Modelling Diagenesis in Archaeological Bone

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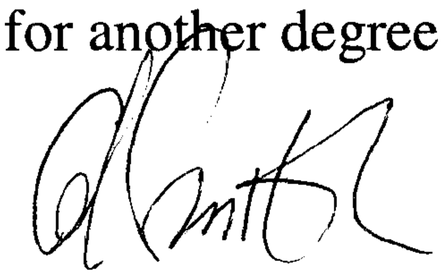
Thesis submitted in partial fulfilment of the requirements for the degree of PhD

Fossil Fuels and Environmental Geochemistry
University of Newcastle-upon-Tyne

April 2002

Declaration

I hereby certify that this thesis is all my own work, except where otherwise stated, and that it has not been submitted for another degree at this or any other university.



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Contributions

Part 1: **Bone, Taphonomy and Archaeological Bone**

100% contribution.

Part 2: **Modelling biomolecular deterioration in archaeological bone**

2.1: 100% contribution.

2.2: 70% contribution. All the database manipulation, modelling, research in to soil/air temperatures and palaeoclimate is my own work. Laboratory experiments were carried out by Angela Gernaey, James Roberts. The manuscript was written by myself and Matthew Collins.

2.3: 80% contribution. Majority of the manuscript was written by myself, all the modelling work was carried out by myself.

Part 3: **Measuring diagenesis in archaeological bone**

3.1: 100% contribution.

Soil analysis was carried out by Anders Nord. Miranda Jans carried out all the histological analysis. All other diagenetic parameters were measured by myself or Christina Nielsen-Marsh.

3.2: 80% contribution. The data manipulation, and manuscript have been entirely written by myself.

3.3: 90% contribution. The manuscript was written by myself. Archaeological data and maps were provided by Paul Arthur.

3.4: 80% contribution. The osteocalcin assay was developed and undertaken by myself, Richard Prigodich helped with laboratory work and assay development and advice, Oliver Craig, also advised.

Part 4: **Synthesis, conclusion, future work**

100% contribution.

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List of Abbreviations

A	Pre-exponential factor
aDNA	ancient DNA
DNA	deoxyribonucleic acid
Ea	Activation Energy
ELISA	enzyme linked immunosorbent assay
FTIR	Fourier transform infrared spectroscopy
Gla	γ-carboxyglutamic acid
HI	Histological index
HgIP	Mercury intrusion porosity
IgG	Immunoglobulin
IRSF	Infrared Splitting Factor
kJ	Kilojoules
LGM	Last glacial maximum
M	Molar concentration
MAT	Mean annual temperature
mDNA	Mitochondrial DNA
MFD	Microscopical focal destruction
mol	Moles
Na_2EDTA	Disodium Ethylenediaminetetraacetate
NCP	Non-collagenous protein
nm	nanometers
Oc	Osteocalcin
PBS	Phosphate buffered saline

PCA	Principal components analysis
PCR	Polymerase chain reaction
ppm	parts per million
Py-GC-MS	Pyrolysis-Gas Chromatography-Mass Spectrometry
T_{eff}	Effective temperature
µm	micron

Acknowledgements

I would like to thank Matthew Collins and Christina Nielsen-Marsh for their energetic and enthusiastic discussions, from which, many ideas in this thesis have been developed. I would also like to thank the members of the Ancient Biomolecules Group, past and present (Oliver Craig, Sam Roberts, Emma Waite, Angela Gernaey, Jennifer Hiller and Kirsty Penkman) for their support and guidance throughout. Everyone else at NRG who has aided me in my work is also gratefully acknowledged. Special thanks must go to Dave Dunbar and Steve Addison at the Central Analytical Service, for all their hard work carrying out elemental analysis and FTIR.

This work was funded as part of a European Union funded project; all those who contributed must be thanked. Special thanks go to Miranda Jans, and Paul Arthur, who have helped with my work in particular. Henk and Eva Kars are also acknowledged for their sterling work on the final report.

I must also thank Gordon Turner-Walker, Richard Prigodich, Peggy Ostrom, Case Vemeer, Eva Valsami-Jones, Andrew Millard and Martin Evison who have all provided samples, advice or analysis.

Finally, I would like to thank all my family and friends for their unstinting support and encouragement throughout, especially my mother, father, grandmother and sister.

Abstract

This thesis presents a series of papers intended to further our knowledge and understanding of bone taphonomy, with special attention given to the survival of organic molecules (collagen and DNA). An integrated modelling and measuring approach has been taken. Initially, the rate of chemical degradation of bone collagen was estimated from data derived from high temperature laboratory experiments. These rates were then extrapolated to realistic archaeological burial temperatures using palaeoclimatic reconstructions. The model was tested using radiocarbon databases using data on location and collagen yield. Despite the simplicity of the approach and the large errors involved, it does appear that the model may be useful for predicting the limits of collagen survival, although most bones have less collagen than the model predicts. A similar model was produced for the depurination of DNA.

The models which are based on rates of chemical deterioration, highlight the role that temperature may play in biomolecular preservation, however they fail to address the complexity of bone taphonomy. A study of bones from a site in southern Italy (Apigliano) identified bone with no evidence of microbial attack, but otherwise severe diagenesis (including little or no collagen) – so called 'pre-fossilised' bone. In a wider study of 200+ archaeological bones excavated form 40 sites across Europe this 'pre-fossilisation' was identified as one of four possible diagenetic states, the others being, 'microbial attack', 'complete dissolution' and 'good preservation'. These states of preservation are defined by characteristic sets of diagenetic parameters, although the environmental and taphonomic conditions that control these states of preservation are less well defined. In addition to the standard diagenetic parameters measured, the preservation of osteocalcin (a potential proxy for DNA) was investigated using immunological techniques.

Part 1

Bone, taphonomy and archaeological bone

1 Introduction

The subject of this thesis is the taphonomy of archaeological bone. Bone, or more correctly its semi-fossil, or fossil counterpart, is a ubiquitous biological material at many archaeological sites. As such it is a useful source of information for archaeologists. The study of bone can provide data on a number of levels, for example morphological analysis can provide data on the evolution of species, or domestication of animals. From the analysis of bone assemblages it may be possible to infer farming strategies, or site formation processes, and chemical analysis of bone protein or DNA can be used to make inferences about the diet of organisms, or the genetic relationships between species. Thus bone is potentially a useful resource for reconstructing a number of aspects of the past, although not all bone can be used for all of the above analyses. This is because bone undergoes physical and chemical changes in the period between an organism's death and the point at which it is excavated and analysed by archaeologists. These changes can be extremely varied and are dependent on the events that occur during this period. The result of these events is that the bone excavated is different to its original form, and thus information at a number of levels can be lost.

The study of the passage of a bone from dead organism to the point of excavation is called taphonomy and first developed as a sub discipline of geology, and palaeoecology, with the main techniques being macroscopic analysis of bone in an effort to make palaeoenvironmental interpretations. New scientific techniques extracting and analysing biomolecules (e.g. stable isotope analysis of collagen or ancient DNA studies) are being increasingly applied to archaeological and fossil bone, pushing back the boundaries of what we can learn about ancient bones from their chemical analysis. This has not only led to new areas of study and new knowledge, but new problems. Problems such as - How do taphonomic processes affect the results of the analyses that are carried out? How long do these biomolecules last in the archaeological and geological record? The result is that taphonomy must also encompass the study of bone at the physico-chemical level in order to understand how these changes may affect the new types of data being generated, and answer these fundamental questions.

Taphonomy is by its nature a complex field, as it is the study of *all* the processes that occur to an organism after death and up until the point of excavation (or even analysis). The variety and possible number of interactions of these processes is bewildering in its complexity. Whilst an attempt must be made to account for all these processes it is impossible to gather data for all aspects, some of which may be transient. This thesis will focus primarily on the degradation of bone proteins (collagen and osteocalcin), and DNA, however the nature of bone taphonomy is that these areas cannot be studied in isolation, thus mineral changes to the bone and their relationship to the alteration of the organic molecules must also be discussed. The complexity of the processes occurring and the effects that they can have on the bone make taphonomy an inexact science, in addition the timescales over which these processes occur make it very difficult to study them. This complexity necessitates the use of models (conceptual and numerical) to help simplify the processes behind what is happening during taphonomy.

This thesis combines a number of approaches to the study of bone taphonomy. In order to understand the changes that occur in bone during this process it is necessary to follow the path of bone from the living bone of an organism, through death, decomposition and burial, excavation and analysis. Section 1 describes the properties of bone *in vivo*, the key processes of taphonomy, and thus the properties of archaeological bone. In addition the bone as an archaeological resource is discussed, and how taphonomy can affect the information available to the archaeologist. An account of how these specific changes are recognised and measured is also given.

The second part of the thesis applies a modelling approach to bone diagenesis. Simple mathematical models have been developed in an attempt to describe the limits of degradation for two biomolecules (DNA and collagen). The models are based on high temperature laboratory experiments, and palaeoclimatic reconstructions in northern Europe, and Australia. Descriptions of the two models (collagen and DNA) and their results are given in the two papers in Part 2.

Part three is a summary of research carried out as part of the 'European Bone Degradation Project' (ENV4-CT98-0712) (Kars & Kars 2001). The main aim of the project was to measure diagenetic parameters of archaeological bone excavated from across Europe in order to classify the state of preservation. This data was then compared to that of the soil characteristics of the sites to determine relationships between the soil chemistry and bone preservation. Additional site specific, archaeological and environmental information was included in the interpretation. A brief description of the project is given, followed by three papers that cover: the main data from the project, the unusual state of preservation at one site in particular, and the use of an enzyme linked immunosorbent assay (ELISA) technique to investigate osteocalcin preservation. The first paper entails an overview of the diagenetic trends observed in the data, and interpretation of these trends in terms of the site taphonomic and soil data. The second paper discusses the unusual state of bone preservation at the site of Medieval Apigliano. The bone at this site appears to exhibit signs of fossilisation after less than 1000 years of burial. The possible reasons for this occurrence are discussed in the paper. The final paper in this section details the use of an ELISA technique applied to the cattle bone excavated in the project. The survival of bovine osteocalcin is discussed in relation to the other diagenetic parameters for the cattle bones.

The final part includes a synthesis of the two approaches given in parts two and three. Following the discussion the conclusion of the thesis is reached and suggestions for future research are made.

1.1 Physical and Chemical Description of Bone

The subject matter of this thesis is archaeological bone, more specifically how and why it degrades in the soil environment. To understand these processes a number of physical and chemical properties of the bone are measured and compared to those of fresh bone. To fully appreciate what these measurements mean the physical and chemical structure of bone must be understood. Bone is a complex physical and chemical composite consisting mainly of the protein collagen and a mineral similar to a carbonated hydroxyapatite, in close association. It makes up the majority of vertebrate hard tissue, i.e. the skeleton. Whilst the structure and properties of bone are a function of its composite nature, it is simplest to describe it in terms of its various components. A detailed overview of bone structure and chemistry is given in Lowenstam & Weiner (1989), a brief summary is given here.

1.1.1 Collagen

Approximately 30% of the bone (by weight) is protein. Type I collagen accounts for approximately 90% of the bone protein in mature healthy bone. Collagen, like bone as a whole, has a hierarchical structure. At the smallest level of organisation collagen is made up of a conservative pattern of amino-acid residues. Every third residue is a glycine, and this is often bound to a proline residue. This configuration in the primary structure gives the protein secondary structure the ability to coil tightly and rigidly, and for each coil to fit into tight association with other collagen molecules. This association is further strengthened by cross-linking between the molecules.

Type 1 collagen actually exists as a macromolecule constructed from three alpha chains, two $alpha_1(I)$ chains and one $alpha_2(I)$ chain. The alpha chains are left handed helices, and are twisted together around each other along a common axis in a right handed coil to form a type I collagen macromolecule about 300 nm in length and 1.5 nm in diameter. The ends of the molecule, telopeptides, are non helical.

These molecules are ordered into fibrils where the molecules are aligned in a staggered fashion so that the structure is offset, repeating itself every 67 nm. Each molecule is over lapped by another approximately every 27 nm, and there is a gap before the next in line every 41 nm. Fibrils can then be bound into fibres and in turn the fibres into fibre bundles.

In physiological solution collagen is an insoluble protein, however as it is denatured, e.g. by heating or chemical bond breaking, the rigid collagen structure fails and the molecule becomes soluble gelatine, a transformation with important archaeological implications.

1.1.2 Mineral

Bone mineral is usually described as a non-stoichiometeric form of calcium hydroxyapatite (unit cell is given as $Ca_{10}(PO_4)_6(OH)_2$), sometimes referred to as bioapatite. Bioapatite is non-stoichiometric as it is up to 5-10% calcium deficient and has a number of other ions substituted into the structure e.g. OH^- substituted with F^-,

and Ca^{2+} with Pb^{2+}, Sn^{2+} and Sr^{2+}. Significantly carbonate ions (both carbonate CO_3^{2-} and bicarbonate HCO_3^-) are present in bioapatite at approximately 3-5%. These carbonate ions can be structural i.e. substituted for phosphate ions in the lattice, or adsorbed onto the crystal or hydration layers. Bioapatite crystals are very small (2-5 x 40-50 x 20-25 nm) which means that the structure of bone has a very large surface area, an important feature in the physiological role of bone in the body. This large surface area means that the crystals are very reactive, an important property affecting bone taphonomy in the *post mortem* environment.

The bone mineral is embedded within the collagen fibre structure, and possibly within the gap zone of the collagen fibres. The crystals in bone *in vivo* are small, either plate like or needle shaped, the size of which increases to a maximum with the age of the individual. There can also be crystal size variation between species.

1.1.3 Non-collagenous proteins

Bone protein is dominated by collagen, and thus the other proteins that are endogenous to bone are normally described together as non collagenous proteins (NCPs). This group includes: sialoproteins, proteoglycans, phosphoproteins, osteonectin and osteocalcin (bone Gla protein). The roles of all the NCPs are not fully understood, most seem to play a role in mineralization, remodelling of the bone, and demineralisation. Other proteins can be associated with the bone, such as immunoglobulins and blood serum proteins, e.g. albumin.

Osteocalcin (or bone Gla protein) is the second most abundant protein in bone, constituting 10-20% of the NCP fraction and has been extracted from archaeological and palaeontological bone (e.g. Muyzer *et al.*, 1992, Ajie *et al.*, 1992, Collins *et al.*, 2000). It is a small protein, unlike collagen, and is only approximately 50 residues long containing an unusual amount of the γ-carboxyglutamic acid residues (Gla), although like collagen it is structurally conserved. Osteocalcin lacks a stable conformation in solution, but in the presence of Ca^{2+} (e.g. bone mineral) the Gla rich region forms an alpha–helical structure and binds strongly (Hauschka, 1986); it is also known to bind to collagen (Prigodich & Vesely 1997).

1.1.4 Physical structures of bone (microstructure and porosity)

There are two main structures in bone at the microscopic level; cortical or compact and trabecular or cancellous bone. Cortical bone makes up the shafts of the long bones whereas the ends of the long bones are trabecular bone. These types of bone are at a microscopic level made of lamellar bone, i.e. they are made of discrete layers of bone. As bone is formed, e.g. in new growth or repairing a fracture, the bone lacks the lamellate organisation. This type of bone is called woven bone, and contains more mineral than lamellar bone.

A common form of the lamellar structure is Haversian bone seen in cortical bone, where the lamellae (layers of parallel mineralised collagen fibres) are arranged concentrically around a central Haversian canal. The Haversian canals run parallel to the length of the bone, and are connected to the medullary cavity and each other,

carrying blood vessels, nerve fibres and connective tissue; they are also connected transversely to the periosteum. Surrounding the Haversian canal are the concentric rings of the lamellae, within which are a number of osteocyte lacunae. Osteocytes are bone cells that maintain the bone matrix, and are contained within the osteocyte lacunae, which are interconnected by a system of canaliculi. Haversian bone is constructed of repeating units of Haversian canals surrounded by several layers of lamellae, each unit is called an osteon. Not all bone has the Haversian system, e.g. trabecular bone, where the lamellae are arranged in simple rows or folds.

Bone is a porous structure due to its network of blood vessels and osteocyte lacunae and canaliculi, and the small size of the mineral crystals, it has an internal surface area between 85-170m^2g^{-1}. Nielsen-Marsh & Hedges (1999) estimated the pore volume of modern bovine long bone to be ~ 0.0445cm^3g^{-1}. Assuming a density of ~1.9gcm^{-3} this is about 8% by volume similar to figures for modern human specimens (5-10%, although increasing with age) given by Feik *et al.*, 1997.

1.1.5 DNA

Deoxyribonucleic acid (DNA) is an essential part of all living organisms, and bone tissue is no exception. DNA is the hereditary code molecule, a copy of which is passed on from parent to daughter cells during cell division. Chemically DNA is a relatively simple molecule, comprised of two strands of DNA running counter parallel coiled into an alpha-helix. Each strand consists of a deoxyribose sugar-phosphate 'backbone', attached to which are a sequence of bases, which are in turn bonded to the bases of the opposite strand. DNA contains only four bases the purines (guanine and adenine) and the pyrimidines (cytosine and thymine). The bases are arranged on the strands so that a base attached on one strand will only bond with another on the opposite of a specific type, so that guanine only bonds with cytosine and thymine only adenine. The long strands of DNA (55-250 million base pairs long) are contained in the cell nucleus in the form of chromosomes. These structures are also bound in proteins for extra protection.

In addition to the nuclear DNA other cell organelles contain DNA, notably the mitochondria. The DNA contained in the mitochondria is inherited mainly through the maternal line, and is required for the reproduction of the mitochondria. Sequences of mitochondrial DNA (mDNA) are often the target molecule for ancient DNA research, as they are present in high numbers. Cells may contain 800 or more mitochondria, and each mitochondrion may contain around 10 copies of its DNA, increasing the chance of survival of a particular sequence.

DNA is constantly damaged *in vivo*, although many mechanisms exist to repair this damage, as DNA is so important to healthy cell reproduction. On death, however these enzymatic repair systems will no longer be maintained, and autolytic enzymes are likely to cause the rapid degradation of DNA in most tissue.

1.1.6 Lipids

The majority of lipid found in bone will originate from bone marrow, or from the flesh around the bone. Evershed *et al's.*, analysis of modern bone showed that both acyl lipids and cholesterol can be solvent extracted from the bone, with the yield of

acyl lipids reducing with distance from the marrow cavity (Evershed *et al.*, 1995). Cholesteryl esters can also be found, which may originate from the blood or meat associated with the bone. Bone will also contain phospholipids and calcium acidic phospholipid-phosphate complexes.

1.2 Taphonomy

'Bones' recovered from archaeological and palaeontological sites were once part of living organisms, but at the point of study are quite different to the bones *in vivo*. The changes that occur during this passage are the subject of this thesis, and are often termed taphonomy, or diagenesis. Both of these terms are borrowed (or arguably misappropriated) from palaeontology, where such studies originated. An overview of taphonomy and diagenesis is given briefly here, but for more detail see Lyman (1994).

Taphonomy in its broadest sense is the study of what happens to organisms after they die. The term was originally coined by J. A. Efremov, (taken from the Greek meaning the laws of burial) to describe the study of what happens to animals after they die, and how they become fossilised, i.e. their passage from the biosphere to the lithosphere. The study of taphonomy deals mainly with degraded bones and teeth, and shells found in (or on the surface of) the soil, or similar deposit, these being mineralised body tissues, and thus having the greatest longevity in the biogeosphere under most circumstances (N.B. only bone is considered in this thesis). Although taphonomy is often used nowadays to describe the study of the passage of other biological tissue (e.g. plants) into the fossil record. Taphonomy can be considered a sub discipline of palaeontology, or archaeology, dealing with fossilised or partially fossilised bones and teeth, concerned with the physical and chemical transformations to the bone. In essence it is the study of the fossilisation process, and not the fossils themselves, although the study of these processes can reveal much about the fossil. Fossilisation in these terms is not however useful as fossilisation is only one extreme end-point of the taphonomic process. If we consider taphonomy as the processes that occur to an animal after its death, it is apparent that these processes are numerous and varied, and that it may be useful to further subdivide taphonomy (see Figure 1.2-A). It is also apparent that the 'end-point' of these processes is in most cases somewhat artificial as it is often defined as the point of excavation or discovery of the bone or tooth, the alternative being the point at which the bone or tooth is destroyed as a recognisable unit.

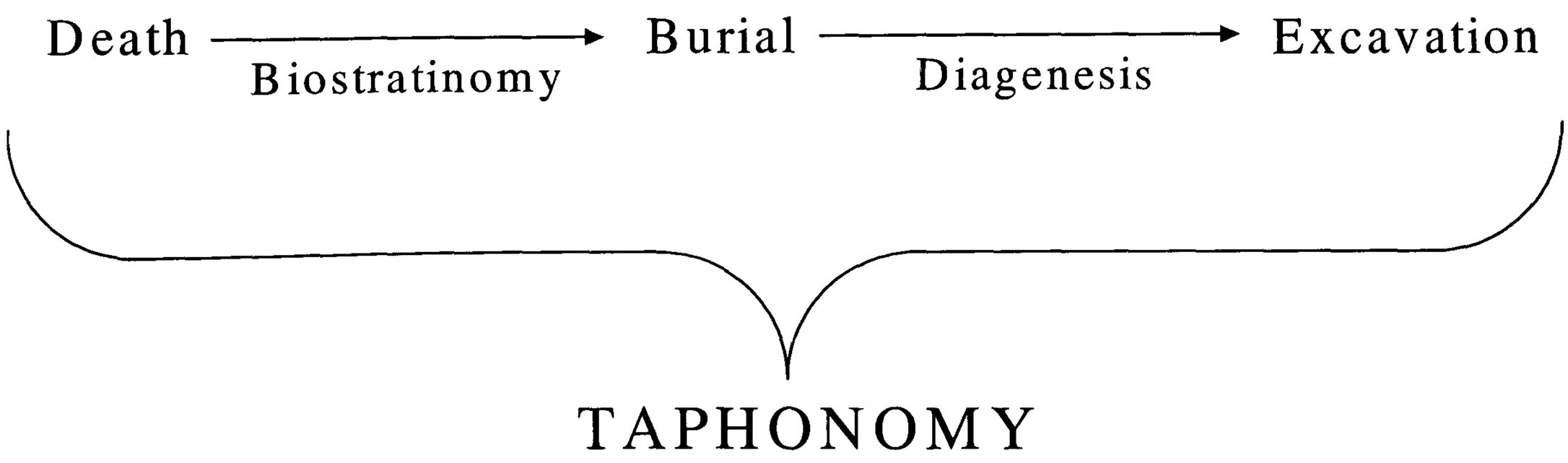

Figure 1.2-A. The taphonomic process

Figure 1.2-A shows the key points in the study of taphonomy. The precise timing of many of the events in the taphonomic history of a fossil may be difficult to define. For example burial may take some time for an antelope killed on an African plain, however over archaeological or geological timescales this may be negligible; in contrast to the deliberate burial of a human which is well defined. The division between biostratinomy and diagenesis is in some ways misleading, as in order to understand the formation of the archaeological and palaeontological record all aspects should be considered. The distinction between the two is more an accident of the history of the two sub disciplines.

Biostratinomy was born from palaeontology from the need to know how the fossil record is formed. It has thus been largely preoccupied with specimens of geological age, e.g. animal death assemblages, and early hominid remains. This has traditionally involved the study of macroscopic and microscopic changes to bones and teeth, their movement prior to deposition, and the structure and formation of death assemblages and how these reflect the living populations from which they originate. Diagenesis is a sedimentological term referring to the chemical changes (both mineralogical and organic) that occur to sediments as they mature. Arguably tooth and bone (and any other part of an animal) once they have been deposited in the soil become a sedimentary unit, or a part of one, and thus the processes that occur to them are by definition diagenetic. Thus the study of the post burial (diagenetic) changes to bone have developed from the soil science and geochemistry disciplines, and tend to concentrate on changes at the molecular level, e.g. mineralogical transformations, interaction with the soilwater, changes to the organic matter in bone. Both biostratinomy and diagenesis are discussed in more depth below.

Many aspects of the taphonomic process may appear negligible, but may result in lasting effects on the bone, and it is thus important to consider all events that may have an impact on the bone. Strictly, the process (or sequence of events) starts with the death of the organism, however to understand the end result of the process it may be important to understand the starting material. A general account of bone has been given above, but the state of a bone in an individual may depend on the species, age, sex and state of health. The mode of death will also have an effect on the bone. Death can occur in a number of ways; old age, disease, violent trauma, to mention but a few, some of these may have a direct effect on the bone. For example, violent trauma may involve the fracturing of bones.

1.2.1 Biostratinomy

The initial stages of taphonomy, from death to burial, can be known as biostratinomy. The impact of the immediate post-mortem period on bone has not been studied in any detail. At a cellular level, the cells die and undergo autolysis. It is assumed that the bone cells (osteocytes, osteoclasts, and osteoblasts) will undergo this process, breaking down their own cellular structures. The overall effect of this on the bone will be negligible. The autolysis of the surrounding soft tissues may have more impact, if they are not removed, producing an acidic environment. The breakdown of the body as a whole may have an important role, releasing gut bacteria into the tissue in general (Child 1995). These may play a role in microbial attack of the bone. These latter stages are the result of allowing a corpse to rot. Further to the body's own decay mechanisms microbes and fungi will populate the body as a food source, indeed

the bodies own gut flora may play a part in this. Arthropods will use the body as a food source and as shelter (Payne 1965, & 1968). These breakdown processes can be rapid, even in temperate climates, leaving a body skeletonised in a matter of days to months (depending on the season). Permafrost conditions may in effect halt the decay of the body for hundreds of thousands of years. The direct effect of these processes on the bone may be difficult to perceive, with the exception of microbial attack (if indeed this is the mode of microbial attack of bone), although removal of the flesh will leave the bones exposed to weathering and disarticulation of the skeleton. It should be noted that for a body that is interred soon after death these processes can also occur, and they can thus also be described as diagenetic.

A number of processes can occur before the onset of the decay of a body, which prevent it. As noted above one mode of death is via violent trauma, one example of this is predation. Predation will result in the majority of the soft tissue being removed from the bones, either by the predator, or by subsequent scavengers. This may limit opportunities for arthropods, and microbes and fungi to colonise the corpse, but again may leave the bones exposed, particularly to microbial and fungal attack. The effects of predation and scavenging on bone can be quite marked. Predation can leave bite marks on the bone, or have a large impact on the bone when it has passed though the gut of an organism. Gnawing is also a common feature of bone that has been scavenged. Predation and especially scavenging may be a source of disarticulation of the skeleton. Bones can also be trampled and crushed under foot, moving and damaging the bone.

Humans as taphonomic agents are known to have a variety of effects on bone. Humans can be predators, although cut marks from tools may be a more obvious mark than biting or gnawing of the bone. They may also use bone and tooth as a raw material for manufacturing artefacts. Humans also have a variety of methods for disposing of fellow humans. Burial and cremation are common, although the conditions of burial can vary greatly. Cremation and burning of bone has dramatic effects on the physical and chemical properties of bones and teeth (e.g. McKinley & Bond, 2001).

In addition to the effects of other organisms on the bone, abiotic factors can have significant effects on the bone. Weathering (wind/rain abrasion, UV light) can cause bone to move around, polish and crack through shrinkage, and frost action (freeze thaw) may also cause cracking. These factors can alter the mineral and protein contents of the bone (Tuross *et al.*, 1989). Additionally environmental factors such as rivers can play a major role in transporting bone away from its original site of deposition before depositing the bone downstream, perhaps cracking and polishing the bone *en route*.

Biostratinomy thus deals mainly with the spatial movement of bones and teeth, and the physical changes that occur mainly on the macro and microscopic scale. Many of the above processes will have severe effects on the bone, and many bones, especially from natural deaths, will not survive to be buried, unless they occur in depositional environments. Some circumstances may ensure burial of a body, such as deliberate human burial, however this does not ensure long-term survival in the archaeological or fossil record.

1.2.2 Diagenesis

The condition of the bone when it enters the soil will determine how it behaves once it is there. Bone may be relatively intact in the case of a deliberately interred human, or very degraded if it has been exposed on the surface for a length of time. Some of the taphonomic agents that are prevalent during biostratinomy are also relevant to the diagenetic arena, as plant roots, soil microbes, fungi and arthropods can all potentially cause damage to the bone, although arguably the most significant agent of decay in the soil is soilwater. Exposed bone in the soil will interact with the ground water, the extent of which is dependent on the hydrological regime around the bone and the state of the bone itself, significantly the porosity, and thus hydraulic conductivity of the bone (Hedges & Millard, 1995). Soilwater will reflect the soil in which it is in, in terms of chemistry (e.g. pH, dissolved ions) and this in conjunction with the hydrological regime will determine the type and rate of changes that occur to the bone mineral. Mineral alteration can vary from recrystallisation of the hydroxyapatite phase, to alteration of the mineral phase to another type, via exchange of ions between the soilwater and the bone mineral, or complete dissolution of the bone mineral. In addition to the mineral changes the collagen will degrade. Mineralised collagen is a very resistant material, but after mineral dissolution (or perhaps reorganisation) collagen will degrade more quickly and will be exposed to potential microbial attack (Nielsen-Marsh *et al.*, 2000). These changes are discussed in more detail later.

1.3 What is 'Archaeological bone'?

Part three of this thesis focuses on the measurement of certain physico-chemical characteristics of bones recovered from archaeological sites. This material 'archaeological bone' differs from its biological counterpart in that it is by definition the end result of a number of transformations (taphonomic processes, detailed above) that change it from a living bone in an organism to an 'archaeological bone' recovered from an archaeological site. This distinction may be obvious, but it is important – the material found at archaeological sites is *not* bone. The effects of the taphonomic process on bone can vary greatly, from bone preserved in permafrost, which can be preserved like fresh bones, to complete destruction of the bone, so that the archaeologist would never know that it existed. In most cases bone is recovered in a state between these extremes, and in many circumstances archaeological bone is physically and chemically different to its original *in vivo* state.

Fresh (unfleshed) bone is whitish in colour, and is a very strong tough material. It is encased in the periosteum, an outer membrane, giving it a waxy appearance. The bone may also contain blood vessels and marrow in the medullary cavity. This contrasts to most archaeological bone that is often stained by the soil, will hardly ever contain marrow, or other adhering soft tissue, and is often softer and more friable than fresh bone. The reasons for this change in the bone are that the bone has fundamentally altered both physically and chemically, as a result of the taphonomic process. The bone mineral has become more crystalline, or may have changed to a slightly different mineral phase (e.g. brushite, or francolite), the collagen has deteriorated and been leached from the bone, the bone has become more porous (with the exception of lithified material), and the bone may have damage to the histological

structure caused by microbial attack. This can leave the bone physically weaker than fresh bone. These changes are detailed in section 1.5 and are caused by the agents that act during the taphonomic process.

1.4 Bone as an archaeological resource

Bone is a valuable archaeological resource because it is one of the most frequently recovered biological materials from archaeological sediments, and can provide a lot of data about human and animal populations and activities.

Most human material found at archaeological sites will be from deliberately interred individuals, and is thus the most direct evidence of human activity at a site. Firstly, the burial itself provides data on the burial rites conducted by the society. Someone must have buried the individual in the manner that they have for some reason. Secondly the occupant of the grave presumably reflects the past society in some way. These interpretations may be aided by the biological data and/or cultural data from the burial.

Bone itself can provide data at a number of levels, the morphological and metric characteristics of a bone can be used to infer the species, age, sex, and physical stature of the individual. This data has applications for palaeoanthropology, reconstructing palaeodemography, and archaeozoology. In the case of the latter bone can be used to infer domestication of species, or the demographic structure of a cull assemblage may be characteristic of a certain animal husbandry strategy (e.g. dairying), and of course this has palaeodietary implications. In addition, at a macroscopic level bone can be used as a source of palaeopathological data, where a number of medical conditions, such as tuberculosis, leprosy, syphilis, neoplasms, and osteoporosis leave lesions or other traces on the bone. Bone can also provide direct evidence of trauma to individuals, showing injuries, such as fractures, or healed fractures.

Bone is an invaluable resource for evolutionary biologists as fossil bone is sometimes the only evidence for extinct species. The biostratonomic marks left on the bone can be used to interpret site formation process, and thus provide evidence on the formation of the fossil record. Natural death assemblages (assuming the formation processes are understood) provide data for palaeoecological reconstruction (Shipman, 1981, Behrensmeyer, 1991).

Bone is also a source of data at the molecular level that can be used to complement the macroscopic data. The organic molecules in bone can be analysed for a variety of purposes. The isotopic ratios of both carbon and nitrogen in the bone collagen are used for palaeodietary inference and carbon for radiocarbon dating (Ambrose & De Niro, 1986, Hedges & Law, 1989). Osteocalcin remaining in the bone may be of debatable value for the same purposes (Ajie, *et al.*, 1991, Burky *et al.*, 1998), but may prove useful for molecular evolutionary analysis (see Ostrom *et al.*, 2000). Associated proteins such as albumin and immunoglobulins have been detected in ancient bone using immunological techniques (e.g. Catteneo *et al.*, 1992a and b). The ability of immunological techniques to distinguish between species makes them a potential tool to determine the species of fragmentary bone, where diagnostic parts are missing.

DNA has been extracted from bone and been used to provide data to help answer a number of archaeological questions. DNA can be used to identify the sex of remains that is otherwise difficult morphologically, e.g. where diagnostic parts of the skeleton are missing, or in infants (e.g. Götherström *et al.*, 1997; Mays & Faerman, 2001), and used to reveal patterns of kinship amongst individuals (Götherström *et al.*, in press a). Ancient DNA (aDNA) extracted from bone has also been used to provide data on the genetic relationship between modern humans and Neanderthals (Krings *et al.*, 1997, Ovchinikov *et al.*, 2000, and Krings *et al.*, 2000). Bone is also a source from which to extract DNA from foreign organisms, such as that of leprosy, tuberculosis and malaria (e.g. Haas *et al.*, 2000; Taylor *et al.*, 1996).

Bone lipids have been a somewhat overlooked resource, although recent studies have proposed their utility for isotopic analysis to complement stable isotopic studies of bone collagen. Cholesterol has the ability to survive intact in archaeological bone (unlike collagen), thus the isotopic signal of cholesterol is known to be faithful to the dietary one. It also provides information on an alternative metabolic pathway to that of protein (Stott & Evershed 1996; Stott *et al.*, 1997; Stott *et al.*, 1999). Like DNA, bone is also a source of lipids from foreign organisms (Gernaey *et al.*, 2001).

Bone mineral can also be analysed for chemical information (see Lee-Thorp & van der Merwe, 1991 and refs therein). Unless bone is preserved exceptionally the organic fraction is the most labile, and will not survive in very old fossils, thus the above avenues of investigation are lost. Stable isotopic analysis of carbon from the mineral carbonate has been used to infer palaeodiet of fossil hominids (e.g. Lee-Thorp *et al.*, 2000). Bone strontium (both the isotopic ratio and Sr:Ca) is another possible source of palaeodietary information potentially revealing trophic levels of species with in food chains, although there is some debate surrounding this work (Sillen & Sealy, 1995). Oxygen isotopes from bone phosphate have been similarly used to make inferences about palaeoclimate (Iacumin *et al.*, 1996) Although tooth enamel is the preferred substrate for these analyses, as tooth enamel biogenic signals are apparently less prone to diagenetic change than those of bone. Bone can also be used for Uranium series dating Millard & Hedges, 1995.

Bone contains a wealth of information that can be used to make inferences about past individuals, societies, economies, and the palaeoenvironment in which these are set, however the taphonomic process can completely destroy, alter and obscure many of the potential sources of information contained in bone; this is discussed below.

1.5 Taphonomic effects on bone

The taphonomic history of a bone will affect its physical and chemical properties. Bone retains information at a number of different levels, from the macroscopic to the chemical level, and different states of preservation will potentially result in the different levels of information being lost or obscured. For example, a fully lithified fossil may contain perfect morphological and histological consistency with its original form, however the mineral phase will be entirely different to the original, and there will be no collagen remaining, allowing morphological analysis but not chemical analysis. Contrast this with a site where the bone retains some collagen, and maybe DNA, but is friable or broken, or where the bone has been destroyed in its entirety.

All these outcomes can be observed in the spectrum of preservation types of archaeological bone, and they all have different outcomes for the potential data available to the archaeologist. It is thus important to understand how different taphonomic events affect or bias the bone, in order that they may be accounted for in interpreting the information from archaeological bones, or bone assemblages.

The archaeological and fossil record is the direct result of the various taphonomic processes that have occurred at the site from which the material has been excavated. For the sake of simplicity a number of aspects of bone diagenesis (and how they are assessed) are described below: detailing the physical and chemical changes that occur to bone during the taphonomic process, and how they are measured. However, it should be noted that the divisions are artificial and the processes that cause them can act on many aspects of the bone at once.

