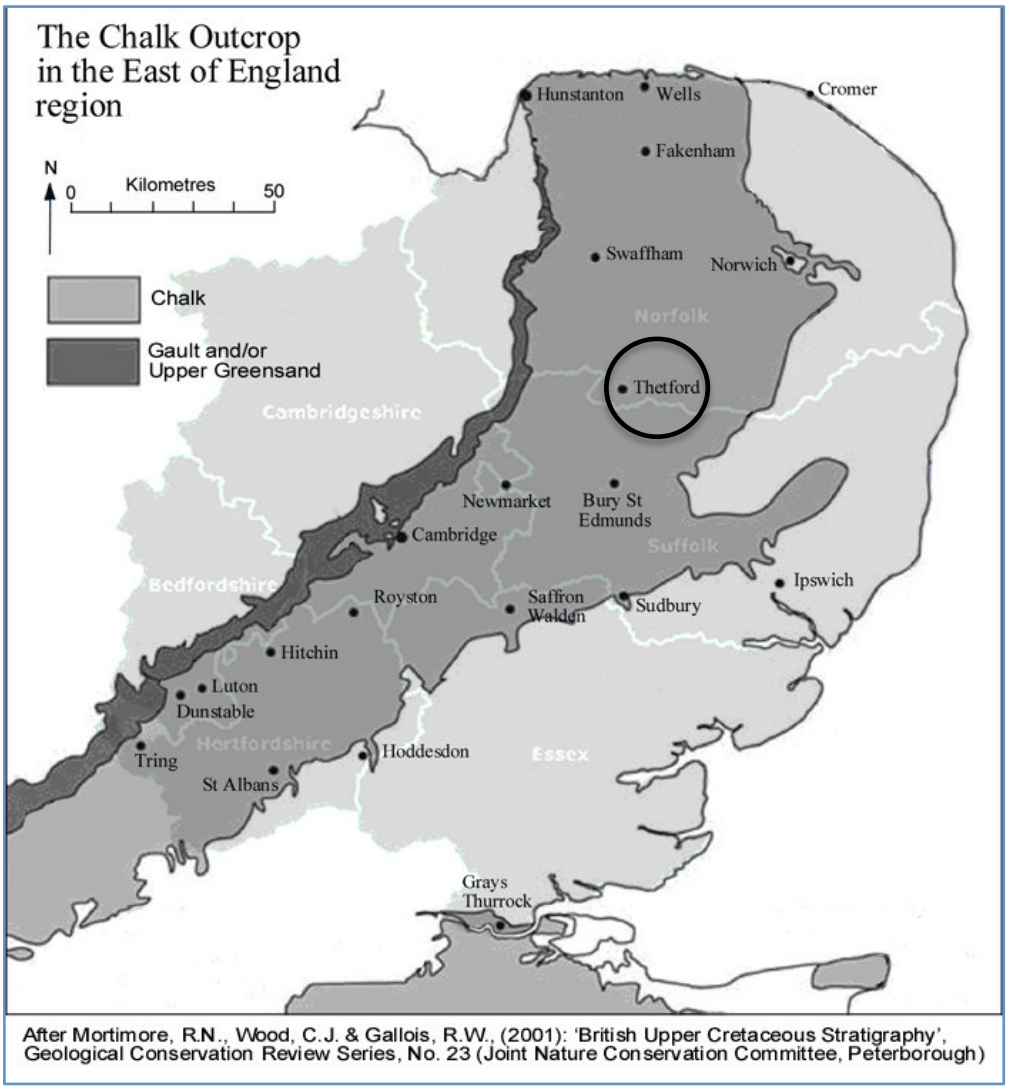

Figure 31: Geological map of the East of England (Mortimore, 2001). The circle indicates the approximate location of Snarehill Hall in Brettenham.

7.4 Norfolk/Snarehill bone and teeth samples:

The sampling of bone and teeth was undertaken at the archive of the Norfolk Museums and Archaeology Service Unit in Norwich, UK. The skeletons were provided by the Archaeology Service and chosen for their completeness and good preservation state. One long bone (femur or humerus) and one molar were taken from each individual for both DNA and isotope analysis.

No	Skeleton	Age class	Sex	Bone	Teeth
1	**NAU 116**	Middle Adult (35-50yrs)	M	L Humerus	3rd molar
2	**NAU 119**	Young Adult (20-35yrs)	F	R Femur	3rd molar
3	**NAU 130**	Old Adult (50+yrs)	M	L Femur	3rd molar
4	**NAU 153**	Middle Adult (35-50yrs)	F	L Femur	3rd molar
5	**NAU 173**	Young Adult (20-35yrs)	F	R Femur	3rd molar
6	**NAU 203**	Middle Adult (35-50yrs)	M	L Femur	3rd molar
7	**NAU 212**	Young Adult (20-35yrs)	M	L Femur	3rd molar
8	**NAU 246**	Middle Adult (35-50yrs)	M	R Femur	3rd molar
9	**NAU 273**	Young Adult (20-35yrs)	F	R Femur	3rd molar
10	**NAU 297**	Young Adult (20-35yrs)	M	L Femur	3rd molar
11	**NAU 322**	Middle Adult (35-50yrs)	F	L Femur	3rd molar
12	**NAU 325**	Middle Adult (35-50yrs)	M	L Femur	3rd molar
13	**NAU 378**	Middle Adult (35-50yrs)	M	L Humerus	3rd molar
14	**NAU 393**	Middle Adult (35-50yrs)	F	L Radius	3rd molar

Table 19: Bone and teeth samples from the excavation site at Snarehill Hall in Brettenham/Norfolk.

7.4.1 Snarehill soil samples

Eight soil samples were collected from the excavation site itself and from different points within the grounds of Snarehill Hall for a representative sample set of the location.

Approximately 300 g of subsoil was collected into paper bags for analysis and locations recorded with GPS as shown below (Table 20).

Snarehill soil sample	GPS location (WGS 84)
SH1	N 52.41706 / E 0.78026
SH2	N 52.41706 / E 0.78056
SH3	N 52.41691 / E 0.78051
SH4	N 52.41678 / E 0.77865
SH5	N 52.41823 / E 0.77927
SH6	N 52.41822 / E 0.77923
SH7	N 52.41560 / E 0.78164
SH8	N 52.41058 / E 0.84118

Table 20: Snarehill soil samples with according GPS locations

7.5 DNA analysis

The specific interest in this instance is the identification of potential Anglo-Saxon immigrants from Germany or Viking immigrants from Scandinavia (Denmark and Sweden) recovered from the burial site at Snarehill Hall using mtDNA markers. The commonly encountered fragmented nature of DNA in archaeological human tissue requires the use of mitochondrial DNA markers as discussed earlier in chapter 2 ("Ancient DNA"). The greater abundance of mtDNA present in human cells increases usually the recovery of sufficient DNA for further analysis and it also allows to examine questions of ancestry and migration of peoples throughout history as it is passed on from generation to generation through the maternal line.

7.5.1 DNA analysis

The analytical procedures have been previously described in chapter 5 ("Materials and Methods" - "DNA analysis"). Hence this chapter will only present the outcome of DNA analysis.

7.5.2 DNA results for Norfolk of bone and teeth

Multiple DNA extracts from the Norfolk bone samples were used to assess the efficiency of the different extraction protocols as previously described in Chapter 5. Regretfully, none of the protocols tested produced any detectable DNA after quantification so that the ancestry of the burial population could not be resolved.

7.5.3 Conclusion and future work

DNA from ancient samples is notoriously challenging to extract because of its degraded state and low copy number. Additionally, often the presence of PCR inhibitors introduced into skeletal remains from the burial environment is co-extracted during DNA extraction and prevents the amplification of target DNA. Due to the age of the sample material which is estimated to be ~1000 years old DNA degradation and/or PCR inhibitors seem the most likely scenario for the unsuccessful outcome. However, obtaining correct results also depends to a high degree on the method of extraction and/or parameters used for PCR amplification. Often it is a matter of trial and error to identify the actual cause why a specific method has not produced the anticipated results since there is a range of different factors involved in each step of aDNA analysis that can be responsible for a certain effect.

However, there are several technical options that could be considered to improve results provided sufficient sample material was available. One way to increase DNA yields is to use more bone material and teeth for extraction. Where only a low concentration of DNA is present, increasing PCR amplification cycles could also be an effective approach to increase DNA copies. However, there is also the risk involved that “foreign” modern DNA is co-amplified, which would override the original signal of degraded DNA. Sometimes also increasing the injection time and/or voltage on the sequencer can improve signal intensity when low concentrations of DNA are analysed.

It would also be interesting and worthwhile to test if new analytical techniques could possibly improve DNA recovery to produce the results that were anticipated to provide the ancestry information in the Norfolk case study.

7.6 Strontium and lead isotope analysis

The objective of this study was to analyse the elemental content and isotope composition of strontium and lead in bone and teeth and to investigate geographic origin and migration of the individuals at Snarehill Hall. The analytical steps were already described in Chapter 5. The results thereof will be discussed in the following sections.

7.6.1 Results

Bone (n=14) and teeth (n=14) from 14 individuals from the Snarehill excavation site were cleaned, processed for and analysed by ICP-MS for their Sr and Pb concentrations, respectively, and measured for their Sr and Pb isotope ratios by MC-ICP-MS as described in the "Materials and Methods" chapter 5.

Table 21 below shows the accumulated Sr and Pb concentration/isotope data for the Norfolk bone/teeth samples. Most samples were analysed in triplicate unless stated otherwise in the table.

Sample ID	Sample Type	n	mean Sr (ppm)	n	mean 87Sr/86Sr	n	mean Pb (ppm)	n	206Pb/204Pb	207Pb/204Pb	208Pb/204Pb	206Pb/207Pb
NAU116/B	Bone	3	185 ± 2	3	0.70814 ± 0.00001	3	18 ± 0.3	3	18.52 ± 0.01	15.70 ± 0.01	38.63 ± 0.05	1.180 ± 0.0002
NAU116/T	Enamel	2	79 ± 2	3	0.70836 ± 0.00006	2	3 ± 1	3	18.51 ± 0.07	15.66 ± 0.08	38.53 ± 0.25	1.182 ± 0.002
NAU119/B	Bone	2	216 ± 17	3	0.70809 ± 0.00001	2	5 ± 0.3	3	18.48 ± 0.01	15.64 ± 0.01	38.47 ± 0.03	1.181 ± 0.0003
NAU119/T	Enamel	2	104 ± 5	3	0.70830 ± 0.00002	2	1 ± 0.01	3	18.53 ± 0.05	15.70 ± 0.03	38.62 ± 0.05	1.181 ± 0.003
NAU130/B	Bone	2	178 ± 7	3	0.70813 ± 0.00003	2	18 ± 2	3	18.48 ± 0.02	15.64 ± 0.02	38.47 ± 0.07	1.182 ± 0.0005
NAU130/T	Enamel	2	114 ± 6	3	0.70864 ± 0.00003	2	1 ± 0.4	3	18.56 ± 0.07	15.70 ± 0.03	38.62 ± 0.13	1.183 ± 0.003
NAU153/B	Bone	3	236 ± 18	3	0.70808 ± 0.00001	3	25 ± 1	3	18.49 ± 0.03	15.69 ± 0.05	38.57 ± 0.11	1.178 ± 0.002
NAU153/T	Enamel	1	74	1	0.70852	1	3	3	18.52 ± 0.06	15.71 ± 0.06	38.61 ± 0.13	1.179 ± 0.002
NAU173/B	Bone	3	221 ± 1	3	0.70814 ± 0.00001	3	28 ± 2	2	18.50 ± 0.01	15.63 ± 0.01	38.43 ± 0.02	1.184 ± 0.0001
NAU173/T	Enamel	1	97	2	0.70868 ± 0.00003	1	7	3	18.57 ± 0.05	15.70 ± 0.05	38.64 ± 0.16	1.183 ± 0.001
NAU203/B	Bone	3	188 ± 3	2	0.70821 ± 0.00003	3	43 ± 1	2	18.48 ± 0.01	15.64 ± 0.01	38.47 ± 0.02	1.182 ± 0.0002
NAU203/T	Enamel	1	115	2	0.70866 ± 0.00004	1	2	2	18.53 ± 0.05	15.68 ± 0.03	38.57 ± 0.10	1.181 ± 0.001
NAU212/B	Bone	3	189 ± 1	3	0.70818 ± 0.00001	3	36 ± 1	3	18.47 ± 0.01	15.63 ± 0.01	38.44 ± 0.03	1.182 ± 0.0005
NAU212/T	Enamel	3	117 ± 4	2	0.70845 ± 0.00001	3	4 ± 0.3	2	18.52 ± 0.05	15.67 ± 0.08	38.57 ± 0.24	1.182 ± 0.003
NAU246/B	Bone	3	186 ± 3	3	0.70807 ± 0.00003	3	15 ± 0.7	3	18.51 ± 0.04	15.67 ± 0.05	38.57 ± 0.15	1.181 ± 0.001
NAU246/T	Enamel	1	108	1	0.70878	1	1	2	18.68 ± 0.02	15.83 ± 0.04	38.94 ±0.07	1.180 ± 0.002
NAU273/B	Bone	3	202 ± 2	3	0.70825 ± 0.00001	3	34 ± 3	3	18.48 ± 0.01	15.61 ± 0.003	38.40 ± 0.01	1.183 ± 0.0001
NAU273/T	Enamel	2	126 ± 2	2	0.70872 ± 0.00001	2	3 ± 0.2	2	18.49 ± 0.01	15.62 ± 0.01	38.44 ± 0.03	1.184 ± 0.001
NAU297/B	Bone	3	240 ±1	2	0.70822 ± 0.00003	3	11 ± 0.2	2	18.56 ± 0.02	15.72 ± 0.01	38.75 ± 0.03	1.180 ± 0.001
NAU297/T	Enamel	3	118 ± 1	2	0.70867 ± 0.00002	3	2 ± 0.02	2	18.52 ± 0.02	15.69 ± 0.05	38.59 ± 0.13	1.181 ± 0.002
NAU322/B	Bone	3	199 ± 1	2	0.70816 ± 0.00004	3	15 ± 0.5	3	18.51 ± 0.01	15.64 ± 0.01	38.52 ± 0.03	1.183 ± 0.0004
NAU322/T	Enamel	1	62	2	0.70910 ± 0.00001	1	8	3	18.50 ± 0.04	15.66 ±0.03	38.52 ± 0.12	1.181 ± 0.001
NAU325/B	Bone	2	227 ± 10	3	0.70820 ± 0.00003	2	83 ± 3	3	18.49 ± 0.01	15.63 ±0.01	38.45 ± 0.03	1.183 ± 0.0003
NAU325/T	Enamel	3	148 ± 2	2	0.70865 ± 0.00001	3	10 ± 0.5	3	18.50 ± 0.10	15.69 ± 0.03	38.59 ± 0.16	1.179 ± 0.005
NAU378/B	Bone	2	284 ± 31	3	0.70824 ± 0.00003	2	75 ± 8	3	18.46 ± 0.01	15.63 ± 0.001	38.44 ± 0.01	1.181 ± 0.0001
NAU378/T	Enamel	2	140 ± 1	3	0.70870 ± 0.00003	2	20 ± 0.3	3	18.51 ± 0.02	15.67 ± 0.01	38.58 ± 0.04	1.181 ± 0.001
NAU393/B	Bone	3	222 ± 1	3	0.70813 ± 0.00001	3	17 ± 1	2	18.46 ± 0.01	15.62 ± 0.002	38.41 ± 0.01	1.181 ± 0.0003
NAU393/T	Enamel	3	79 ± 2	2	0.70851 ± 0.00002	3	3 ± 0,04	2	18.56 ± 0.01	15.70 ± 0.05	38.67 ± 0.15	1.182 ± 0.004
Snarehll soils		0	no data	7	0.70805 ± 0.00011	0	no data	8	18.14 ± 0.07	15.63 ± 0.03	38.14 ± 0.09	1.161 ± 0.004
Snarehill water			0.3685 ± 0.0009	3	no data		0.0004±0.0007	3	no data	no data	no data	no data
NIST 1486		5	293±9	11	0.70940 ± 0.00002	5	1.4±0.1	10	19.10 ± 0.08	15.71 ±0.03	38.77 ± 0.11	1.216 ± 0.005

Table 21: Sr and Pb concentration and isotope ratio composition data for Norfolk bone and teeth enamel samples including Snarehill soils and water samples as well as the NIST1486 bone meal standard.

Results of the measurements for reference standard NIST1486 bone meal and the certified values are listed in Table 22. The reproducibility was determined by multiple analysis' (7x) of separately digested samples.

Element	**NIST 1486 Bone meal Certified (µg/g)**	**NIST 1486 Bone meal Measured (µg/g)**
Sr	264 ± 7	293 ± 9
Pb	1.335 ± 0.014	1.44 ± 0.13

Table 22: NIST 1486 Bone meal standard reference material certified values for Sr and Pb versus measured values (µg/g).

7.7 Results of Sr concentrations and isotope results for bone and teeth

7.7.1 Sr concentrations

The measured Sr concentrations for the Norfolk bone samples show values of 178 to 284 ppm (212±29, 1σ), while concentrations for enamel were between 74 to 148 ppm (106±25, 1σ).

Sr levels in human bone reported in the literature are very variable and range between 40 and 740 ppm (Turekian and Kulp, 1956, Hoogewerff et al., 2001, Ezzo, 1994, Mays, 2003, Thurber et al., 1958, Beard and Johnson, 2000). In a worldwide survey Thurber et al. (Thurber et al., 1958) compiled Sr data of human bone and reported values of 105-344 ppm and a mean of 172 ppm across all continents. Mays et al. (Mays, 2003) also published similar concentrations for modern bone (100-300 ppm), and Beard et al. (Beard and Johnson, 2000) (100-500 ppm), which were analysed from comingled human remains from the Vietnam War. From the latter study also Sr concentrations in enamel were determined to be in the range 75-280 ppm.

The Sr concentrations determined in human enamel are consistent with data analysed in archaeological populations (50 to 150 ppm) (Evans et al., 2006b, Evans and Tatham, 2004), and which are also similar to concentrations found in modern teeth (Underwood, 1977).

7.7.2 $^{87}Sr/^{86}Sr$ for bone and enamel

Sr ratios from bone and enamel including NIST 1486 bone meal standard reference material and Snarehill soils are plotted and shown in Figure 32.

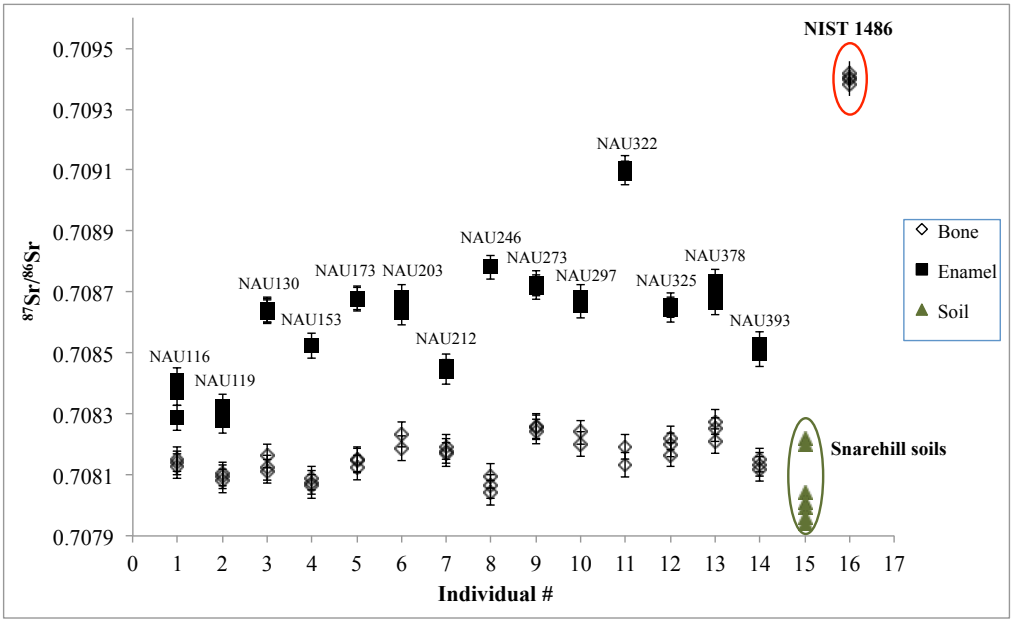

Figure 32: Strontium isotope data for the Norfolk bone/tooth enamel samples (+/- 0.00004, 2σ) incl. Snarehill Hall soil samples and NIST 1486 bone meal standards. Error bars represent the measurement uncertainty 2σ of NIST 1486.

Isotope ratios for Norfolk bone show very little variation with the lowest value of 0.70807 and the highest with 0.70825 among individuals. Strontium ratios for tooth enamel lie within a wider range between 0.70830 and 0.70891 and are generally higher than in bone across all samples.

Sr ratios between bone and enamel vary significantly (t-test: $p<0.05$). The greatest difference between bone and enamel is seen in NAU322 (0.70816 in bone vs. 0.70910 in enamel).

The Snarehill soil samples (n=8) fall within the same range as the Snarehill bone collection with isotope ratios ranging from 0.70794 to 0.70822. The similarity of Sr ratios thus raises the question if all the bone samples have exchanged Sr with the soil and only the enamel represent original values.

7.7.3 Reference data for $^{87}Sr/^{86}Sr$

Fortunately, there have been some efforts recently to produce isotope maps, which can be used as valuable reference material for forensic and archaeological provenancing applications (Evans et al., 2010, Voerkelius et al., 2010, Frei and Frei, 2011, Frei and Frei, 2013).

UK:

The Sr isotope map from Evans et al. (Evans et al., 2010) is based on measurements of different sample types such as water, plant, soil, shells and bone material from Britain. Hence it presents the bioavailable Sr fraction, which is particularly useful in characterising the geographic origin in bone and teeth. The reference data for strontium isotope ratios for the Snarehill Hall area gives a very narrow range between 0.708 and 0.709 (Figure 33).