One very important outcome of the taphonomic process is the complete destruction of the bone, or possibly more importantly differential preservation of bone. Preferential loss of small bones may give a bias to larger species in bone assemblages. This can impact on demographic interpretations if juvenile or female bones are preferentially lost if they are more gracile. Systematic loss of certain skeletal elements may give false evolutionary information in fossil specimens. A good example of the impact of differential preservation is the discovery of the Neanderthal hyoid bone, a small and presumably labile bone (Arensburg *et al.*, 1989). The discovery of this bone has led to reappraisals of Neanderthal linguistic abilities, and of course has repercussions for the definition of the human condition.

A better knowledge of the effects of taphonomy can help to account for such biases in the fossil record. The physico-chemical changes to bone need to be understood to account for their effects on the quality of data derived from archaeological bone. Furthermore an understanding of taphonomic changes can help guide policy in the realms of research and cultural resource management. A better knowledge of how biomolecules survive in bone will allow researchers to target resources on studying bones with greater chances of yielding them. If the effects, on the bone, of changing a site's environment are known then this will enable decisions to be made about archaeological site management.

The following sections detail some of the key taphonomic changes that occur in bone.

1.5.1 Microbial attack –histological damage

Microbial attack of bones is a well documented phenomenon and has been known about for at least two decades (Hackett, 1981). Garland describes four types of microscopic focal destructions (MFDs) observed under light microscopy in archaeological bone, which are attributed to the incidence of bacterial and/or fungal attack (Garland 1987). MFDs are areas of the histological structure that have been damaged by microbiological agents, which have characteristic shapes. They apparently follow the histological structures of the bone, i.e. parallel to the Haversian system, and contours of the lamellae, although this is difficult to judge as they are observed in cross section. They appear as holes or pitted areas in the histological structure of the bone, and often have a hypermineralised edge. The types of MFD are

distinguished by their diagnostic shapes which may be dependent on the type or types of organisms responsible.

Despite the longevity of these studies a number of key questions remain unanswered about the histological damage observed in archaeological bone. Which species of organism create the MFDs, how do they do this, and where do they originate? When does this process take place, does it happen immediately after death, how long does it continue? If it stops, why does it stop, and can it be restarted?

As stated above the soft tissue of a corpse is usually decomposed rapidly after death, but the mineralised state of bone means that it is somewhat more robust, the mineral protecting the protein from enzymolysis. Child proposed a scheme where micro organisms will initially feed on the soft tissue, then invade the bone structure through the natural spaces. Although the system may at first be aerobic, microbial growth leads to consumption of the oxygen and thus anaerobic conditions. Under anaerobic conditions proteins will be fermented, a by-product of which is the production of fatty acids. This may lower pH, dissolving mineral, exposing more collagen to enzymolysis. *In extremis* this may lead to complete dissolution of the bone (Child, 1995).

A number of bacterial and fungal species have been observed in and isolated from archaeological bone (Marchiafava *et al.*, 1974; Piepenbrink 1986), some or all of which may have been responsible for the original destruction of the bone. Child suggests *Clostridium histolyticum* as a bacterium likely to be involved in bone degradation, as it is an anaerobic bacterium that produces specific collagenases (Child, 1995). Jackes *et al.*, (2001) report the presence of (but were unable to isolate) bacteria in Mesolithic bone from Portugal, which is tentatively assigned to *Clostridium* species, stating that *Clostridium* is found in both the soil and mammalian intestines.

The presence of bacteria in Mesolithic bone does not necessarily mean that the process of microbial degradation is currently taking place, especially as the bacteria could not be isolated; indicating that they may not still be viable. Furthermore, the sample was taken from a museum collection, and thus the damage caused by the bacterium could potentially have happened post-excavation. The precise commencement and duration of microbial attack does however remain an important question. Bell *et al.* (1996) observed MFDs in bone three months post-mortem, although this bone was deposited in animal scat, which may be a highly microbial taphonomic pathway. Yoshino *et al.* (1991) have observed microbial attack in bone that has been interred for only 15 years, demonstrating that microbial attack can and does occur quickly post-mortem, but the length of time this continues is not known. The extent of microbial attack in bone varies (see below); this may be due to the diagenetic conditions of the bone or the length of time of exposure. Based on the distribution of estimates of the extent of attack (Histological Index), and assuming that the process is time dependent Hedges *et al.* (1995) estimate that the process is usually complete by about 500 years. If this is correct then the extent of attack is controlled by an extraneous factor (perhaps a change in the environment). Jackes *et al.*, (2001) suggest that the extent of microbial attack may be curtailed by structures in the bone, where the microbes can no longer colonise fresh areas of bone, and expire in their own acidic waste products. Conversely, if microbial attack is assumed to be a

relentless process then the extent of attack is determined by the rate of attack and the time of excavation or analysis, if this were true one might expect *in situ* bacteria to be observed more often.

Whatever the precise mode and extent of microbial attack, it is clear that the action of these microbes is deleterious to the physical and chemical integrity of the bone, removing collagen and reprecipitating mineral. The importance of microbial attack and its potential effects on mineral and organic studies of bone are critical. Furthermore, microbial attack will lead to increased porosity and thus surface area of the bone, making the bone more liable to dissolution in ground water (Hedges & Millard, 1995). In order to assess the effects of microbial attack, Hedges *et al.*, (1995) developed a histological index to semi-quantify the extent of microbial attack in bone sections. This has since been revised, see Millard, 2001. This has proved to be a useful tool in assessing the state of preservation of bone as a whole, correlating with a number of other diagenetic parameters (Hedges *et al.*, 1995, Nielsen-Marsh & Hedges 2000), and the ability to amplify DNA from bone samples (Colson *et al.*, 1997). More details of the method can be found in the above references and Part 3 of this thesis.

1.5.2 Mineral changes

As mentioned above bone mineral *in vivo* consists of small thermodynamically unstable crystals maintained by the homeostatic process of the body. After death homeostasis is no longer maintained, and the crystals are liable to change. In archaeological bone the most significant changes in the mineral will be brought about by interaction with the ground water (notwithstanding physical removal of mineral via biostratonomic processes).

The effects of the ground water on bone will be controlled by its chemistry (pH, dissolved ions) and the hydraulic regime at the site (Hedges & Millard, 1995), both of which may in turn be controlled to some extent by the local geology. This interaction will cause three key changes to occur in the bone: dissolution of the mineral, change in crystallinity of the mineral, and the absorption of exogenous ions.

Mineral dissolution is the loss of bone mineral - the mineral is dissolved in the ground water, and transported away from the bone. Dissolution of the bone mineral leads to an increase in the porosity of the bone, which in turn exposes more surface area of the bone to interact with ground water. This process can be self perpetuating and ultimately catastrophic, as has been demonstrated in laboratory experiments by Pike & Nielsen-Marsh (2001). Dissolution of the bone mineral is dependent on the severity of the soilwater and hydraulic regime, however if it occurs its logical conclusion is the total destruction of the bone (as the collagen is exposed to other destructive factors).

If the environment of the bone is more benign then only partial dissolution of the smallest crystals or recrystallisation of the bone mineral are likely to be the main processes of mineral alteration. These phenomena result in an overall increase in the crystallinity of the bone mineral. The term crystallinity is used to cover a number of properties pertaining to the crystals that make up the bone mineral, these include crystal size, order and perfection. Whilst the notion of crystallinity remains somewhat

vague, measurements of archaeological and palaeontological bone crystallinity have been used to gauge the extent of mineral change during the taphonomic process. Changes in crystallinity have been assessed using a crystallinity index of a powdered sample of bone, measured either by the X-ray diffraction pattern of the sample (e.g. Hedges *et al.*, 1995, Person *et al.*, 1995), or using the phosphate contour in the infrared spectrum of the powder after it has been incorporated into a potassium bromide pellet (Weiner & Bar-Yosef, 1990).

In this thesis crystallinity index is measured using Fourier transform infrared spectroscopy (FTIR). The use of FTIR enables the identification of other mineral phases, e.g. diagenetic calcite, and also semi quantitative assessment of the carbonate:phosphate ratio. It has been noted that carbonate substitution in the lattice does effect the crystallinity of apatite, thus the two factors are related (Newesley, 1989). Nielsen-Marsh & Hedges (2000) note that there is a relationship between the carbonate:phosphate ratio and the crystallinity index of archaeological bone but that it is slightly different to that of the manufactured apatites observed in Newesley (1989).

Both the methods of measuring the crystallinity index can be problematic. With XRD sample orientation and homogeneity can reduce precision in the measurements, whilst the amount of sample preparation (grinding) has been observed to create systematic error in the infrared crystallinity index (Surovell & Stiner 2001).

In a precipitative environment exogenous ions will become incorporated into the bone mineral lattice as recrystallisation occurs. The presence of diagenetic calcite ($CaCO_3$) in the bone can be observed in the infrared spectrum as a peak at $713cm^{-1}$ (in transmittance). Sodium, strontium, magnesium and the rare earth elements are often incorporated, as well as uranium and fluorine. Some of these substitutions have been studied in detail, as they are significant for archaeological interpretations and studying fossilisation processes. The effect of uranium uptake in fossil bone is benign to the bone survival, but the diagenetic mode of uptake is of interest for the application of uranium series dating (Millard & Hedges 1996). The incorporation of rare earth elements has also been investigated as a tool to investigate the formation of fossil bone assemblages (Trueman, 1999). The isomorphic nature of bioapatite means that these substitutions can occur without detriment to the stability of the lattice. Indeed the observed increase in crystallinity of archaeological bone mineral indicates that the mineral is more stable. The nature of the bone mineral will thus reflect the most stable form that the mineral can take depending on its environment, be that brushite, hydroxyapatite or fluorapatite. If the bone mineral is ultimately unstable it will dissolve.

1.5.3 Collagen degradation

The abundance of something in the archaeological record has more to do with taphonomy than it does with its original abundance prior to entering the archaeological record. This is true for most artefacts and is equally true for biomolecules. Bone collagen is the most abundant archaeological protein, this is due in part to the fact that it is a resilient structure, but also because it makes up ~20-25% of bone, and in this mineralised form it is even more resilient.

Collagen has been the focus of much biomolecular archaeological research, because of its abundance. Collagen can be easily extracted from archaeological bone (by dissolving the mineral) and quality checked using the carbon:nitrogen ratio of the residue, or the amino-acid profile. The ease of extraction and abundance mean that biomolecular techniques applied to the analysis of bone do not need large samples. This, and the fact that it can be quality assured, has lead to it being the substrate of choice for both radiocarbon dating and palaeodietary studies (Hedges & Law 1989, Ambrose, 1990).

Collagen can be degraded principally via two mechanisms the action of soil microbes and via chemical hydrolysis of peptide bonds. The action of microbes on bones is discussed above, where by they bring about small areas of destroyed bone (MFDs) where the collagen is destroyed and the mineral restructured (Jackes et al., 2001). Collagen is a chemically resistant substance, and the rate of decay will be greatly increased by enzymolysis. Child discusses the various enzymes and enzyme complexes likely to be involved, specific collagenases and proteases that may have collagenase activity. It is believed that whilst the collagen mineral association is still intact these enzymes will not be able to work properly, i.e. they can only begin to work when the bone is partially demineralised (Child 1995).

The chemical degradation of collagen will also occur via peptide bond hydrolysis. This process is anticipated to be much slower than enzymatically catalysed collagen degradation at ambient temperatures. The rate of collagen hydrolysis is dependent on a number of factors, although key factors are the availability of water, the temperature of the reaction and the pH of the solution. In bone it is usually assumed that the pH of the system is approximately neutral as bone mineral dissolution will buffer lower pH (Bada & Shou, 1980), and only under the driest conditions will water not be available for reaction. The role of temperature is discussed in Part 2 of this thesis.

The result of both the enzymatic and chemical hydrolysis is that the collagen is reduced to shorter random coil water soluble gelatine, which is leached from the bone. This results in a reduced amount of collagen, increased porosity (see Nielsen-Marsh & Hedges 1999) and reduced mechanical strength (Turner-Walker & Parry, 1995). The effect on the utility of collagen as a substrate for radiocarbon dating and stable isotope analysis is negligible until the amount of collagen is reduced to ~2-5% of the original amount (Hedges & van Klinken 1992, Ambrose, 1990, Tuross & Stathoplos, 1993). Below this value the 'collagen' fraction of the bone is less pure, as observed by deviation in the carbon:nitrogen ratio of this fraction from that of pure collagen (approximately 3.2, atom %).

Another change that can be observed in the collagen fraction is the racemisation of the amino-acid residues. The amount of racemisation in the aspartic acid in collagen was first investigated as a possible dating technique (Bada, 1985); however the kinetics of racemisation made the system difficult to understand especially in collagen depleted bone. It has since been proposed as a screening tool for ancient DNA analysis (Poinar *et al.*, 1996), however the value of this has also been challenged (Collins *et al.*, 1999).

1.5.4 Degradation of NCPs, in particular osteocalcin

Non-collagenous proteins (NCPs) have been less extensively studied than collagen mainly because they are in such small quantities *in vivo*, that after diagenesis, they would survive only in amounts that would be impractical for analysis. As a result of the dearth of research into the survival of other NCP fractions in archaeological bone only the degradation of osteocalcin will be considered here in any detail.

Despite the structural integrity of collagen and the relatively labile nature of the NCPs it has been noted that NCPs are abundant in collagen depleted bone (Masters 1987). It has been suggested from the results of laboratory experiments and analysis of archaeological and palaeontological bone that osteocalcin (Oc), or at least parts of the molecule, can survive for long periods in bone, perhaps even millions of years (Collins *et al.*, 2000, Muyzer *et al.*, 1992), longer than the expected longevity of collagen. The preferential survival of Oc over collagen has lead to the investigation of Oc as an alternative substrate for stable isotope analysis and radiocarbon dating in collagen depleted bone (Ajie *et al.*, 1991, Ajie *et al.*, 1992 and Burky *et al.*, 1998), although there is apparent discrepancy between the collagen and Oc values in collagen depleted bone. The differential survival of the two protein fractions is presumably due to different preservation mechanisms. Oc has been shown to be chemically very stable in bone heating experiments (Collins *et al.*, 2000) and is known to bind tightly to the mineral phase (Haushcka, 1986), which may offer protection from biological degradation, inhibiting enzymolysis, and contribute to its chemical stability. It has been noted that increasing crystallinity of bone from modern taphonomic assemblages and archaeological bone (Tuross *et al.*, 1989, Collins *et al.*, 2000) is associated with decreasing Oc content, supporting the mineral protection theory. Oc can also bind to collagen, which may be an alternative survival mechanism, although presumably not in collagen depleted bone.

Not withstanding the studies mentioned above, there have been few investigations comparing Oc survival with other diagenetic parameters of the bone, especially the role of micro organisms, this is investigated in chapter 3.4.

As a result of the small amounts of NCPs expected in archaeological bone they have mainly been investigated by the use of immunological techniques. These techniques rely on the immune response reaction, where a detecting protein (an antibody) is able to 'recognise' a specific target molecule (an antigen). Such reactions can be very specific so that the antibody may pick out one particular protein or a group of similar proteins, and they are very sensitive, being able to detect nanogram amounts of protein. Immunological techniques are ideal for investigating small amounts of archaeological proteins, being both highly specific and sensitive for the target molecule. Immunological techniques have been used to investigate the survival of albumin and immunoglobulin (IgG) in experimentally buried and archaeological bone. It has been demonstrated that albumin remains immunologically detectable for longer than IgG in the same archaeological bone, suggesting that albumin is more resistant to decay. The survival of these proteins in bone has not been extensively studied, in comparison to other diagenetic parameters of bone, although it has been suggested that the age of the specimen and the interaction with water will have a degenerative effect on the protein (Catteneo *et al.*, 1992b; Catteneo *et al.*, 1995).

1.5.5 DNA Degradation

The recovery of DNA from ancient samples has been a revolution for evolutionary biologists and archaeologists alike. Archaeological bone has been the focus of many ancient DNA (aDNA) studies, principally because bone is ubiquitous at archaeological sites, and is thus an obvious choice for an ancient DNA reservoir. The polymerase chain reaction (PCR) has enabled tiny amounts of surviving DNA to be amplified in to practical amounts for analysis, expanding the utility of aDNA analysis. PCR is however a double-edged sword, for as well as enabling the amplification of tiny fragments of aDNA it can also amplify tiny amounts of modern DNA contamination which can be hard to eliminate from the laboratory even under the most stringent conditions, especially for work involving human DNA. The importance of such work to provide data for some of the 'big' archaeological questions is not in doubt (Krings *et al.*, 1997) but the field remains dogged by a lack of controls for contamination (Cooper & Poinar 2000).

Excellent reviews of DNA degradation can be found in Lindahl (1993) and with specific reference to DNA in ancient samples Hofreiter *et al.*, (2001), a brief description is given here with particular reference to bone.

It is anticipated that under most circumstances following the death of the organism the cells' own enzymes (nucleases) will cause the rapid destruction of DNA. Only under exceptional circumstances (perhaps rapid desiccation, or low temperature) will this process fail to destroy all DNA. It seems reasonable to suggest that any DNA that survives this process, and remains relatively intact for thousands of years must be subject to some form of special preservation mechanism. Tuross (1993) noted that DNA has an affinity for hydroxyapatite, and suggested that DNA survival may be prolonged by binding to bone mineral (see also Okazaki *et al.*, 2001). DNA preserved in bones from Pompeii has been stained, and it is concentrated in the osteocyte lacunae (Guarino *et al.*, 2000), suggesting that the DNA has been retained in the cellular region of the bone, although this may be a function of the unusual taphonomic circumstances of the site.

Whilst it may be that there are special circumstances under which aDNA will survive in bone, the usual case is that aDNA cannot be extracted and amplified for analysis. The reasons for this may be two-fold; firstly, because the DNA has been destroyed, so that only short fragments remain, that will not prove useful to extract, or secondly that the DNA has become bound in the bone in some manner that it may not be extracted or analysed by conventional means. Assuming that some DNA has survived the initial autolytic process (which it may well, as the conditions for this are probably good for bone preservation in general), then the longer term bone taphonomic factors may then play a part in DNA diagenesis specifically. Similarly to collagen it is likely that DNA will be degraded principally via microbially mediated decay, or by chemical means (mainly hydrolytic reactions). Hagelberg *et al.*, (1991) demonstrated that it was more difficult to extract and amplify DNA from microbially attacked bone than bone that had not been microbially attacked. Colson *et al.* (1997), went further, quantifying the amount of microbial damage using the Oxford histological index (Hedges *et al.*, 1995). They noted that whilst DNA could usually be amplified from bone with HI5 (perfect histology) and could never be amplified from bone with HI0 (no histological features remaining). It was difficult to predict the outcome of DNA

analysis from bone with HI scores between these values. It is unlikely that microbes are directly digesting DNA, but maybe cosuming already degraded DNA if it is available to them. The concomitant effect of microbes: lowering pH, altering the structure and crystallinity of bone may have secondary effects on the DNA preservation.

Chemical degradation of DNA occurs mainly by hydrolysis and oxidation. Lindahl states that the most likely degradation pathway for hydrated DNA in ancient samples is depurination (cleaving of the purine bases from the sugar phosphate backbone); this causes further damage and fragmentation of the DNA (Lindahl, 1993). In bone the rate of depurination may be slowed two-fold by the presence of hydroxyapatite, however it will still be the fastest degradation pathway assuming there is sufficient water for the reaction to proceed. Oxidative damage, although slower, can have different effects on aDNA analysis. Evidence of oxidation products of DNA bases has been detected in ancient tissues using gas-chromatography mass spectrometry. High levels of hydantoins (oxidation products of the pyrimidine bases, thymine and cytosine) were observed in samples from which DNA could not be amplified (Höss *et al.*, 1996). It is believed that hydantoins may interfere with the PCR reaction thus rendering amplification of the DNA impossible. This is one example of where the DNA may be preserved in the sample (albeit altered), but is irretrievable by current extraction methods. The use of different sample preparation treatments may be able to release DNA bound in macromolecular complexes in samples, liberating it and making it available for analysis (e.g. Poinar *et al.*, 2001), although such procedures have yet to be applied to bone samples.

The result of DNA degradation has been demonstrated empirically to be single strands of DNA usually 100-300 base pairs long. Despite the amount of research into aDNA recovered from bone there has been little research into its degradation, probably due to the difficulties in quantifying the amount of DNA in the sample, and the technical difficulties of the work in general. A number of studies have however proposed that other features of bone degradation are related to DNA survival, and thus measuring these proxies may prove to be useful screening tools for DNA survival.

Götherström *et al.*, (in press b) investigated the effect of increasing crystallinity of the bone mineral on the ability to amplify different length DNA fragments, in both heated modern bone powders and archaeological samples. The probability of DNA amplification was decreased with increasing crystallinity index, adding weight to the crystal binding theory of DNA preservation. Similarly the degradation of collagen in bone has also been investigated as a method of assessing DNA preservation (see Poinar *et al.*, 1996 but also Collins *et al.*, 1999, and Poinar & Stankiewicz, 1999, for racemisation and pyrolysis gas-chromatography techniques). From these studies it is suggested that DNA remains well preserved when collagen is, although this may be as a result of the conditions for good organic preservation rather than a mechanistic preservation link between the two molecules.

There is much debate as to what the survival limits of DNA are in the archaeological record, and often a temporal limit (100kya) is placed on this (Höss, 2000; Wayne *et al.*, 1999), although it is noted that survival will be a function of both time and the environment. Smith *et al.* (2001) presented a model to calculate the relative rates of DNA depurination at a number of archaeological sites across Europe, accounting for

the age and approximate palaeoclimatic history of each site. This produces a conceptual limit on DNA survival, the 'thermal age' that combines both the age and temperature of the site into an estimate of the amount of damage to the DNA. Whilst the model cannot account for factors such as microbial damage, if all things are equal, the relative amount of damage can be compared. This concept is taken up and expanded in Chapter 2.3. There is still no real idea of how long extractable (analysable) DNA will survive in ancient bone. The study by Krings *et al.*, 1997 was well controlled (reproduced in another laboratory), and thus it is assumed that the limit is ~40-50kya in temperate climates, perhaps extending to 100kya in permafrost sites. Although it should be noted that by using hybridisation techniques it may be possible to reveal DNA surviving as very short fragments in 500kya bone (Geigel, 1997)

1.5.6 Porosity changes

Bone porosity has been identified as a key factor in the diagenesis of archaeological bone. Arguably the key to bone survival is the preservation of intact mineral. If the mineral can remain relatively unaltered organic molecules will survive well in the archaeological record. For longer-term survival as fossils it is apparent that the depositional conditions must be benign for the mineral phase as fossils are basically mineral psuedomorphs of the original bone. Mineral diagenesis is controlled by interaction of the mineral and the soilwater, the extent of which will be controlled by the hydrological regime of the site and the pore structure of the bone (Hedges & Millard, 1995).

The pore structure of archaeological bone has been investigated by water sorption porosimetry and mercury intrusion porosimetry. Using the water sorption technique Hedges *et al.* (1995) have revealed that archaeological bone is more porous than its modern counterpart, and that the porosity increases in line with other diagenetic parameters (e.g. nitrogen loss). The water sorption technique is able to measure three basic properties of the porosity defined as: total porosity, microporosity (pore volume in pores with < 4nm radii), and macroporosity (pore volume in pores with > 4nm radii). These changes to the pore structure are interpreted as the result of: (1) dissolution and recrystallisation of the mineral through interaction with the ground water, (2) the action of bacteria and fungi (microscopic focal destructions and tunnelling), and (3) loss of collagen. It should be noted that under some circumstances archaeological bone might become less porous, where the natural pores are in-filled with calcite or other debris from the soil, this is probably true of fossil bone. An inverse trend has been identified between the macro and microporosity measurements where, as microporosity decreases macroporosity increases in archaeological bone. Nielsen-Marsh & Hedges (2000) interpret the increase in microporosity as being associated with collagen loss, however increases in macroporosity are possibly due to mineral dissolution. Studies investigating pore structure using a nitrogen adsorption technique, however, indicate that both macro- and microporosity may increase with worsening bone preservation (De La Cruz Baltazar 2000)

Water sorption analysis provides only limited data about the changes to the pore structure in archaeological bone. Using mercury intrusion porosimetry (HgIP) Nielsen-Marsh & Hedges (1999) demonstrated that very distinct changes to the pore

structure occur when protein is removed from the bone in the laboratory using hydrazine hydrate. They also demonstrated that archaeological bone can have a large increase in the pore volume of pores with radius 100-10000nm possibly due to microbial attack of the bone. This level of detail revealed by mercury intrusion porosimetry has shown this technique to be a powerful tool for extending our knowledge of the pore structure of archaeological bone. HgIP is used in this thesis to investigate the porosity of archaeological bone see Part 3.

1.5.7 Lipids

Lipids have not been studied in this thesis, nor have they been the subject of extensive diagenetic study elsewhere, although for completeness their degradation will be discussed. Modern bone contains two major groups of lipids acyl lipids and cholesterol (and similar compounds). In the studies that have been carried out using gas chromatography-mass spectrometry and gas chromatography-isotope ratio mass spectrometry, only the cholesterol group has been found in significant quantities in archaeological specimens (although this may be a function of the specific taphonomic conditions for the sample sets). The cholesterol carbon skeleton is itself very resistant to decay and is thus a useful substrate for palaeodietary studies. The cholesterol degradation products may yet reveal the nature of the taphonomic history of the bone, with some bones containing predominately oxidative degradation products, and others reduced cholesterol products. These patterns may be linked to the depositional conditions of the bone, but studies comparing the lipid profiles with other more widely used bone diagenetic parameters have yet to be carried out. Cholesteryl esters can also be found, which may originate from the blood or meat associated with the bone (Stott & Evershed 1996, Stott *et al.*, 1997, Stott *et al.*, 1999). Exogenous diagenetic lipids have also been observed in archaeological bone, e.g. diploptene, most likely originating from sedimentary bacterial cell membranes (Evershed *et al.*, 1995).

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Part 2

Modelling biomolecular deterioration in archaeological bone

2 Modelling Diagenesis

Part 2 of this thesis details models designed to describe the degradation of collagen and DNA in archaeological bone. The models are based on a number of assumptions that may not be entirely realistic in all archaeological bone, but must be simplified in order for the model to be manageable. The first assumption is that the two biomolecules degrade principally by hydrolysis reactions. The rates of the reactions have been measured in laboratory experiments, and then extrapolated to lower temperatures equivalent to those found in the soil. The reaction rates are temperature sensitive and thus to model the amount of hydrolysis in buried ancient bone the changes in the soil temperature, and palaeoclimatic perturbations must also be considered. Some of these precepts are considered here.

2.1 Kinetics of collagen hydrolysis and DNA depurination

Collagen is a protein molecule and at its most fundamental level is made up of a chain of amino acid residues bound together by peptide bonds. Peptide bonds can be broken when water reacts with the protein, hydrolysis (see Figure 2.1-A). At the point of hydrolysis the protein chain is severed, at the most extreme this results in a macromolecular protein being broken down into all of its constituent amino acids. In collagen this process breaks down the rigid insoluble collagen structure to a more amorphous water soluble gelatine. Collagen hydrolysis is slow (especially for mineralised collagen), but the rate of the reaction can be increased at higher temperatures or by acid or alkali catalysis (Rudakova & Zaikov, 1987). The rate of collagen hydrolysis can also be catalysed by enzymatic activity.

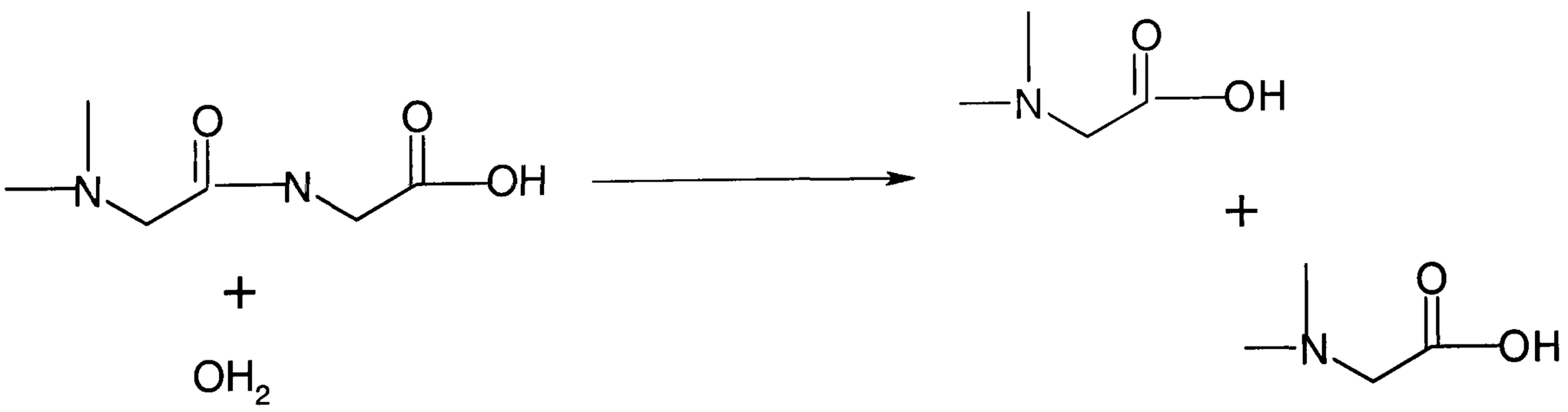

Figure 2.1-A. Hydrolysis of two glycine molecules

DNA depurination is also a hydrolytic process. This reaction causes the removal of the purine bases from the sugar phosphate backbone of the DNA molecule. This further destabilises the sugar phosphate backbone causing it to break, resulting in ever decreasing lengths of DNA as the reaction is allowed to proceed. Again, like collagen hydrolysis the rate of DNA depurination maybe influenced by: enzymatic activity, temperature, and pH. Both reactions require water to be present in the system,

however both molecules have water associated with them and only under the most arid conditions will the reactions be stopped.

The rate of a reaction can be described mathematically. For example for the collagen/gelatinisation reaction

$$\text{Collagen} \longrightarrow \text{Gelatine}$$

Equation 2.1-a

the rate of the reaction can be defined by either the rate of collagen loss, or the rate of formation of gelatine as the reaction proceeds with time thus (assuming there is excess water)

$$d[G]/dt = -d[C]/dt$$

Equation 2.1-b

These can be measured experimentally in bone by either the loss of the acid insoluble fraction, or the the increase in the water soluble gelatine in the supernatant, following either reaction is suitable. Therefore (assuming a first order reaction) the rate of collagen loss is described thus

$$-d[C]/dt = k_1[C]$$

Equation 2.1-c

where k_1 is the rate constant.

The amount of collagen at any one time can be predicted by

$$[C]_t = [C]_0 e^{(-k_1 t)}$$

Equation 2.1-d

Most rates of chemical reaction are temperature sensitive, and will increase as temperature increases. Arrhenius described the temperature dependence of the rate coefficient as

$$k_2 = Ae^{(-Ea/RT)}$$

Equation 2.1-e

where

A = the pre-exponential factor
Ea = the activation energy of the reaction
R = the gas constant
T = temperature (in Kelvin)

Both A and Ea can be determined from a plot of $ln\ k_2$ over $1/T$ (an Arrhenius plot), where $ln\ A$ is the *y-axis* intercept and $-Ea/R$ is the slope of the line.

Using Equation 2.1-d & Equation 2.1-e rates of collagen hydrolysis and DNA depurination at any given temperature can be calculated and the amount of hydrolysis that has occurred at any given time. However, in order to predict the amount of collagen and DNA hydrolysis in archaeological bone it is necessary to know the thermal history of the bone. Assuming that most archaeological bone has spent a considerable period buried in the soil a knowledge of thermal properties of soils is required. The thermal regime of the soil is determined by the properties of the soil and by the air temperature, thus in order to estimate this for archaeological bone a knowledge of palaeoclimatic changes is necessary also.

2.1.1 Modelling soil and cave temperatures

Modelling soil temperatures can be problematic due to the complex number of factors that can influence the temperature of the soil at any depth. The most important factor is the amount of energy that the soil absorbs, i.e. the amount of solar radiation that it receives at the surface. This energy will permeate the soil depending on the thermal diffusivity of the soil, which is in turn dependent on a number of other factors, e.g. the soil type, compaction, the amount of water. The soil temperature will increase according to its specific heat capacity (which is again a function of the soil itself). As the energy is propagated through the soil, it is converted to heat, raising the soil temperature, thus the amount of energy received decreases with depth. The mean annual soil temperature is normally constant and equal to that of the mean annual air temperature; good estimates of mean soil temperature can be made from local weather stations (Kusada & Achenbach, 1965; Toy *et al.,* 1978). Modelling soil temperature is complicated by diurnal and seasonal fluctuations in temperature. Diurnal fluctuations last for short periods only and in most soils only the first few centimetres will be affected. The seasonal change in the air temperature is paralleled more strongly in the soil. It can be approximated by a sine wave function where the amplitude of the temperature variation decreases with depth, and a phase lag, that also increases with depth.

Accurate modelling of the amplitude variation is important for making estimates of the amount of a chemical reaction that has taken place if the reaction is temperature sensitive. As the rate of the reaction may be exponentially related to the temperature the magnitude of the increase in the rate during the summer will be greater than the magnitude of the decrease in the rate during the winter. Thus if the amount of reaction is calculated over a year the apparent average temperature for the amount of reaction is greater than the actual mean annual temperature of the environment. We call this the 'reaction effective temperature' and it is unique for every reaction. Obtaining data to model the variation of temperature in soil with depth is difficult

because the number of variables involved means that a general model cannot be applied. Furthermore, the most important factor is the thermal diffusivity, which is normally derived empirically from measuring the temperature of soil at different depths. Predicting burial temperatures for archaeological sites is complex as the soil properties will change spatially and temporally. For example, the depth of burial may change as well as the amount of water in the soil (which will affect the thermal properties of the soil). Predicting how these factors may interact over archaeological time scales is difficult and would require a detailed knowledge of the site hydrology as well as palaeoprecipitation levels. At best reasonable estimates based on air temperature and soil temperature relationships and quantitative palaeoclimatic reconstructions can be made.

Frozen ground and snow cover can cause large deviations from the sinusoidal soil temperature model given above, due to latent heat effects in the phase transitions of water to ice (and vice versa) and the insulating and reflective qualities of snow. Put simply, as air temperature drops the soil loses heat to the air and the soil water will also lose heat energy, although there is no change in the temperature as there is a phase change from water to ice. Only if there is a sufficiently long cold period will the temperature of the ice start to change. Similarly in a subsequent warm season much of the sun's radiation will be spent thawing the ice not, heating the soil; again only if the period is long enough will the soil temperature increase. The result is that whilst there is much energy transfer between the soil and the air, the temperature of the soil will remain at approximately zero – the 'zero curtain' effect. In addition to this snow cover may exacerbate the problem. Snow will retard the thawing process as it has a high albedo and will act as a heat sink as it thaws. It will also insulate the ground during cold periods so that the soil temperature will not drop as much as the air temperature.

In reality, the situation is more complex as other factors must be considered when accurately modelling the soil temperature at depth of permanently frozen ground. These factors include the depth of the frozen layer of the soil, and the thickness of the active layer (the near surface region that thaws and freezes most frequently). Thus in cold areas where the soil is frozen, the extreme seasonal oscillation of the air temperature may not be paralleled in the soil temperatures mainly due to the zero curtain effect (Zhang *et al.*, 1997). This will affect both the mean and amplitude of the soil temperature. Thus in permafrost and snow covered regions large deviations from the model given above are expected, however without specific measurement these factors cannot be accounted for in a general model.

Caves are an important archaeological resource partly because they appear to provide favourable preservation conditions for bone. This may in part be due to their unusual micro-environment which is different to most other archaeological sites. One unusual feature of caves is the temperature regime. Cave air temperatures are controlled by the flow of air into and out of the cave, and are thus dependent on the air temperature outside of the cave. In caves with two entrances at different levels the 'chimney effect' is observed. This occurs when there is a difference in the air temperature, and thus density inside and outside of the cave. Air will be drawn into or out of the cave so that equilibrium is maintained, thus the temperature of the deep cave air remains equivalent to the mean air temperature outside of the cave (Bogli, 1980). There is more air movement near the cave entrances thus there will be greater temperature

fluctuation in these areas, however the amplitude of variation tends to fall exponentially with distance from the cave entrance. Furthermore, for a bone buried in a cave sediment, variation in temperature will be further dampened (see above). Some caves will not follow this simple model, e.g. a downward sloping single entrance cave will tend to trap cold air at the bottom. For modelling purposes, here caves have been defined as a special case where the burial temperature is constant, and equal to the mean air temperature outside of the cave. Assuming there is no deviation around this mean, the effective temperature for reactions is equivalent to the mean annual temperature.

2.1.2 Modelling palaeoclimate

Having established that the burial temperature of archaeological bone in both open and cave sites is dependent on the air temperature of the site, in order to model the entire burial temperature history accurately quantitative estimates of palaeoclimate must be used.