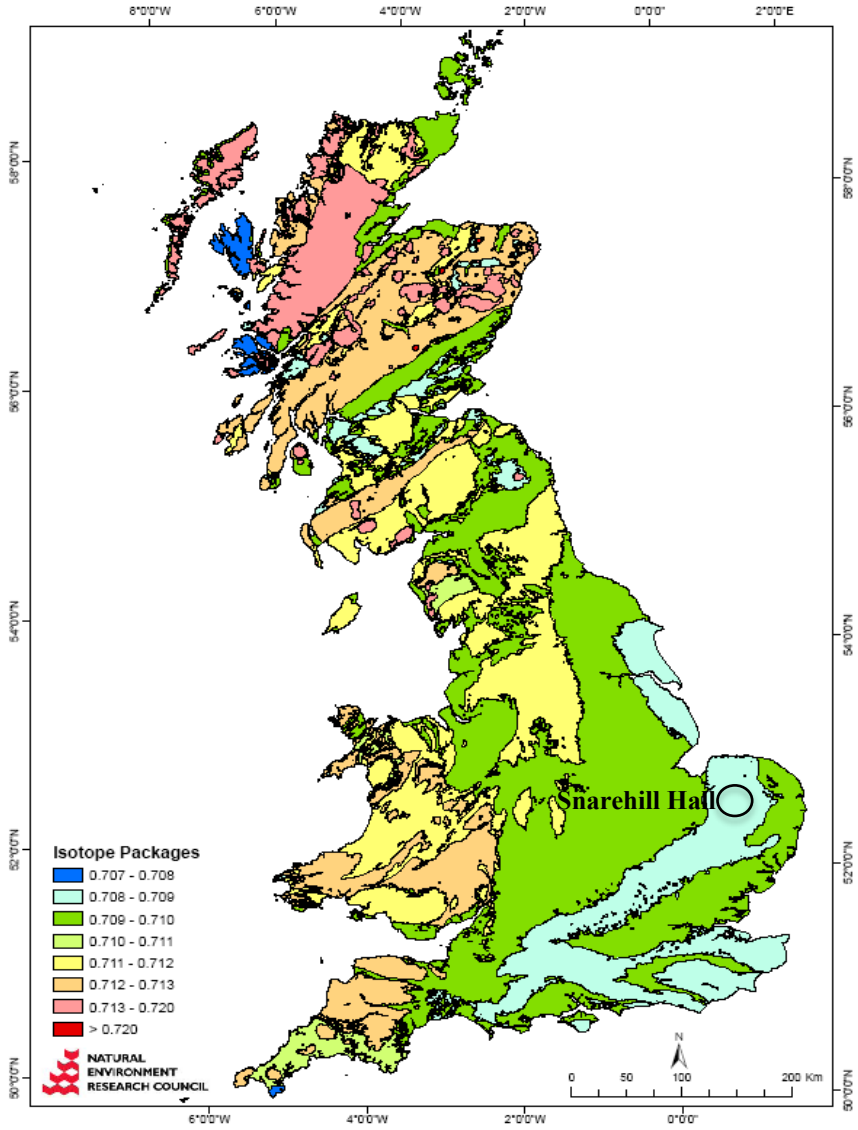

Figure 33: Map of biosphere strontium isotope variation across Britain (Evans et al., 2010). The circle indicates the approximate location of Snarehill Hall.

The strontium isotope values for the Norfolk bone and teeth samples measured in this study correlate with the reference values reported by Evans et al. (Evans et al., 2010) with a mean of 0.70814 for bone and 0.70862 for enamel.

Another valuable source of Sr isotope data has been compiled from all over Europe (Voerkelius et al., 2010) by analysing 650 different mineral waters. The Sr concentrations analysed from the UK/Norfolk were between 0.70701 and

0.70900, which overlaps with the data from Evans et al. (Evans et al., 2010). Isotope ratios for Northern Germany (Schleswig-Holstein) and Denmark lie within the range of 0.70901 to 0.71100 although two "outliers" from inland Denmark show identical ratios to those found in Norfolk (Figure 34).

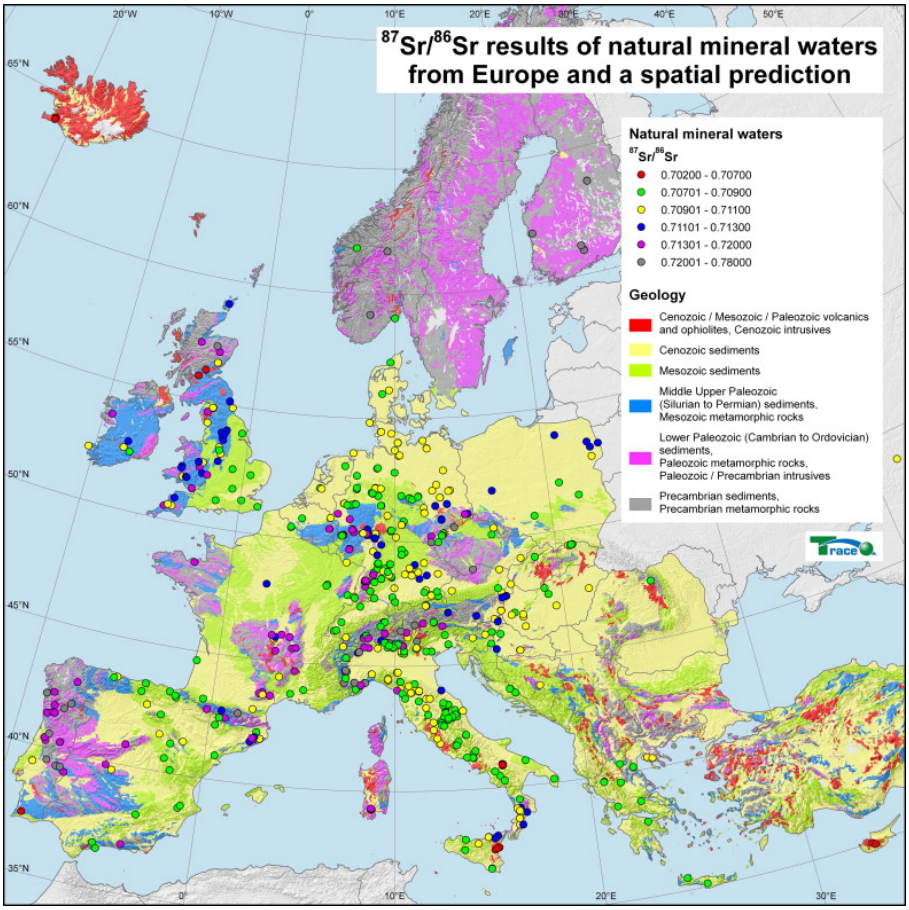

Figure 34: Map with $^{87}Sr/^{86}Sr$ results of natural mineral waters from Europe (Voerkelius et al., 2010)

Denmark

Two studies on the Sr isotopic composition of Danish surface waters have been published by Frei et al. (Frei and Frei, 2011, Frei and Frei, 2013), and constitute useful reference data for a potential Viking origin among the human remains

from Norfolk. Sr isotope values reported for Danish surface waters range from 0.7078 to 0.7125 (average 0.7096 ± 0.0016).

In a different study by Frei et al. (Frei and Price, 2012) Danish fauna and human enamel data were measured, which showed a range from $^{87}Sr/^{86}Sr$ 0.70717 to 0.71185 with an average of 0.70919 for the fauna samples while for human enamel the range covered $^{87}Sr/^{86}Sr$ from 0.7086 to 0.7110 with an average of 0.7098.

Sample type	Sr ratios	Country	Reference
Water, plant, soil, shells and bone	0.708-0.709	East Anglia/UK	(Evans et al., 2010)
Mineral water	0.70701-0.70900 0.70901-0.71100 0.70901-0.71100 0.70701-0.71100	East Anglia/UK Northern Germany Denmark Netherlands	(Voerkelius et al., 2010)
Surface water	0.7078 to 0.7125	Denmark	(Frei and Frei, 2011, Frei and Frei, 2013)
Fauna and Human enamel	0.70717-0.71185 0.7086-0.7110	Denmark	(Frei and Price, 2012)
Human tooth enamel	0.7078-0.7108	Denmark/Jutland	(Price et al., 2012c)
Red deer antler	0.70907	Northern Germany /Jutland	(Grupe, 2011)

Table 23: Accumulated Sr isotope ratios published from UK, Denmark and Northern Germany by different authors as referenced.

Strontium isotope data (enamel) reported from Viking assemblages are consistently higher than 0.7092 (Chenery et al., 2014, Symonds et al., 2014), which correlates with the more radiogenic underlying ancient geology prevalent in most of Scandinavia.

7.8 Results for Pb concentrations and isotope data of bone and teeth

7.8.1 Pb concentrations

The results for lead concentrations in bone show a broad range from 5 to 83 ppm (30±23, 1σ) and in enamel 1 to 20 ppm (5±5, 1σ) (Table 21). The elevated levels of Pb measured in Norfolk bone and teeth are well above normal physical background lead levels reported for archaeological bone material. Several studies have measured pre-historic bone and concluded with similar values for averaged "natural" Pb concentrations of <0.65 μg g^{-1} (Drasch, 1982, Ericson, 1991, Shafer et al., 2008). Ericson et al. (Ericson, 1991) determined averaged Pb concentrations from pre-historic bone from North American Pecos Indians (~1400 A.D.) of 0.65 ppm and Shafer et al. (Shafer et al., 2008) determined a similar value in their analysis of Iron Age bone. Archaeological bone that has been exposed to environmental Pb exhibits concentrations usually in access of 1 ppm (Ericson, 1991, Gulson, 2008). Historically excessive lead levels of up to 300 ppm (Montgomery, 2010b) have been documented during the Roman Empire as a consequence of the intense mining activity (Shafer et al., 2008) and the use of Pb in the production of pipes, pewters, tableware, beverage containers and many more household utensils (Wittmers, 2002). For comparison, modern bone values are between ~3-60 ppm lead (Montgomery, 2010b), which have been mainly the result of Pb additives in gasoline and Pb added to wall paints released into the environment in industrialised countries (Gulson, 2008).

A number of studies have shown that the original Sr and Pb signature is best preserved in enamel because its dense inorganic matrix remains largely unaffected by diagenetic alterations (Budd et al., 2001, Budd et al., 2004b, Montgomery et al., 2005a). Budd et al. 2004 (Budd et al., 2004b) reported lead concentration in pre-historic teeth (5500 BP) of 0.04 to 0.4 ppm and stated that natural lead concentrations at any period were approximately 0.5 to 1 ppm, even in modern British populations where values of 1 ppm were reported. However, teeth from Romano-British populations revealed significantly elevated Pb levels of ~40 ppm (Budd et al., 2004b), which reflects the intense ore exploitation and use of leaded products.

The elevated lead levels observed in Norfolk bone (5-75 ppm) and teeth samples (3-20 ppm) are more comparable to those documented for the preceding Romano-British period or modern Pb concentrations and hence suggest that these individuals were exposed to increased environmental lead levels (eg. from mining and/or use of lead utensils) rather than natural geologic lead during their lifetime.

7.8.2 Lead isotope results

$^{206}Pb/^{204}Pb$ lead isotope ratios for bone ranged from 18.46 to 18.56 whereas for enamel values were between 18.49 and 18.68, and $^{208}Pb/^{204}Pb$ isotope ratios were between 38.41 to 38.75 in bone and 38.44 to 38.99 in enamel, respectively. $^{206}Pb/^{204}Pb$ isotope ratios for the majority of bone and enamel samples were largely coherent and confined within a narrow range from 18.46 to 18.51 ($^{208}Pb/^{204}Pb$ 38.41 to 38.57). Only a single enamel value (NAU246) had a slightly higher $^{206}Pb/^{204}Pb$ of 18.68 ($^{208}Pb/^{204}Pb$ 38.94) compared to all other sample types. One bone sample (NAU297) exhibited $^{206}Pb/^{204}Pb$ of 18.56 ($^{208}Pb/^{204}Pb$ of 38.75) and is found just outside the top end of the reported literature data.

Snarehill soil samples were distinctly lower than the values for the bone/enamel samples. $^{206}Pb/^{204}Pb$ isotope ratios ranged from 18.01 to 18.21 (mean 18.14±0.07), and $^{208}Pb/^{204}Pb$ from 37.97 to 38.24 (mean 38.14±0.09), respectively.

The measured $^{206}Pb/^{204}Pb$ isotope ratios for each individual are given in Figure 35, including the average value for the Snarehill soil samples (n=8).

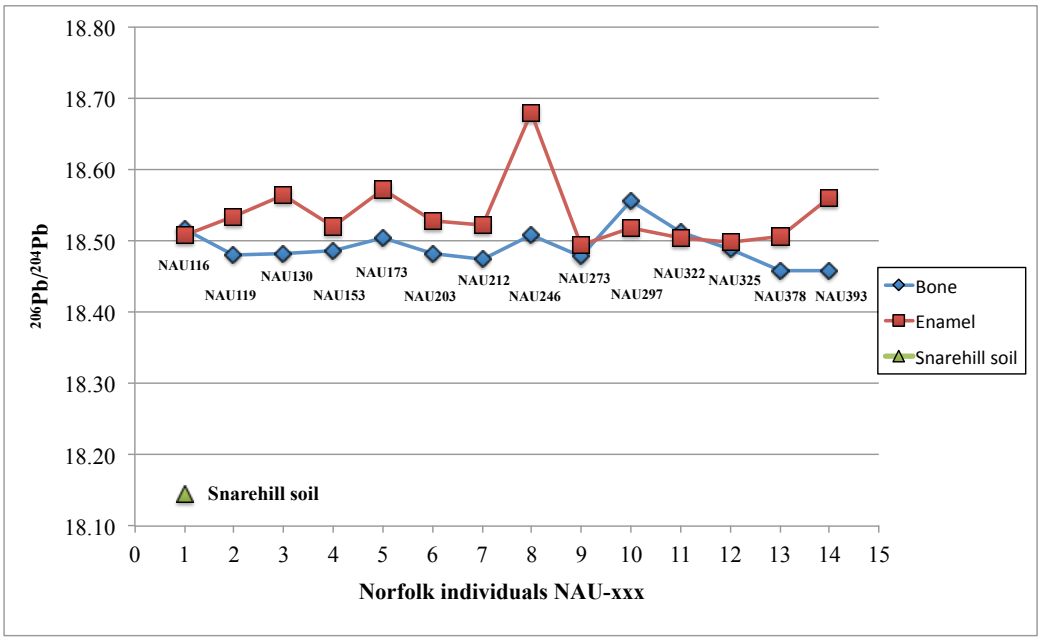

Figure 35: The plot shows the $^{206}Pb/^{204}Pb$ for the Norfolk bone and teeth samples including Snarehill soils. The values represent the mean values for each individual and soils analysed.

Figure 36 below shows the plotted lead isotope results for the Norfolk bone, enamel and soil samples in comparison to literature data from historical teeth from the UK (Kamenov and Gulson, 2014) and estimated range of Pb isotope ratios for anthropogenic British ores (Montgomery et al., 2005a).

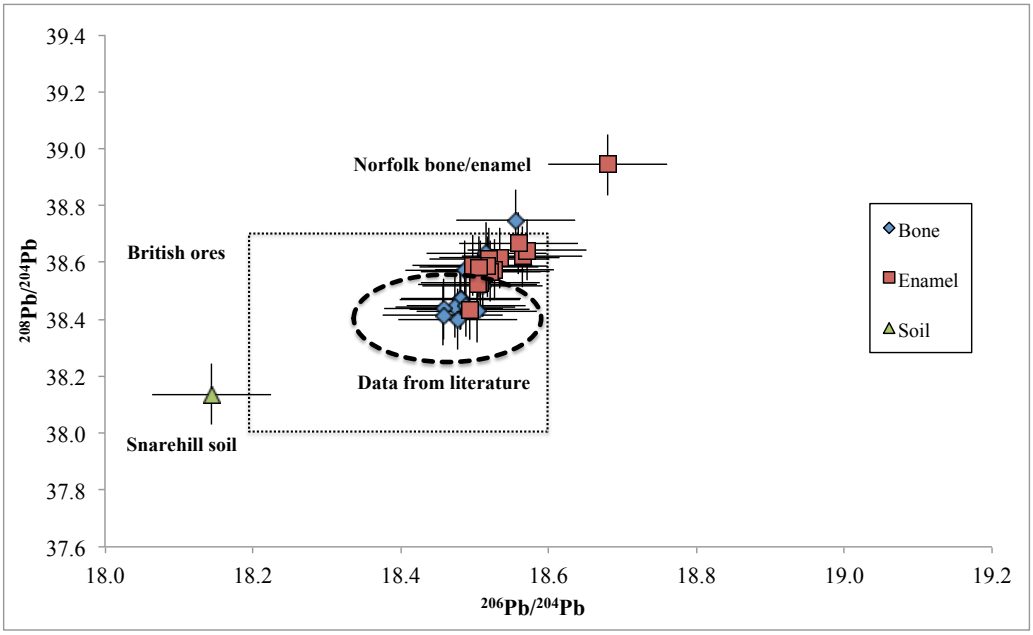

Figure 36: The plot shows the average Pb isotope ratios ($^{208}Pb/^{204}Pb$ and $^{206}Pb/^{204}Pb$) for the Norfolk bone, enamel and soil samples. Error bars represent 1 σ of NIST 1486 (n=10). The doted ellipse indicates the reported range for historical teeth from the UK, Rome and Norway (Kamenov and Gulson, 2014) and the dotted box the estimated British ore Pb isotope ratios (Montgomery et al., 2005a).

7.8.3 Pb isotope literature data

Literature data selected from Montgomery et al. (Montgomery et al., 2005a) in Table 24 represent the estimated range of Pb isotope ratios for anthropogenic British ores in comparison to the measured Norfolk bone and enamel values.

Pb isotope ratio	Estimated range of English ore values	Measured Pb ratios from Norfolk bone/tooth enamel
$^{206}Pb/^{204}Pb$	18.2 - 18.6	18.46 - 18.68
$^{208}Pb/^{204}Pb$	38.0 - 38.7	38.41 - 38.94
$^{206}Pb/^{207}Pb$	1.165 - 1.190	1.178 - 1.184

Table 24: Estimated Pb isotope ratios from Britain (Montgomery et al., 2005a) in comparison to the Norfolk bone and enamel samples.

Sample type	Pb ratios	Reported values	Country	Reference
Ore	$^{206}Pb/^{204}Pb$ $^{208}Pb/^{204}Pb$ $^{206}Pb/^{207}Pb$	18.2 – 18.6 38.0 – 38.7 1.165-1.190	Britain	(Montgomery et al., 2005a)
Historic teeth	$^{206}Pb/^{204}Pb$ $^{208}Pb/^{204}Pb$	18.44±0.10 38.44±0.16	UK and Rome, Norway	(Caulfield et al., 2015)
Agricultural soils	$^{206}Pb/^{207}Pb$	<1.174-1.191 1.174-1.245 1.174-1.245 <1.174-1.191	East Anglia Denmark N/Germany Netherlands	(Reimann et al., 2012)
	$^{207}Pb/^{208}Pb$	0.4046-0.4074 0.3978-0.4074 0.4046->0.4074 0.4046->0.4074	East Anglia Denmark N/Germany Netherlands	

Table 25: Accumulated Pb isotope ratios from various sources as referenced in the table.

The published lead data from agricultural soils from locations across Europe is an important source of geochemical reference data for isotopic provenancing. In this study Reimann et al. 2012 (Reimann et al., 2012) demonstrated that the Pb in European soils at present still represent the natural origin apart from a few unexplained local anomalies. The lead data for the area of East Anglia derived from this study showed lead concentrations between 15.4 and 23.3 mg/kg and the $^{206}Pb/^{207}Pb$ isotope ratios between 1.174 and 1.191. However, this area also contains a small pocket of $^{206}Pb/^{207}Pb$ below 1.174. The equivalent $^{206}Pb/^{207}Pb$ from the Norfolk study ranged from 1.178 to 1.184 for bone and 1.179 to 1.184 for enamel. Snarehill soils had a $^{206}Pb/^{207}Pb$ of 1.161 (±0.004).

7.9 Results for Rare Earth Elements from bone and teeth

The rare earth elemental concentrations (ppb) of the Norfolk bone and enamel samples are shown in Table 26 below.

Sample ID	La	Ce	Pr	Nd	Sm	Eu	Gd	Tb	Dy	Ho	Er	Tm	Yb	Lu	Σ REE (ppb)
NAU116/B	34.78	53.05	6.41	25.41	4.24	18.81	7.24	1.05	4.15	0.88	2.41	0.15	2.42	0.48	**161**
NAU116/T	14.09	26.81	4.80	<DL	<DL	5.91	<DL	2.45	8.00	3.04	4.68	3.11	<DL	<DL	**73**
NAU119/B	41.83	56.66	7.40	29.91	7.90	18.30	8.59	1.10	4.77	0.88	2.72	0.49	2.36	<DL	**183**
NAU119/T	9.24	10.96	1.47	6.67	<DL	2.22	<DL	<DL	<DL	<DL	<DL	0.11	<DL	<DL	**31**
NAU130/B	39.53	53.03	6.75	27.78	4.68	19.25	8.31	1.11	4.60	0.82	3.36	0.48	2.54	<DL	**172**
NAU130/T	4.23	16.81	0.73	4.29	<DL	<DL	<DL	0.84	3.11	<DL	<DL	0.24	<DL	<DL	**30**
NAU153/B	36.70	40.32	5.30	20.69	5.67	38.11	6.09	0.79	3.44	0.74	2.20	0.16	1.51	0.20	**162**
NAU153/T	<DL	178.73	<DL	<DL	<DL	<DL	<DL	1.80	<DL	<DL	<DL	<DL	<DL	<DL	**181**
NAU173/B	90.23	123.43	17.69	70.39	13.50	27.60	16.43	2.34	12.34	2.38	6.53	0.78	4.99	1.01	**390**
NAU173/T	106.64	656.43	15.17	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	**778**
NAU203/B	23.14	38.65	4.46	19.66	3.94	20.50	5.35	0.80	3.02	0.61	1.90	0.26	1.44	0.38	**124**
NAU203/T	6.80	5.67	0.81	6.58	<DL	1.18	<DL	<DL	1.68	0.29	1.06	<DL	<DL	<DL	**24**
NAU212/B	108.59	171.33	22.10	89.93	16.74	21.48	19.19	3.00	14.88	2.98	7.16	1.01	6.33	0.90	**486**
NAU212/T	11.07	12.69	<DL	<DL	<DL	<DL	<DL	1.82	<DL	<DL	<DL	<DL	<DL	<DL	**26**
NAU246/B	129.17	223.49	27.66	104.29	19.95	17.50	21.05	2.72	12.90	2.81	7.35	0.97	5.87	0.87	**577**
NAU246/T	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	101.25	<DL	<DL	<DL	<DL	<DL	**101**
NAU273/B	123.97	222.51	26.88	103.64	17.91	20.38	17.84	2.38	11.53	2.35	5.64	0.73	5.22	0.82	**562**
NAU273/T	17.99	25.04	3.06	11.85	2.25	2.79	3.69	0.59	2.24	0.35	1.56	0.15	0.73	0.32	**73**
NAU297/B	34.89	45.49	6.99	26.34	6.04	19.98	6.25	1.07	5.28	0.88	2.90	0.16	2.62	0.56	**159**
NAU297/T	6.86	5.92	1.28	3.24	1.00	0.83	2.11	0.30	0.99	0.25	0.28	<DL	0.40	0.35	**24**
NAU322/B	31.47	43.48	6.19	26.39	5.57	23.34	5.88	0.83	3.72	1.09	2.29	0.14	2.53	0.51	**153**
NAU322/T	<DL	137.19	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	12.46	<DL	<DL	<DL	**150**
NAU325/B	28.72	39.52	4.03	30.35	5.11	21.96	<DL	1.74	7.47	<DL	<DL	<DL	<DL	<DL	**139**
NAU325/T	39.87	21.38	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	2.08	**63**
NAU378/B	62.38	70.99	10.24	41.29	8.44	17.32	10.08	1.77	7.28	1.82	6.71	0.88	6.29	0.93	**246**
NAU378/T	21.08	15.49	3.54	16.78	1.55	1.07	5.36	0.42	1.42	0.00	1.40	0.11	<DL	<DL	**68**
NAU393/B	151.03	206.45	30.45	131.70	26.06	31.05	26.85	4.09	21.80	4.37	11.90	1.56	9.29	1.66	**658**
NAU393/T	<DL	31.69	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	**32**
NIST1486	21.46	43.99	4.25	20.60	7.86	90.85	9.62	0.97	4.56	1.09	2.99	0.37	2.85	0.25	**212**

Table 26: REE concentrations (ppb) in Norfolk bone ("B") and enamel ("T")

The enamel samples had total REE concentrations in the lower range from 24 to 181 ppb except for NAU173/T that was significantly above (778 ppb) all other enamel levels.