Palaeoclimate can be inferred from a number of proxy data sources ranging from tangible macroscopic plant remains to oxygen isotope ratios in ice cores, however none of these can directly give data on the temperatures of the past. The type of proxy data used in reconstructions is normally defined by the question being asked. The oxygen isotope data measured in ice cores from both poles can be interpreted as being records of global, or hemispherical climate fluctuations, but can only be used as a general guide (GRIP Members, 1993; Jouzel *et al.*, 1987). The changes observed in the GISP ice cores (Johnsen *et al.*, 2001) may be reflected in the climate of continental Europe (i.e. general climatic episodes), but the detail of the climate in specific areas will be lost, i.e. the magnitude or rate of change may differ significantly between the two, or some episodes may only be local to one area. The number of data sources and the ways in which they can be used is numerous, however very few palaeoclimatic reconstructions provide numerical estimates of climate temperatures. Global Climate Models (GCMs) have been established in order to retrodict palaeotemperatures and precipitation (e.g. Gates, 1976; COHMAP members, 1988; Hewitt & Mitchell, 1997), although the quality of the predictions is very dependent on a number of interacting variables and the geographic resolution of the data may be low. Equally, to produce a geographically wide general model of hydrolysis reactions site-specific palaeoclimatic reconstructions would prove impractical.

The models described in parts 2.2 and 2.3 rely heavily on palaeoclimate reconstructions of northwest Europe for the Pleistocene and Eemian periods produced by the EPECC project (Vandenberghe *et al.*, 1998; Huijzer & Vandenberghe, 1998; Witte *et al.*, 1998; Coope *et al.*, 1998; Aalbersberg & Litt, 1998). These reconstructions are based mainly on pollen core and coleopteran assemblage data, although geomorphic and periglacial feature data (such as ice wedges) are used where available. Temperatures are reconstructed using uniformitarianist principles based on a knowledge of the temperature ranges that the species occupy today. Trees and coleoptera respond to climate differently in that coleoptera adapt more quickly (i.e. they are more mobile and reproduce more quickly) than trees to climatic changes. They can therefore provide data on short-lived climatic events that may not be observed in pollen data. Their use in conjunction with pollen reconstructions allows them not to be over interpreted. This multi-proxy approach has allowed the

reconstruction from one data source to be constrained by data from another source. Periglacial features are relied upon mainly during the coldest periods, where bioproduction is very low. The result has been the production of reconstructed mean annual and maximum and minimum temperatures across a transect of northwestern Europe, where the data allows this. To supplement this data quantitative palaeoclimate data from southern France has also been used (Guiot *et al.*, 1993). This study uses similar techniques to those described above and extends the geographical range of palaeoclimatic reconstruction further south in Europe.

The thermal history of a fossil (and thus the palaeoclimate) plays an important role in the survival of bone and biomolecules in bone, particularly in northern Europe where the changes in climate have been extreme. A brief description of the palaeoclimate of northern Europe is given here, based on the EPECC reconstructions (Vandenberghe *et al.*, 1998). Palaeoclimatic terminology can be confusing as the same periods often have different names in different countries. Approximately 130kya the climate of Europe was in an interglacial phase (the Eemian), and the climate was not unlike that today, only perhaps hotter, becoming less oceanic towards the end of the phase. The Eemian was followed by a period that consisted of a series of alternating stadials and interstadials. The period after the Eemian is termed the Herning stadial, a cold period with continental climate where mean July temperatures were less than 10°C, which was followed by the Amersfoort-Börup Interstadial. This period was perhaps 4°C cooler than the Eemian, with a continental climate that had a west-east gradient in the winter. This was followed by the Rederstall Stadial in which it was too cold for forest growth in northern Europe. A short interstadial followed (the Odderade) similar to the Amersfoort-Börup.

The Weichselian glacial period followed consisting of 3 main cold phases the Early Pleniglacial, the Hasselo Stadial and the Late Pleniglacial. These date from approximately 74-59kya, 41-38kya and 27-13kya respectively, with the latter period encompassing the period of maximum glaciation. During this period the temperature gradient was north-south, with mean summer temperatures below 10°C, and winters as low as -20°C. There is evidence of a brief warm period (50-41kya) where temperatures may have been similar to those today.

The period between 14-9kya marks the transition between the Weichselian and the current interglacial (the Holocene); the temperature gradient appears steeper in this period, especially from southern England to Sweden, possibly reflecting the effects of warm Atlantic waters and the ice-sheet. The Dryas events (~14-13kya and 11-10kya) are periods where the climate turned cold abruptly. At about 10kya the climate ameliorated to the temperatures of the current interglacial.

The Holocene has been a relatively stable climatic period compared to the large excursions described above. It is treated in the models as a constant period of temperature and is derived from modern day recorded temperatures. This is an oversimplification as there are well known fluctuations in temperature during this period (e.g. Roman and Medieval warm periods, and 'the Little Ice Age' see Lamb, 1982). Whilst the Holocene is recognised as a generally warm period with some fluctuation there is a dearth of quantitative palaeotemperature data for the period, and it is thus modelled as period of constant temperature.

2.1.3 Sources of Error

It is clear that exactly modelling the thermal history of a fossil is so complex as to be impossible due to the number of variable factors that would need to be understood and recorded. Much of the data that would be required is not available now, and would be almost impossible to retrodict into the past. Thus, a simplified modelling approach has been taken. The price of simplification is a less realistic model, as assumptions and generalisations have to be made that cannot be practically checked. Nevertheless, the models can be tested against real data to test if they describe aspects of the biomolecular decay in archaeological bone. A number of possible sources of error are likely to occur so that the model will not be able to cover all possible situations. The biggest source of error will come from the temperature estimates made, e.g. deviations from the simple model of cave temperatures, or errors in the palaeoclimatic data used. The palaeotemperature data is only approximate, the authors themselves concede that they may have underestimated the magnitude of extreme temperatures, erring on the side of caution in their interpretations; nor was the data intended for the use it is put to here. Thus in both the models presented below there is scope for sizable errors.

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2.2 *Improving the analysis of ancient collagen: establishing and testing a quantitative chemical model of collagen degradation*

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2.2.1 Abstract

We present here a synthesis of practical laboratory and theoretical modeling studies aimed at determining the practical limits of bone collagen survival in the geosphere. Bone collagen has been heated in laboratory experiments to determine the relationship between temperature and the rate of the collagen/gelatinisation phase change. The results of these experiments have been used to extrapolate the rates at global burial temperatures to estimate the limits of bone collagen survival on a global scale. This model has been tested by reconstructing the thermal histories of over one thousand radiocarbon dated bones with known ages and collagen yields. The test reveals that nearly all the bones dated contain less collagen than the model predicts, indicating that alternative modes of collagen loss must play a significant role in collagen degradation in the geosphere. They also reveal that the temperature of the buried bone will be a more significant factor than the age of the bone in determining collagen survival.

Keywords

Collagen, Bone, Gelatinisation, Modelling

2.2.2 Introduction

Collagen is one of the most important proteins for geochemical analysis. The importance of collagen as a material and as an archaeological resource stems from its abundance and relative stability. Analysis of radiogenic and stable isotopic composition of collagen is used for dating and trophic analysis, respectively (Hedges & Law 1989; Ambrose 1990). Collagen is the most common protein in many invertebrates and all vertebrates; in dentine and bone, hydrated collagen comprises more than 60% of the volume. To assess the practical limits of archaeological applications, it is necessary to understand the limits of geochemical preservation.

The purpose of this investigation was to establish these limits on a broad, global scale. Of all the highly unstable biopolymers investigated by archaeological chemists, the survival of collagen, specifically mineralized collagen, has the potential to be one of the simplest to predict. The chemical degradation of collagen represents one of the most profound changes undergone by any protein from a highly crystalline water insoluble collagen to water soluble random coil gelatine. Disruption of the helical structure is a co-operative effect initiated in an unstable region of the helix (Miles *et al.*, 1995). The instability may be caused by elevated temperature and/or chain scission.

To provide a global predictive model for chemical degradation, microbial processes must be ignored. Microbial processes are known to play a very significant role in bone and dentine deterioration (Piepenbrink, 1986; Grupe, 2001; Child, 1995; Jackes *et al.*, 2001) and appeared, at first, to make the whole approach fruitless. Two factors encouraged us to continue. Firstly, the nature of mineralized collagen means it is protected from biodeterioration (Krane, 1970), we can therefore explore the theoretical limit of collagen survival and expect to see our predictions confirmed in bones which display no evidence of microbial alteration. Secondly, we can test the model using large data-sets of ^{14}C dated bones to assess the extent to which the trends predicted in the model are confirmed or overwritten by a microbial signature.

In intact collagen, helix disruption is believed to be focused in the gap zone which contains the regions of instability in the helix and provides the space into which the helix can unravel (Miles *et al.*, 1995). In mineralized collagen (bone and dentine), key physical properties of the protein are modified by the growth and extension of crystals within and across this gap zone (Landis, 1995), stabilising the helix and raising the temperature required to melt the helix from 69°C to ~ 150°C (Kronick & Cooke, 1996). This intimate association of mineral and protein also protects the collagen from enzymolysis (Krane, 1970), not least because many of the sites for enzymatic cleavage are within regions of the helix exposed in the gap zone. Despite the apparent complexity of collagen preservation in the environment, we assume in all subsequent discussion that enzymolysis of collagen must be preceded by either partial mineral dissolution or gelatinisation and therefore we cannot model it simply.

Two radiocarbon laboratories (Oxford and Groningen) use similar methods of extracting and isolating collagen (see Gillespie *et al.*, 1984; Hedges & Van Klinken, 1992; Hedges & Law, 1989) and routinely report collagen yield along with radiocarbon date. Different methods yield different proportions of collagen and we have only reported data from the two most common methods. These have been

calibrated one against the other and a correction factor used to determined collagen contents. This has provided 1244 ^{14}C-dated collagen yields from identified locations for the study.

We have predicted collagen loss by establishing the temperature dependence of collagen loss in mineralized tissue, and making predictions using global estimates of soil burial temperatures from modern soil temperature data and quantitative palaeoclimatic reconstructions. We have tested the predictive value of our model using collagen yield data from radiocarbon laboratories, and attempted to assess the global limits of collagen preservation.

2.2.3 Materials and Methods

2.2.3.1 Artificial diagenesis experiments

Bone powder was prepared from compact bone of cow tibias, which had been defatted in acetone and fractured into large pieces. Bone fragments were milled under liquid nitrogen. The milled bone was size-fractionated over plastic sieves to isolate a < 63μm fraction (bone powder).

In the first set of experiments (performed by Dr A. Gernaey), bone powder was hydrated to 95% RH by exposure at 20°C to the vapour from a saturated $NaSO_3$ solution, until a constant weight was obtained. The hydrated powders were loaded into annealed glass tubes which were flushed with argon and flame sealed (wet bone experiments). Tubes were heated in ovens at temperatures of 95°C, 85°C, 75°C, 65°C and 55°C for periods of up to 18 months. At set periods, tubes were removed and frozen until use. In a second set of experiments (performed by Mr S. Roberts) powders were heated in excess water in capped tubes.

Samples from both experiments were demineralized using the protocol described by Collins & Galley (1998) to determine the proportion of the residual insoluble fraction.

2.2.3.2 The radiocarbon data-set

The whole radiocarbon data set comprises almost 2500 bones dated at the Oxford and Groningen laboratories. After rejecting all data deriving from unusual extraction techniques, those with no given location and those beyond the range of calibration (all the ^{14}C dates given here are the mean date and have been calibrated using the INTCAL98 calibration, to the nearest ten years (Stuiver *et al.,* 1998)), less than half were used in the study. This selection meant that the data-set used only 1244 bones, each with given locations, collagen yields, and calibrated ages up to approximately 24000 cal BP. This requirement limits the testing of our model much beyond the last glacial maximum.

Collagen extraction procedures at the radiocarbon laboratory have been modified over time to improve the quality of collagen extracted. The initial method involved alkali extraction of 'humic substances' from the collagen, followed by gelatinisation (Longin, 1971). An improvement of this method used an ion exchange step, but

resulted in a reduced yield. A range of archaeological samples with varying collagen yields were prepared by the each of the two methods and their yields compared. As anticipated, ion exchange reduces the yield of collagen (typically by some 40 %, Figure 2.2-A). All yields reported from ion exchange extraction were multiplied by 1.67 to account for the reduction. Data on yields from a modified version of these methods (such as the inclusion of a solvent extraction phase) were not included in the analyses.

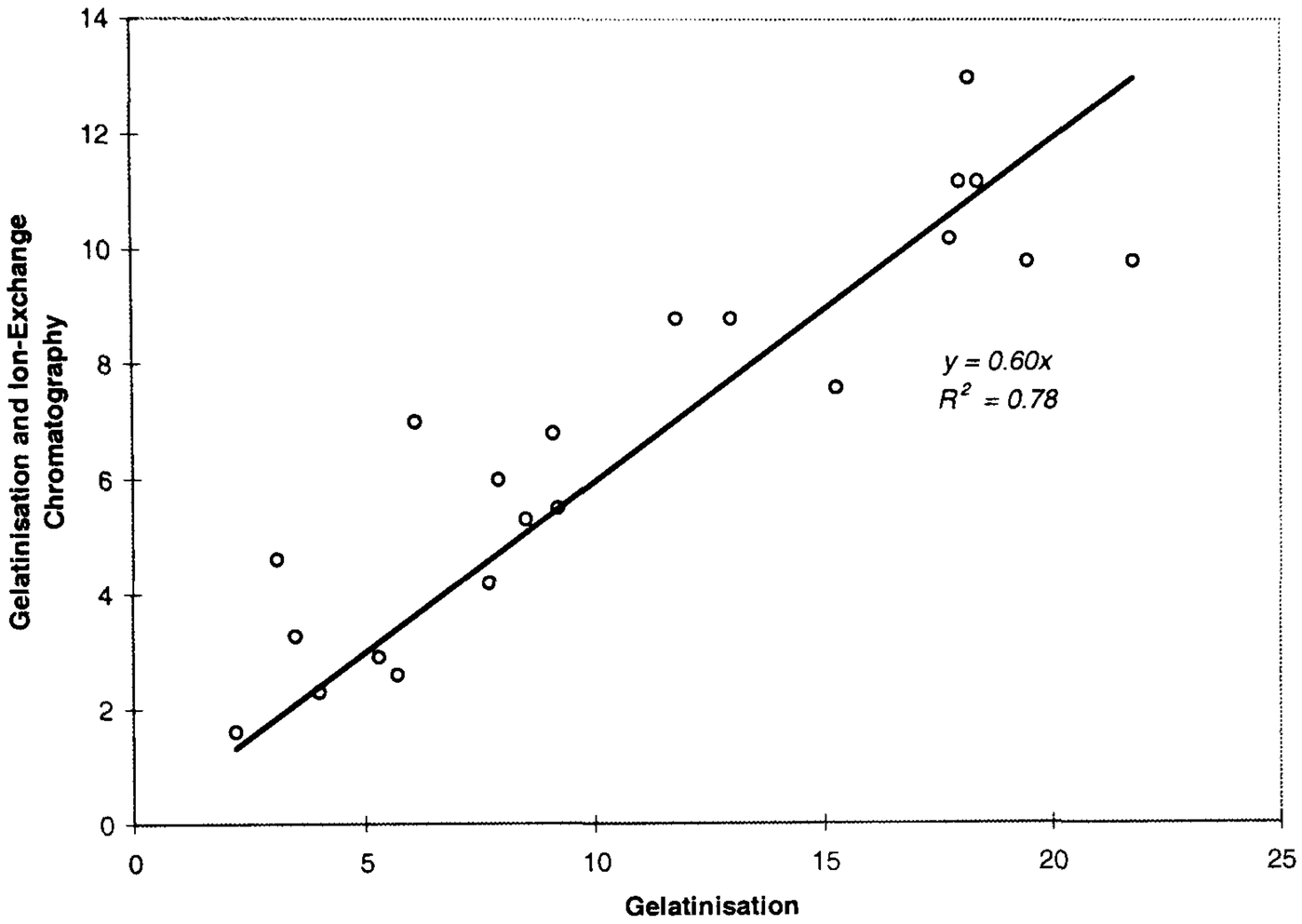

Figure 2.2-A.Correction for difference in 'collagen' extraction yield between gelatinisation extraction and gelatinisation followed by ion-exchange chromatography.

Collagen yields are reported relative to dry weight of bone. Where bone is poorly mineralized (as in the case of neonate bones), or more commonly has undergone mineral loss (as is the case in bone from acidic peat bogs) this will have the effect of increasing the collagen content (e.g. a bone from Solway Moss has a collagen content of 80%). It was not possible to correct for mineral loss and less than 2% of all collagen yields reported were higher than the 23% typical of bovine compact bone. In subsequent analyses, these were considered to have in excess of 100% of their collagen surviving.

Using the temperature dependence of collagen gelatinization established from the laboratory study (see below), and estimates of the thermal histories of the bones, we have attempted to estimate the amount of gelatinisation in the radiocarbon dated bone collagen and compared our estimates with the actual yields. In order to do this we need to know the thermal regime of the soil and the palaeoclimate of the site from which the bone has been excavated. Producing a precise model for each bone would be impossible, thus, we have produced a simplified model that may be used on a global scale, not a site-specific approach.

2.2.3.3 Modelling thermal history

We have modeled two types of sites open and cave sites as they will have two different types of thermal regime. The thermal history of a buried fossil in an open site can be modelled accurately only if a number of variables are known. The key factors in soil temperature are the amount of incident radiation, the thermal properties of the soil and the depth of the soil, although there are a number of other local factors that may influence the temperature regime (Krarti *et al.*, 1995). It has been demonstrated that the mean annual temperature of soil is roughly constant, and that seasonal fluctuation around the mean is controlled by that of the climate. Seasonal variation is propagated in the soil and can be modeled as a sine wave, but it is dampened with depth, dependent on the thermal properties of the soil. Good estimates of the mean annual soil temperature can be taken from local weather stations (Kusada & Auchenbach, 1965; Toy *et al.*, 1978), although again local effects can cause large deviations from this simple model (e.g. snow cover). The degree of seasonal fluctuation that a fossil will experience will be controlled by its depth, the fluctuation in the climate, and the thermal properties of the soil. For most sites, this information will be unobtainable, both for the present and for the past. The amount of fluctuation is important as the rate of collagen gelatinisation is related exponentially to the temperature. Thus, the rate of reaction increases during warm periods more than it decreases in colder periods. The net effect is that there is more reaction in a system with temperature fluctuation than one with a flat mean and thus the apparent effective temperature (T_{eff}) of a fluctuating system is greater than that of a stable system (T_{mean}) (see Wehmiller *et al.*, 2000).

As a simple model, deep cave air temperatures can be approximated to the local mean annual climate temperature, and considered to deviate little from this mean value (Bogli, 1980). This may be an over simplification for all caves, as many will deviate due to local conditions, but it serves the purpose of a general global model, avoiding the necessity of temperature data for every cave. Fossils within cave sediments can thus be modelled differently from those in open sites, the difference being that fossils within cave sediments will experience little fluctuation in temperature. Even in caves that do not fit this model exactly, and show some variation in temperature, the fact that fossils are buried within the cave sediment will mean that any temperature fluctuations will be dampened, and hence negligible in most cases. This means that T_{eff} will be equal to MAT for cave fossils and thus, for fossils recovered from caves, but not rockshelters, T_{mean} is used.

In addition to modeling the temperature fluctuation in the soil, the thermal history of the soil must also be considered, i.e. the palaeoclimate. We have used a simple palaeoclimate model where the Holocene (the last 10.4 kyr) is considered to be a constant temperature. This is an over simplification as it disregards the 'Little Ice Age' and the 'Medieval warm period', and post industrial warming, however these perturbations are small compared to the major climatic fluctuations during the Pleistocene. We also lack widespread quantitative palaeoclimatic reconstructions for the Holocene.

Holocene temperatures were estimated using climatology data compiled in the years 1987 and 1988, the ISLSCP Initiative I (Meeson *et al.*, 1995) has made global estimates of the temperature of ground below 50 cm depth (Deep Soil Temperature).

This was calculated from surface temperature, using the expression.

$$T_{(d)}^{\,n} = (1\text{-}c)T_{(o)} + c(a\,T_{(s)}^{\,n} + b\,T_{(s)}^{\,n-1})$$

Equation 2.2-a

Where $T_{(o)}$ denotes the mean annual surface temperature, $T_{(s)}^{\,n}$ and $T_{(s)}^{\,n-1}$ are the surface temperature for month *n* and previous month *n-1*, a and b are constants defining the temperature phase lag (a = b = 0.5) and c is a constant describing the amplitude damping (c = 0.77). Using this data we estimated the mean temperature (T_{mean}) and the total reaction over the 24 month cycle to make an estimate of the effective temperature (T_{eff}) at each grid point. A Holocene temperature could be assigned to each bone in the database for which we had a location, a T_{eff} for open sites and T_{mean} for cave sites. No correction is made for altitude, beyond the resolution of the ISLSCP data-set.

For sites older than 10.4kyr it is essential to account for palaeoclimatic variation when considering the thermal regime of the soil. Here, we have attempted to produce thermal histories for specific regions, based on published quantitative palaeoclimatic reconstructions. The major climatic events modelled are the Dryas events, and Bolling and Allerod stadials and Last Glacial Maximum (LGM). A limitation of this approach is that only certain geographic regions can be modelled, that is, those regions where such studies have been carried out. At present, we have used quantitative palaeoclimatic reconstructions covering the entire period back to 24000 BP for north western Europe (British Isles, Northern France), Germany, Poland and for Southern France (Huijzer & Vandenberghe, 1998; Witte *et al.*, 1998; Coope *et al.*, 1998; Aalbersberg & Litt, 1998; Guiot *et al.,* 1993). The first three reconstructions are part of an integrated project using a variety of sources (e.g., pollen data, beetle data, periglacial features) from a number of sites to reconstruct palaeoclimate in northern Europe (the regional distinctions are our own). The latter uses similar methods (pollen and beetle data) from cores further south in France. The reconstruction from the latter, although methodologically similar, uses a different time scale to the aforementioned studies, and has been adapted to fit this time scale. These reconstructions provide various data, including estimates of maximum and minimum temperatures of the coldest and warmest months, and estimates of the mean annual temperature either directly, or by difference from the estimates of means of the warmest and coldest months. From these data, we have created curves of mean annual temperature for the above regions.

The palaeotemperatures mentioned above are estimates of mean annual temperature (MAT), and not estimates of past effective temperatures (palaeoT_{eff}). For open sites it is observed that MAT and T_{eff} are related, but T_{eff} can differ considerably from MAT due largely to seasonality in the climate. This is of particular importance as the palaeoclimatic data suggest large increases in seasonality, particularly in the colder phases of the Pleistocene. The seasonality in the climate is propagated in the soil matrix, but is dampened to an extent due to the thermal properties of the soil, and the

depth of burial. Without data on the soil properties at each location and how this effects the dampening of the seasonal variation it is impossible to estimate the T_{eff} from the MAT effectively. Without an estimate of palaeoT_{eff} comparisons would have to be made between palaeoMAT and current T_{eff} for open sites. To resolve this problem a crude method of estimating the palaeoT_{eff} is given below. This is not an ideal solution, but does address some of the problems, such as seasonality and dampening that are encountered when comparing MAT and T_{eff}. In turn it carries with it a number of assumptions, these are: that the dampening present today in northern Europe is similar to that of the past, and is geographically similar in this region. This uniformitarianist assumption has to be made, although it may well be untrue in that the dampening effect depends on the soil type, water in the soil, and the depth, all factors that will no doubt have varied temporally. It also assumes that the air temperature and soil surface temperatures are the same, and that seasonal changes in the air temperature are translated directly into the soil, with some dampening. This may not be true in areas of permafrost where there are extreme air temperatures which are not always observed in the soil, owing to snow cover providing insulation, and the thermal properties of water freezing into permafrost, the so called 'zero curtain' effect (Zhang *et al.*, 1997).

An attempt to model the effective temperature at the last glacial maximum (LGM T_{eff}) has been made. It is possible to estimate the decline in temperature amplitude from the surface down to a depth of one metre using measured soil temperature data. The amplitude dampening was approximated to an exponential curve of temperature with depth for 12 sites in northern Europe (where data was collected to at least 1m, and at least four depths) using data from Chang (1958) (average R^2 value = 0.98). The amount of dampening at these sites was then modelled from depth 0-1m. If these sites are considered typical of northern Europe then the average dampening of amplitude (maxima to minima) at 1m is 69% (c.f. Equation 2.2-a). For four of these sites climatic temperature data is available (from weather stations) for the same periods when the soil temperature data was recorded via the LDEO/IRI Data Library (Baker *et al.*, 1994). This data was used to compare the amplitudes of temperature for the modelled soil profiles at 0m, and the recorded air temperatures, (air temperature amplitude = 1.1801x (soil surface amplitude) - 2.66, $R^2 = 0.76$, n=4). The dampening effect of 69%, above, has been applied to the palaeoclimatic data of mean air temperatures, and associated amplitude variations to produce what is in effect a palaeoclimatic T_{eff} estimate curve, going back to 25000 years BP see Figure 2.2-B. For sites older than 10.4 kyr in northern Europe the Holocene temperature is taken from the T_{eff} data calculated from the ISLSCP data set. For periods before 10.4 kyr the difference between the sites Holocene T_{eff} and the difference between the regional average Holocene T_{eff} and the regional LGM T_{eff} is calculated, all other temperature fluctuations are calculated relative to this. Bones that are older than 10.4 kyr from outside the geographic regions described above have not been modelled.

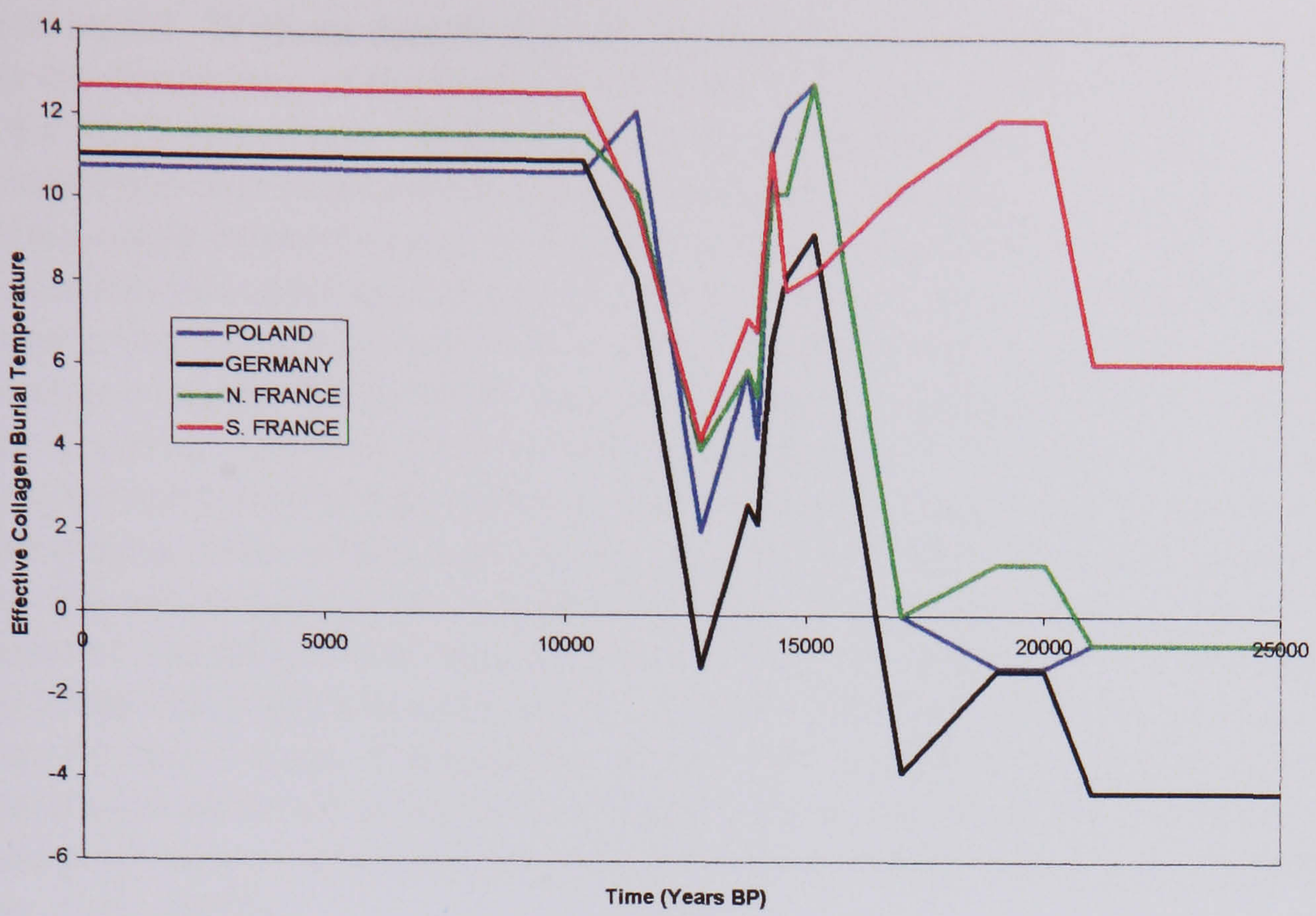

Figure 2.2-B. Regional collagen gelatinisation effective palaeotemperatures for Northern Europe (*Ea*=173 kJmol^{-1}).

To estimate the size of the errors compounded by the simplified approach we have taken, estimated T_{eff} and MAT data and compared these with published data on soil and air temperatures respectively (Meeson *et al.*, 1995; Baxter, 1997; Wehmiller *et al.*, 2000; Saheb Al Quattan pers comm.; Baker *et al.*, 1994).

2.2.3.4 Calculation of Collagen Thermal Age

To be able to compare the predicted amount of collagen to the measured amount between sites we have used the concept of the 'collagen thermal age'. This is estimated by integrating collagen loss over thermal history of the sample and expressing this as the time required to achieve the equivalent amount of collagen loss at a constant 10°C ($_{yrs@10°C\ collagen}$).

2.2.4 Results & Discussion

2.2.4.1 Temperature dependence of collagen loss

The weight loss experiments were run in two phases on separate occasions 75-95°C and 55-75°C by Dr A. Gernaey. There is a clear offset between the two sets of data (Figure 2.2-C). At the time of the initial development of the model only the 75-95°C dataset was available and thus all further analysis (e.g. calculations of reaction effective temperature) has been made using an activation energy (*Ea*) of 173.2kJmol^{-1} and a pre-exponential factor (*A*) of 2.11x10^{19} s^{-1} derived from this data. The kinetic parameters derived from the 55-75°C experiments are *Ea* = 155.3kJmol^{-1} and *A* = 3.11x10^{16}, and a composite trend through both sets of data gives *Ea* = 174.4kJmol^{-1}

and $A = 2.93x10^{19}$. This gives an idea of the error that may be involved in estimating the rates of these reactions at high temperatures (see also section 2.2.8).

Despite the possible errors it is clear that the activation energy is very high compared with other reactions leading to bimolecular deterioration significantly higher than the consensus activation energy for peptide bond hydrolysis (92 kJ mol^{-1}) Collins & Galley (1998).

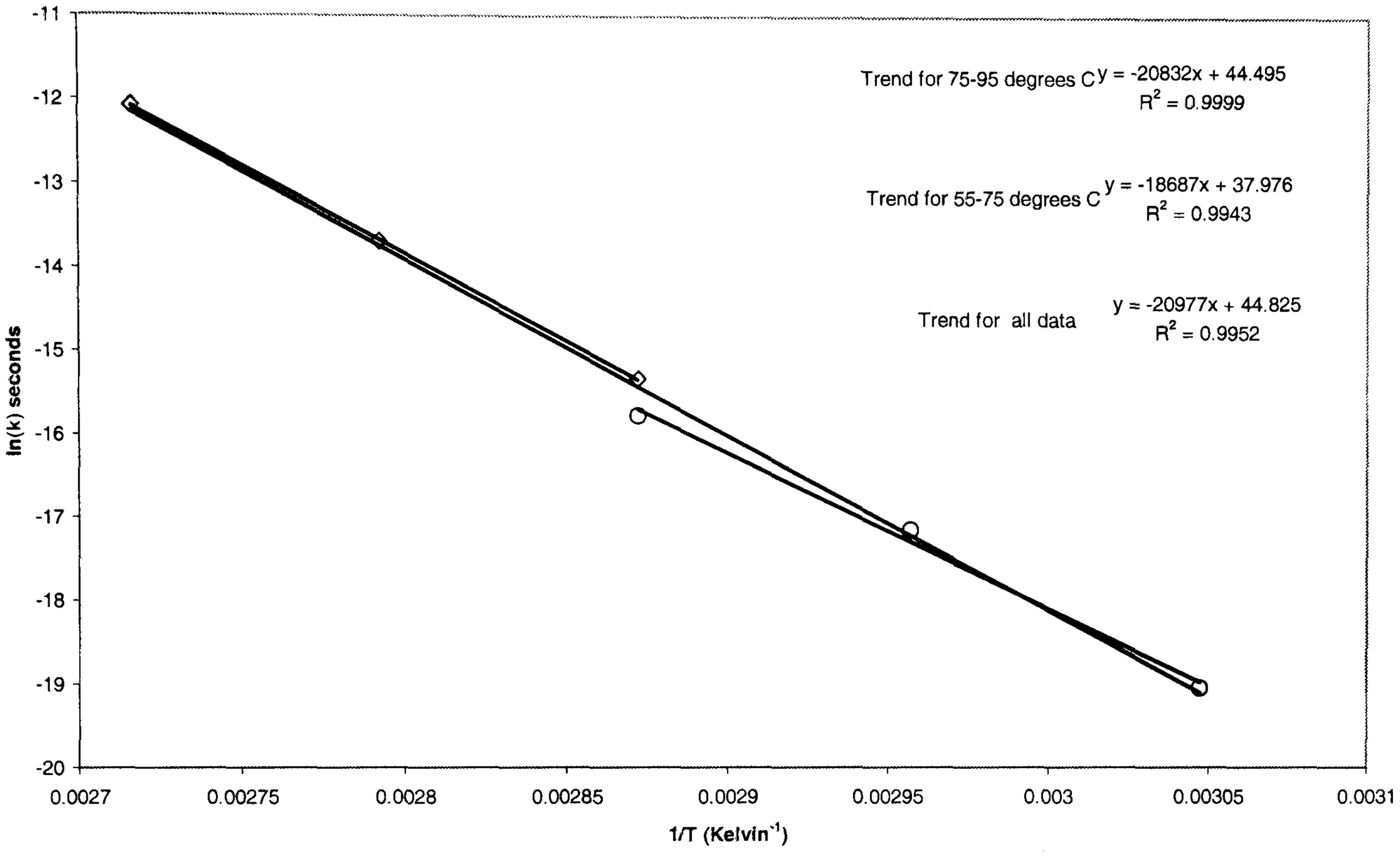

Figure 2.2-C. Arrhenius plot of experimentally determined rates of gelatinisation estimated from the loss of the residual insoluble fraction.

2.2.4.2 Comparison with previous studies

The activation energy estimated from this study is much higher than that derived from data given in Ortner *et al.*, (1972), but slightly lower than the value reported by Von Endt & Ortner (1984) (Table 2.2-i). Estimates of half-lives at 20°C and 8°C based upon these three alternative estimates appear different. In the case of this study and Von Endt & Ortner (1984), the differences are primarily due to a lower pre-exponential constant in the latter study.

The differences between the three studies are surprising, given the apparent simplicity of the experiment. We suspect that the differences are in large part explicable by experimental design. We have used freezer milled powders and measured the amount of acid in soluble residue, whereas the other studies have processed bone as powder or chunks at ambient temperatures and measured the release of soluble nitrogen. The measurement of insoluble fraction, or soluble N should not induce error as they are simply measuring different aspects of the same reaction perhaps the differences are produced during the sample preparation.

Method	range	E_a	A	R^2	n	t ½ yrs	t ½ yrs	Authors
	°C	kJ mol^{-1}				@ 20°C	@ 8°C	
release of soluble N	100-140	132.1	2.02×10^{13}	0.968	5	370	3,700	Ortner *et al.*, 1972
release of soluble N	100-130	183.3	1.00×10^{20}	0.998	3	100,000	2,500,000	Von Endt & Ortner 1984
loss of insoluble fraction	95-55	173.2	2.11×10^{19}	0.995	5	7,600	158,000	this study

Table 2.2-i. Comparison of kinetic data with earlier studies

2.2.4.3 Applying the global model

Having obtained a relationship between temperature and the rate of gelatinisation, it is possible to use this to explore the survival of collagen in archaeological bones. The hypothesis that the chemical degradation of collagen is a rate controlling step in the survival of collagen is testable. Correction for temperature should reveal a trend in the persistence of archaeological collagen with time. Furthermore, as temperature dependence is known, it will be possible to estimate the effect of seasonality and palaeoclimatic cooling on the loss of collagen. The effect of seasonality is accounted for in our T_{eff} estimates, all T_{eff}s are calculated using an *Ea* of 173.2 kJ mol^{-1} and a pre-exponential constant (*A*) of $2.11x10^{19}s^{-1}$. In order to test a number of the approximations in the model described above we have compared a number of aspects of the temperature estimates used and compared these with published data.

If reported collagen yield, corrected for method of preparation, is plotted against radiocarbon age, the trend of collagen yield against time is obtainable. At best, however, there was only a weak correlation between collagen yield and radiocarbon age (see Figure 2.2-D, but when the yield is plotted against collagen thermal age ($_{kyrs@10℃\ collagen}$), which takes into account differences in the T_{eff} experienced by different samples, a much clearer trend emerges (as can be seen from Figure 2.2-E). In almost all cases the yield of collagen is significantly lower than the maximum predicted collagen yield for bones of any temperature-corrected time, indeed most bones contain less than 50% of the predicted yield (see Figure 2.2-F). This distribution could have a number of explanations; (i) the rate of gelatinisation cannot be extrapolated to lower temperatures without incurring an error, i.e., our calculated rate is too slow, (ii) the predicted temperatures are systematically too cold although this is unlikely (see below), or (iii) other modes of collagen loss must play a significant role in collagen depletion in the geosphere, e.g. microbial attack. If we assume that the model is robust and that the first two explanations are incorrect, this finding demonstrates that chemical degradation alone does not account for the observed loss of collagen. With time there is an increasing apparent yield of collagen, indeed it is striking how much variation in collagen there is in the very youngest material. We have therefore succeeded in calculating an "upper bounding limit" for collagen survival, but this upper bounding limit fails to explain the majority of the variation observed in the burial environment.

Figure 2.2-D. Collagen yield vs. time for radiocarbon dated bone. o = open sites □ = cave sites.

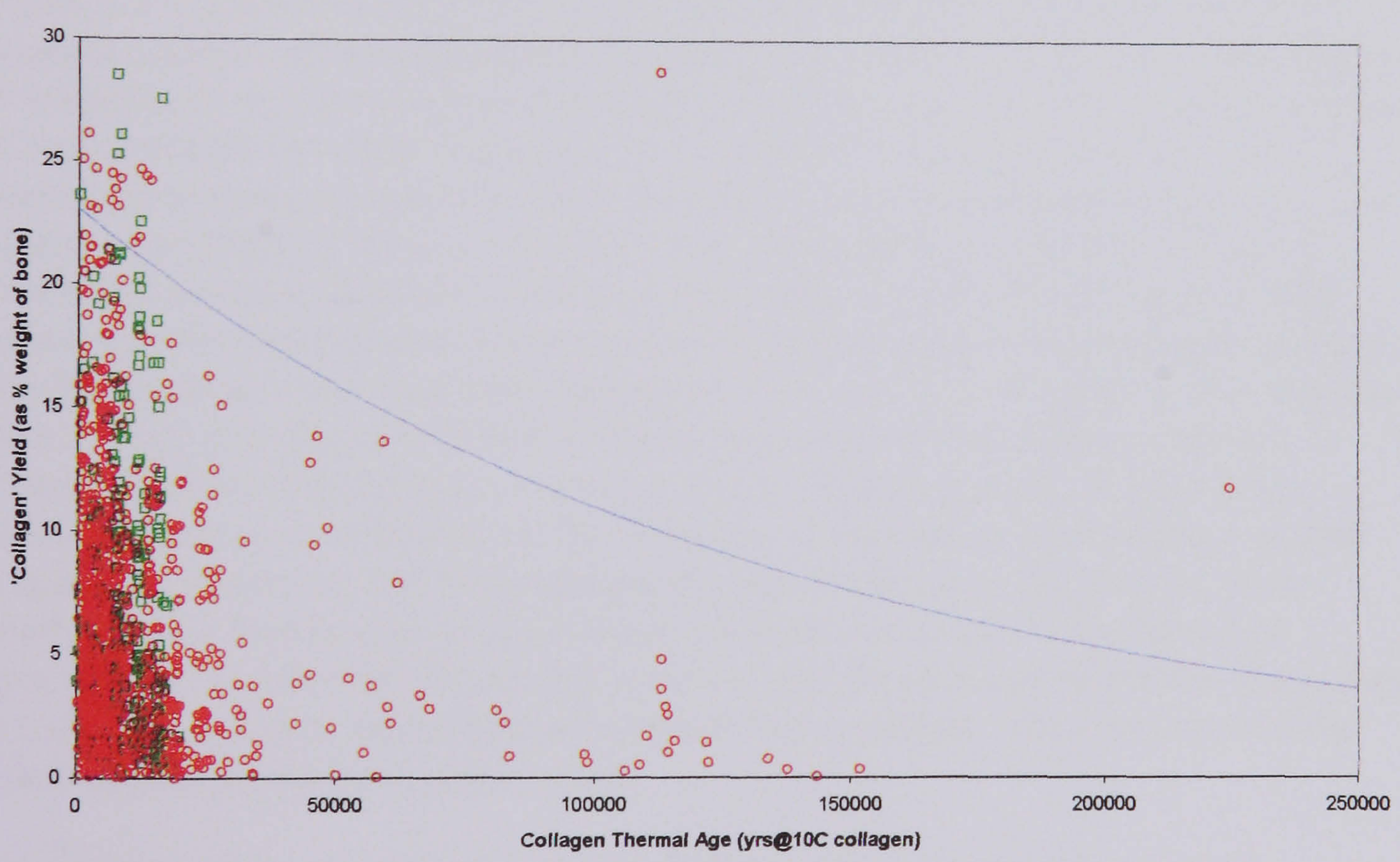

Figure 2.2-E. Collagen yield vs. collagen thermal age @10°C after temperature correction. Theoretical maximum yield is given for 10°C is shown. o = open sites □ = cave sites

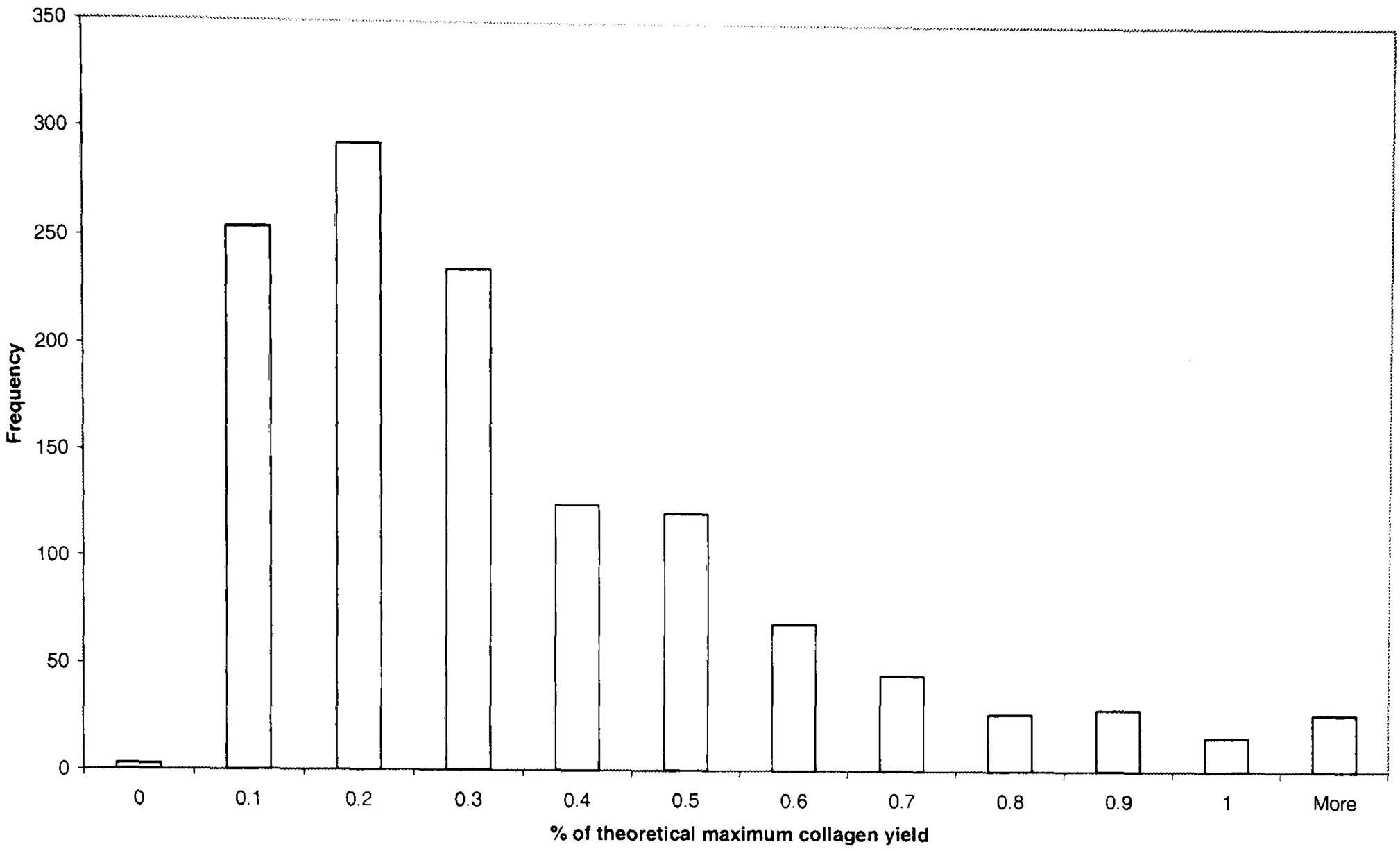

Figure 2.2-F. Frequency distribution of collagen yields relative to theoretical maximum.

The predictions can also be used to identify geographic trends in the survival of bone. Figure 2.2-G shows a map of predicted collagen levels after 11.5 kyr of burial at constant collagen effective temperature (using a first order reaction rate). The control of temperature on the distribution of yields can clearly be seen with the predicted loss of bone collagen in higher temperature environments. On a global scale, it is interesting to study areas of the world with particularly dramatic survival clines. The relative abundance of bone-producing sites in South America, the Rift Valley of Africa and Southern Australia relative to other regions on these continents is well known but this study provides data that can be used to suggest that collagen survival may be essential to the long term integrity of bone, and thus its survival in to the fossil record. Loss of collagen will both decrease the tensile strength of the material (Turner-Walker, 1995) and increase the porosity (Pike & Nielsen-Marsh, 2001). These two processes will increase the reactivity of the system by permitting greater exchange of fluids and will thus increase the rate of change in the mineral phase, whether this is fossilization or dissolution, the latter lowering the likelihood of preservation. In addition, dissolution reactions and the speed of microbial attack may be increased at higher temperatures compounding the effect. This may explain the distribution of fossils on a global scale.

The upper bounding limit represents the maximum theoretical collagen yield assuming that all of the deterioration is due to chemical deterioration. If the estimates are correct, then very well preserved bones should contain collagen at levels predicted by the rates. However in a recent study of medieval Italian bones (see Chapter 3.3), well preserved histological material has lost almost all its collagen, thus under some (as yet unknown circumstances) collagen loss can be accelerated.

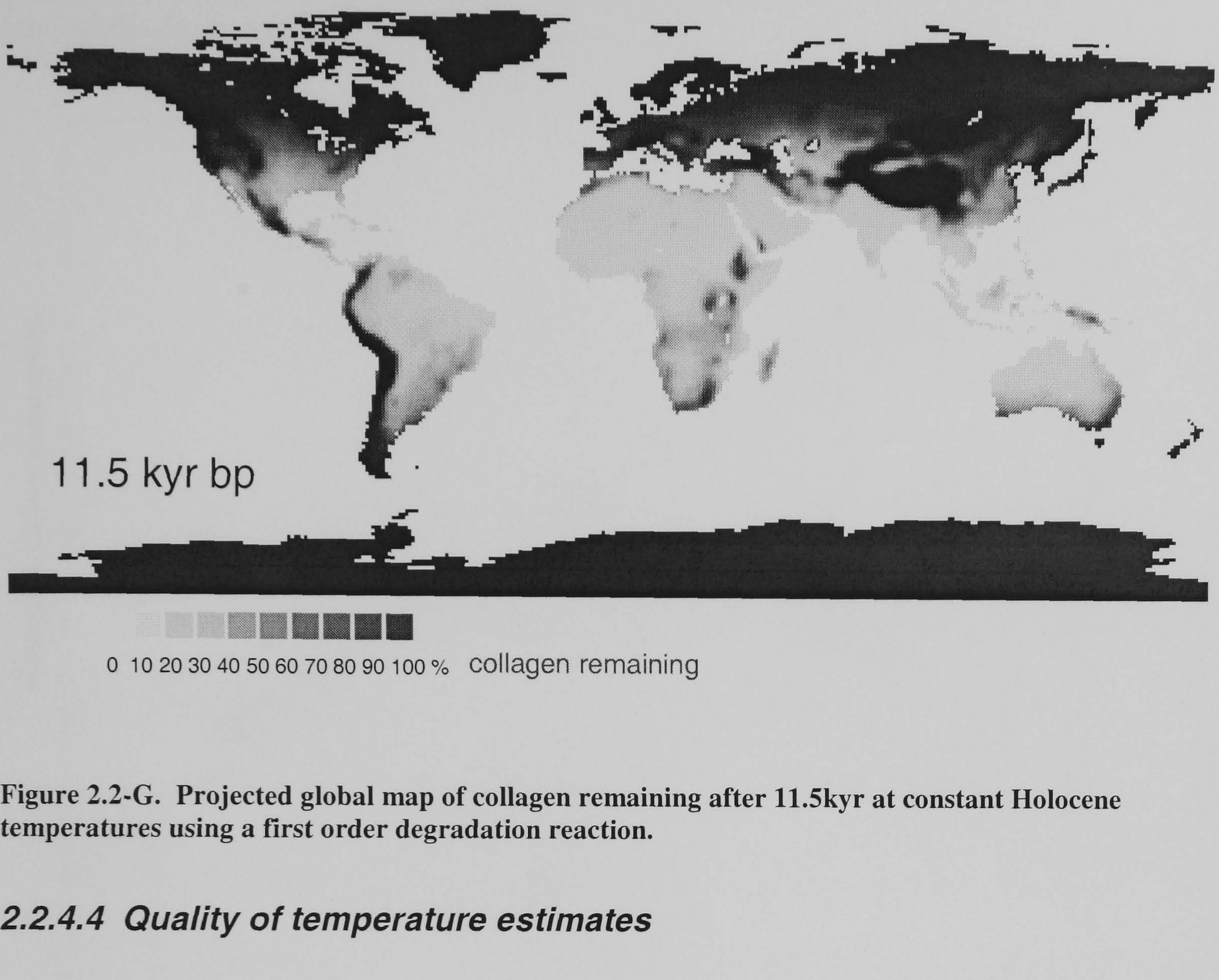

Figure 2.2-G. Projected global map of collagen remaining after 11.5kyr at constant Holocene temperatures using a first order degradation reaction.

2.2.4.4 Quality of temperature estimates

Estimates of T_{mean} and T_{eff} derived from ISLSCP were compared with; MAT estimates from long-term records and T_{eff} calculated directly from recorded soil temperatures, respectively (see Figure 2.2-H and Figure 2.2-I). Considering the number of possible errors that may affect the estimates of global temperatures the estimates can be considered reasonable, although in both the MAT and T_{eff} estimates there is an apparent trend of underestimating higher temperatures and overestimating lower temperatures. If these are general trends across the reconstructed temperatures then we are likely to be under estimating collagen loss in higher temperature sites and over estimating those in colder sites, although the errors would have to be quite large to alter the interpretations given above. Wehmiller *et al.*, 2000 demonstrated that down the eastern seaboard of America there is a stronger correlation between the latitude of the site and T_{eff} than there is for the air temperature of the site and the T_{eff}. This is intuitive, as the T_{eff} will mainly be governed by the seasonality of the site, which will be strongly related to the latitude. Thus for making predictions, if a relationship can be established for the T_{eff} and the latitude this will prove useful, however these relationships may only be true for certain geographic regions and are not true on a global scale. The errors in the deep soil temperatures from the ISLSCIP data set may be larger than those from a more geographically discrete area due to extremes of altitude which are not resolved in the ISLSCIP data set, which takes an average temperature for the whole 1° by 1° grid square. Our analysis reveals that in the absence of regional 'corrections', T_{mean} produces better correlations with global estimates of MAT. Similarly, T_{eff}, although it too displays considerable errors at individual sites, provides errors acceptable within this broad pattern of collagen

survival. Deviation from our model can be caused by local effects such as shaded ground or extreme soil thermal properties. These problems may be compounded beyond the Holocene by significant climate change.

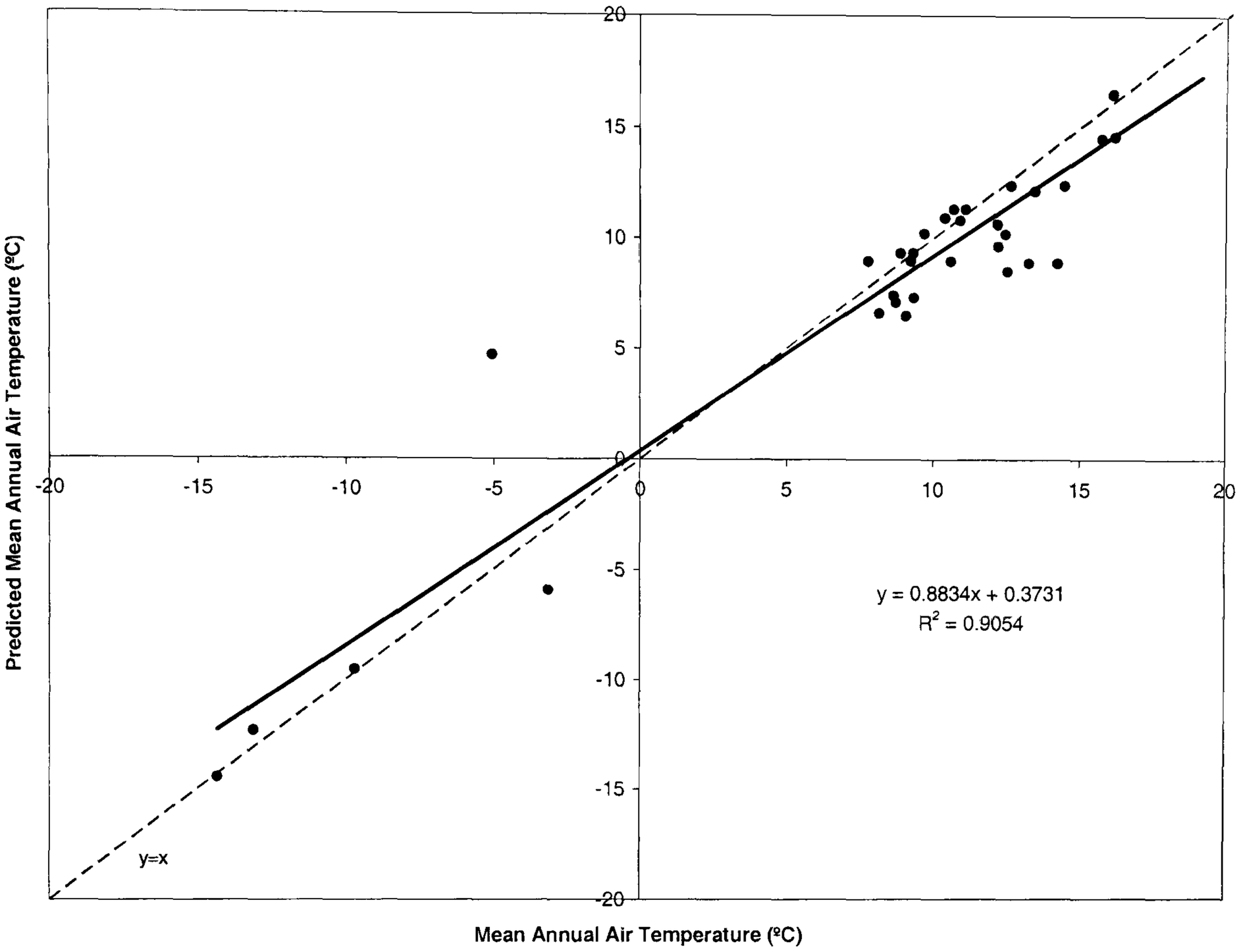

Figure 2.2-H. Comparison of air temperature estimates vs. recorded air temperatures.

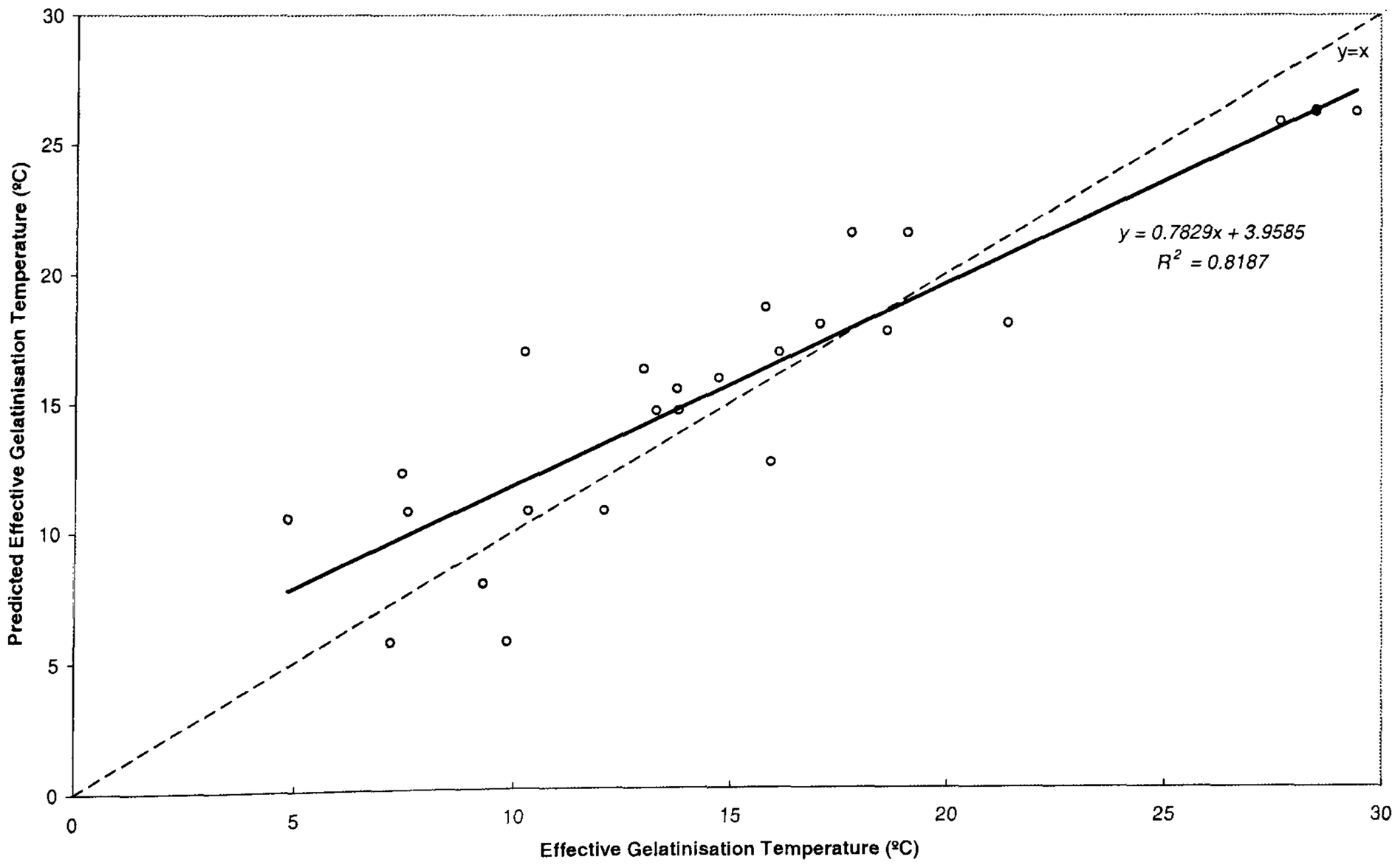

Figure 2.2-I. Comparison of soil temperature estimates vs. soil temperatures (converted to effective gelatinisation temperatures).

2.2.5 Conclusions

We have succeeded in establishing kinetic data for the collagen gelatinisation reaction at high temperature in the presence of water (Ea = 173.2 kJmol^{-1}, $A = 2.11 \times 10^{19}$). If this data is used to predict rates of gelatinisation at likely burial temperatures for archaeological bone, then it is apparent that bone collagen survives less well than anticipated from the model. If the model does not contain a significant systematic bias that creates this effect and is a realistic model of the chemical loss of collagen, we can conclude that other modes of collagen loss must play a significant role in the removal of bone collagen from the geosphere. Microbial attack of archaeological bone is a ubiquitous phenomenon and will remove collagen, however bones that show no evidence of microbial attack can also show apparent rapid loss of collagen (see Chapter 3.3), although the precise mechanism is not clear. Even if there are other modes that can remove collagen, not anticipated in this model it would be expected that some bones would lose collagen as predicted, and the fact that very few bones have more collagen than predicted leads us to believe that we may have predicted an upper limit to collagen survival. If this is so, we anticipate that collagen will not survival beyond the Holocene in the tropics.

2.2.6 Acknowledgements

CS was funded by the EU (ENV4-CT98-0712). The LDEO/IRI Data Library is acknowledged for facilitating access to climatic data. The original collagen weight loss data was supported by NERC grant GST/02/1017.

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2.2.8 Annex to Improving the analysis of ancient collagen: establishing and testing a quantitative chemical model of collagen degradation.

Recent results from similar heating experiments comparable to those described in part 2.2 (McNultey *et al.*, 2002, and Steffi Ritz-Timme pers comm.) have lead to a reassessment of the kinetic parameters of collagen gelatinisation and thus collagen survival. The rates of collagen loss in these experiments are similar to those observed by Ortner *et al.*, 1972; and Von Endt & Ortner 1984. If these data are plotted together on an Arrhenius plot (Figure 2.2-J) then the *Ea* of collagen gelatinisation may be higher at 180.8kJmol^{-1}; however, the absolute rate of collagen loss is lower. The alteration in activation energy is negligible in terms of calculating effective gelatinisation temperatures, for example for a thermal regime of 10°C ± 20°C (using a sinusoidal model) the effective temperature is greater by only ~0.2°C using the *Ea* of 180.8kJmol^{-1}.

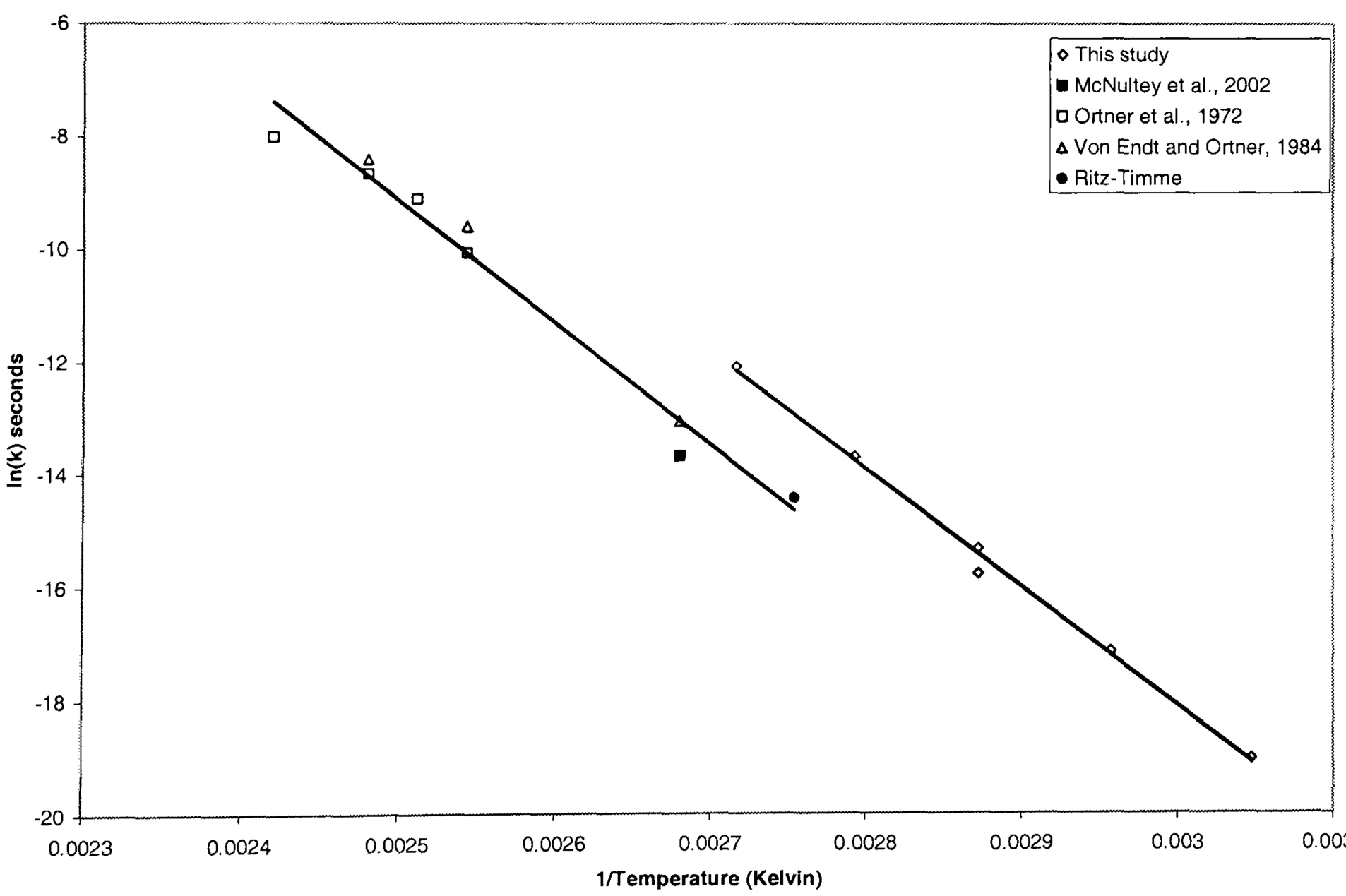

Figure 2.2-J. Arrhenius plot comparing high temperature rates of bone collagen degradation from various studies.

The effect of this on the absolute rate of collagen gelatinisation at likely effective temperatures (10-30°C) can be seen in Figure 2.2-K. The limit of collagen survival is predicted here for different temperatures, clearly if the slower rate is correct collagen

will survive more than 10 times longer than predicted from the previous rate. The reason for the discrepancies in the two sets of data is not clear, however, the experiments described in part 2.2 used freezer milled bone powders. It is possible that homogenisation under liquid nitrogen damages the collagen in some way (e.g. disruption of the structure through rapid ice crystal formation) making the collagen more labile in subsequent heating experiments.

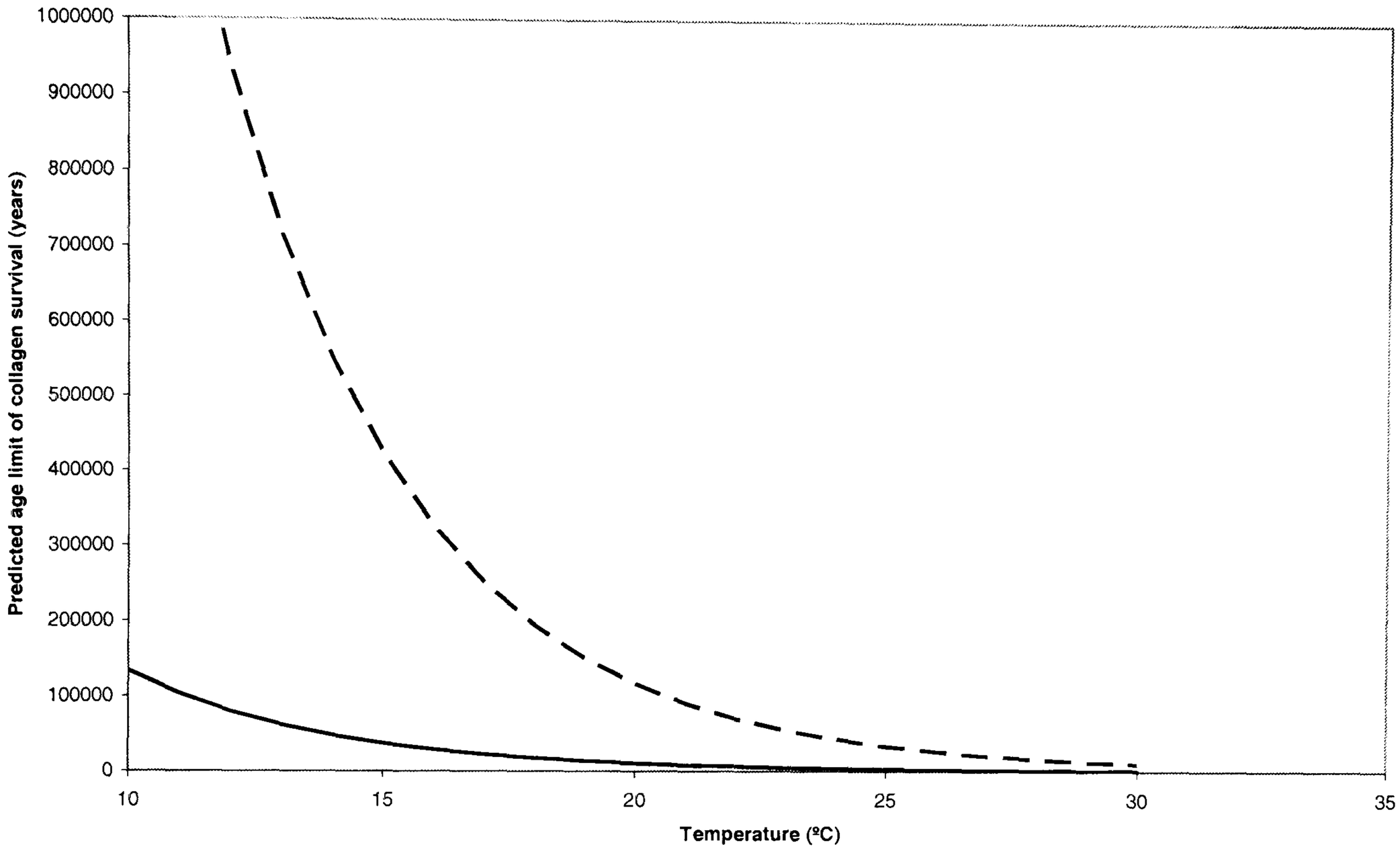

Figure 2.2-K. Predicted maximum limit of collagen survival with temperature. Solid line *Ea* = 173.2kJmol^{-1}, *A* = 2.11x10^{19} s^{-1}; dashed line, *Ea* = 180.8 kJmol^{-1}, *A* = 4.51x10^{19}s^{-1}.

It is clear that the temperature dependence of the reaction is high (in the region of 150-180kJmol^{-1}) however, errors in this can make significant changes to the absolute rates when extrapolated to lower temperatures, i.e. likely burial temperatures. The high temperature rates require further confirmation from low temperature data. Considering radiocarbon dated bone from the Oxford and Groningen database it is observed that there is an apparent dearth of old bones from hot sites (where as charcoal is dated from such sites). If we assume that this is the effect of temperature on the collagen yield it seems reasonable to suggest that the oldest material from the hottest sites will indicate the limits of collagen preservation, or thereabouts. Using bone of known calibrated age, collagen yield and our temperature estimates it is possible to estimate the rate of collagen loss from these old hot bones (Table 2.2-ii). When these are plotted on an Arrhenius plot (plotted for both Mean annual air temperature and effective temperature) it is clear that rates extrapolated from the high temperature experiments approximate to actual rates of collagen loss in bone (Figure 2.2-L). It should be noted that these actual loss rates maybe too high as bone can also lose collagen through other mechanisms, therefore rates of chemical loss alone will plot lower on the *y-axis*. Considering all this data the *Ea* of collagen gelatinisation is probably ~160kJmol^{-1}.

Site	Country	^{14}C Age	Calibrated age (y)	Mean Annual Temperature°K	Teff*	Collagen Yield$
Shum Laka	Cameroon	7150	7950	294.36	293.73	17.125
Abu Hureya	Syria	8180	9040	290.37	294.43	0.345
Toe, Irian Jaye	Indonesia	2930	3140	293.14	297.36	4.81
Anse	Seychelles	125	100	299.8	299.5	4.13
Cuello	Belize	3750	4140	298.15	298.25	3.211

Table 2.2-ii. Selected bones from the Oxford and Groningen database. * Effective temperature calculated with $Ea = 173.2kJmol^{-1}$, $A = 2.11x10^{19}\ s^{-1}$. $ Collagen yield as % dry weight of bone corrected for extraction method (see Chp2.2).