The total REE contents in bone were generally higher (124 to 658 ppb) than in enamel but for the majority of bones still relatively low (124 – 183 ppb). Five samples (NAU173B, NAU212B, NAU246B, NAU273B, NAU393B) had higher REE concentrations between 246 and 658 ppb suggesting possible

contamination from the soil. Overall, bone showed a remarkably uniform behaviour in comparison to enamel as shown in Figure 37. The REE concentration for the bone meal reference standard NIST 1486 was determined with a mean concentration of 212 ppb (n=9).

Cerium was proposed to be a particularly good indicator for contamination from the soil as it readily exchanges for Ca^{2+} ions in the hydroxyapatite lattice (Feng et al., 2005, Deer, 1983). In the Norfolk samples the Ce in bone ranged from 40 to 223 ppb while in enamel from 6 to 656 ppb. Four out of the 14 bone samples and two out of the 14 enamel samples contained >150 ppb Ce, which is above biogenic levels (Hoogewerff et al., 2001). These Ce concentrations therefore suggest that apart from these five samples mentioned above, no major ion exchange between bone and the soil has occurred.

The spider diagram below (Figure 37) shows the distribution of the shale normalized REE (Taylor and McClennan, 1985) concentrations of the Norfolk samples. The relatively uniformly distributed REE pattern among the samples indicates that this assemblage has been exposed to similar or identical conditions in the burial environment. REE concentrations in enamel are generally found below that of bone resulting from the greater structural resistance to diagenetic modifications.

The samples are slightly more enriched in MREE (Sm to Dy) than LREE (La to Nd) or HREE (Tb to Lu). Both sample types display a positive Eu anomaly, which is more pronounced in bone than in enamel, and only very small negative Ce anomalies in the majority of samples except for NAU130/T and NAU173/T that exhibit positive Ce anomalies.

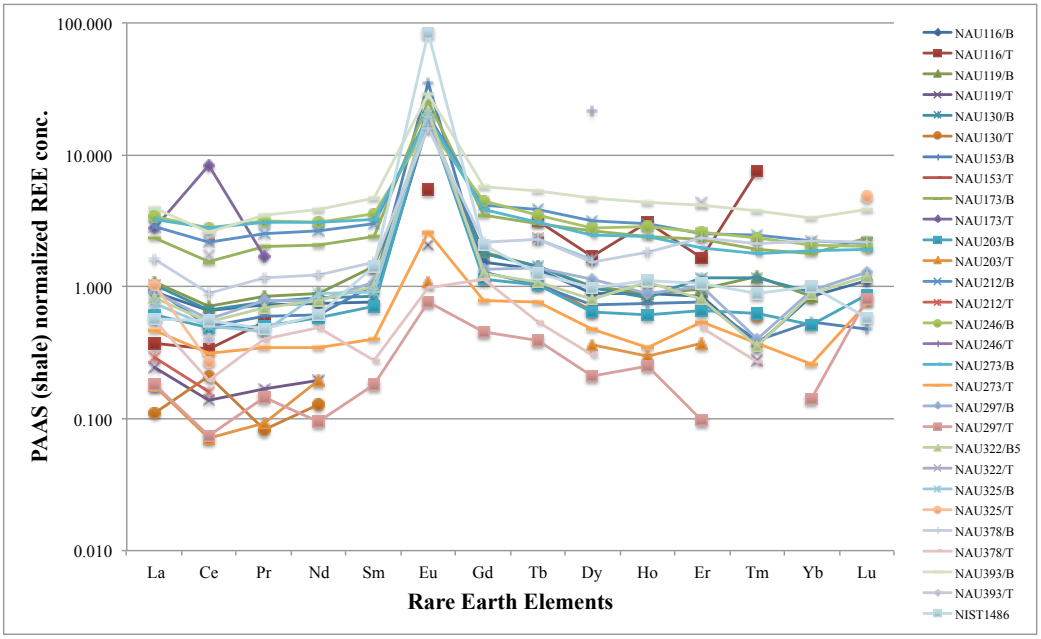

Figure 37: Spider diagram of REE composition in the Norfolk bone and enamel samples (ppb, PAAS (Post-Archean Australian Average Shale) normalized (Taylor and McClennan, 1985)).

All REEs are trivalent except for Ce and Eu, which are both sensitive to redox reactions and as a result can also occur in different states. Under oxidizing conditions Ce can be oxidized from a trivalent (Ce^{3+}) to tetravalent (Ce^{4+}) state, whereas Eu^{3+} can be reduced to the divalent state (Eu^{2+}) under highly reducing conditions (MacRae et al., 1992). The resulting fractionations can be observed as Ce and Eu anomalies, respectively. Negative Ce anomalies are an indication of oxidizing environments and positive Ce anomalies indicate reducing environments (Elderfield and Pagett, 1986, Wright et al., 1987). Similarly to Ce, Eu anomalies occur due to redox reactions. The distribution of the different Eu states is a function of pH. In acidic and near-neutral soils Eu^{3+} prevails exhibiting a negative Eu anomaly whereas in alkaline and strongly alkaline pH environments Eu^{2+} dominates leading to a positive anomaly (Sverjensky, 1984). The relatively high Eu anomalies as seen within the Norfolk samples have been suggested to be typically found in bones from reducing conditions possibly

caused by microbial action of organic matter during early diagenesis (Trueman et al., 2003).

The proposed two main mechanisms responsible for REE fractionation between water and bone apatite are a) through adsorption cation exchange and b) lattice substitution (Reynard et al., 1999). Reynard et al. (Reynard et al., 1999) introduced the use of a plot (La/Yb vs. La/Sm) to distinguish between these two mechanisms. Adsorption is associated with "early diagenesis" whereas recrystallization substitution with "late diagenesis" (Reynard et al., 1999). Reynard et al. (Reynard et al., 1999) argued that the substitution mechanism would only have little effect on La/Yb ratios while it would increase if adsorption takes place, and vice versa in regard to the effect on La/Sm ratios. Either mechanism, however, would result in a significant increase in MREE.

To visualize the fractionation pattern between LREE, MREE and HREE in the Norfolk bone and enamel samples La/Sm ratios were plotted against La/Yb ratios (PAAS shale normalized REE (Taylor and McClennan, 1985) as shown below.

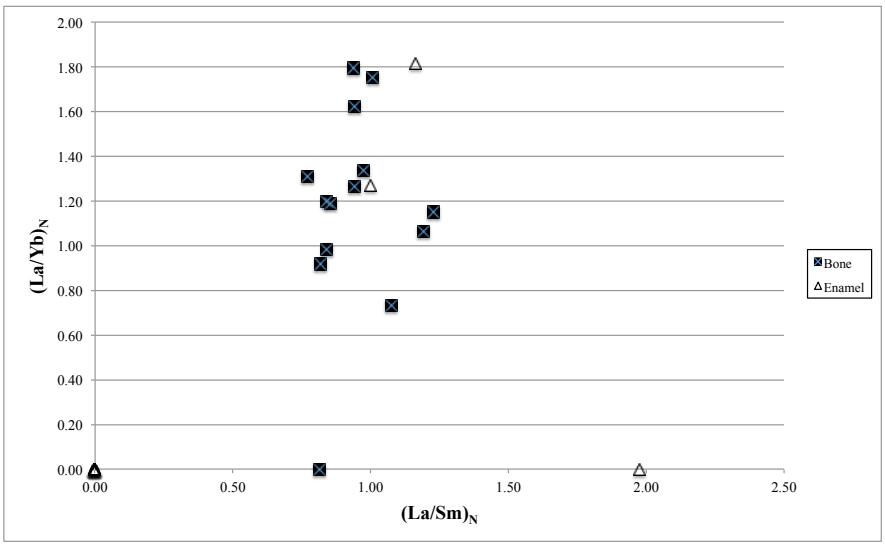

Figure 38: The diagram illustrates the fractionation pattern in the Norfolk bone and enamel samples illustrated as shale normalized REE concentration ratios of LREE (La/Sm) vs. HREE (La/Yb).

The fractionation pattern observed in the Norfolk bone shows a largely increasing upward trend of HREE concentrations (~0.7 to 1.80) and a relatively static concentration of LREE (~0.8 to 1.20) compared to the HREE. This pattern suggests that REE have been incorporated during early diagenesis through adsorption rather than substitution as described by Reynard et al. (Reynard et al., 1999). Substitution would have resulted in a significantly greater fractionation between LREE and HREE resulting in a pronounced enrichment of MREE apparent as a "MREE bulge". One bone sample, NAU325/B did not exhibit any signs of LREE fractionation but the same level of HREE enrichment as all other bone samples. Enamel was hardly affected by any REE fractionation except for NAU273/T and NAU297/T, which were both enriched in HREE similar to that of bone. A single enamel sample NAU378/T shows a high enrichment in LREEs (La/Sm 1.82) and a complete absence of HREE.

Apart from REEs, uranium (U) has also been suggested to be a relatively good indicator of diagenetic changes in bone as concentrations in living bone are very low ~0.1 ppm but can increase to >1000 ppm in fossilized bone (Trueman and Tuross, 2002). The concentrations in the Norfolk bone, however, were extremely low both in bone and enamel with values ranging from 0.0 to 0.9 ppm (data not shown).

7.10 Discussion Sr and Pb combined

The East of England has encountered a constant influx of immigrants from continental Europe during all time periods. The Anglo-Saxon period lasted for six centuries (5^{th} to 11^{th} century) where immigrants from Northern Germany and later (8^{th} century) also Vikings from Scandinavia constituted the majority of settlers. The aim of this study therefore was to investigate whether the 14 human remains excavated at the site could reveal potential immigrants from the respective countries by Sr and Pb isotope analysis and to constrain their geographical origin. Although burial sites of the Viking period have been

frequently discovered and identified based on the presence of grave goods such as weaponry, jewellery or other artefact indices, most of the time the absence of skeletal remains has significantly limited the possibility of isotope analyses and thus the amount of available isotope data (Montgomery et al., 2014). Only a few smaller studies were successful in retrieving skeletal material from the Viking Age (Pollard et al., 2012, Budd et al., 2004a, Hall et al., 2008, Chenery et al., 2014, Montgomery and Evans, 2006, Buckberry et al., 2014) that could be used for comparison in this study.

7.10.1 Strontium

The results for Sr isotope ratios from bone form a relatively homogenous group clustered within a narrow range (0.70807 to 0.70825). According to the available reference data from Evans et al. (Evans et al., 2010) and Voerkelius et al. (Voerkelius et al., 2010) these values fit into the characteristic Sr composition that is typical for the soils in this region.

The apparent bias towards the Snarehill soil values could lead to the assumption that the Sr ratios measured in bone are the result of diagenetic alteration. To verify potential contamination from the burial environment REE analysis was incorporated in the analysis. The total REE concentrations (Figure 37) for the majority of bones (n=8) ranged from 124 to 183 ppb except for five samples, which had increased levels of REE (246 to 658 ppb). As normal REE levels in living bone are ~100 ppb (Trueman et al., 2006), and in uncontaminated buried bone generally <1000 ppb (Trueman and Tuross, 2002), the results suggest that most of the bone was not greatly effected by ion exchange or bulk contamination from the burial environment with the exception of the five mentioned before. However, although an elevated REE content could be observed in these five outliers they did not display any closer proximity to the soil in the graph or significantly different behaviour compared to the uncontaminated bone samples of the same burial population. Furthermore, if the bones had been extensively contaminated it probably would also have resulted

in an increased Sr uptake from the burial soil. But the Sr concentration in any of these five samples was within the normal range of uncontaminated bone (189 to 222 ppm). Therefore it seems that the REE concentration range seen in these samples (246 to 658 ppb) did not have a major impact on the quality of the isotope data in this study.