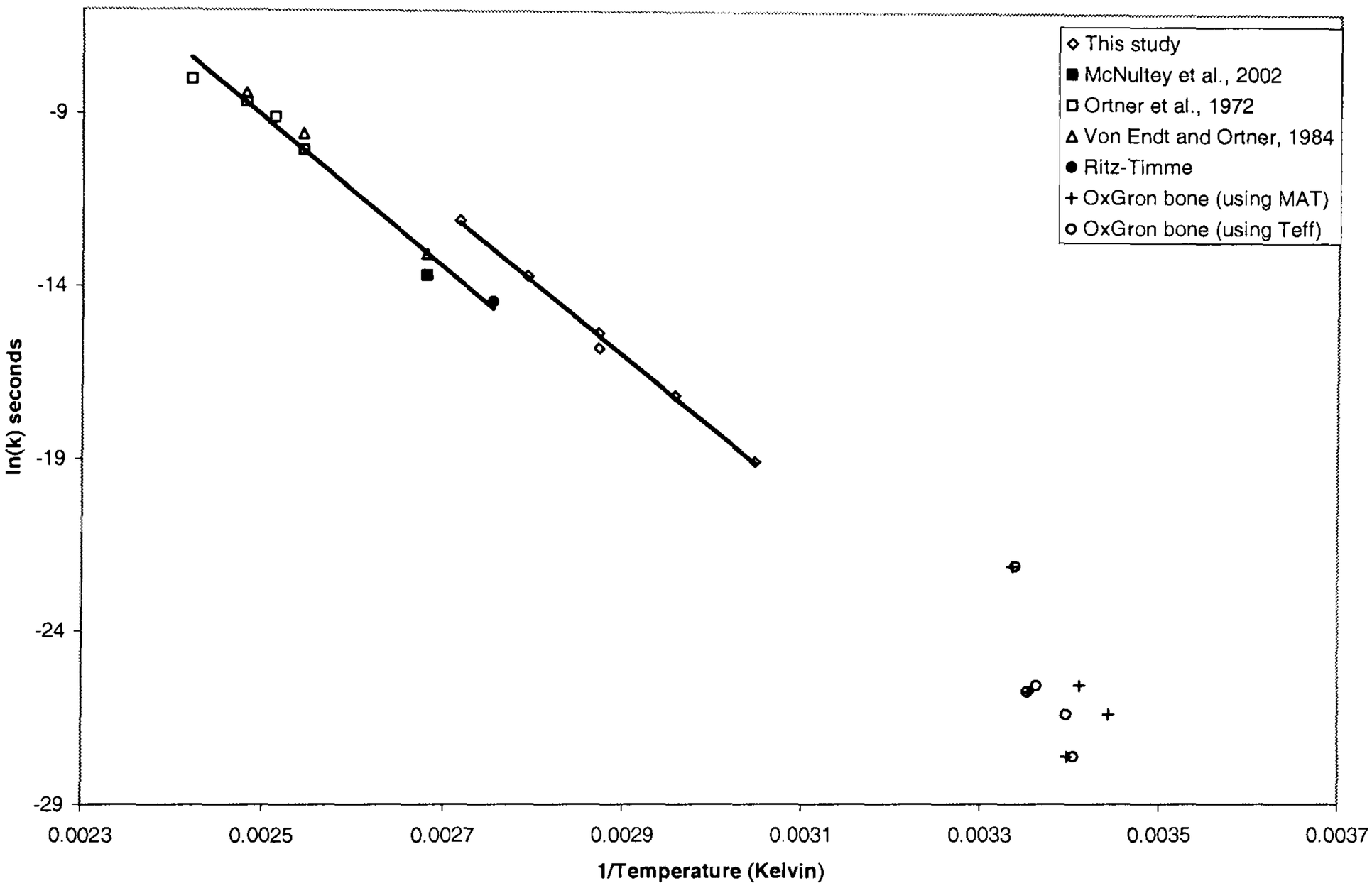

Figure 2.2-L. Arrhenius plot comparing observed rates of collagen loss from bone in the Oxford and Groningen database with high temperature experiments. The low temperature bone data has been plotted with both mean annual temperature of the site, and a collagen gelatinisation effective temperature, using $Ea = 173.2kJmol^{-1}$, $A = 2.11x10^{19}\ s^{-1}$.

Chapter 2.2 has considered collagen degradation based on an activation energy of $173kJmol^{-1}$, whereas it can be suggested from the data from other experiments and the Oxford and Groningen radiocarbon database that the rate of chemical collagen loss may be slightly less temperature sensitive and absolutely slower. This does not significantly detract from the findings of chapter 2.2. It should be noted that whatever the absolute kinetic data, it is evident that collagen gelatinisation is a highly temperature sensitive reaction. Thus temperature will play a more significant role in collagen preservation than in other decomposition reactions (such as isoleucine epimerization) which have markedly lower activation energies (of ~ $130kJmol^{-1}$). It

has been demonstrated that the effect of different *Ea* (173.2 and 180.8kJmol^{-1}) on the effective collagen gelatinisation temperature over the range of likely burial temperatures is negligible, and thus also on the effective temperature used in chapter 2.2. The distribution graphs too will be slightly different, so that most bones will contain even less collagen than predicted by the possible fast rate in chapter 2.2, however the shape of the distribution will remain unaltered.

2.2.9 References

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2.3 The thermal history of human fossils and the likelihood of successful DNA amplification.

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2.3.1 Abstract

Recent success in the amplification of ancient DNA (aDNA) from fossil humans has led to calls for further tests to be carried out on similar material. However, there has been little systematic research on the survival of DNA in the fossil record, even though the environment of the fossil is known to be of paramount importance for the survival of biomolecules over archaeological and geological timescales. A better understanding of aDNA survival would enable research to focus on material with greater chances of successful amplification, thus preventing the unnecessary loss of material and valuable researcher time. We argue that the thermal history of a fossil is a key parameter for the survival of biomolecules. The thermal history of a number of northwest European Neanderthal cave sites is reconstructed here and they are ranked in terms of the relative likelihood of aDNA survival at the sites, under the assumption that DNA depurination is the principal mechanism of degradation. The claims of aDNA amplification from material found at Lake Mungo, Australia, are also considered in the light of the thermal history of this site.

Keywords

Ancient DNA, Neanderthal, Modern Humans, Lake Mungo, Thermal History

2.3.2 Introduction

The phylogenetic relationships between modern humans (*Homo sapiens*) and other species of *Homo* are a source of much debate (e.g. Mellars, 1999). A recent approach to collecting data to investigate the relationships is to isolate genetic material from fossil specimens (e.g. Krings *et al.*, 1997; Ovchinnikov *et al.*, 2000; Krings *et al.*, 2000, for Neanderthals; Adcock *et al.*, 2001 for early Australians), and compare this with modern human DNA. Initial successes have led to calls for further genetic sequences, from different geographic locations and age ranges (Höss, 2000; Adcock *et al.*, 2001), however, the quality of aDNA studies in general, has recently been brought into question (Cooper & Poinar, 2000). The isolation of DNA from fossils is a destructive process, and when dealing with valuable material such as fossil humans it is important that a precautionary approach is taken. Assessment of the likelihood of a sample containing amplifiable DNA should be a prerequisite for such work, maximising returns from researcher time, funding, and most importantly, the valuable and irreplaceable resource of the fossils themselves.

It has been observed that DNA appears to survive best in cold dry environments such as permafrost, or high altitude caves, and biochemical studies suggest it is unlikely to survive for more than 100 000 years (Wayne *et al.*, 1999). Yet, there has been little systematic work on the long-term survival of DNA in the fossil environment, and consequently assessments of the prospects of DNA survival remain anecdotal. The need to find further fossil human DNA sequences from wider geographical and temporal ranges is compelling. The proposed upper bound to survival of 100,000 years encompasses both the extinction of the Neanderthals, and the diversification of modern humans, but the oldest successful amplifications are from permafrost, not sites of human occupation.

Pääbo & Wilson (1991) used a theoretical approach, based upon the rate of depurination of DNA at 15ºC and pH of 7.5 in physiological solution, to suggest that it would take approximately 100,000 years to destroy all the DNA that could be usefully extracted from a 1 g chloroplast sample. Using a similar approach (see Appendix A), it is possible to calculate an approximate mtDNA copy number for fresh bone to be about 2.5×10^{11} per gram of bone ($0.3 \times 10^{11} - 10 \times 10^{11}$, see Table 2.3-i). Using the rate constants for DNA depurination from Lindahl & Nyberg (1972), a theoretical limit for the survival of 105bp fragments (the size amplified from the Neanderthal type specimen, Krings *et al*, 1997) in 1g of bone at 15˚C at pH 7.4 would be ~ 5.7kyrs, extending to ~ 105kyrs at 0ºC.

While it is intuitive that fossils from cold environments will have better biomolecular preservation than those from hot climates, and that younger fossils will be better preserved than older ones, the distinction between an old and cold fossil and a young hot one is more difficult to assess.

		value	comment
1	Volume occupied by osteocyte lacunae	1.5% of bone	HGIP of human bone (NB higher values are seen in bird bone)
2	Volume occupied by osteocyte lacunae	1.7-2.8%	http://www.orl.med.umich.edu/orl/archgroup/a6.htm
3	Size of individual osteocyte lacunae μm	11.7 –17.4 length 4.8 – 6.6 width 3 – 3.4 height	http://www.orl.med.umich.edu/orl/archgroup/a6.htm
4	Hence volume of osteocyte	200 μm^3	
5	Volume of osteocyte occupied by mitochondria	20%	in liver cells values of 15 – 25 % reported
6	Skeletal Density of modern bone gcm^{-3}	1.4 – 1.7	increases ultimately to 3.0 due to loss of collagen and water
7	Hence numbers of osteocytes per gram modern bone	5×10^7	assuming a density of 1.47 gcm^{-3}
8	No of mitochondria per cell	1000	Ranges from 800 – 1500 depending upon values given in (5)
9	Hence no of copies of mitochordria per gram	5×10^{10}	
10	No of copies of mtDNA per mitochondria	5	literature values range from 2 – 20
11	No of copies of mtDNA per gram of bone	2.5×10^{11}	estimate from 3×10^{10} - 1×10^{12} using the extremes given in (5) and (10)

Table 2.3-i. Estimation of mtDNA content in compact bone

The preservation of biomolecules in the fossil environment is complex, especially in bone (Collins *et al.*, in press). In brief, bone degradation is considered to occur mainly by two processes; one rapid, mediated by microorganisms and fungi (see Bell *et al.*, 1996), and the other, chemical degradation which is a relatively slow process. For skeletal material to become part of the fossil record it is likely that microbial attack will have to be excluded. If microbial taphonomy is inhibited, then the two major chemical pathways that will lead to DNA destruction are condensation (e.g. glycation of nucleobases, Pischetsrieder *et al.*, 1999) and hydrolysis of the purine bases (Lindahl & Nyberg, 1972). The importance of cross-linking in the survival of DNA has not been investigated in detail so far (see Poinar, 1999). Principal factors that influence the rate of hydrolytic depurination are pH, amount of chemically available water and temperature. The first two factors are less significant in bone as bone itself exerts a substantial buffering effect between pH 4 - 9 (Bada & Shou, 1980), and the pore size distribution of bone encourages water retention (Hedges & Millard, 1995). Deep burial will buffer temperature fluctuation, but only around an annual mean, and thus temperature is likely to play a substantial role in defining the envelope of DNA survival (Smith *et al.*, 2001). Here we present a more detailed account of our assessment of the thermal history of fossil hominid sites from Northern Europe, and use this to rank sites according to their thermal age. The thermal age relates the time / temperature function at different sites using the temperature dependence of DNA depurination estimated in aqueous solution. A comparison is made between the DNA depurination thermal ages of sites in NW Europe and Lake Mungo, a site in Australia where controversial claims have been made for the recovery of ancient DNA (Adcock *et al.*, 2001; Cooper *et al.*, 2001).

2.3.3 Methods

The thermal regime of a fossil is governed by two major factors, the mean temperature, and the variation about this mean, both of which will vary over time due to climatic changes. Thus to reconstruct the thermal history of a fossil, data must be obtained for both the modern day temperature of the site, and the palaeotemperature.

For a fossil buried in open ground the thermal regime of the fossil is assumed to be the same as that of the surrounding soil. It has been demonstrated that good estimates (±1°C) of mean soil temperature can be taken from local weather stations mean air temperature (MAT) data (Kusada & Achenbach, 1965; Toy *et al.*, 1978), but that the thermal regime (i.e. extent of seasonal fluctuation in the soil) is difficult to predict. The amount of seasonal fluctuation in the burial environment, and hence the *effective* MAT for a chemical reaction (see Wehmiller *et al.*, 2000) will be controlled by many factors including; burial depth, the fluctuation in the climate, the thermal properties of the burial environment (Krarti *et al.*, 1995) and local effects (e.g. vegetation or snow cover). Consequently, assessment of the thermal history of open sites is difficult as such information is not usually available nor can it be readily extrapolated back into the past.

In the cave environment the MAT is generally considered a good approximation of local deep cave air temperatures (Bogli, 1980). Deep cave temperatures are also known to be quite static and prone to little seasonal variation, also humidity in caves is high, and not prone to large fluctuations. For a fossil buried in a cave sediment, any fluctuations around the mean value will be dampened further by the cave sediment. It

should be considered however that there is more fluctuation at the cave entrance in both temperature and humidity.

We have attempted to reconstruct the thermal history of Northwest European Neanderthal cave sites, and that of the early anatomically modern human site of Lake Mungo in Australia, by combining both modern day temperature data, and palaeoclimatic evidence.

2.3.3.1 Thermal Model for Northwest European Neanderthal cave sites

Modern day mean annual temperatures of the Neanderthal cave sites listed below have been estimated from weather station data near to each locality (see Table 2.3-ii). For simplicity, and due to a lack of quantitative palaeoclimate data the, Holocene is considered to be a stable climatic period with constant temperature back to 10.4kyr BP. Temperatures for this period were estimated from sequences of continuous data (years - decades) from a weather station in the locality of each Neanderthal site (Baker *et al.*, 1994). Only mean temperatures are used in this model as there is assumed to be no variation about the mean.

Altitudes of the cave sites in metres have been estimated from GTOPO30 (Gesch *et al.*, 1999) using a weighted average of the four nearest values to the location unless the correct altitude is known (italics, Table 2.3-ii). For eleven sites where the altitude is known, the best fit relationship between GTOPO30 estimates and true altitude is given by

altitude estimate = 1.07 x *known altitude* +10.42m.

The correlation is highly significant, $R^2 = 0.977$, $n = 11$

A thermal lapse rate with altitude of 6°C/km has been used to account for differences in altitude between the cave site and the nearest weather station.

Pleistocene thermal histories of the sites have been reconstructed by applying one of four different regional models, (i) northern France and the Benelux countries, (ii) Germany (iii) Poland (based on data in Aalbersberg & Litt, 1998; Huijzer & Vandenberghe 1998; Coope *et al.*, 1998; Witte *et al.*, 1998) and (iv) Southern France (Figure 2.3-A). The thermal history of southern France has been reconstructed using quantitative data from Guiot *et al.* (1993) compiled using methods similar to those of the other studies. In cases where palaeoclimatic data are incomplete, palaeotemperature values have been assigned based on similar climatic periods for the same region, or values in similar regions for the same period.

The average difference between modern mean annual temperature taken from the ISLSCP data set (Meeson *et al.*, 1995; Sellers *et al.*, 1995) and that of the reconstructed mean annual temperature for the last glacial maximum (LGM) has been calculated for each region. To assign each site a temperature for the last glacial maximum, the average LGM/Holocene difference is subtracted from the Holocene temperature for each site. All other differences between Holocene and palaeotemperatures are calculated relative to this LGM/Holocene difference. Thus each site has a palaeotemperature curve based on its current (Holocene) temperature,

and its regional palaeoclimatic curve. There is an implicit assumption in this approach; that current differences in temperature caused by geographic location etc. are constant with time. In part this assumption is true, as the main differences in temperature between sites (especially within one region) will be due to differences in altitude, these differences are maintained in this model (although this does assume that the thermal lapse rate remains constant). Conversely the palaeoclimatic evidence suggests that the temperature gradient across Europe, currently north-south, has fluctuated in the past, in both direction and inclination, and local effects such as vegetation cover and local weather conditions will have changed, these factors will affect both inter- and intra- regional variation, and are not accounted for in the model. The reconstructions also ignore the possibility that geothermal heating may have elevated cave temperatures during periods of reduced precipitation in the Pleistocene.

An alternative climate model for southern France has been used, for comparative purposes, based on the climate curve produced by Van Andel (1997); essentially a reconstruction based on palaeoclimatic data from Les Echets (Guiot *et al.*, 1989). This model is not confined to the chronology of the data above, but only covers the last 60kyr. This model has been used as a comparison to see how thermal ages may differ using alternative palaeoclimate models.

During periods of glaciation it is likely that cave deposits underwent periods of freezing. The relationship between air temperature and permafrost surface temperature is difficult to predict due to the zero curtain effect (Zhang *et al.*, 1997). However, the rate of depurination in frozen samples is thought to be weakly temperature sensitive (Osborne & Phillips, 2000) so this problem is somewhat circumvented. For the purposes of modelling we have taken a nominal effective temperature of 0°C for fossils in permafrost, i.e. where the model predicts a temperature of 0°C or less.

The thermal age of Mezmaiskaya has also been reconstructed, although we lack quantitative palaeoclimatic data for this region. A reconstruction has therefore been made using a constant Holocene temperature. (N.B. Assuming that the temperature in this region cooled during the Pleistocene, the thermal age of this site is likely to be overestimated.)

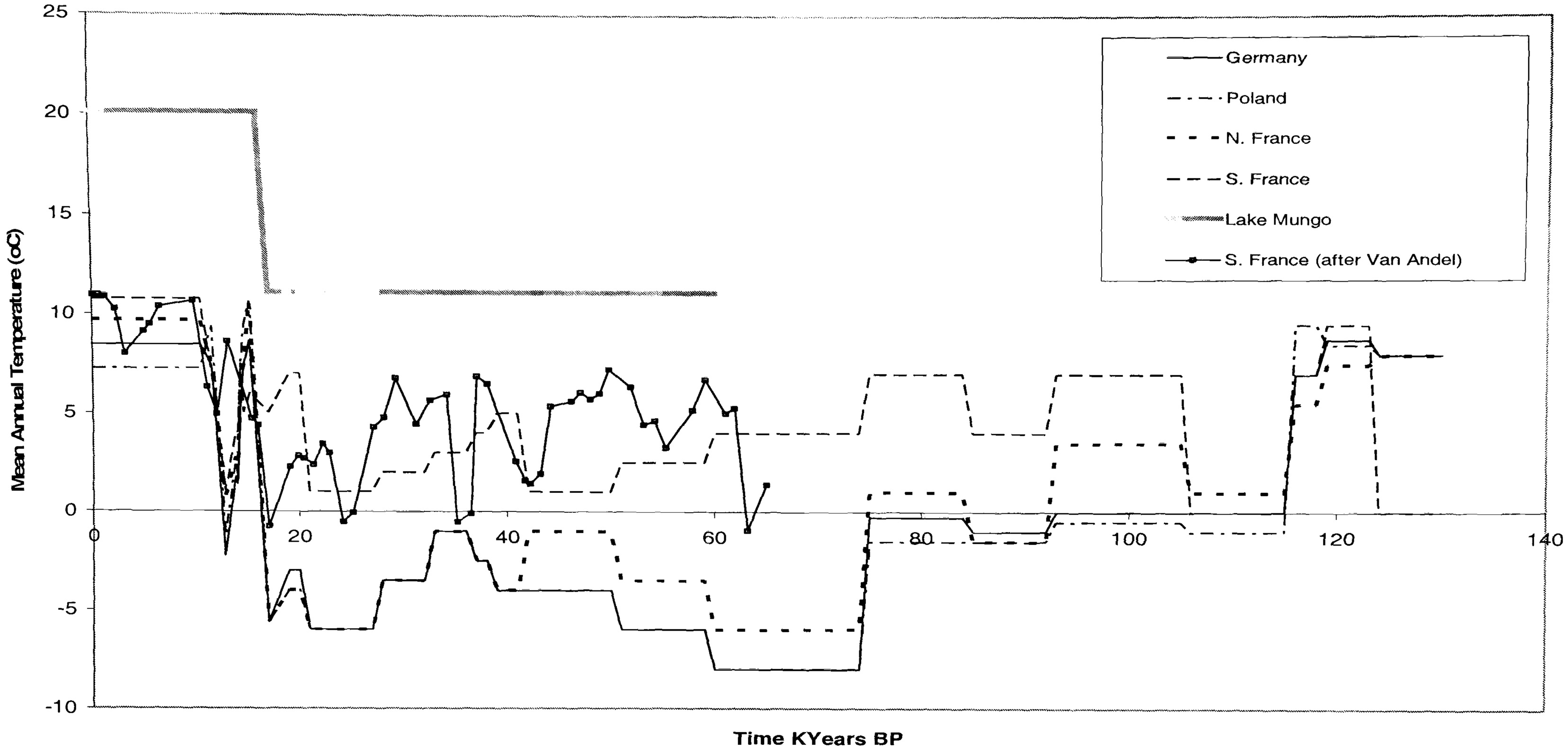

Figure 2.3-A. Regional palaeoclimate models for Northwest Europe and Lake Mungo Australia. Holocene (0-10.4kyrBP) temperatures are the average mean air temperature taken from the ISLSCP data set (Meeson *et al.*, 1995; Sellers *et al.*, 1995) for Germany, Poland, S. France and N. France. S. France alternative based on Van Andel, 1997, and Lake Mungo on Ambrose, 1984 and Miller *et al.*, 1997.

2.3.3.2 Thermal model for Lake Mungo

The thermal history of Lake Mungo has been reconstructed using measured sediment temperatures from Lake Mungo (Ambrose, 1984) and reconstructed palaeotemperatures inferred from isoleucine epimerisation rates from ratite eggshells (Miller *et al.*, 1997). The data for burials at 1.5m at stations 1 and 2 (Ambrose, 1984) were used to calculate the effective burial temperatures. *Effective DNA depurination temperature* (T_{eff}) is the constant temperature equivalent which causes the net equivalent depurination that will have been accrued by the DNA over one year, taking into account seasonal temperature fluctuation and using a temperature dependent reaction having an activation energy of E_a 127kJ mol^{-1} (Lindahl & Nyberg, 1972). Total annual reaction was calculated using a sinusoidal wave model using the mean annual temperature and variation about this mean for modern temperatures and palaeotemperatures (see Appendix B for an example of the calculation of thermal age). The palaeoclimate of Southern Australia is markedly different to that of Northwest Europe. A model based upon amino acid epimerisation data indicates a 9°C warming after 16kyr BP with no other significant excursions (Miller *et al.*, 1997). We have therefore used a simple palaeoclimatic model using the present day effective temperatures calculated from sediment temperatures for the period 16-0kyrBP, which was preceded by a period 9°C cooler than the present arithmetic mean annual temperature, from 60kyrBP-16kyrBP.

2.3.3.3 Rates of biomolecular deterioration

The rate of DNA depurination at each site has been calculated as the integration of the site's palaeoclimatic curve, the age of the fossils at the site, and the activation energy (E_a) of the depurination reaction (Lindahl & Nyberg, 1972). To enable comparison sites are ranked using 'thermal age', where the calculated rates, are normalised to what the rate would be at 10°C (see Appendix B). Where sites have multiple occupancy or have not been accurately dated a number of alternative thermal ages are given corresponding to different actual periods of burial.

2.3.4 Results and Discussion

The results of this analysis can be seen in Table 2.3-ii. The Feldhofer cave site from which DNA has been amplified is ranked as ten of thirty-nine, with a thermal age of ~19 $_{kyr\ @10°C}$. If our estimate of the Holocene temperature is 1°C in error at this site, the thermal age is approximately 3 $_{kyr\ @10°C}$ (~16%) different. The sequence of ages given for each site reveals an important feature of the thermal history, for example, at Feldhofer if a fossil is considered to be 50kyr, 45% of the thermal age is accumulated over the first 40kyr of deposition, and 55% of the thermal age is accumulated during the last 10kyr. Recent unpublished radiocarbon determinations have directly redated the fossil humans to about 40 kyr (Schmitz & Thissen, 2000). These ages may be minima, but this redating to 40kyr will reduce the thermal age by only 2 $_{kyr\ @10°C}$. Furthermore, if a fossil was deposited 74 kyrBP at Feldhofer, the thermal age would only be ~3 $_{kyr@10°C}$ older than a fossil deposited at 50kyrBP. This reveals the importance of considering the entire thermal history of a fossil, and the effect of the warming during the Holocene. This also indicates that the temperature at which the fossil is held during the Holocene is of key importance to biomolecular survival, and

that the absolute age of the fossil may be of negligible importance if the fossil is kept very cold. Using the alternative climate model based on Van Andel (1997) a southern French site with a current estimated temperature of 12.2°C (e.g. Marillac) will have its thermal age reduced by approximately 1 $_{\text{kyr @10°C}}$ at 50kyr, and by 2 $_{\text{kyr @10°C}}$ at 74 kyr (3-5%). This demonstrates some of the error involved in estimating thermal age, and highlights the importance of the climate model.

The thermal age of Mezmaiskaya (also a site where aDNA has been successfully amplified (Ovchinikov *et al.*, 2000) is likely to have been overestimated here, as we have not modelled any cooling during the Pleistocene, although it is still lower than that of Feldhofer cave. This is probably due to the altitude of the site (1.3km), keeping the site cold in comparison to many other sites.

To validate the approach, a similar model has been tested by comparing published data on DNA amplification success and failure with the thermal ages of fossils (Smith *et al.*, 2001). This model used a lower freezing cut–off point at -3°C, and slightly different kinetic parameters, however the relative rankings of the sites were the same as those produced by the model presented here. Despite the inherent uncertainties of the reconstructed thermal histories, the 'thermal age' is remarkably effective in predicting DNA survival. In this analysis, Feldhofer cave is the site with the largest thermal age to yield amplifiable DNA, thus we would predict that material from sites with greater thermal age than Feldhofer less likely to yield amplifiable aDNA.

Using the rate of DNA depurination for physiological solution (at pH 7.4) from Lindahl & Nyberg (1972) and our estimate of mtDNA copy number (Table 2.3-i), at 10°C the last copy of a 105bp fragment in 0.4g of bone (the same sample and fragment size used in Krings *et al.*, 1997) would be expected to be destroyed after 14kyrBP. Thus the thermal age limit predicted, ~19 $_{\text{kyr @10°C}}$ is beyond what may be estimated from the copy number calculation. Copy number estimates of 105bp fragments from the Feldhofer bones however, estimate approximately 2.5 - 3.75 x 10^3 copies remaining per gram of bone (Krings *et al.*, 1997), using the same parameters above, this would suggest that the rate would need to be approximately 4.9 times slower than the rate at pH 7.4 at 10°C. The rate of depurination at pH 7.88 is approximately twice as slow as that at pH 7.4, and is a more realistic pH for reactions in bone, furthermore, Lindahl (1993) reports a two-fold reduction in depurination rate when adsorbed to apatite. Marguet & Forterre (1998) report that rates of depurination are retarded for both double stranded and single stranded DNA in the presence of chloride salts, due to their direct interaction with purine nucleotides; thus elevated salt concentrations may also reduce the predicted rates of DNA deterioration. Differences in absolute rates will not affect the relative rankings of the sites, but would compromise predictions of the remaining copy numbers (c.f. Pääbo & Wilson, 1991); absolute rates of DNA deterioration are therefore not estimated.

Site	Age kyr	Palaeoclimate Model	Altitude (m)	MAT °C Holocene	Thermal Age (kyr@10°C)	Thermal Age (kyr@10°C) at				
						10 kyr	32 kyr	50 kyr	74 kyr	130 kyr
Ochoz	NA	P	524	6.9		5	11	13	17	32
Kulna	NA	P	514	7		6	11	13	17	33
Sipka	NA	P	432	7.1		6	11	14	17	33
Spy	NA	NF	179	8.9		8	13	16	19	34
Subalyuk	NA	P	416	8.2		7	13	16	19	38
Engis	NA	NF	172	9.3		9	14	16	20	35
Fond de Foret	NA	NF	183	9.5		9	14	17	20	37
La Naulette	NA	NF	168	9.6		9	15	17	20	37
Soulabe las Maretas	NA	SF	754	8.9		8	13	16	21	38
Neanderthal	50	G	128	10.1	19	10	16	19	22	43
Saint Brelade	NA	NF	*10*	11.1		12	19	21	25	47
Arcy sur Cure	32	SF	*125*	11.4	21	13	21	26	34	61
La Chaise (B.-Delauney)	NA	SF	393	10.4		11	18	22	28	50
Malarnaud	NA	SF	475	10.5		11	18	22	28	51
Rigabe	NA	SF	400	11.4		13	21	26	34	61
Regourdou	NA	SF	196	11.6		14	22	27	35	63
Rochelot	NA	SF	141	11.9		14	23	29	37	67
Montgaudier	NA	SF	128	12		15	24	29	38	68
Caminero	NA	SF	131	12.1		15	24	30	39	70
Fontechevade	NA	SF	*120*	12.1		15	24	30	39	70
Petit Puymoyen	NA	SF	131	12.1		15	24	30	39	70
Chateauneuf sur C	NA	SF	102	12.2		15	25	31	39	71
Hortus	NA	SF	*390*	12.2		15	25	31	39	71
Marillac	NA	SF	106	12.2		15	25	31	39	71

Site	Age kyr	Palaeoclimate Model	Altitude (m)	MAT ºC Holocene	Thermal Age (kyr@10°C)	Thermal Age (kyr@10°C) at				
						10 kyr	32 kyr	50 kyr	74 kyr	130 kyr
Rene Simard	NA	SF	103	12.2		15	25	31	39	71
Montmaurin	NA	SF	355	12.3		15	25	31	40	72
Quinzano	NA	SF	71	12.5		16	26	32	42	75
Orgnac	NA	SF	*320*	12.7		17	27	34	43	78
Pech de l'Aze	NA	SF	169	12.7		17	27	34	43	78
La Masque	NA	SF	150	12.8			17	28	34	44
La Chapelle aux Saints	NA	SF	149	12.9		17	28	35	45	80
Combe Grenal	NA	SF	108	13.1		18	29	36	47	81
Lezetxiki	NA	SF	266	13.3		19	31	38	49	84
Monsempron	NA	SF	71	13.3		19	31	38	49	88
Roc de Marsal	NA	SF	75	13.3		19	31	38	49	88
Grotta del Principe	NA	SF	*12*	14.7		24	40	49	64	88
La Chaise (Abri Suard)	130	SF	393	10.4	50	11	18	22	28	115
Caverna delle Fate	NA	SF	*280*	15.2		26	44	54	70	50
Lazaret	NA	SF	*25*	16.1		31	52	64	83	126
Mezmaiskaya	32	Holocene	*1310*	5.7	14	4	14	N/A	N/A	149

Table 2.3-ii. Ranking of European Neanderthal Cave sites based upon thermal age. MAT, Mean Annual Temperature; NA, Data not available. Palaeoclimate model: P=Polish, G=German, NF=Northern France, SF=Southern France. NB. DNA has been successfully amplified from material from Feldhofer (Krings *et al.*, 1997) and Mezmaiskaya (Ovchinikov *et al.*, 2000), an unsuccessful attempt has been made from material from La Chaise Abri Suard (Cooper *et al.*, 1997)

Given the technical problems with amplifications from the Neanderthal material and the variable state of bone preservation, we would suggest that in the absence of convincing screening methods (see Collins *et al.*, 1999) 19 $_{kyr\ @10^{o}C}$ is a sensible thermal limit. Note however that this does not mean that all sites with thermal ages lower than Feldhofer *will* contain amplifiable aDNA, as other factors may accelerate deterioration (Ovchinnikov *et al.*, 2001; Collins *et al.*, in press). Furthermore, by amplification of shorter fragments it is possible to extend the temporal range further. Thus reports of the successful amplification of an 80bp fragment from a cave bear 80kyr – 100 kyr at Scladina Cave (Loreille *et al.*, 2001) with a thermal age of ~20 $_{kyr\ @10^{o}C}$ are consistent with the results from Feldhofer (considering the actual rate of DNA degradation at Feldhofer is slower than we predict).

Using data from Ambrose (1984), a number of thermal models for the Lake Mungo site can be established (Table 2.3-iii) based upon differences in temperature recorded at different times and different depths. Although the range of calculated 'thermal ages' for a 60kyr fossil is large (133-216 $_{kyr\ @10^{o}C}$), they are an order of magnitude higher than that of Feldhofer cave. If Feldhofer cave lies close to the technical limit of amplification at 19 $_{kyr\ @10^{o}C}$ it would seem highly unlikely that there would be amplifiable DNA in the Lake Mungo fossils. Even if the site is only 40 kyr old as has been suggested (Gillespie & Roberts, 2000; but compare Grün *et al.*, 2000), the thermal age is reduced by 16% at most, still much higher than that of Feldhofer. The extreme thermal age at the site, the poor state of preservation of the material, acknowledged as being too fragmentary to sex reliably (Brown, 2000; Thorne & Curnoe, 2000), and having 'negligible organic preservation' (Gillespie & Roberts, 2000 p. 727) indicate that DNA preservation at the site as a whole is likely to be poor. This coupled with the concerns raised over the technical aspects of the Lake Mungo sequences (Cooper *et al.*, 2001) suggest that the reports of amplification of aDNA from Lake Mungo should be treated with caution until the work can be better substantiated, e.g., repeated in another laboratory. If DNA can be extracted from fossils with thermal ages as high as that of Lake Mungo the potential for finding aDNA in other samples, particularly many of the Neanderthal sites, are greatly increased.

Station	1	1	2	2
Year	82/83	83/84	82/83	83/84
Mean Soil at 1.5m depth Temperature*	20.1	19.1	20.6	19.8
Soil Temperature at 1.5m depth Amplitude*	2.5	1.7	7	5.9
Effective temperature after 16kyr BP	20.4	19.2	22.5	21.2
Effective Temperature before 16kyr BP	11.4	10.2	13.5	12.2
Thermal age (kyr@10C) if 60 kyr	162	133	216	176
Thermal age (kyr@10C) if 40 kyr	137	112	189	152

Table 2.3-iii. Modelled thermal ages of Lake Mungo fossils if buried at 1.5m. *Data taken from Ambrose (1984)

2.3.5 Conclusion

The application of molecular techniques to fossil materials is an expanding field, and can provide invaluable data on the relationships between modern humans and their fossil relatives. At present only a few authentic sequences of fossil hominid DNA have been reported. Without a better understanding of the survival of DNA in the fossil record, valuable fossils may be damaged and destroyed and much time wasted in the search for more aDNA sequences. The thermal history of fossils is a useful indicator for the likelihood of survival of biomolecules in the fossil record. If DNA depurination is considered to be the key mechanism of DNA degradation in fossil bone material (excluding the role of micro-organisms, which may be a prerequisite for long term survival Trueman, 1999) then the thermal history of the site will be a key parameter in defining the state of DNA preservation. The analysis of the thermal history of northwest European cave sites from which Neanderthal remains have been recovered, and subsequent conversion in to a relative amount of DNA degradation, indicates that few other north west European Neanderthal cave sites are likely to yield amplifiable DNA using present techniques. Furthermore when the relative amount of DNA degradation at Lake Mungo is compared with that of north west European cave sites we would predict that the likelihood of amplifying authentic endogenous DNA from fossils from this site is very low. If this is true, then the authenticity of this sequence must be brought further into doubt (Cooper *et al.*, 2001).

2.3.6 Acknowledgements

The LDEO/IRI Data Library is acknowledged for facilitating access to climatic data.

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Part 3

Measuring diagenesis in archaeological bone

3 Measuring diagenetic parameters of archaeological bone

This section consists of three papers detailing the measurement of diagenetic parameters of bone excavated from a number of European archaeological sites. This work was carried out as part of a major European Union funded initiative entitled *The Degradation of Bone as an Indicator in the Deterioration of the European Archaeological Property*, a brief description of which is given below. The analytical measurements made on the bone were based on those described by Hedges *et al.* (1995), and designed to be comparable to them. Considering the number of samples analysed simple and cost effective measurements of bone diagenetic parameters were essential. The bone diagenetic data produced is analysed here describing three major diagenetic pathways, and the effects that they have had on the physico-chemical aspects of the bones measured. They are also discussed in terms of their archaeological contexts and the environmental and soil conditions. The main subject of this thesis is the discussion of the diagenetic parameters of bone, most of which were measured at Newcastle University. Macroscopic and microscopic examination of the bone was carried out at Rijksdienst voor het Oudheidkundig Bodemonderzoek (ROB) in the Netherlands by Miranda Jans, who has developed the new histological methods of analysis. This thesis by necessity discusses the results of the histological index (Hedges *et al.*, 1995; Millard, 2001) measured at ROB and the cracking index developed at ROB (Jans *et al.*, in press), however further detail of the histological preservation is beyond the scope of this thesis.