The total REE concentrations in enamel were between 24 and 778 ppb (mean 300 ppb, n=14). The majority (n=11) of enamel were equally below the commonly accepted limit of ~100 ppb (Trueman et al., 2006, Trueman and Tuross, 2002) and therefore are most likely to represent the original Sr signature.

However, the interpretation of Sr isotope ratios from archaeological bone is always problematic if the Sr signature of the bone and that of the burial environment is near-identical or even identical. In such an instance, it will be difficult to distinguish to whether the Sr isotope signal is diagenetic or not. A mixing model between bone and groundwater is attempted below (Figure 39) to illustrate the consequences of diagenetic Sr impact.

Mixing signal between Norfolk bone and groundwater

In order to illustrate the potential consequences of an increased diagenetic uptake of Sr into the Norfolk bone a mixing model between the bone sample NAU378 with the highest concentration of Sr (~285 ppm) was plotted against different fractions of groundwater uptake. The Snarehill water samples analysed had Sr concentrations of 0.3685 ± 0.0009 (n=3).

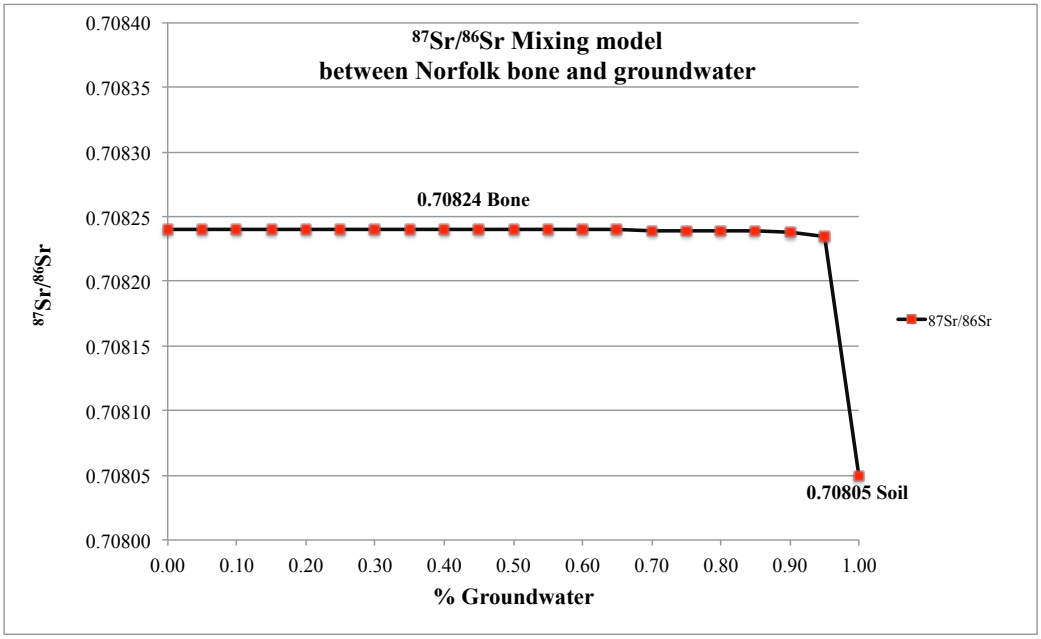

Figure 39: Mixing model between Norfolk bone NAU378 (Sr concentration ~285 ppm, $^{87}Sr/^{86}Sr$ 0.70824±0.00003, n=2) and groundwater (Sr concentration ~0.4 ppm).

Assuming the individuals did not migrate and that the enamel results represent the pure uncontaminated bone, then using 0.4 ppm Sr in the groundwater and a Sr isotope composition of 0.70805 reflecting the local soil, the mixing calculations show that ~100 % of the Sr in the bone would need to have exchanged with the groundwater to achieve the observed values in the bone. In this case with ~0.4 ppm in groundwater and roughly 300 ppm in bone, this would imply that 750 equal volumes of water would be required to flush the dietary signal out of the bone and replace it with the groundwater Sr isotope composition. This would have led to extreme leaching of some of the other elements and enrichment of elements like uranium, which could not be observed in the Norfolk bones. The more likely explanation seems that the Sr isotope ratio in the bone is reflecting the ante-mortem dietary signal.

Local or non-local?

The comparison of the Sr isotope data based on the bone results from the individuals buried within the grounds of Snarehill Hall with the available literature material suggests a local origin. However, similar values have also been determined in Denmark as "outliers" in inland Denmark's ((Frei and Frei, 2011, Frei and Frei, 2013, Price et al., 2012c). The literature data based on the study of mineral waters (Voerkelius et al., 2010) also showed two localities of a similar Sr isotope ratio in the Netherlands but at the same time also a slightly higher isotope ratio is found there as well. The overlap of the similar lithologies and thus indistinguishable Sr isotope ratios between these regions makes it very difficult to disregard any of these areas as potential origins of these individuals.

Tooth enamel values (0.70830 and 0.70891) in contrast show a much greater variability, with the majority (n=8) being within 0.7086 and 0.7087. The lowest ratios of ~0.70830 were only found in two samples, and two in the mid-range with 0.70840 to 0.70850. One single individual (NAU322) had an elevated enamel value (0.70910) compared to the other Norfolk samples, which suggests that this individual lived in a slightly more radiogenic and/or older lithology during childhood. Potential candidate areas that exhibit Sr isotope ratios >0.07090 include areas with heavier Jurassic clay soils towards central and Northern parts of Britain. For example, a similar result was also published from the 5th to 7th century A.D. cemetery at West Heslerton/North Yorkshire (Budd, 2003) from juvenile enamel with a mean value of 0.7090 +/- 0.0008 (2s, n = 13). To date, enamel values between 0.7066 and 0.7144 have been recorded in archaeological human tooth enamel in Britain (Montgomery 2002), which reflects the isotopic variability of the country. Although higher Sr isotope ratios are not exceptional for England, they are unusual for the soils found in Norfolk. Such higher Sr isotope ratios, however, are also commonly found on continental Europe. The "outlier" from Snarehill would be consistent with values (>0.7090) reported for human enamel from Denmark or Northern Germany (Price et al., 2012b, Frei and Price, 2012) and could possibly indicate that this individual was raised there and moved to Britain later in life.

Furthermore, if a population lived in the same environment and sourced the same food and water it is expected to exhibit a fairly homogeneous isotopic pattern. In this case this one "outlier" is significantly different to the "local" Sr signature, which implies that this individual had access to a different food and water source during enamel formation than the majority of the other individuals in this environment of the site.

While bone values form a relatively homogenous population among all individuals, tooth enamel values give a different impression. Values here are not only highly variable among individuals themselves but also very distinct from bone within each individual, apart from two (NAU116 and NAU119). The isotopic composition of these two sample types in these two individuals is closer than the ones observed in any of the other individuals but still distinct. Assuming that the bone tissue has not been significantly altered post-mortem, the distance between bone and teeth Sr isotope ratios in all individuals justifies the interpretation that the burial population as a whole spent their childhood, at least until completion of dental development, in areas with Sr compositions slightly more radiogenic than that associated with the local soil signature found at the site. However, the 'non-local" enamel Sr signatures are too diverse to be assigned to one specific origin. It seems more likely that they have come from various locations within the UK itself or even continental Europe. A probable origin could indeed be Denmark as documented by comparable enamel data (Frei and Price, 2012, Price et al., 2012c). Again, also Northern Germany and even the Netherlands cannot be ruled out due to partly overlapping Sr compositions with East Anglia. Hence it seems very likely that they are not of local origin.

7.10.2 Lead

Lead mining in Britain commenced as early as 49 A.D. during the Romano-British period (Montgomery, 2010b). The ore deposits were located mainly along the West and North of Britain, with none in the East Anglian region. The

rich deposits were intensively exploited to produce lead-glazed pottery, cooking utensils, lead pipes, jewellery and other everyday goods without being aware of the toxicity of lead. Lead poisoning therefore occurred very commonly. Evidence for elevated Pb levels in human skeletal material has come from a number of studies (Budd et al., 2001, Budd et al., 2004b, Montgomery, 2010b, Kamenov and Gulson, 2014). Lead levels during the Romano-British time peaked in the 4th century followed by a decrease from the 5th to 7th century before starting to rise again during the 8th to 11th century (Montgomery, 2010b).

Data from archaeological human bone of people who lived in uncontaminated environments suggests that Pb concentrations were no more than 1 or 2 ppm (Faure, 1986), and that any higher concentration derives from either excessive ingested amounts or contamination after burial. Grandjean et al. (Grandjean, 1973) published bone lead data from humans who lived in what is now Denmark from 4000 B.C. to 1700 A.D., which contained even less than 1 ppm. Lead concentrations measured in teeth (including dentin and enamel) from a Neolithic and Bronze Age burial in Southern England have been published with values ranging from 0.15 to 0.68 ppm (Montgomery et al., 2000) and 0.34±0.23 ppm from an Anglian cemetery (5th to 7th century) in West Heslerton/Yorkshire (Montgomery et al., 2005a), which is in strong contrast to the lead results obtained from the Norfolk samples (5 to 75 ppm in bone, 3 to 20 ppm in teeth). Such concentrations are thus beyond natural levels (<1 ppm) and can only be explained by anthropogenic lead contamination in vivo.

It is acknowledged that bone is highly susceptible to diagenetic alteration because of its high organic content and porous structure. Thus archaeological bone tends to take up Pb and other ions from the burial environment. If this were the case the bone Pb isotope ratios would be similar to that of the burial soil. However, in the Norfolk study bone ratios are very distinct from the local soils, which suggests that the exchange must have already occurred during the lifetime of the individuals. The archaeological report from the Snarehill site does mention the find of a copper ring, however, there is little else evidence of any other Pb source, which would explain the high Pb levels in this burial

assemblage. The regional Cretaceous Chalk geology itself has very low Pb concentrations and can therefore be excluded as source, which leaves it to speculations if the Pb burden accumulated in vivo could have derived from the use of lead glazed pottery, pots and household ware and/or from occupational exposure to lead.

The source of Pb is most likely 'homemade" as the isotopic composition correlates with the isotope signatures reported from British ore deposits ($^{208}Pb/^{204}Pb$: 38.0–38.7) (Montgomery et al., 2005a). $^{208}Pb/^{204}Pb$ ratios for bone were between 38.40 and 38.75 and for enamel between 38.44 and 38.94. Apart from a single enamel sample NAU246 ($^{208}Pb/^{204}Pb$ mean 38.94, n=2), all bone and enamel samples are found within the estimated Pb isotope range of British ores. The slightly higher $^{208}Pb/^{204}Pb$ observed in the enamel of NAU246 is not representative for the local lithology and cannot be attributed to any Pb source commonly found in the UK or even Europe based on the available literature data. $^{206}Pb/^{204}Pb$ and $^{206}Pb/^{207}Pb$ ratios from Pb ores throughout the world range between 16.0-18.5 and 1.19-1.25, respectively (Komarek et al., 2008, Hansmann and Köppel, 2000). Only the Mississippi Valley ore deposit in the USA has significantly higher Pb ratios ($^{206}Pb/^{204}Pb$: >19). The extreme opposite of very low Pb ratios ($^{206}Pb/^{204}Pb$: 16.0-16.1) is only common for the Pb ore deposit from Broken Hill and Mt. Isa in Australia (Hansmann and Köppel, 2000). Since the enamel Pb isotope ratio in individual NAU246 ($^{208}Pb/^{204}Pb$ of 2.086), could not be assigned to any of the local or continental Pb isotopic compositions in the probable candidate regions its origin remains unclear.