The first paper is an overview of the principal bone diagenesis findings of the project, describing three main diagenetic outcomes for bone. Examples are given from key sites displaying typical forms of the diagenetic modes and possible explanations are given regarding what is known about the taphonomic histories of the bones. The first paper mentions one of the modes of diagenesis as a type of 'pre-fossilisation' of the bone. This theme is taken up in the second paper which describes the state of preservation of bone at the key site of Apigliano. Some of the bone preserved at Apigliano displays some of the most extreme cases of the 'pre-fossilisation' phenomenon. One of the most striking features of this preservation is the apparent rapid loss of the collagen without evidence of microbial attack. The amounts of collagen are compared to those anticipated by modelling hydrolysis of the bone collagen. Possible explanations for this peculiar type of preservation are discussed. The final paper describes the immunological assay (using a novel rapid antigen extraction technique) of the amount of bovine osteocalcin remaining in the cattle bone excavated for the project. The results are discussed in terms of the diagenetic states described above and previous work on the survival of osteocalcin in the archaeological and geological record.

3.1 The Degradation of Bone as an Indicator in the Deterioration of the European Archaeological Property

The main objectives of the project were to investigate the state of preservation of bone in the archaeological record under different burial conditions in Europe. The project was carried out by a consortium of government, museum, university and commercial researchers from the Netherlands, Britain, Sweden and Italy. The focus of the work was to excavate bone from a variety of archaeological sites throughout these countries, define the state of preservation of the bone in terms of diagenetic parameters, and to relate the state of bone preservation to the current soil conditions (both physical and chemical). In addition, the general environment of the site, e.g. the hydrology, the history of land use was considered and of course the archaeological aspects of the bones were considered, such as the age of the burials the type of burial, the species, sex and age of the individuals. The aim was to identify relationships between the various bone preservation variables and the soil, environmental and archaeological parameters of the bone. These relationships could then be used to develop cultural heritage management policies. For example, it would prove useful to be able to predict what the effects on the archaeology would be of changing the land use of a site, or maintaining an *in situ* preservation brief. The outcomes for bone can be quite varied where information can be lost at a number of levels in the bone.

In the course of the project over 200 bones have been analysed from more than 40 archaeological sites, ranging in geographic and temporal range from Medieval Turkey and Italy, to Medieval and Roman Britain and the Netherlands to the Neolithic of northern Sweden. The aim of this thesis is not to present all the data from the project, in the papers below, but to summarise and highlight the key diagenetic trajectories. A full project report can be found in (Kars & Kars, 2001).

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3.2 Diagenesis of bone from the European Holocene.

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3.2.1 Abstract

'Bone' preserved in the soil is a valuable resource to archaeologists, providing a wealth of information on humans and animals at both the individual and population level, via macroscopic examination and chemical analysis. Both these types of data can be altered or even destroyed by the changes that occur to the bone after the death of the individual, and before the analysis of the bone in the laboratory. The changes that occur in the depositional environment are termed diagenesis. Although archaeological bone diagenesis has been the subject of numerous studies, and a number of general trends have been observed, many of the processes that occur are still not fully understood in terms of when they occur, and the effects that they have on the bone. Many studies are limited by the number of, the type of, or the age of the sites studied. We present here the measurement and analysis of a number of diagenetic parameters of over 200 bones excavated from more than 40 sites in 5 different European countries.

Keywords

Bone Diagenesis, Diagenetic Parameters, Fossilisation, Microbial attack.

3.2.2 Introduction

'Bone' or archaeological or fossil bone as we shall call it here, is a valuable resource for archaeologists, and palaeontologists. It provides the most direct evidence of human and animal presence at a site. Physical and chemical examination of fossil bone can reveal a remarkable amount of data about the bone that can be extrapolated to information about the living organism, and the activities at the site. The amount and validity of this information is in part dependent on the state of preservation of the material.

Fossil bone excavated from archaeological and palaeontological sites has undergone a number of transformations in the period after death and prior to excavation. Understanding these transformations is crucial as; (a) they alter the physical and chemical properties of the bone, which may affect the outcome of analyses carried out on them, (b) they are crucial to understanding the local site formation processes, and (c) they aid our understanding of the formation of the fossil record as a whole, having important conservation and heritage management implications.

The transformations that occur in fossil bone are dependent on a large number of interacting factors. This means that the states of preservation of (or conversely kinds of diagenetic changes to) fossil bones can be varied, ranging from well preserved bone, to that which is completely destroyed, and many levels between these extremes. Understanding the processes that have created any one fossil bone is thus complex.

This complexity has lead to a number of ways of studying fossil bone. Most studies rely on 'measuring' or defining one or more properties of the bone, and comparing this to the values of fresh modern samples to ascertain how or how much the property has altered. Bone is a composite material composed of protein (90% of which is collagen) and mineral (a biologically formed non-stoichiometric calcium hydroxyapatite, with 3-6% carbonate incorporated). Changes to the protein and mineral fractions are usually measured by either simple nitrogen determination of the whole bone (or collagen extraction) for protein; and by measuring the increase in the crystallinity of the mineral phase using either infrared spectroscopy (Weiner & Bar Yosef, 1990) or X-ray diffraction (Person *et al.*, 1995). The preservation of proteins other than collagen has also been studied (Tuross, 1993), as have the elemental (Trueman, 1999) and isotopic (Lee-Thorp, & van der Merwe, 1991) compositions of bone mineral. In addition to the chemical examination of bone the physical degradation is also monitored. Macroscopic examination of fossil bone can reveal much about the history of a fossil, e.g. whether it has undergone predation, butchering or burning events. Micromorphological preservation of the bone is often assessed using light or scanning electron microscopy to view the incidence of *post mortem* microbial and fungal attack on the bone (Hackett, 1981). Alteration to the pore structure of bone has been investigated, using water and mercury intrusion porosimetry (Nielsen-Marsh & Hedges, 1999).

The list of parameters above is by no means exhaustive and for logistical reasons many studies of bone diagenesis tend to measure only one or two of the above parameters, and/or focus on an assemblage from one site. This can limit the study to one particular species, to one particular time period, and one particular set of environmental circumstances, making a synthesis of general diagenetic trends difficult.

Hedges *et al.*, (1995) used the approach of measuring simple 'diagenetic parameters' of bones from three bone assemblages from sites with different environments and ages in attempt to answer a number of questions about the fundamental changes that occur to bone in the fossil record. They measured the mineral (crystallinity increase) protein, and porosity changes, and semi-quantified the damage to the histological structure. This study played an important role in revealing some of the underlying patterns of bone diagenesis, however, the results posed as many questions as they answered, as it was still difficult to discern the mechanisms of the changes involved. Further work using a similar approach (Nielsen-Marsh & Hedges, 2000), has extended and clarified this original work. Some of the concerns of the above studies are that the majority of the bones studied are from northwest European sites, from temperate climatic zones, and date from the Holocene. They may thus be representative of general diagenetic trends of bone (if such trends exist) deposited under such circumstances, but may not reflect the possible variation of bone overall. Another concern is the appropriateness of the parameters measured - Are they adequate to describe the processes of fossil bone diagenesis? Do they provide useful information with enough accuracy and precision?

As part of a major European funded initiative on bone degradation (Kars & Kars, 2001) a number of diagenetic parameters were measured on over 200 bones from more than 40 sites in 5 different European countries. The diagenetic parameters used have been selected to be, in part, comparable to those used by Hedges *et al.*, (1995) and Nielsen-Marsh & Hedges (2000), and thus supplement these data sets. They have also been extended to facilitate more detailed measurements (see below). The geographic and temporal range of the material is similar to these previous studies with many of the bones excavated from temperate northern European Holocene sites, although the geographical range of sampling extends to southern Italy and Turkey. As well as being used to supplement and test the data produced in other studies the results are also discussed with reference to their impacts on information that may be gleaned form analysis of the bone.

3.2.3 Materials

Modern bovine tibia was obtained from a local butcher. The periosteum and marrow were removed with a scalpel. The bone was then sawn into chunks and defatted for 24 hours in acetone. The chunks were then freezer milled under liquid nitrogen into a powder. Chunks and powder were used as the standard modern bovine bone for subsequent analysis, unless otherwise stated.

Archaeological bone was excavated from a number of sites throughout Europe (Britain, the Netherlands, Italy, and Sweden). The age of the material ranged from *c*6000BP to *c*200BP, it was recovered from a variety of different depositional contexts (graves, rubbish pits, ditches, living surfaces) and contained various skeletal elements (see Kars & Kars, 2001 for more details). Of the 223 bones analysed, complete data sets were produced for 196, the data can be found in appendix C. Brief overviews of key site archaeology and geological settings can be found in appendix D.

3.2.4 Results and Discussion

The data is summarised here, using principal components analysis (PCA), simple bivariate plots and examples from key sites, highlighting the most important findings, the full data set can be found in Kars & Kars (2001). Details of the methods are given in the relevant sections.

3.2.4.1 Principal Components Analysis (PCA)

Principal components analysis has been used to simplify the large and complex data set generated, by the nature of this simplification data is lost, however it is useful to try to identify 'diagenetic trajectories' or modes of diagenetic alteration. PCA was carried out on 196 samples for which all analyses had been carried out. A number of variables were excluded from the analysis, as they are known to correlate well with each other (e.g. %N and % 'collagen'), or because of anticipated skewing of the data (e.g. C:N ratio of the collagen, where extreme values may over emphasise the importance of this factor, which has a specific relationship to collagen yield [see below]). The histological index was converted to a % of the original structure remaining (see Millard, 2001), rather than the category score for PCA. Table 3.2-i, shows the correlation matrix (r values) for the parameters used in the PCA.

	% 'collagen'	Crystallinity Index	Carbonate Phosphate ratio	Bulk Density (gcm-3)	Skeletal Density (gcm-3)	Pore volume (0.01-0.1µm diameter)	Pore volume (>0.1µm<10 µm diameter)	Pore volume (>10µm-70µm diameter)	Histological Index
Crystallinity Index	-0.5501	-	-	-	-	-	-	-	-
Carbonate Phosphate ratio	0.5676	-0.6062	-	-	-	-	-	-	-
Bulk Density (gcm-3)	0.5570	-0.2746	0.4917	-	-	-	-	-	-
Skeletal Density (gcm-3)	-0.2410	0.3014	-0.1577	0.1930	-	-	-	-	-
Pore volume (0.01-0.1µm diameter)	-0.5190	0.3178	-0.3679	-0.4320	0.5045	-	-	-	-
Pore volume (>0.1µm<10µm	-0.4444	0.3098	-0.3965	-0.6255	0.0208	0.0723	-	-	-
Pore volume (>10µm-70µm diameter)	-0.2974	0.2523	-0.3070	-0.5445	-0.0292	0.2309	0.2381	-	-
Histological Index	0.3512	-0.1282	0.3113	0.5357	0.1537	0.0847	-0.7234	-0.1304	-
Cracking Index	-0.2570	0.3462	-0.3037	-0.2529	0.3074	0.6530	-0.0787	0.2401	0.3576

Table 3.2-i. Correlation Matrix (r-values) of diagenetic parameters for European Holocene bones

The correlation matrix demonstrates that many of the parameters co-vary, but none of the correlations have high statistical significance. Principal component 1 (accounting for ~39% of the variation) appears to represent diagenesis in a very general sense

(Figure 3.2-A). It correlates strongly with decreasing values for 'collagen' (see Table 3.2-ii), carbonate phosphate ratio, and bulk density, it also shows some inverse correlation with the splitting factor and the porosity components (particularly the two smaller pore diameter ranges). Principal component 2 (~22% of the variation) appears to discriminate between bones with histological attack and those without, although other parameters also correlate contributing to the discrimination, i.e. the amount of cracking and the skeletal density. Notably the pore volume in the small pore range (~0.01-0.1μm diameter pores) is positively correlated with this factor, and that in the mid pore range (>0.1μm<10μm pore diameter) negatively correlated. This factor can be interpreted as displaying two quite different modes of bone diagenesis; 'fossilisation' and microbially mediated degradation.

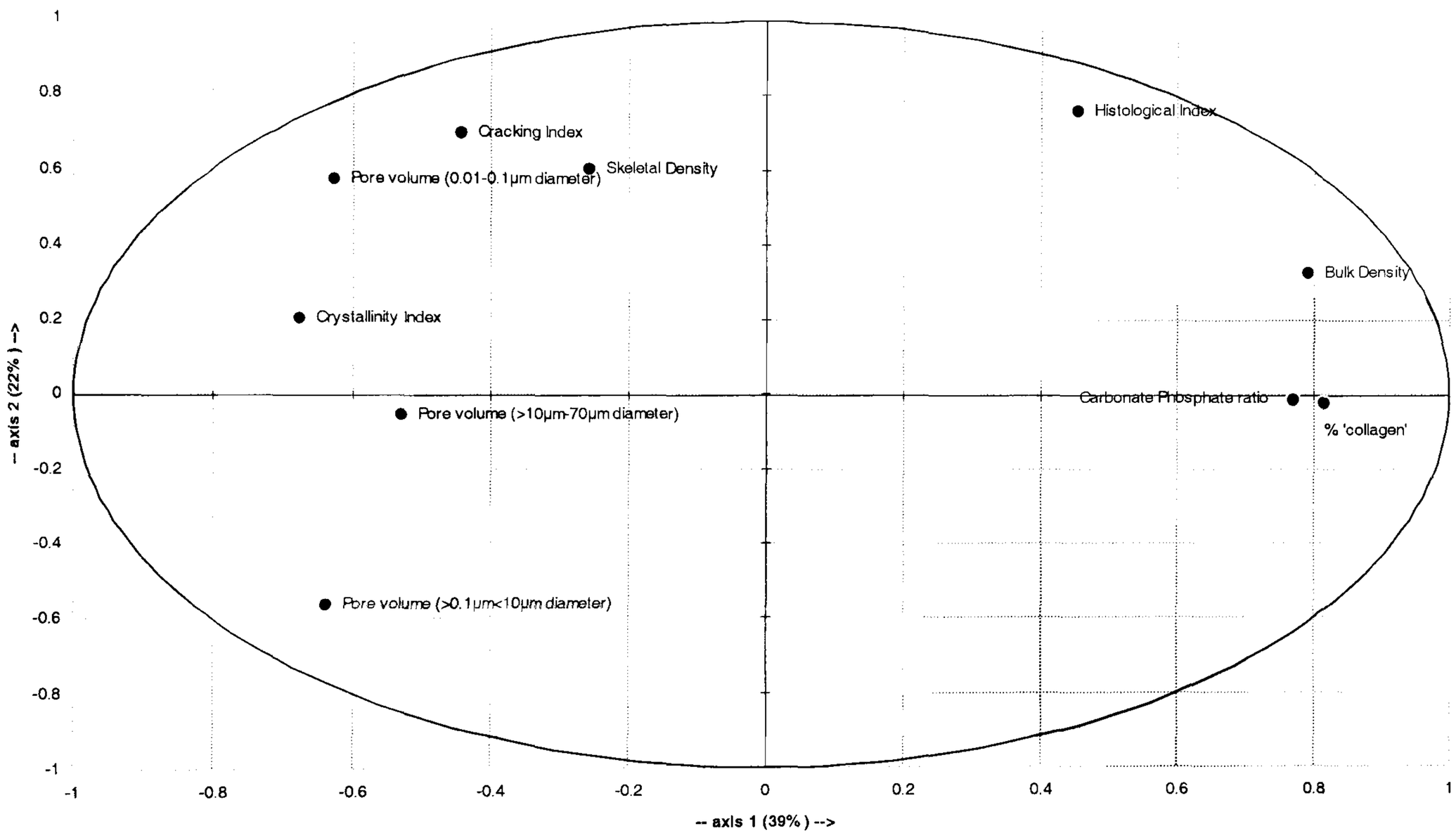

Figure 3.2-A. Correlations circle for principal components 1 and 2 after PCA of European Holocene bone diagenetic parameter data.

A further 10% of the variation in the data can be explained by Principal component 3. The main discriminating factor in component 3 appears to be porosity in the larger pore range (>10μm-70μm diameter pores), and a negative correlation with the skeletal density. This may represent dissolution of the bone.

The above explanations of the principal components can be further illustrated by plotting the principal components values of bones from key sites (Figure 3.2-B).

	factor 1	factor 2	factor 3	factor 4	factor 5	factor 6	factor 7	factor 8	factor 9	factor 10
% 'collagen'	0.8141	-0.0218	0.1685	0.0342	0.1008	0.4610	-0.1818	0.2126	0.0797	0.0009
Crystallinity Index	-0.6758	0.2036	-0.2892	0.5365	0.0368	0.0659	0.2726	0.2195	0.0463	-0.0045
Carbonate Phosphate ratio	0.7698	-0.0135	0.1231	-0.3671	0.1722	-0.0373	0.4651	0.0924	-0.0359	-0.0086
Bulk Density (gcm-3)	0.7900	0.3285	-0.3896	0.1981	0.1348	-0.0250	-0.0212	-0.0833	-0.1057	0.1991
Skeletal Density (gcm-3)	-0.2594	0.6031	-0.5503	-0.2651	0.4086	0.0713	-0.1007	-0.0238	-0.0251	-0.1112
Pore volume (0.01-0.1µm diameter)	-0.6273	0.5761	0.0772	-0.4084	-0.1182	-0.1025	-0.0695	0.1950	0.1273	0.1354
Pore volume (>0.1µm<10µm	-0.6376	-0.5614	-0.2075	-0.1785	0.0798	0.3296	0.1487	-0.1939	0.1462	0.0857
Pore volume (>10µm-70µm diameter)	-0.5310	-0.0541	0.5937	0.1431	0.5754	-0.0685	-0.0449	-0.0133	-0.0392	0.0515
Histological Index	0.4549	0.7633	0.2340	0.1883	-0.0031	-0.0280	0.0832	-0.1954	0.2704	-0.0340
Cracking Index	-0.4434	0.6988	0.3100	-0.0280	-0.2108	0.3193	0.1032	-0.0999	-0.2263	0.0021
% of variability	0.3889	0.2235	0.1134	0.0789	0.0622	0.0450	0.0381	0.0233	0.0185	0.0082

Table 3.2-ii. Correlations (r-values) between diagenetic parameters and the Principal components for European Holocene bones.

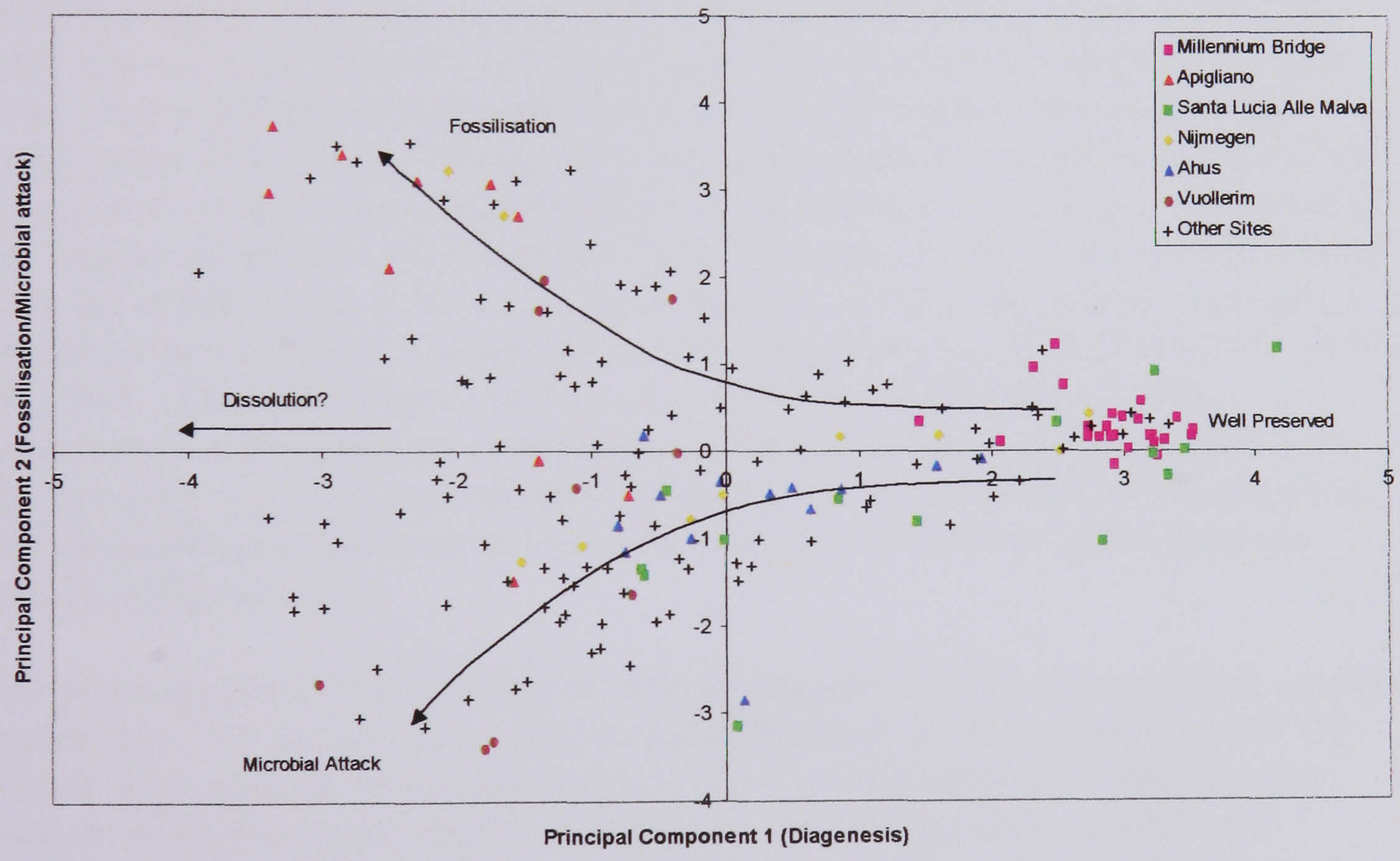

Figure 3.2-B. Principal component scores (PC2 vs. PC1) for bone from the European Holocene. Possible interpretations given.

Detailed descriptions of the sites can be found in appendix D. Well preserved bone from Millennium Bridge, Santa Lucia alle Malve and Nijmegen, have positive principal component 1 (PC1) values, and either neutral, or slightly positively PC 2 values. The main mode of diagenesis at Åhus and Santa Lucia alle Malve (for bone that is not well-preserved) is microbial attack. This is represented by the trajectory from the well-preserved bone to the double negative area of the plot. The opposite trajectory is that of 'fossilisation', represented by Apigliano (although some bone from this site does show a microbial attacked trajectory). The site of Vuollerim shows an interesting contrast in preservation; the main mechanism for bone degradation at this site is quite severe microbial degradation, however three bones plot on the fossilisation trajectory. They have high histological preservation; very high crystallinity index (the infrared spectra are similar to those in Stiner *et al.*, 1995 for the highest burnt category) and they have very low collagen levels. There is also archaeological evidence of burning of the bones (charring, found in hearth structures). This obviously suggests that these bones have experienced a high temperature event (in excess of 500°C), which produces a type of bone preservation similar to the 'fossilisation' of Apigliano bone (see Chapter3.3). The site of Nijmegen is unusual in that it has examples of well-preserved bone, and bones on the microbial attack, and fossilisation trajectories. This is somewhat misleading as Nijmegen actually has three periods within the site (see Appendix D). Bone from the Roman layers appears to be 'fossilising', whilst the Later Medieval material (1600-1800AD) is undergoing microbial attack. Material excavated from the earlier medieval layers (1500-1700 AD), appears to be generally well preserved. The soil properties at the site do not reveal major differences that might account for the differences observed in the preservation of the bone. The fact that the middle period of the site also has the best preservation would suggest that the length of interment is not a factor. One possible explanation is different pre-burial factors for the material. For example, the material from the middle phase at Nijmegen includes infant burials. It has been suggested that microbial attack of bone may be initiated by endogenous gut flora (Child 1995, Bell *et al.*, 1996). The undeveloped gut flora of infants may explain the excellent preservation of these burials compared to others at the site. It is interesting to note that nowhere else is well-preserved bone found with fossilised material, suggesting that the conditions that result in fossilisation are harsh and never allow bones to remain well preserved.

The proposed dissolution pathway is not well supported by the data, in that it can be argued that there are few bones that do not show some skewing towards either the 'fossilisation process' or microbial attack. This could be because conditions that promote bone dissolution are also conducive to microbial attack, and thus the microbial element has a weighting on the PCA plot. Moreover if the dissolution conditions are severe, then the logical conclusion is complete dissolution of the bone, and thus no data.

The interpretation of the PCA can only give a general idea of the diagenetic processes, as by its nature it simplifies the data, and indeed sometimes the interpretation using only PCA can be slightly misleading. Specific diagenetic parameters and their relationships to others are dealt with below.

3.2.4.2 Protein

It is normally considered that collagen will be lost by one of two principal methods in the burial environment, either through the action of bacteria and fungi, or through chemical loss mainly hydrolysis of peptide bonds (Child, 1995). The effects of bacteria and fungi can be seen under light microscopy as tunnels and microscopic focal destructions (MFDs), thus in bone with perfect histology (HI=5) it is assumed that collagen is lost slowly via chemical hydrolysis (von Endt & Ortner 1984). These two mechanisms are not mutually exclusive, thus whilst microbial attack occurs chemical hydrolysis will also proceed, albeit at a considerably slower rate.

3.2.4.2.1 Whole bone nitrogen content

Bone samples were cleaned and ground in an agate pestle and mortar to a fine powder. The amount of nitrogen in the whole bone powder was measured using a Carlo Erba 1106 Elemental Analyser in accordance with the manufacturer's instructions. Analyses were carried out in duplicate (mean values are given).

3.2.4.2.2 % 'Collagen'

Bone shards (dimensions less than 0.5x0.5x0.5cm) of known weight (less than 0.06g) were demineralised in 2mls of 0.6M HCl overnight in 2 ml eppendorf tubes. The tubes were then centrifuged (at 6000 rpm for 5 minutes). The acid was decanted, and the remaining acid insoluble residue was washed by the addition of 2mls of distilled water and a second centrifugation. The wash was repeated twice more. The acid insoluble fraction was then oven dried overnight at 65°C, and weighed. The carbon and nitrogen values of a sample of the acid insoluble fraction were then obtained using a Carlo Erba 1106 Elemental Analyser as above. Elemental analysis was carried out in duplicate. The percent 'collagen' is calculated by multiplying the weight of the insoluble fraction (as a percentage) by the amount of nitrogen in the insoluble fraction (as a percentage), this is then divided by the amount of nitrogen in modern bone powder insoluble fraction (16.74%, n=20). The C:N ratio (as atom percent) was calculated to assess if the insoluble fraction is collagen (Ambrose, 1990).

3.2.4.2.3 Histological index and 'collagen' yield

Figure 3.2-C shows a plot of histological index vs. collagen yield. This plot shows a general decline in collagen yield with decreasing histological index. In bone with HI 5 there is a bivariate distribution with bones with either high 'collagen' yields or low 'collagen' yields, this is also seen in the data of Hedges *et al.*, (1995) and Nielsen-Marsh & Hedges (2000). The former can be explained as well-preserved bones. It would seem counter intuitive for histologically perfect bones to have lost all their collagen unless they are very old, or have been maintained at a high temperature (perhaps burnt or cooked).

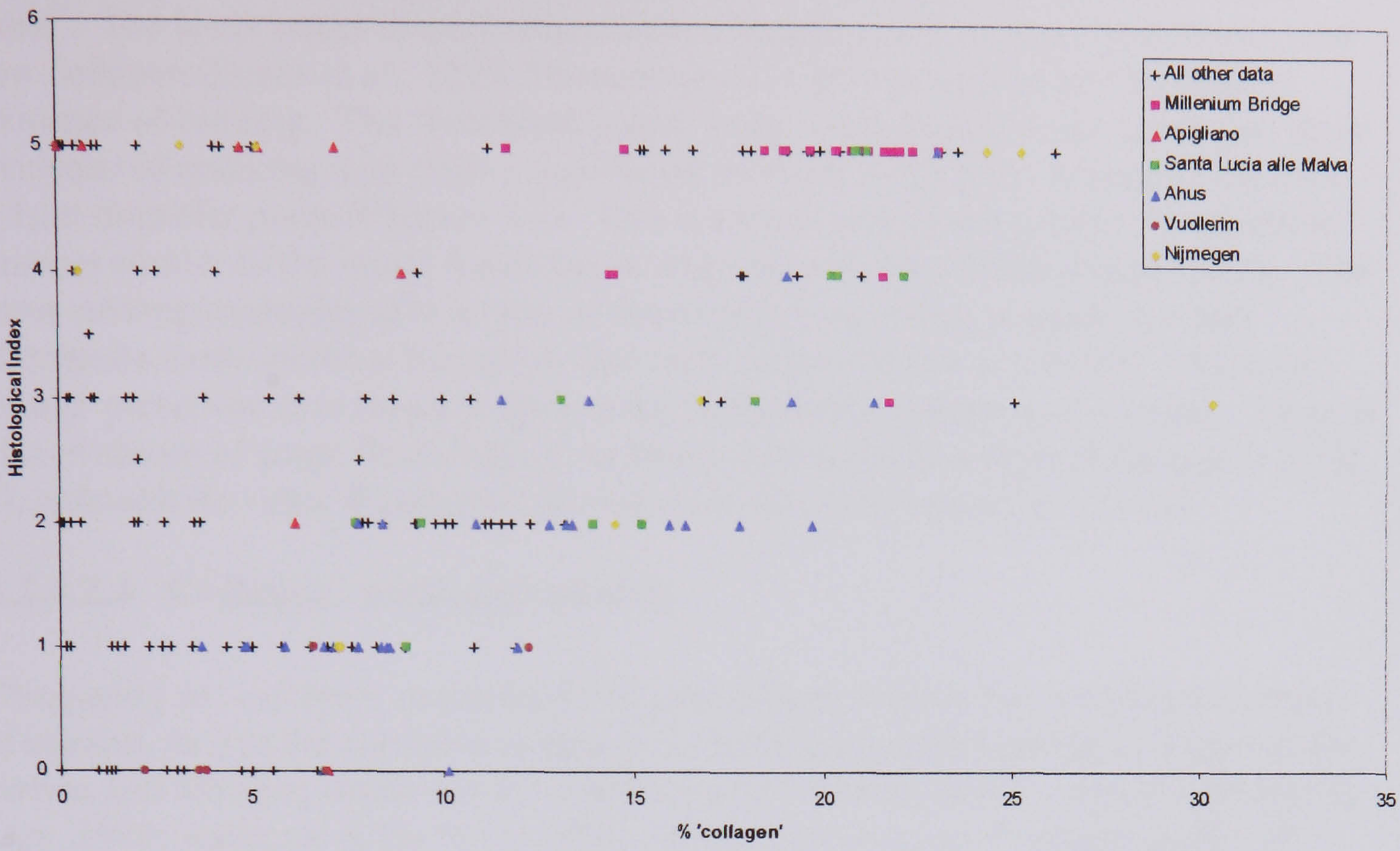

Figure 3.2-C. Histological Index vs. % collagen for European Holocene bones

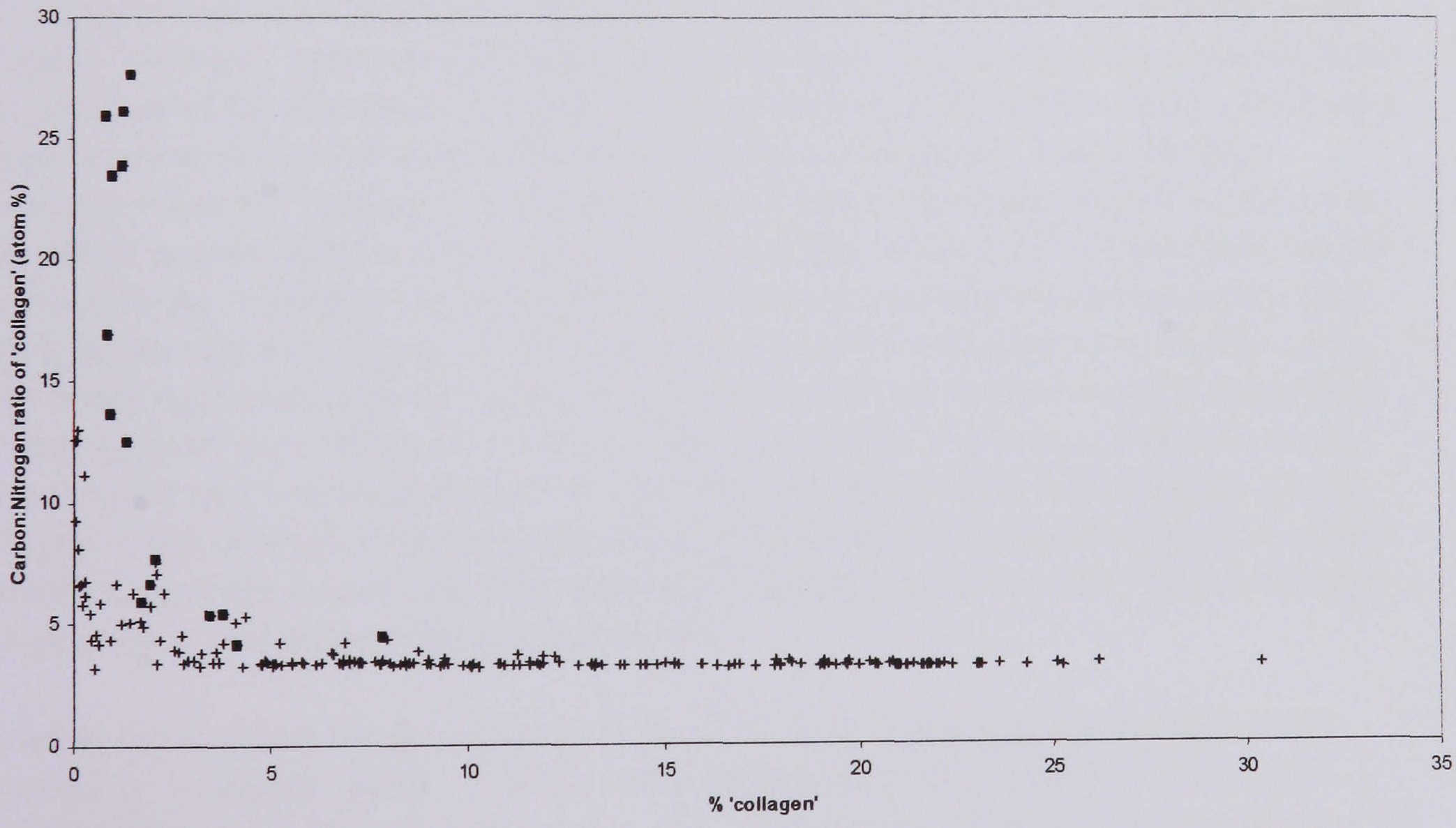

Figure 3.2-D. Carbon:Nitrogen ratios of 'collagen' from European Holocene bones. ■ From Ypenburg, + all other sites. (Not all data is shown).

The latter group contains two distinct types of preservation, burnt and 'fossilised bone'. The burnt bones display characteristic features such as high crystallinity, and low collagen (Stiner *et al.*, 1995, Shipman *et al.*, 1984) as well as archaeological evidence of burning. The 'fossilised bones' share these characteristics, but also show evidence of cracking, and often a significant increase in the pore volume in the 0.01-0.1μm diameter pores (Chapter 3.3). This bone has undergone a rapid 'fossilisation' process similar to the initial fossilisation stage described by Pfretzschner (2000). The most striking examples of this type of preservation are found at Apigliano and Hierapolis, both southern European sites with carbonate rich sediments. However, similar preservation is found at sites in the Netherlands, Britain and Sweden. There is also evidence of some 'fossilisation' in bones with some histological damage (i.e. HI 4), although the other diagenetic parameters of this bone are not as altered.

3.2.4.2.4 'Collagen' yield and quality

The quality of 'collagen' extracted from archaeological bone has long been a source of interest, as it is the substrate of choice for both radiocarbon dating of bone and for carbon and nitrogen stable isotope measurements in palaeodietary studies (Hedges & Law, 1989; Ambrose & De Niro, 1986). It has been suggested that the quality of 'collagen' extracted from archaeological bone is dependent on the amount of 'collagen' in the sample and that for quality control purposes in palaeodietary studies the carbon:nitrogen ratio of the 'collagen' should be in the range of 2.9 – 3.6 (Ambrose, 1990). For radiocarbon dating a number of methods can be employed to ascertain the quality of the extracted 'collagen', however as a rule of thumb meaningful dates can usually be obtained from bones with greater than 2-5% of the original 'collagen' remaining (Hedges & van Klinken 1992). The data obtained from the analysis of the European bones here is in agreement with these studies, showing a large increase in the C:N ratio with lower levels of 'collagen'. The C:N ratio increases when the 'collagen' yield drops below about 5% of the weight of the bone, this effect is even more marked when the yield drops below ~2%. One reason for the increase in the 'collagen' nitrogen ratio may be contamination from soil humic and fulvic acids. Humic substances will increase the carbon:nitrogen ratio significantly. The bones represented by full squares in Figure 3.2-D are from the site of Ypenburg, where many of the C:N ratios are high. The bone at this site was in a poor state of preservation and was stained dark brown. The site was coastal with shallow graves dug into sand, with an overlying peat layer. One explanation for the high C:N ratios and staining of the bones would be that humic substances have been washed through the peat layer and accumulated in the bone.

Another explanation for the increase in the C:N ratio is just a general effect of the decreasing 'collagen' yield. There is some evidence for this, Figure 3.2-E, shows a detail of Figure 3.2-D, with a reduced *y-axis*. There is an apparent increase in the C:N ratio with decreasing 'collagen' yield, although the majority of the points still fall between the quality parameters of Ambrose, 1990. It may be that as the 'collagen' yield decreases the amount of actual collagen decreases, and thus the 'collagen' fraction becomes dominated by other persistent proteins. It has been noted that the Gla-rich region of osteocalcin has a strong affinity for collagen (R. V. Prigodich, pers comm.), if Gla residues are bound to the collagen this may have the effect of increasing the C:N ratio (see Masters, 1987).

3.2.4.3 Mineral Changes

Changes to archaeological bone mineral can take on a variety of forms, loss of the mineral (dissolution), transformation of the apatite to another form e.g. brushite in acidic conditions or francolite in lithified fossils, or just changes in the existing bioapatite crystals. Bone mineral in vivo has very small thermodynamically unstable crystals, which in the geosphere become increasingly crystalline (i.e. larger, more perfect, better ordered, with less strain), this is measured by the crystallinity index.

The crystallinity of the mineral fraction was measured using infrared spectroscopy of bone powder (~1mg) crushed into a KBr (60 mg) pellet. The crystallinity index or infrared splitting factor (IRSF) is calculated using the splitting ratio of the phosphate *v*4 doublet at 567 and 605cm^{-1} (Weiner & Bar-Yosef, 1990). The infrared spectrum was also used to identify the presence of other mineral phases.

Another major change is the loss of carbonate from the crystal matrix. This has not been measured directly, but by using the ratio with the phosphate (C:P). The carbonate:phosphate ratio (C/P) was also calculated from the infrared spectrum using the peaks at 1415cm^{-1} (CO_3^{2-}), and 1035cm^{-1} (PO_4^{3-}). This measurement can be compromised by collagen that also absorbs in the 1415cm^{-1} region, and is thus only used as semi-quantitative measure.

The relationship between the crystallinity index (as measured by infrared splitting factor) and the C:P ratio has been observed before (Nielsen-Marsh & Hedges 2000). The data here is a useful supplement as it not only increases the number of samples analysed, but also extends the range of crystallinity index values upwards, displaying an otherwise unknown feature of the relationship. Figure 3.2-F shows a plot of C:P ratio vs. crystallinity index (IRSF) the majority of the data falls in a broad line similar to that shown by Nielsen-Marsh & Hedges, (2000), however at crystallinity in excess of 4.0 - 4.1 the relationship appears to break down. Above these values the C:P ratio no longer decreases, it remains at approximately 0.1. This change in the relationship between the C:P and crystallinity index appears to signify that a crucial change has occurred in the mineral. It could be that the 0.1 C:P ratio value indicates the loss of all the carbonate from the bone, and this value is simply a methodological artefact, or that the value is real, but approximates to the lowest possible value, either way when this value is reached the splitting factor is

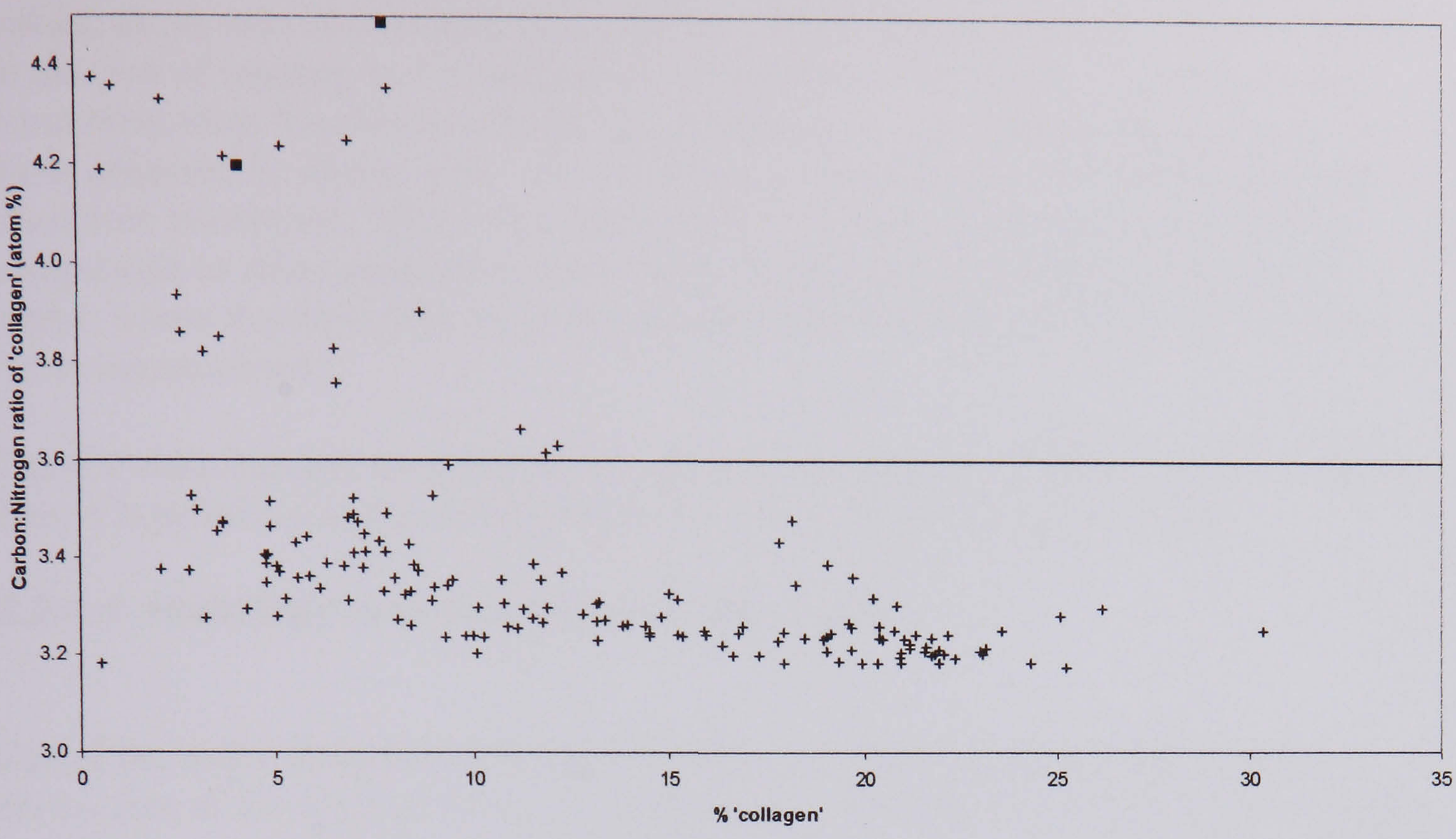

Figure 3.2-E. Carbon:Nitrogen ratios of 'collagen' of European Holocene bones, detail. ■ Ypenburg, + all other sites, the line represents the upper limit for quality control in stable isotope studies proposed by Ambrose, 1990. (Not all data is shown).

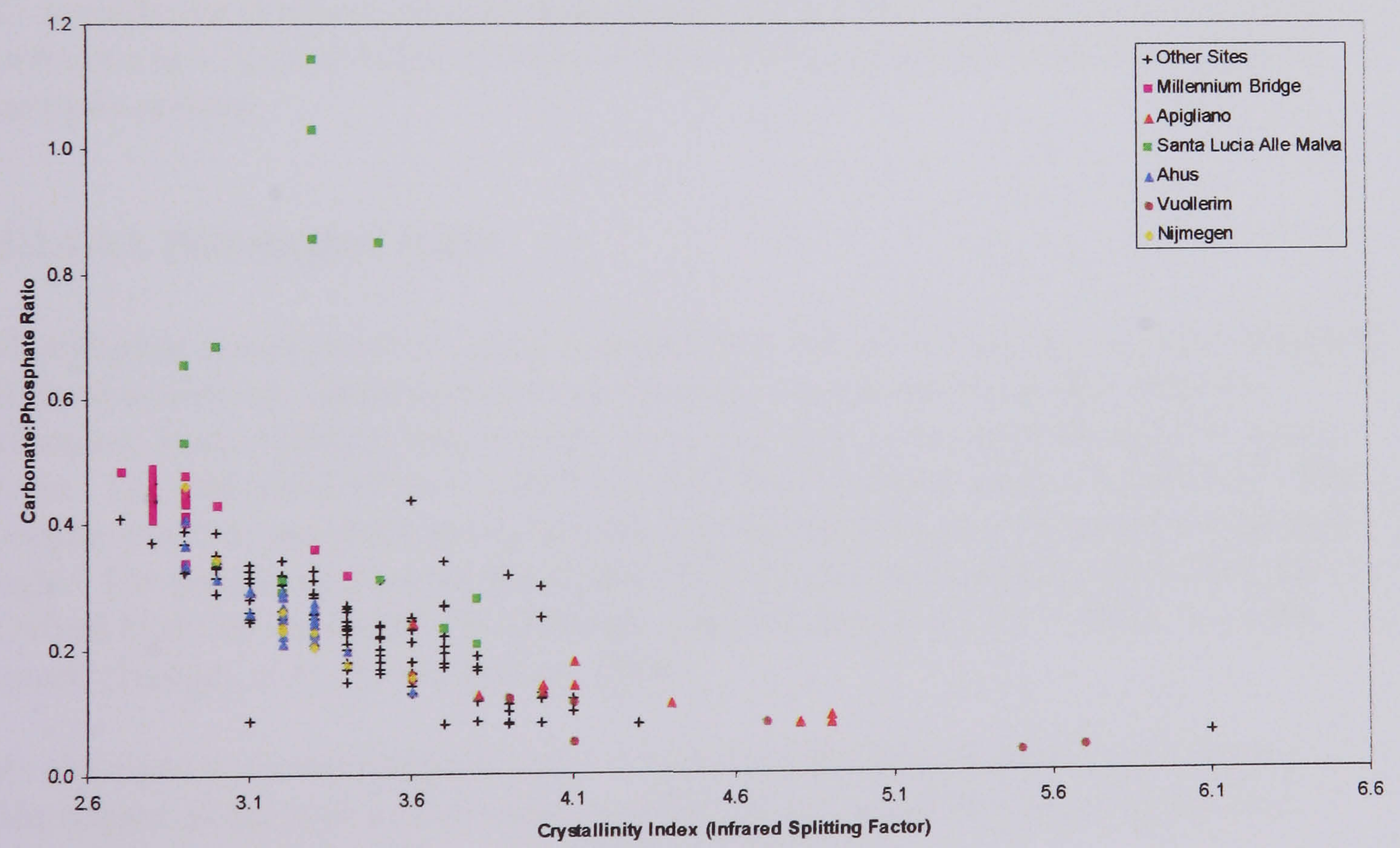

Figure 3.2-F. Carbonate:Phosphate ratio vs. Crystallinity Index measured from Fourier Transform Infrared Spectroscopy for European Holocene bones.

no longer controlled by the loss of carbonate in the same manner it is at lower IRSF values, as the loss of carbonate is negligible. This critical point seems only to occur in the case of burning and 'fossilisation' of the bone. The infrared spectra for the burnt bone from Vuollerim indicate severe changes to the mineral phase similar to those observed by Stiner *et al.*, 1995 who suggest that the mineral phase may be β-tricalcium phosphate. (N.B. the splitting factor of burnt bone may not be directly comparable to other measurements because the phosphate doublet has become a triplet, where the third peak may decrease the intensity of those used in the splitting factor measurement).

The C:P ratio can also increase by the addition of calcite from the burial environment. This is highlighted by the material from the site of Santa Lucia alle Malve.

3.2.4.4 Histology and microscopic structure

One of the major diagenetic changes that occurs in archaeological bone is the destruction of the original histological structure. The main destructive factor is attack by microbes and fungi, this type of histological destruction is in the form of tunnelling through the bone. This tunnelling causes localised areas of the bone structure to be destroyed, removing the collagen and reprecipitating the mineral in hypermineralised areas at the edge of the areas of destruction (Hackett, 1981). The extent of this destruction varies in archaeological bone such that some bones remain apparently unchanged and others have no visible histological features remaining. In an attempt to quantify the extent microbial attack Hedges *et al.*, 1995 (revised Millard, 2001) defined a histological index of bone, which could be assessed from polished thick sections of bone.

3.2.4.4.1 Histological Index

Histological preservation has been assessed here using the histological index obtained from thin sections. Bone sections for histology were prepared as described by Hermann *et al.*, (1990). Samples were sawn from the bone, and embedded in epoxy resin. The embedded bone was then cut into 30μm sections using a microtome. The section was then mounted on a glass microscope slide and viewed under transmitted light. The preservation of the bones histological features were described using the Oxford Histological Index (Ox HI) score, ranging from 5 = >95% intact, 0 = <5% intact, (Hedges *et al.*, 1995; Millard, 2001).

As anticipated, the histological index correlates with other diagenetic parameters. The results of the histological index analysis are discussed in relation to the other diagenetic parameters in the other sections of this paper.

3.2.4.4.2 Cracking

In addition to the histological index scoring, microfissures (small cracks on the scale of an osteon) were also observed and quantified (the cracking index) by counting the approximate number of osteons with and without cracking. The number of cracked

osteons is then expressed as a percentage (Jans *et al.*, in press). Cracking of the bone microstructure has been observed as a significant process in the early stages of fossilisation (Pfretzschner, 2000).
The incidence of cracking in the bones studied here can be quite variable, however it is evident at high concentrations in bone that has HI5, low collagen yields and high crystallinity index, (see Chapter 3.3), which are also characteristic of fossilised bone, hence the categorisation of these bones as 'pre-fossilised'. The cracking is most likely the result of shrinkage of the bone, perhaps as the collagen is lost, and the mineral restructured, or it could be that after these processes have occurred the bone is more prone to cracking, i.e. it is more brittle. It may also indicate that this process is the result of predominantly dry conditions, although this is difficult to assess. Burnt bone shares many of the properties of pre-fossil bone, however the cracking is less prominent in burnt bones. Lower amounts of cracking observed in bones with lower histological index, may be the result of the cracks becoming obscured as more of the histological structure is destroyed.

3.2.4.5 Porosity changes

Using water sorption porosimetry Hedges *et al.*, (1995) demonstrated that the porosity of archaeological bone is greater than that of modern bone and that this increase correlates with other diagenetic parameters (e.g. nitrogen loss). By controlling the relative humidity of the atmosphere in which sample is contained they measured three basic properties of the porosity which they defined as; total porosity, microporosity (pore volume in pores with < 4nm radii), and macroporosity (pore volume in pores with > 4nm radii). The changes to the pore structure are presumably the result of; dissolution and recrystallisation of the mineral through interaction with the ground water, the action of bacteria and fungi (microscopic focal destructions and tunnelling), and loss of collagen. It can also be postulated that under some circumstances archaeological bone may become less porous, where the natural pores are in-filled with calcite or other debris from the soil.

Water sorption analysis only provides general information about the changes to the pore structure in archaeological bone. Using mercury intrusion porosimetry Nielsen-Marsh & Hedges (1999) demonstrated that very distinct changes to the pore structure occur when protein is removed from the bone in the laboratory using hydrazine hydrate. Large increases in the pore volume of pores with radius 100-10000nm have also been observed, possibly caused by microbial attack of the bone.

Mercury intrusion analysis has been used here to analyse the pore structure of the bone excavated. The method used is principally the same as that used by Nielsen-Marsh & Hedges 1999, however the treatment of the data has been modified. In brief, chunks of bone (approximately 1g) were oven dried at 85°C for at least 16 hours. The pore structure of the bone was then investigated using mercury intrusion porosimetry (Nielsen Marsh & Hedges, 1999).

Two significant changes have been made to the data manipulation described in Nielsen-Marsh & Hedges, 1999. Firstly, the process of mercury intrusion porosimetry analysis involves forcing mercury into the sample under pressure. With increasing pressure the mercury is forced into pores with a smaller nominal diameter. The size

of the pore diameter can be calculated using capillary law, assuming that the pores are cylindrical, thus

$$D = -\frac{4\gamma \cos\vartheta}{p}$$ **Equation 3.2-a**

where:
D is pore diameter (nm),
p is the applied pressure (psi),
γ is the fluid surface tension (mJ m^{-2}) and
θ is the contact angle between the solid and fluid.

Nielsen-Marsh & Hedges (1999) reported their data using a contact angle of 141° in error. The data reported here uses the contact angle for apatite of 163.1°. This change alters the distribution of the pores, as the pressure:diameter ratio is changed, such that the pore ranged measured is approximately 0.01μm-70μm, and not 2.8 nm to 36,444 nm as stated in Nielsen-Marsh & Hedges, 1999.

Secondly, the pore size distribution data will be plotted here as the porosity [volume of mercury intruded (ml) per ml of bone] vs. nominal pore diameter in microns.

In addition to the pore size distribution analysis mercury intrusion analysis provides data on the bulk density of the sample (the density of the sample as a whole) and the apparent (skeletal) density (the density of the material itself) in gcm^{-3}.

3.2.4.5.1 Porosity Results and discussion

The porosimetry results will be discussed firstly with reference to some of the key changes observed in the porosity of archaeological bone, with examples from key sites. The porosity changes in the European data set as a whole will then be discussed again with reference to key sites.

3.2.4.5.2 Modern and Deproteinated bone

Figure 3.2-G shows the pore size distribution traces for modern cow tibia, and deproteinated modern cow tibia (data taken from Nielsen-Marsh & Hedges 1999 and re-plotted). The samples are not from the same individual.

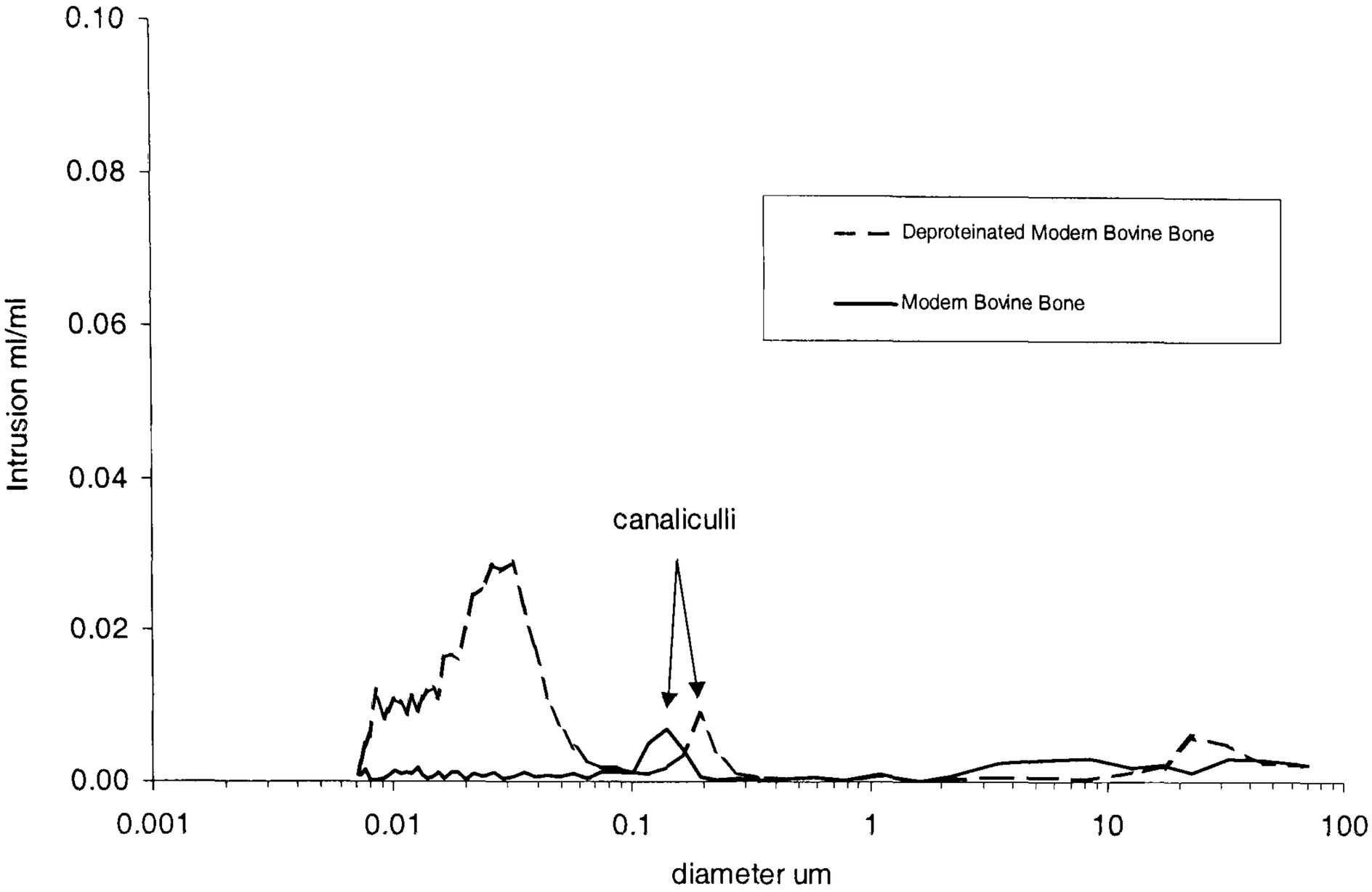

Figure 3.2-G. Mercury intrusion porosimetry traces for modern bovine bone, and deproteinated modern bovine Bone.

The modern cow bone has a relatively even distribution of volume in pores of all diameters measured by this method, with the exception of a peak at ~ 0.15μm, which may represent the filling of the canaliculi with mercury. The deproteinated bone has been treated with hydrazine hydrate to remove the protein from the bone with minimal affect on other diagenetic parameters (e.g. crystallinity index). It is clear that this treatment has had a significant effect on the porosity of the sample. The deproteinated bone shows a vastly increased amount of porosity in the lowest pore diameters. The canaliculi feature appears to have shifted to a slightly higher diameter range, and there is a little more porosity at the higher diameter pores (~ 11μm), although it is not clear if these are significant changes caused by the treatment, or are the result of natural variation in the samples or random experimental errors. The key changes to the deproteinated bone are the removal of protein, and a slight increase in the crystallinity index (IRSF 3.1). It is reasonable to conclude that the removal of the protein (significantly the collagen) in this fashion has lead to the increase in porosity observed.

3.2.4.5.3 Archaeological Porosity

The changes to bone porosity incurred during archaeological bone diagenesis are unique to each bone, however some features and patterns can be identified, and explained in association with other diagenetic parameters. Figure 3.2-H and Figure 3.2-I give examples of some of the most common features of the pore changes observed.

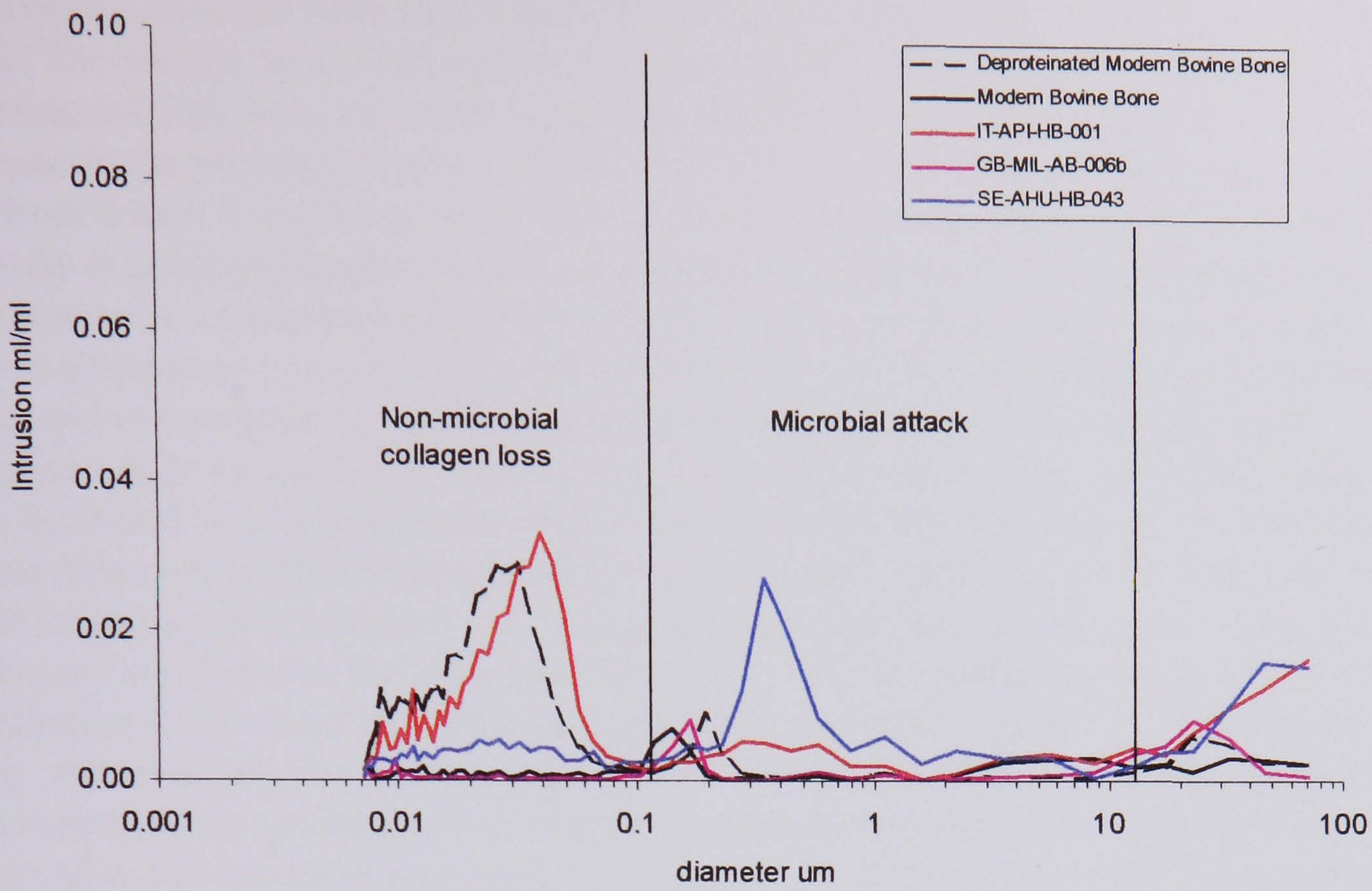

Figure 3.2-H. Typical mercury intrusion porosimetry traces of 'well preserved', 'fossilising' and 'microbially attacked' archaeological bone. Modern bovine bone and deproteinated modern bovine bone are shown for comparison.

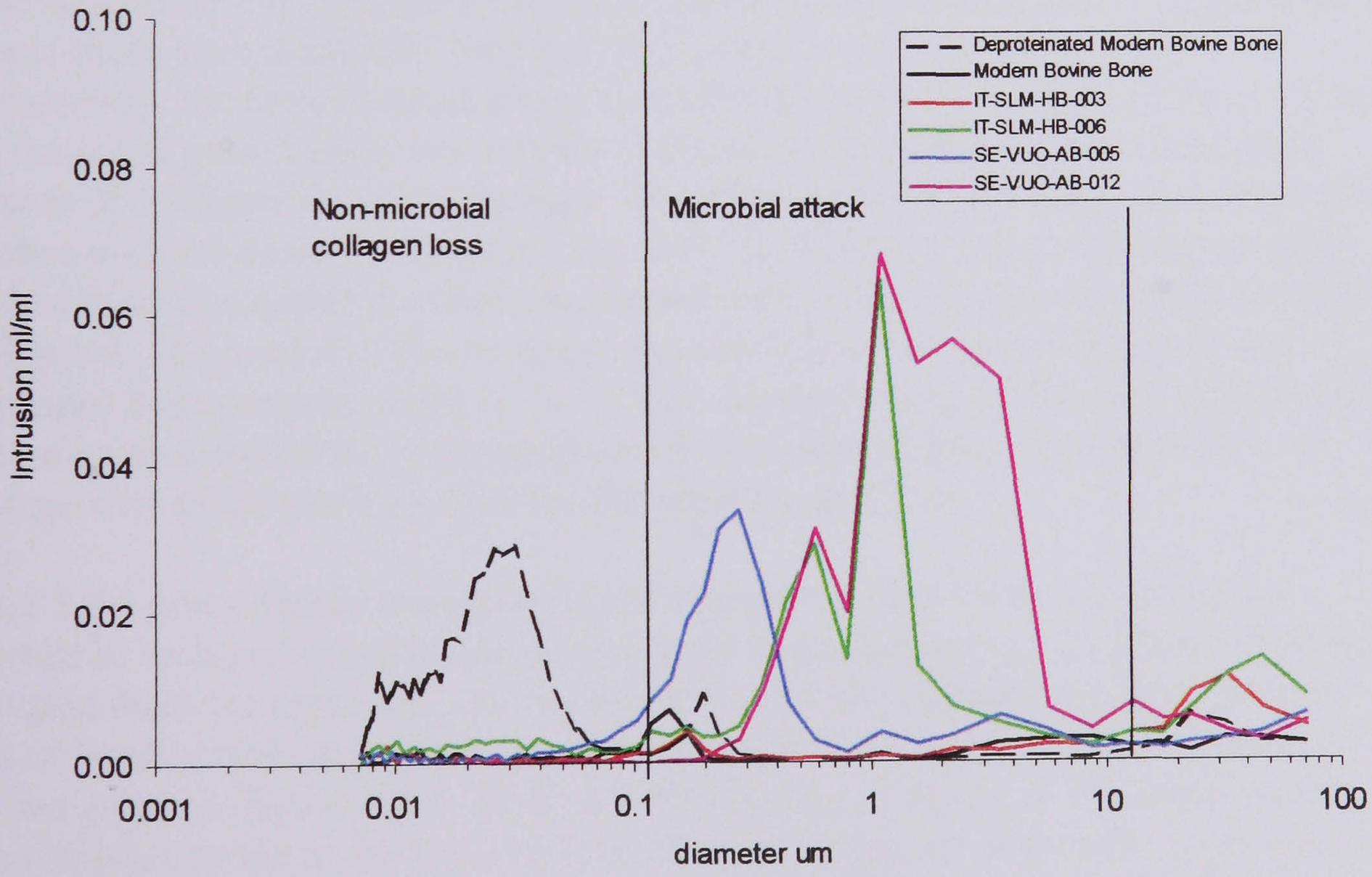

Figure 3.2-I. Mercury intrusion porosimetry traces of selected bones from Santa Lucia alle Malve and Vuollerim. Modern bovine bone and deproteinated modern bovine bone are shown for comparison.

Figure 3.2-H gives examples of well-preserved bone (GB-MIL-AB- 006b), microbially attacked bone (SE-AHU-HB-043), and 'fossilising' bone (IT-API-HB-001). The sample from Millennium Bridge is typical of this site and is virtually indistinguishable from the modern bone standard, with the exception of a small increase in the porosity in pores >8μm. The sample from Åhus is also typical of the site from which it was excavated. Compared to the modern standard the level of porosity is generally higher for all pore diameters. The most striking feature of the distribution is a sharp increase in the volume in pores with diameter 0.12-12.7μm. This is a common (although not exclusive) feature of bones with lower histological index and is interpreted as a direct result of microbial attack. The sample from Apigliano is an example of bone that has been preserved in a 'pre-fossilised' state. This bone and similarly preserved bones show a large increase in pore volume similar to that observed in the laboratory deproteinated bone. These pre-fossil bones have intact histology, low collagen yields and high splitting factors. The lack of microbial attack and similarity to the laboratory deproteinated bone indicate that this porosity distribution is the result of chemical degradation and loss of the collagen from the bone. Alternatively this feature may be the result of crystallinity changes, however the changes in the crystallinity of the pre-fossil bone are generally higher than that observed in the deproteinated bone, which is similar to those observed in microbially attacked bone. Thus if the feature is the result of crystal changes it must also be accompanied by the loss of the chemical loss of collagen. It should be noted that the deproteination-like feature is indicative of the chemical loss of collagen from the bone, and is seldom seen in bone that has been microbially attacked. Bone that has been microbially attacked has lost collagen, but tends to have porosity in the larger pore diameters, suggesting that if the porosity observed in the <0.12μm range is the pore space vacated by the collagen then the microbes must refill this area, or significantly obscure these pore diameters such that the feature is no longer observed. Alternatively, the feature could derive from the different modes of collagen loss, and thus the small pore feature can only be observed in bone that has lost significant amounts of collagen via chemical loss. These descriptions are somewhat subjective, but are a useful way of summarising the data. It should be noted that microbially attacked bone can sometimes have increased porosity in regions other than the one highlighted, and bone that has no histological evidence of microbial attack can sometimes have porosity in the 0.12-12.7μm diameter range. This may reflect a lack of correlation between the observed features and other diagenetic parameters, or heterogeneity in the samples used for the separate analyses.

Figure 3.2-I gives further examples of the changes in pore volume distribution observed in archaeological bones, showing the variation that can be observed in bones excavated from the same site. At the site of Santa Lucia alle Malve there are two types of bone preservation, microbially attacked and well preserved. Examples of each are given in Figure 3.2-I. IT-SLM-HB-006 has evidence of microbial attack, and large intrusion peaks in the 0.12-12.7μm diameter range are observed. Conversely, sample IT- SLM-HB-003, is well preserved and the pore size distribution is scarcely different to that of fresh modern bovine bone. The samples from Vuollerim are a microbially attacked bone (SE-VUO-AB-012) and a burnt bone (SE-VUO-AB-005). The distribution for the burnt bone is somewhat unusual. In most respects the diagenetic parameters for burnt bone are similar to those of pre-fossil bone, however the pore size distribution is quite different. Of the burnt bone analysed here the total porosity of the bones is lower than that of pre-fossil bones. It should also be noted

that the above distribution is not always observed in burnt material. The above distribution may be an example of sample failure (the sample collapsing under pressure) during analysis. This will be likely if the bone is highly crystalline and has lost significant amounts of collagen, and consequently much of its mechanical strength (see Turner-Walker & Parry, 1995).

As stated above, certain patterns of pore distribution appear to be related with certain types of preservation, i.e. well preserved – flat trace, pre-fossil bone – porosity in smaller pore diameters, and microbial attack – volume increase in medium sized pores, although these assignments may be criticised as rather subjective. PCA of all the porosimetry data has revealed that there are significant patterns that can be revealed in the data, and that these variations in the data do correspond with the features described above (see Kars & Kars 2001). It is has been stated above that the explanations for the pore size distributions observed do not always follow the expected patterns. The following section will deal with the relationships between the porosity and the other diagenetic parameters measured, and the interpretation of the PCA. The two key hypotheses alluded to above are that (1) the small pore range is associated with a non-microbially induced collagen loss, a process that occurs significantly in the pre-fossilisation of bones, and (2) increasing porosity in the mid pore range is associated with (if not directly caused by) microbial attack of the bone. PCA of a subset of the data has indicated that the volume of the mid pore range is inversely correlated with the histological index, and that an increase in the small pore volume is associated with the pre-fossilisation process. These hypotheses can be further tested by direct comparison of the porosity data with other diagenetic parameters. For the purposes of the following section the porosimetry trace has been divided into three sections small pores 0.007-0.117μm, mid pore range >0.117-12.7μm, and large pores 12.7-69.7μm.

3.2.4.5.4 Small pores and collagen loss

If the increase in small pore volume and collagen loss are related we would expect to see an increase in the small pore diameter range as collagen is lost from the bone. Figure 3.2-J shows that this is not always the case, but does demonstrate that for 'fossilising' bone from Apigliano the pore volume in pores with 0.007-0.117μm diameter is similar to what might be expected if the collagen is lost through extensive boiling.