Generally, the preservation or loss of the isotopic signature acquired during life will greatly depend on the bioavailable Pb in soil and the in vivo Pb concentration. Hence isotope ratios resulting from a high exposure to lead will be more likely to be preserved in soil with low bioavailable Pb, whereas a very low in vivo isotopic signature will be most certainly altered or even obscured in burial environments with mobile soil lead (Montgomery, 2002).

7.10.3 Rare Earth Elements (REEs)

It is well documented that diagenetic changes in skeletal material, typically in bone, can lead to a significant uptake of trace elements from the burial environment which under extreme conditions has the potential to even overprint the pristine elemental composition (Trueman et al., 2003).

Though not entirely proof, one of the most effective methods to validate the authenticity of the trace metal concentrations in bioapatite is to analyse the content of REE and uranium (Trueman and Tuross, 2002). As previously mentioned, the concentration of REE can increase 3-5 orders of magnitude over geological time scales (Trueman and Tuross, 2002). In modern bone REE concentrations are in the lower ppb range (Trueman and Tuross, 2002, Kohn et al., 1999, Herwartz et al., 2013) hence elevated levels of REE (>1ppm) are a strong indication of post-burial contamination resulting from the REE uptake from the surrounding pore water (Kohn et al., 1999, Trueman et al., 2004, Herwartz et al., 2011). In fossilized bone REE concentrations can increase up to 1000 ppm (Trueman, 1999, Trueman and Tuross, 2002). It is commonly considered that the initial intense uptake of REE into bone occurs shortly after death within the first ten years and can last up to 50000 years (Trueman and Tuross, 2002, Koenig et al., 2009) until bone reaches equilibrium with the pore water or has fully recrystallized (Trueman et al., 2008a) preventing any further bone-pore water exchange. REE gradients during active exchange with pore waters display steep concentration gradients and over time will eventually be evened out when equilibrium is reached. The relatively flat elemental concentration profile observed in the Norfolk samples suggests that no major interaction between bone and depositional environment seems to occur and that the acquired REE signature has stabilized. Early studies suggested that the trace element signature adopted during early diagenesis remains stable (Wright et al., 1987). However, recent research with Lu-Hf isotopes revealed that REE uptake is more dynamic and can continue well beyond early diagenesis (Herwartz et al., 2011, Kocsis et al., 2010) and last to a late diagenetic stage.

As demonstrated by Trueman et al. (Trueman et al., 2011), there are four main factors responsible for the relative distribution of REEs within fossil bone; the Young's modulus (controlling relative adsorption coefficients), the product of the effective diffusion coefficient and the duration of uptake, the ideal ion site radius, and the thickness of the bone. Initial REE fractionation occurs between the bone apatite surface and the surrounding depositional environment and the resulting influx of REE varies depending on the geochemical conditions present in the burial environment (e.g. pH, redox) as well as the nature of the bone itself (Trueman and Tuross, 2002). The structural characteristics of bone such as density and porosity, and the compatibility of the ionic size of the different REE with the Ca sites on the bone apatite crystal surface ultimately determine the REE composition in the individual bone. Following the initial fractionation, REEs are further fractionated within bone itself. Experimental data has shown that during the process of diffusion-adsorption transfer through the bone matrix LREE are progressively lost resulting in the enrichment of HREE in the centre (Koenig et al., 2009, Trueman et al., 2011, Herwartz et al., 2011, Herwartz et al., 2013). This pattern of HREE enrichment has also become apparent in the Norfolk bone samples, and is consistent with frequent observations in bones recovered from different terrestrial depositions and which seems to be independent of the geology (Trueman and Tuross, 2002). HREE enrichment is typically found in alkaline environments (Suarez et al., 2010). The high Eu anomalies seen across all samples also imply that REE enrichment has progressed during reduced conditions typically prevailing in alkaline conditions. This is in accordance with the Cretaceous Chalk geology of the Snarehill site as mentioned in the excavation report (Whitmore, 2001). This finding is coherent with the widely accepted assumption that neutral and alkaline burial conditions are necessary for fossil preservation (Berna et al., 2004) whereas acidic environments would lead to the dissolution of bone apatite.

REE profiles of bone will also depend on the sampling method. Usually the outermost layer of both bone and enamel is cleaned and abraded before analysis to remove contaminants. Particularly LREEs are most concentrated in the outer

bone cortex and tend to decrease towards the centre of cortex. Hence the removal of the outer layer will possibly show a depletion or complete absence particularly of LREEs in concentration profiles. This affect should be considered when interpreting elemental profiles. The observed lack of LREEs in the Norfolk samples might therefore have resulted from the bone preparation procedure applied.

In summary, while REE analysis of the Norfolk samples has revealed some interesting details on the taphonomic history and conditions at the Snarehill site, the concentration levels of REEs were always consistently below 1 ppm, which is generally considered not diagenetically contaminated.

7.11 Conclusion and future work

The objective of the isotope study was to investigate whether Sr and Pb isotope analysis could test if one or more subpopulations were present in the Snarehill burial site, i.e. locals vs. “non-locals”. The answer in this case is not straightforward or sufficiently conclusive to suggest that there were any immigrants from the Continent present at Snarehill Hall. The results obtained from the Norfolk study indicate that all the individuals found at the Snarehill site most likely spent at least their last years locally based on the homogeneity of Sr isotope ratios seen in bone which all fall within the local range typical for the geology of this part of Norfolk. The great variability between enamel and bone samples, however, is a clear indication that a different isotopic composition prevailed in the environment during enamel mineralisation than that characteristic for the local Norfolk isotope signature. Due to overlapping Sr ratios of parts of Britain, Denmark and Northern Germany and even the Netherlands as shown in Figure 40, it is not possible to provide a definite answer in regards to the their childhood residency but there seems to be an implication that they might not have been locals. Similarly, Pb isotopes are not sufficiently distinct from parts of Europe to be able to provide a useful alternative for

discrimination. Other Scandinavian countries as for instance Norway or Sweden as potential Viking origins are unlikely due to the older geologic formation, which is characterised by a higher Sr isotope in Scandinavia compared to Norfolk.

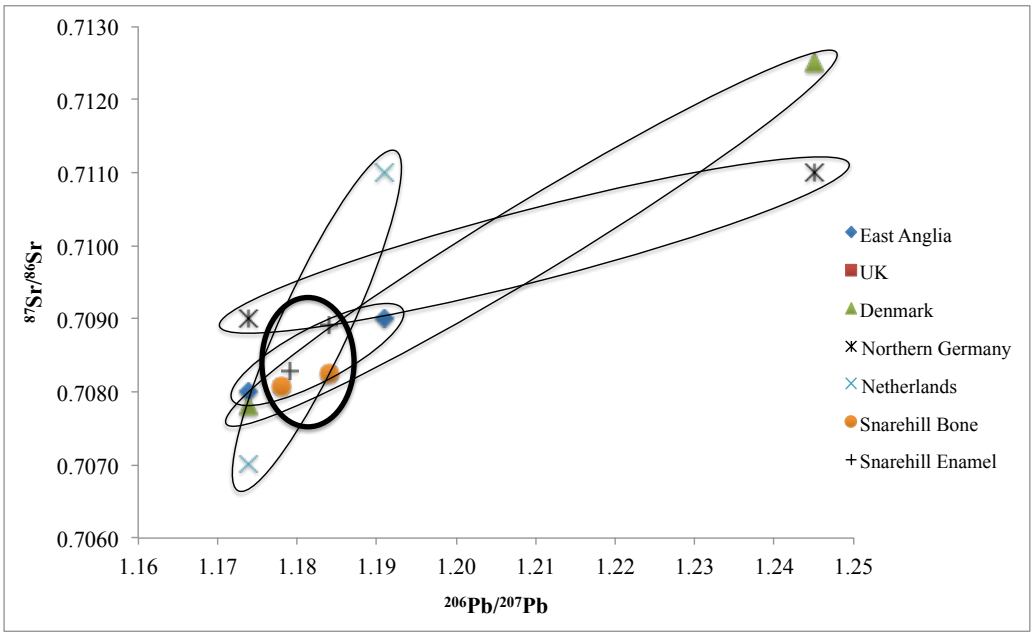

Figure 40: The plot ($^{87}Sr/^{86}Sr$ against $^{206}Pb/^{207}Pb$) shows different literature data for Pb (Reimann et al., 2012, Montgomery et al., 2005b) in comparison to the Snarehill bone and enamel isotope ratios. The Norfolk data is shown within the thickest line of the ellipse.

Environmental data published from Norway and Sweden have $^{87}Sr/^{86}Sr$ of ~0.7092 in coastal regions and inland predominantly between 0.72 and 0.74 (Åberg, 1995, Åberg et al., 1999). Lead data generated for most of Norway and Sweden (Voerkelius et al., 2010) are well above 1.202 ($^{206}Pb/^{207}Pb$).

Human isotope data from Viking populations is very limited and where available mostly restricted to enamel (Montgomery et al., 2014). However, from the few data sets available for comparison a Viking origin of the individuals in Snarehill can be greatly excluded because of the more radiogenic isotope signal

(Symonds et al., 2014, Chenery et al., 2014). Enamel data from Viking assemblages found in Ireland and the Orkneys yield Sr isotope ratios >0.70906 with the exception of one value for a female from the Orkneys that shows an exceptionally low value of ~0.07073. The difference between the female and the two males from the same assemblage has been attributed to increased marine dietary intake in the males as a result of different cultural or geographical origins rather than that the men and women at the site consumed a different diet (Montgomery et al., 2014). The same study by Montgomery et al. compared lead concentrations between the lead polluted environments of medieval England with that of the Viking assemblages. Vikings could be easily distinguished by their very low Pb burden in enamel (<0.5 ppm) whereas individuals from Britain had typically lead concentrations in excess of the natural background level of 0.5 ppm. Based on these findings and the comparative data from this study it seems very unlikely that the Norfolk assemblage is of Viking origin.

The application of REE analysis in ancient human studies is of particular importance as the integrity of the sample material assures the confidence in the results obtained. In this study the majority of skeletal tissues analysed was in remarkably good condition apart from a few exceptions that had elevated REE concentrations. It is not surprising, however, that the enamel was generally better preserved than bone, which is well known and documented in numerous studies. However, putting the REE results in perspective, the concentrations in the Norfolk samples are generally in a relatively low range so that the impact of diagenetic contamination from the soil appears marginal.

The ambiguity of the outcome in regards to the childhood residency during enamel mineralisation highlights the need for alternative or additional techniques that could facilitate the discrimination between geologically identical regions. The creation of more comprehensive global geospatial data in combination with biosphere reference data would be very desirable and essential for enhancing the significance of Sr and Pb isotope data.

Isotope studies with multiple isotopic systems have demonstrated that if one isotopic system fails to provide a clear distinction between geographic origins, others might deliver supportive evidence for a more likely origin (Montgomery et al., 2014). The application of oxygen (and hydrogen) isotope analysis might have provided clearer evidence in regard to local or non-local origins, as the parameters used are distinct from Sr or Pb isotope analysis. The oxygen isotopic composition is determined by the climate and geography, which differs significantly between Scandinavia and Britain. Scandinavian countries are often considerably colder and at higher latitudes, which would become evident in differences of oxygen isotopes. A further great advantage of using oxygen isotopes is the availability of high-resolution oxygen isoscapes (Bowen, 2010b, Bowen et al., 2014). The valuable reference data would greatly facilitate the interpretation of the Norfolk data.

In regard to genetic analysis, it is beyond doubt that this study would have immensely benefitted from DNA data that could have shed more light on the genetic roots of the burial population and thus refined their origins of residency during childhood. Similar to the Spanish case study, future investigations would benefit from the technical advancements that have become available in recent years. Ancient DNA research has become more successful with the introduction of new technologies such as Next Generation Sequencing techniques and new PCR methods that could be applied if these samples would be re-investigated.

Chapter 8

8 Conclusion

Human bones and teeth have been described as an “archive” in the archaeological context, which allows us to gain valuable insights into the individual history of their “owners”. The history recorded in the organic and inorganic matrix of skeletal tissue can be revealed with DNA and isotope analysis, respectively, and can be used to investigate the different aspects in human evolution, migration, cultural development and diet.

The first objective of the interdisciplinary approach chosen for this research project was to combine the well-established method of DNA analysis with Sr and Pb isotope analysis in order to maximize the retrieval of information, which could aid in the identification of ancient and modern human remains.

The re-occurring problem with limited sample material in archaeological and forensic investigations led to the second objective of this study to develop methods that could minimise the amount of sample material required as well as the time and cost for sample preparation and processing.

8.1 Outcomes

The case-specific conclusions have been presented in the relevant chapters. The following section will therefore only briefly summarize the outcomes of this work.

8.1.1 Ancient/Forensic DNA analysis

The well-documented challenges and drawbacks of working with degraded or low concentrations of DNA (Hofreiter et al., 2001, Paabo et al., 2004, O'Rourke et al., 2000, von Wurmb-Schwark et al., 2008, Poinar, 2003, Kirsanow and Burger, 2012) have also emerged as the major stumbling block in this study. Much time and effort was put into the testing of different methods to retrieve sufficient DNA from the bone and tooth samples for the Spanish and Norfolk case studies. The poor quality of DNA encountered in both cases, however, prevented DNA analysis so that, regrettably, no conclusion can be drawn for this part of the research objective.

Since the time that the laboratory work for this thesis was undertaken (2009-2011), the field of ancient DNA research has moved on rapidly. The development of new DNA amplification and sequencing technologies made it possible to recover minute amounts of DNA and also brought about a significant improvement in terms of ease of access and affordability to generate considerably larger amounts of DNA data in less time. The most remarkable innovation in the past decade was the introduction of "Next Generation Sequencing" (NGS) technologies. These instruments produce hundreds of millions base pairs (bp) in parallel within hours. The sequencing of the entire human genome that once took several years and cost several hundred million dollars can now be decoded in a matter of hours and at a cost several tens of thousands of dollars.

The short read length (up to 400 bp) of the NGS technologies has been regarded as a disadvantage in modern DNA studies. For ancient DNA studies, however, this is not an issue as the average fragment size in ancient samples is usually ~50 to ~200 bp long. The new technology also changed and encouraged the development of new methods for sample preparation. The conventionally used PCR method is a uni-plex approach, where one primer pair targets a specific DNA fragment. Newer approaches have used multiplex reactions with several primer pairs to generate multiple amplicons simultaneously. Latest technologies,

such as the RainStorm platform, use up to 4,000 primer pairs in a single reaction simultaneously (Mamanova et al., 2010), which facilitates whole genome sequencing and reduces both cost and time substantially. Developments such as these have significantly benefitted ambitious projects in ancient DNA studies and made it possible to reconstruct genomes of extinct species such as the Neanderthal (Green et al., 2009), the Paleo-Eskimo (Rasmussen et al., 2010) or the wooly mammoth (Miller et al., 2008).

Up until 2013 approximately 124 complete ancient mitochondrial genomes have been published (Paijmans et al., 2013). The first genomes were reconstructed using classical simplex PCR coupled to Sanger sequencing or multiplex PCR and Sanger sequencing (Krause et al., 2008, Krause et al., 2006, Lari et al., 2011, Rogaev et al., 2009, Rohland et al., 2007 and Subramanian et al., 2009). But since then NGS techniques have greatly replaced the traditional methods and produced many more genomes in a short time.

Despite these technological advancements the risk of human contamination poses a constant threat to the authenticity of aDNA results. Vigilant contamination prevention procedures and careful scientific assessment when interpreting ancient DNA data will therefore always remain crucial requirements in this field of research.

8.1.2 Isotope analysis

The application of Sr and Pb isotope analysis to investigate potential migration proved more successful in the Spanish case study. Despite the lack of comparable biosphere data from Villanueva del Rosario it was possible to identify two potential migrants among the burial population.

In the Norfolk case study on human remains from a Late Saxon medieval grave, the results were somewhat inconclusive. The intention here was to identify the presence of possible immigrants from Anglo-Saxon or Viking origin. The

isotopic composition in Norfolk, however, showed resemblance both to other parts of England and to the candidate origin regions in present day Northern Germany, Denmark as well as the Netherlands. These areas all have similar Sr and Pb isotope ratios probably due to the similar geological backgrounds and other regional inputs like Sr from sea-spray from the North Sea. Although no specific geographic origin could be determined, the results interestingly suggested that the burial population was unlikely to be of local Snarehill origin.

8.1.3 Method developments

a)

The development of a new method for the simultaneous extraction of both Sr and Pb from bone and teeth on a single Pb-specific resin proved to be successful. Both elements could be purified in sufficient amounts for successive isotope analysis. The “Two in One” extraction procedure was of great benefit for this study. It saved considerable time for separate sample preparation (Sr and Pb) and separate extraction procedures. It is a convenient and effective approach and could be of great advantage where both elements are required for isotope analysis. Since the specialised extraction resins are also rather expensive it also offers a considerable cost saving.

b)

This work investigated for the first time the feasibility of using the bone residues that are usually discarded after DNA extraction for further Sr and Pb isotope analysis. The first results were promising, as the isotope ratios did not show any significant differences between fresh bone and bone residues, however, a more representative sample number would be needed to validate the results. The advantage of “recycling” sample material is particularly beneficial in studies where both DNA and isotope analysis are of interest. The apparent benefits are

that less destructive sampling is required and that both time and cost could be saved with the combined sample preparation if routinely used.

8.2 General considerations and limitations of Sr and Pb isotope analysis

Sr analysis has proved to be a robust tool for the provenancing of people and materials in many studies as described in the relevant chapters before, but the success of the technique depends on several factors. The bottom line of employing Sr and Pb isotope analysis in provenancing studies is a clear isotopic distinctiveness between geographical regions. As pointed out rightly by Montgomery et al. (Montgomery, 2010a), "*some questions will remain unanswered by strontium isotope analysis, such as the Anglo-Saxon settlement in England…*", simply because the Sr isotopic signatures largely overlap and lack the "uniqueness" required to be distinguishable. Equally problematic are provenancing studies of coastal populations where the universal Sr isotope signature of seawater (0.7092) dominates the terrestrial one hence making coastal populations essentially isotopically identical.

The second major pre-requisite for successful provenancing of humans is the preservation of the original in vivo isotope signature. The major drawback of Sr and Pb isotope analysis of skeletal tissue is its vulnerability to diagenetic overprinting from the burial environment, especially in bone as discussed in previous chapters. Diagenetic alterations in alkaline environments (eg. chalk) are more likely to affect Sr whereas acidic depositions will have a greater impact on Pb due to the different solubility and mobility behaviours of Sr and Pb in the respective environments. Importantly, however, the impact of diagenetic overprinting will also depend on the in vivo Sr and Pb concentrations, respectively. High in vivo concentrations in skeletal tissue will therefore be less effected by low Sr or Pb soil-groundwater concentrations and vice versa (Montgomery, 2002).

The integrity of the sample material of human remains is an essential factor and one of the most reliable methods to control and verify the state of skeletal material is the application of Rare Earth Elements (REE) analysis. The presence of REE in concentrations >1000 ppb are generally a good indication that post-burial contamination has occurred and thus might have altered the original Sr or Pb isotopic signal. Additionally, pre-analytical screening techniques could provide a first evaluation of the integrity of sample material and suitability for further analysis. The most common techniques have been previously discussed in chapter 4 such as measuring the histology index, collagen content, and crystallinity index. Also FTIR (Fourier transform infrared) spectroscopy (Berna et al., 2004) or microscopical examination is frequently applied to detect structural diagenetic alterations in bone and teeth. However, all additional screening applications also require additional sample material, which is usually very difficult to get access to. Sample material from archaeological skeletal material is often precious and therefore most of the time only available in restricted amounts for analytical procedures.

When interpreting isotope data it has to be kept in mind that sources other than the geological background can play an important role in determining the local Sr isotope composition. The input of atmospheric dust, sea-spray and fertilizers, which can travel over long distances and originate from isotopically different Sr and Pb sources can produce mixed isotopic signals and thus complicate the interpretation of isotope data.

Also, the impact of diet on Sr levels in skeletal material has to be taken into account. People tend to have different preferences for food if they have the chance to choose. Especially in modern cultures, where food is imported and exported all over the world and easy to access. The intake of imported food sources especially Ca- and Sr-rich food such as dairy products, leafy greens, legumes and fish and sea-salt, may change the inherent isotopic signal significantly even when consumed in small amounts (Wright, 2005). In ancient human provenancing studies this problem is not as predominant as food was mostly sourced locally. However, in some early cultures salt was traded from

coastal areas to communities that lived further inland and this could result in distinct Sr isotopes ratios between skeletal tissue and the prevailing "local" biosphere (Wright, 2005).

8.3 Suggestions for future work

Although Sr and Pb maps based on bioavailable isotope data are slowly emerging, comprehensive coverage in many European countries and worldwide still has a long way to go. Currently mainly high quality global isoscapes for oxygen and hydrogen (Bowen et al., 2014) are available that are suitable for provenancing purposes. Since it has been acknowledged, that regional variations (Price et al., 2002) or "anomalies" of the biosphere do exist (Reimann et al., 2012), shows clearly the need for more detailed investigations in order to be a useful referencing tool in archaeological and forensic provenancing investigations. Due to the fact that detailed Sr and Pb "isoscapes" on a global scale are almost an impossible task to achieve in the near future, the best approach is the direct sampling of a representative number of biosphere data within the local study area. This would identify regional isotopic variability and thus provide more certainty for interpretation purposes.

The Norfolk study has shown that the Sr and Pb isotope data was not significantly different from other areas within England itself or in the candidate origins on the continent. The lack of isotopic specificity encountered in this study highlights the major limitation of the technique. One way to improve the resolution of "distinctiveness" of an area, however, could include the application of additional isotope systems such as oxygen and hydrogen. Oxygen and hydrogen isotopes have been widely used as a proxy for predicting the climate for a geographical area and as such could complement Sr and Pb isotope analysis effectively. A multi-isotopic approach will generally enhance the possibility to restrict geographic areas as potential place of origin as each isotopic system covers a different aspect (e.g. geology, climate) of a locality. If

different isotopic characteristics happen to overlap for a specific location it could significantly increase the likelihood for a potential place of origin.

The complexity of interactions between geology, biosphere and atmosphere that ultimately determine the specific isotopic composition in human skeletal tissues has not been fully explored yet. There are still abundant opportunities for more in-depth research on the processes involved in these interactions, which hopefully one day will lead to more refinement and higher predictability of geographic origins for provenancing studies. Moreover, dietary input and subsequent deposition of Sr and Pb in bone and teeth need more attention in research. Although it is known that certain nutrients have a greater role in determining a specific Sr isotopic signature, details on metabolic rates, retention or removal, and turnover times of Sr in bone tissue are less well defined. Similar questions also require clarification for the behaviour of Pb in humans to be able to estimate to which extent diet is involved in determining the isotopic signature apart from geological and environmental factors.

To date, Sr and Pb isotope analysis can be successfully applied to exclude certain geographical areas rather than pinpoint to one specific territory. Such information can still significantly contribute to archaeological and forensic questions of residency and migration of individuals, particularly as a complementary tool to DNA analysis.

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