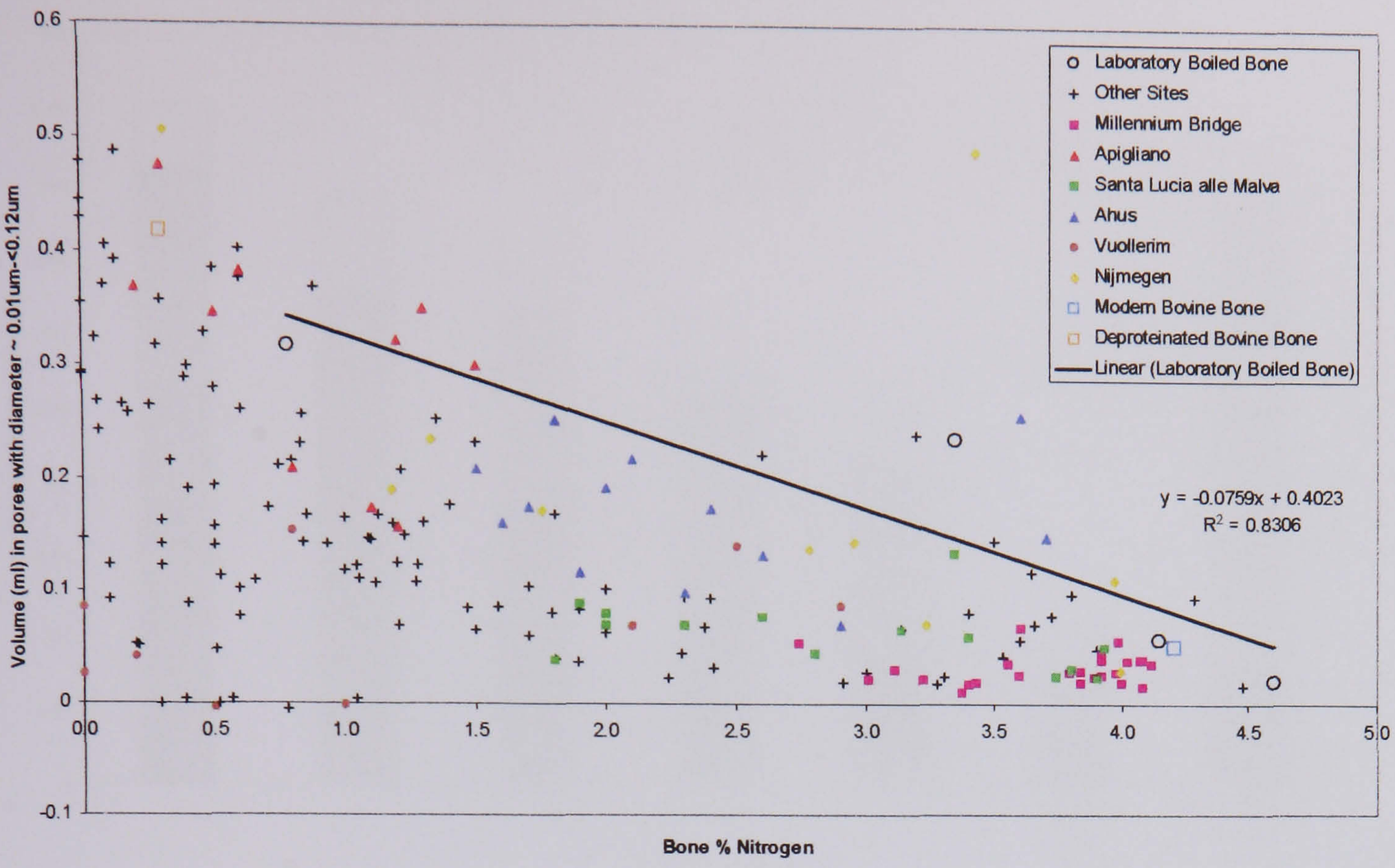

Figure 3.2-J. Porosity in small diameter pores vs. % Nitrogen of the whole bone for European Holocene bones. Laboratory boiled bone data from Roberts *et al.*, in press.

3.2.4.5.5 Medium pores and microbial attack

If the pore volume generated in the mid pore diameter range is associated with the incidence of microbial attack it might be expected that the amount of pore volume generated in this pore diameter range will be correlated with the histological index. Figure 3.2-K shows the average pore volume for each histological index score for the three pore diameter ranges. Note that the histological index score 5 has been split into bones with > 10% 'collagen' remaining by weight, and those with <10% 'collagen' by weight to account for the difference between well preserved and pre-fossil bones in this category. The pre-fossil bone category will also include burnt bone.

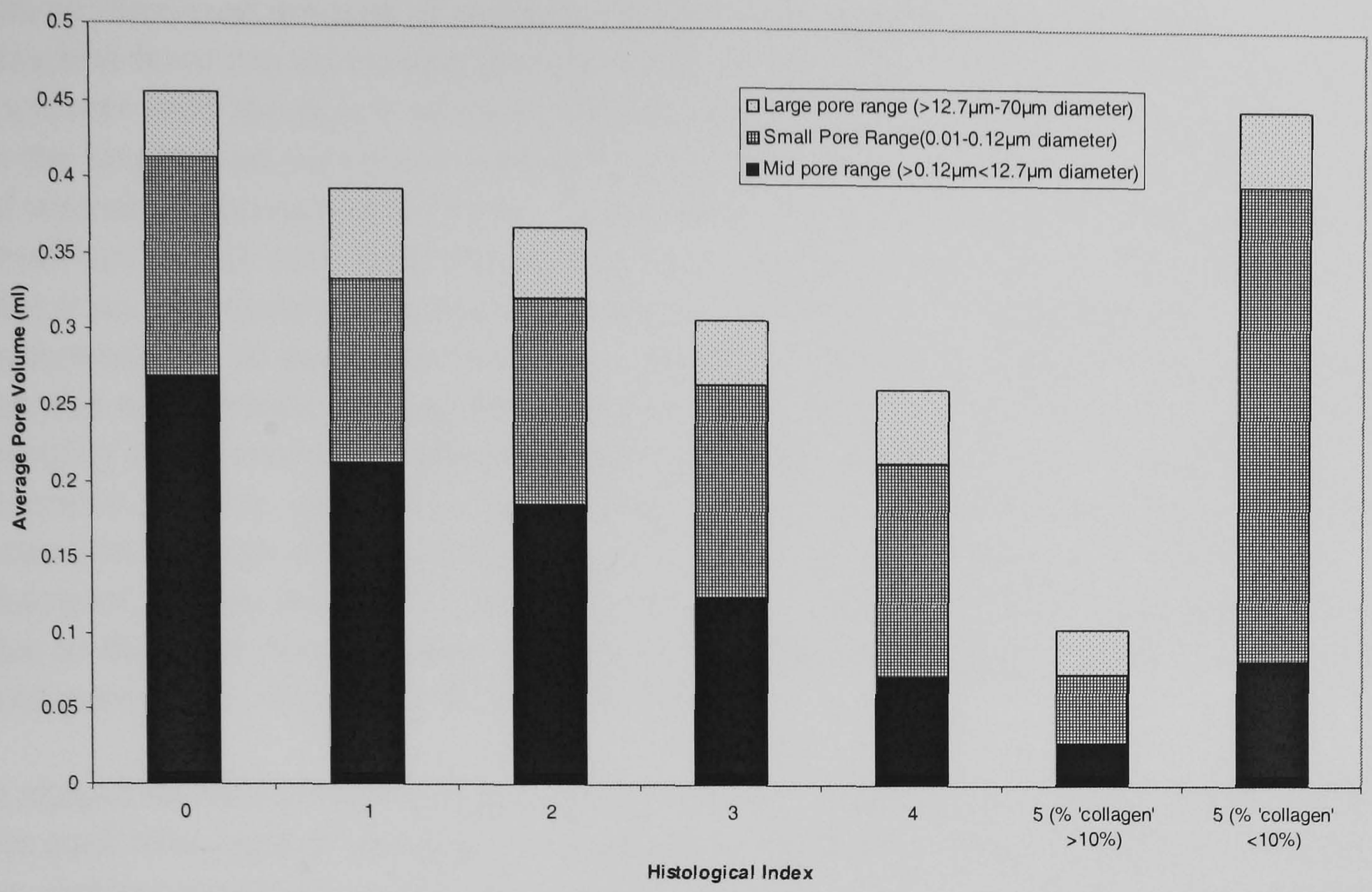

Figure 3.2-K. Average pore volumes for histological categories observed in European Holocene bones.

The average amount of mid pore range porosity increases by almost a factor of ten from well-preserved bone (HI5>10% 'collagen') to bone with HI0. The pore volume in the other pore diameter ranges increases when the bone is no longer histologically perfect, but remains at around the same value for all histological index scores. There is a large increase in the pore volume in the small pores in the pre-fossil bones. Note that the amount of total porosity increases with decreasing histological index, with the exception of the pre-fossil category which has on average total porosity as large as that of HI 0 bones.

Excluding the bones with histology scores of 5 it can be seen that the porosity of bone is greatly increased with increasing extent of microbial attack, and that this increase is almost entirely due to the increase in pore volume in the mid pore diameter range. The contribution of the volume in the small and large pore diameter ranges is almost constant (18–19%) in total, thus the increase in the total porosity is due almost entirely to the increased mid range pore diameter volume increase, which increases with decreasing histological index. In the two histological index 5 categories the amount of porosity is increased greatly in bones with less than 10% 'collagen', with the most notable increase in the small pore diameter range. The amount of large pore diameter range porosity increases in this category, but this is approximately the same amount as for histologically damaged bone.

To summarise the porosity changes, bone with histological index 5 and > 10% 'collagen' remaining has similar pore structure to that of modern bovine bone, i.e. they are well-preserved. Archaeological bone that has undergone either a 'pre-fossilisation process' or microbial attack, has a very different pore structure to modern and well-preserved archaeological bone with, on average, an 18-19% increase in porosity in the small and large pore diameters. In addition to this 'fossilising' bone

has an increased amount of porosity in pores with smaller diameters, and microbially attacked bone has increasing porosity in pores in the mid pore diameter range, that increases with the extent of observed microbial damage. The increase in pore volume in the larger pore diameters is presumably diagenetic, but is independent of the extent of microbial damage to the bone. Considering both the diagenetic pathways, pre-fossilisation and microbial attack, then this change occurs in both, which may mean that it occurs; during either process, maybe as a result of both processes, or independently of each, and that its occurrence is neither favoured by nor subsequently favours either mode of diagenetic change. Microbially attacked bones have increased porosity in the mid sized diameter pores, however the increase in the small pore diameter pores is apparently independent of the extent of microbial attack. Pre-fossil bone (on average) shows a large increase in the porosity of the pores with small diameter, and an increase in the mid pore range. Although his latter increase may be due to the burnt bones in this category, that sometimes display a large increase in the mid pore range, distorting the average value.

It should be noted that the above interpretation is made on the mean porosity values for each histological category. In most cases the distribution of porosity values could be approximated to a normal distribution, however in both HI 5 categories and for HI 4 the distribution is skewed to the lower values (i.e. the interquartile range covers the lower porosity values).

3.2.4.6 Bone Density

Bone density values are calculated as part of the mercury intrusion porosimetry procedure, and are of course related to the porosity of the material. Two measurements are made, the bulk density (the density of the sample as a whole) and the apparent (skeletal) density (the density of the core material).

In general the bulk density of diagenetically altered bone decreases and the skeletal density increases. The bulk density will increase as the bone becomes more porous, the increase in skeletal density is presumably mainly due to the loss of collagen from the bone which is less dense than the mineral phase. Figure 3.2-L shows the predicted plots of both bulk and skeletal density against collagen, assuming a collagen density of $1.105 gcm^{-3}$, and an apatite density of $3.1 gcm^{-3}$. It also shows data from selected sites. The skeletal density values for the archaeological bones are close to those predicted, however the bulk density values are over estimated here. This indicates that pore space must be created in archaeological bone in addition to that vacated by the collagen irrespective of the mode of collagen loss (chemical or microbial), assuming the collagen and mineral densities remain constant. There is an apparent offset between the measured and predicted bulk density values, which may indicate that the mineral density changes in diagenetically altered bone. The burnt bone from Vuollerim deviates greatly from the predicted lines with exceptionally high bulk density for collagen-depleted bone. This may be because some of the pore space has been filled by fusion of the mineral. For other types of diagenetically altered bone, pre-fossil bone has high skeletal density values (although approximate to those predicted), presumably due to the almost complete loss of collagen.

The use of density measurements may be a useful tool in identifying burnt bone (see Shipman *et al.*, 1984; Stiner *et al.*,1995), however the distinction may be more difficult to make if a site contains both burnt and 'fossilising' material.

3.2.5 A European Perspective

The recognition of a number of different diagenetic trajectories has a number of practical uses in terms of heritage management strategies and post excavation treatment. For example, if a site can be established as one that contains predominantly pre-fossilising bone, researchers would be well advised not to use resources on radiocarbon dating bone or stable isotope data from the collagen. However, pre-fossil bone may have the potential to be well preserved morphologically for a long time. Whilst it may be relatively friable pre-fossil bone is in most cases simply a highly crystalline apatitic pseuodomorph of the original bone, thus further diagenetic change is likely to be limited to increased crystallinity of the mineral, or replacement by other more stable mineral phases, i.e. fluorapatite. Thus, in terms of heritage management strategies (for example *in situ* preservation) such material may have the potential to remain unchanged for long periods, if the site environment remains unchanged.

For *in situ* preservation to be an option the diagenetic state of the bone has to be known prior to excavation, thus for heritage managers it would be useful if the diagenetic trajectories could be predicted from a knowledge of other site parameters, e.g. archaeological factors or soil chemistry. For each site in this study, a number of archaeological factors and soil physical and chemical properties were assessed (see Kars & Kars 2001, and Mattsson *et al.*, 1996; Jackson 1965).

Using simplified measures of diagenesis, PC1 as a measure of the general diagenetic state of the bone, and PC2 as a measure of the fossil/microbial trajectory the state of bone preservation at a number of sites has been defined, and compared to soil, archaeological and environmental factors for each site.

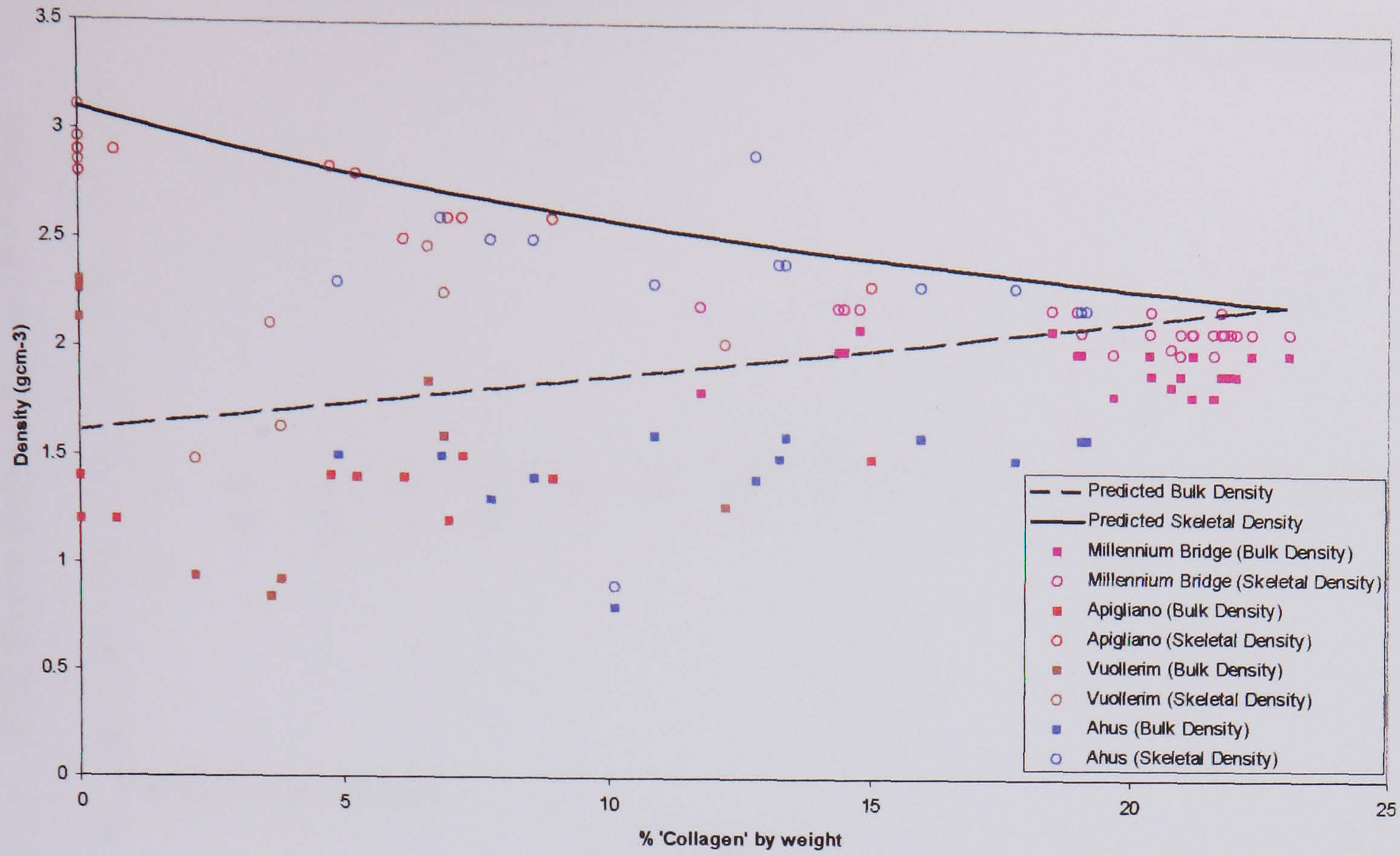

Figure 3.2-L. Predicted and measured density values with collagen loss for European Holocene bones.

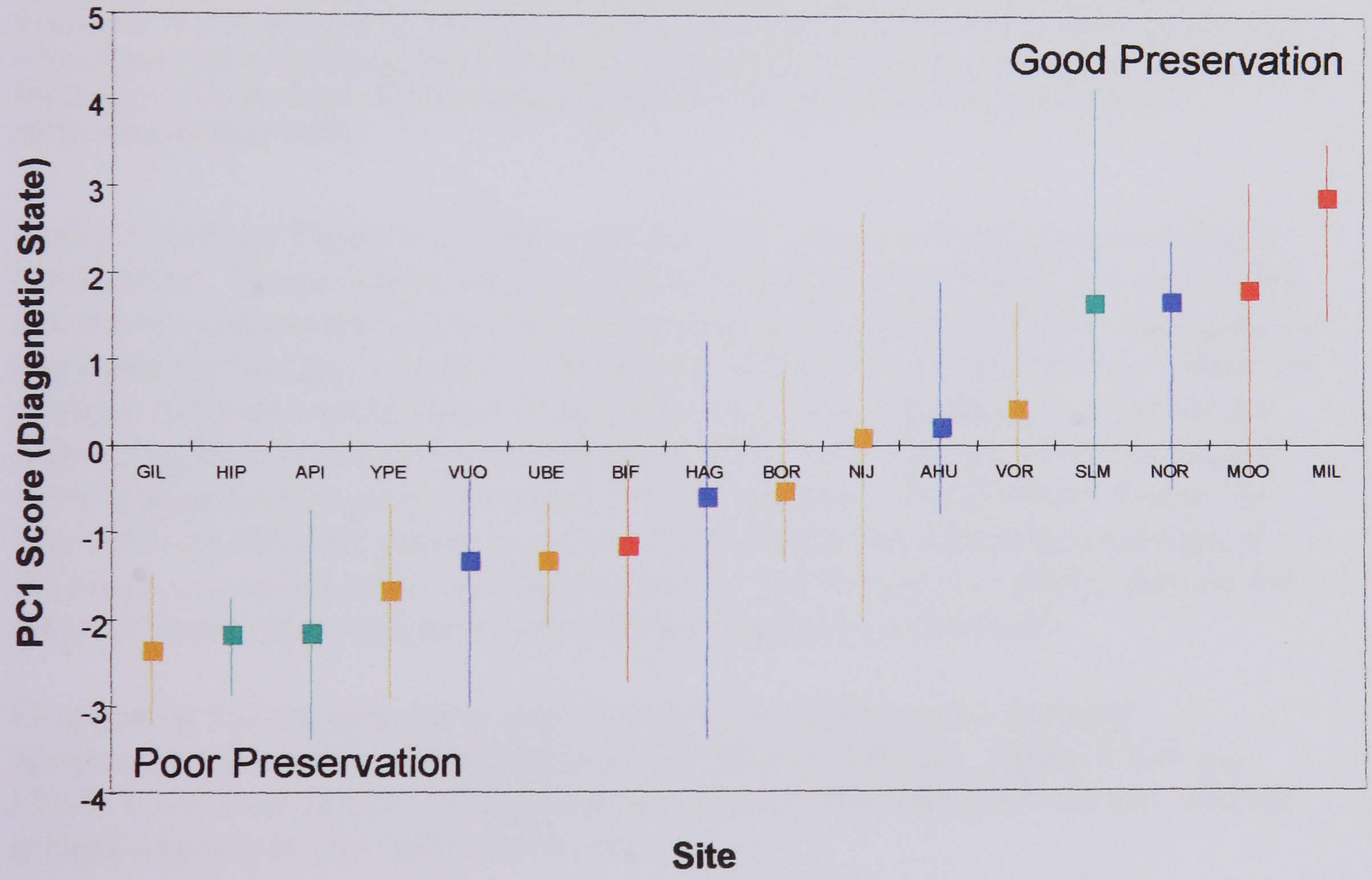

Figure 3.2-M. Range of PC1 scores for individual sites from Europe. Italian sites (Green), Hierapolis (HIP), Apigliano (API), Santa Lucia Alle Malve (SLM); Swedish sites (Blue), Vuollerim (VUO), Häggvik (HAG), Åhus (AHU), Norkköping (NOR); Netherlands sites (Gold), Gilze-Rijen (GIL), Ypenburg-Rijswijk (YPE), Ubbergseveldweg (UBE), Borgharen (BOR), Nijmegen (NIJ), De Vork (VOR); Britain (Red), Bicester (BIF), Moorend Farm (MOO), Millennium Bridge (MIL).

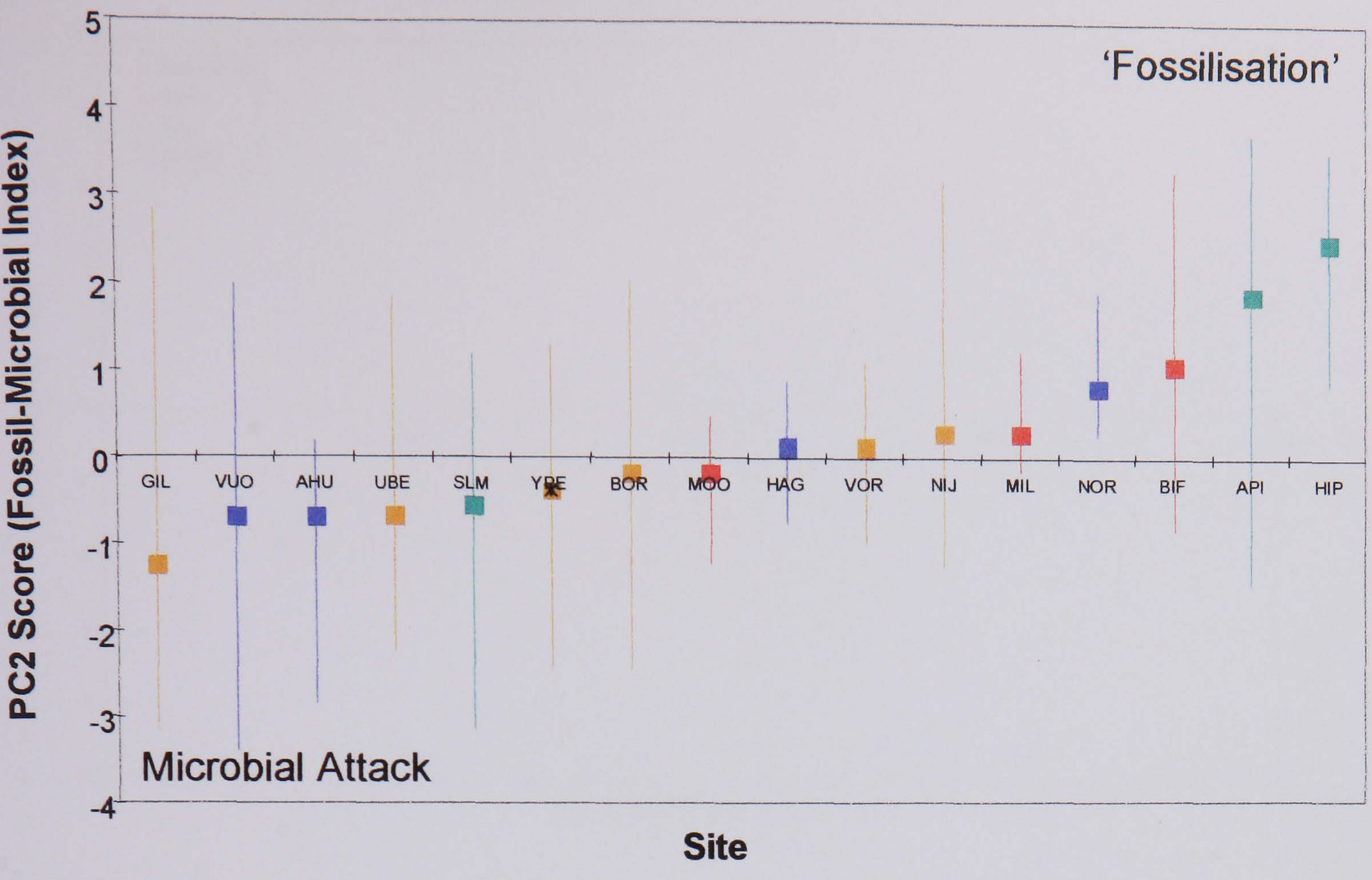

Figure 3.2-N. Range of PC2 scores for individual sites from Europe. Italian sites (Green), Hierapolis (HIP), Apigliano (API), Santa Lucia Alle Malve (SLM); Swedish sites (Blue), Vuollerim (VUO), Häggvik (HAG), Åhus (AHU), Norkköping (NOR); Netherlands sites (Gold), Gilze-Rijen (GIL), Ypenburg-Rijswijk (YPE), Ubbergseveldweg (UBE), Borgharen (BOR), Nijmegen (NIJ), De Vork (VOR); Britain (Red), Bicester (BIF), Moorend Farm (MOO), Millennium Bridge (MIL)

Figure 3.2-M and Figure 3.2-N show the range of values for the diagenetic principal components. The predictive value of these scores is however limited due to the large amount of variation that can be observed at each site, especially in PC2. This analysis highlights the fact that a number of sites both modes of diagenesis can be in effect, for example Apigliano where many of the bones are/or were 'fossilising', but some are undergoing/have undergone microbial degradation, or Vuollerim, where the general mode of diagenesis is microbial attack with the exception of a few burnt bones. At Vuollerim the different pathways are the result of different taphonomic histories of the bones, not soil or environmental conditions. The figures also display that the full range of preservation, and preservation types is seen in every country.

Considering that the sites show large variations in the diagenetic principal components, this makes interpretation at the site level difficult. Figure 3.2-O and Figure 3.2-P show plots of PC1 against the water-soluble calcium in the soil, and the soil pH respectively for individual bones.

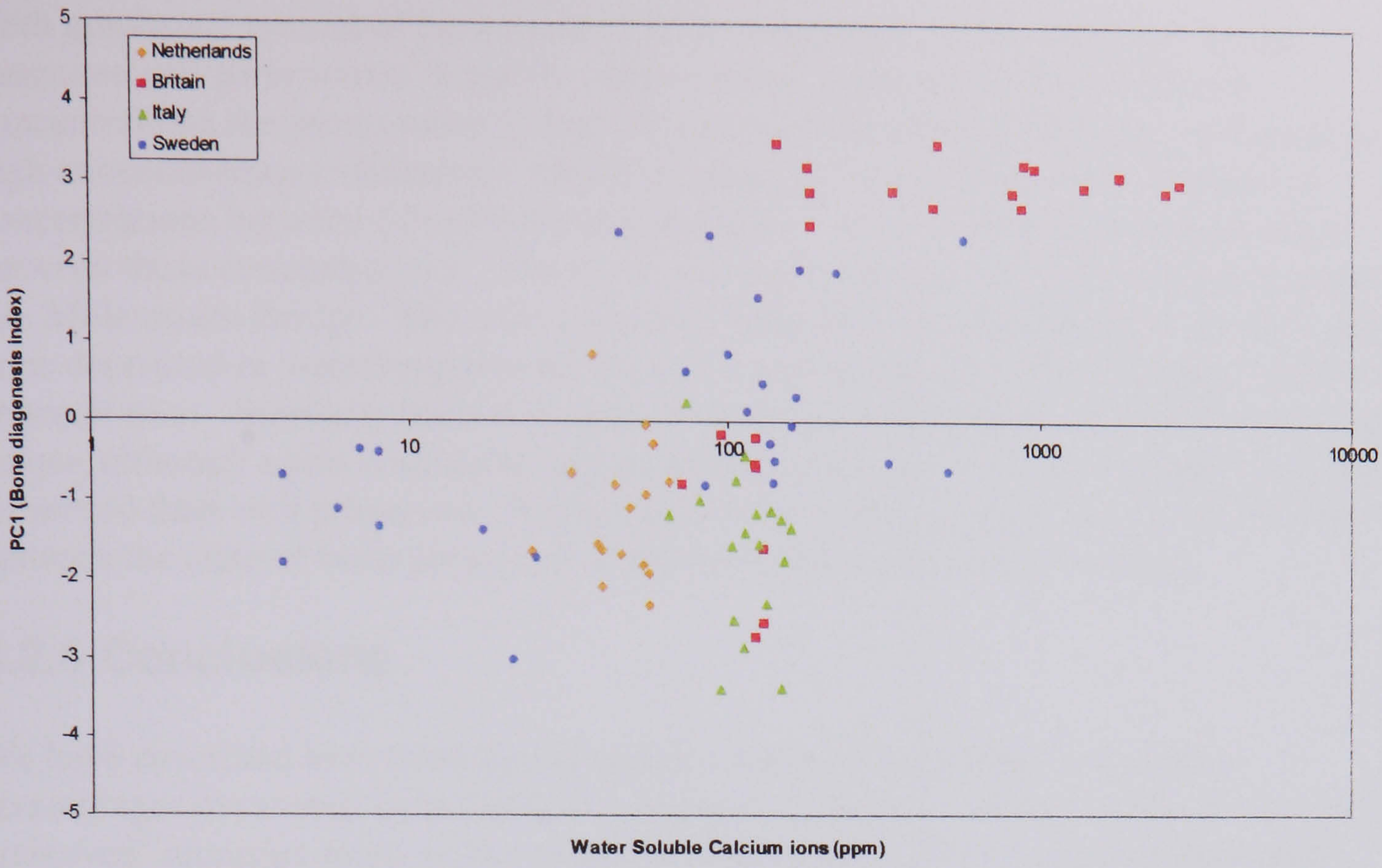

Figure 3.2-O. Principal component 1 scores for individual bones from Holocene Europe, vs. water-soluble calcium content of the soil (ppm), note logarithmic scale.

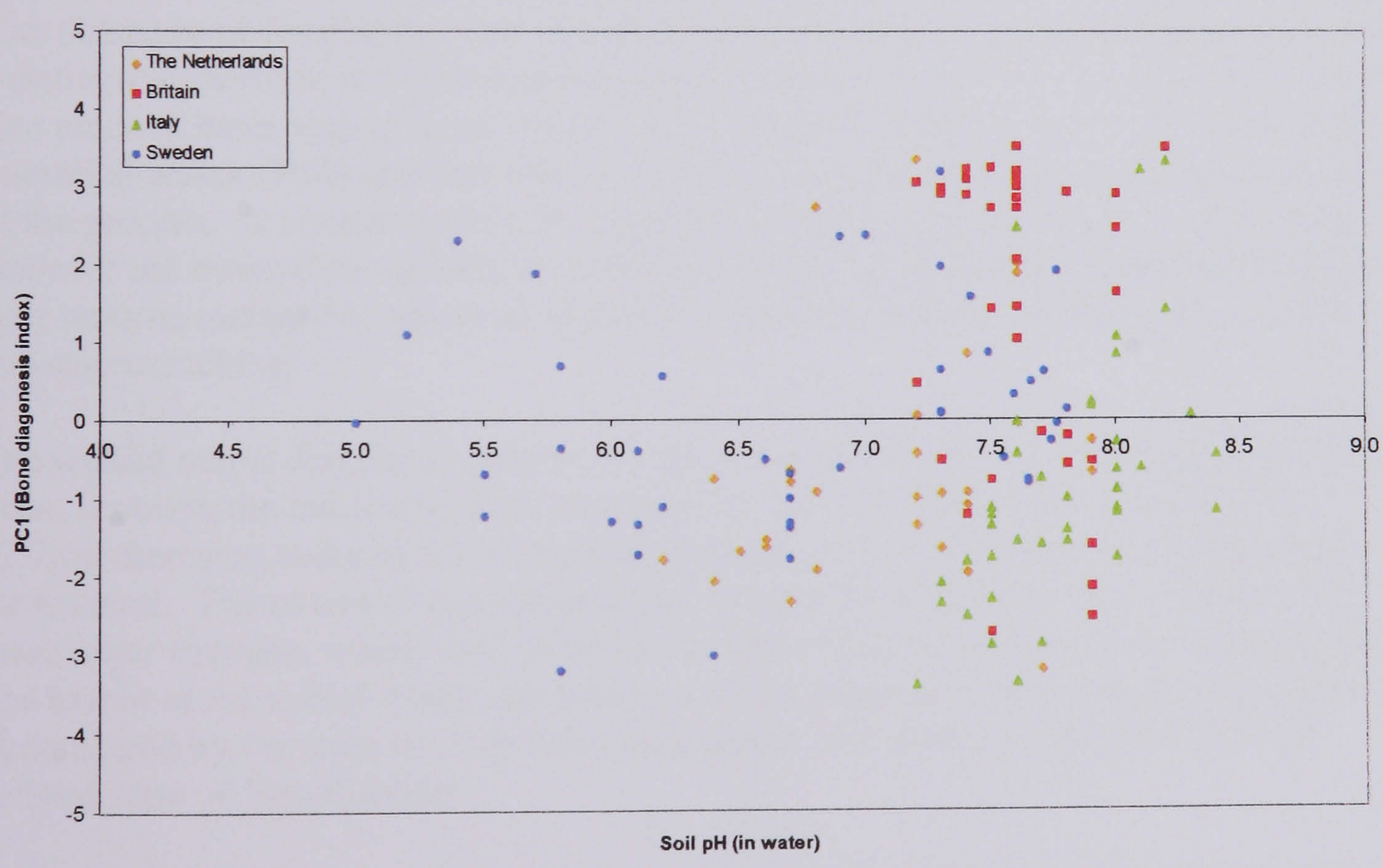

Figure 3.2-P. Principal component 1 scores for individual bones from Holocene Europe, vs. soil pH.

Both graphs are typical of the spread of PC1 values that can be observed for the measured soil parameters. It can be observed that at the extreme calcium ion concentrations the preservation is bad for low concentrations (1-20ppm) and good at high concentrations (>500ppm). However, the majority of sites have calcium ion concentrations between 50-300ppm and display a range of bone preservation states between these concentrations. Moreover, the high calcium levels are recorded at one site Millennium Bridge. The well-preserved bone here may be the result of unfleshed bone deposited in waterlogged conditions in a young site rather than the high calcium concentration. Similarly there is a range of well to poorly preserved bones at most pH values, although again it could be argued that at acidic pH values more bone is poorly preserved than well preserved. Neither dataset provides strong evidence of the link between the state of bone preserved at the site and the soil characteristics.

3.2.6 Conclusions

We have described here three key diagenetic states of bone observed in bone assemblages excavated from Holocene deposits in Europe. Firstly, bone can be 'well-preserved' retaining many of the physico-chemical characteristics of modern fresh bone; or it can be degraded, degraded bone is preserved in one of two distinct ways, either in what appears to be the early stages of fossilisation, or as microbially degraded bone.

'Fossilising' bone, as it is termed here, retains its original histomorphological structure (although with much micro-fissuring), but has very low collagen levels. The pore structure of fossilising bone as determined by mercury intrusion porosimetry has a distinctive increase in volume in the pores of diameter ~0.01-0.1μm diameter, and it also tends to have high mineral crystallinity. Some fossil bone may have some slight microbial attack (HI4) and less extensive mineral alteration, indicating a lesser extent of the process. A similar pattern of diagenetic parameters is observed in burnt bone, however the mineral phase may be entirely altered to β-tricalcium phosphate, and the pore volume increase is observed in pores of greater diameter. Burnt bone also has less microcracking.

The second major diagenetic pathway is microbial attack of bone. Microbial attack of bone, destroys the microstructure, increases the pore volume in pores with 0.12-12.7μm diameter, reduces the amount of collagen, and increases the crystallinity of the mineral. The extent of microbial attack appears to broadly control the extent of these other changes, which tend to be less extreme than those observed in fossilisation. The extent of microbial attack can lead to a broad range of levels of bone degradation as measured by the suite of diagenetic parameters, in contrast to the discrete well-defined state of 'fossilisation'.

It should be noted that a fourth state of 'preservation' could be defined, the complete destruction of the bone. This is difficult to define, as the absence of bone is difficult to determine, moreover it cannot be determined properly by the measurement of the diagenetic parameters. It should be noted that this is however an important (the most common) diagenetic outcome for bone.

The conditions that determine the prevailing diagenetic mode in the soil are difficult to define. All three diagenetic trajectories are observed in the four main regions of

Europe sampled, and often two types of preservation can be found at any one site. Furthermore, there does not appear to be a strong correlation between the type of bone preservation and the physico-chemical properties of the soil adjacent to the bone. It may be that the diagenetic state of bone is determined by earlier pre-burial conditions or events, and that the soil conditions have only a minor influence on the eventual preservation of the bone.

The key change in bone is probably the incidence of microbial attack. We can speculate that the changes that occur in early fossilisation e.g. large crystallinity increases, loss of collagen, may require the structural integrity of the bone to be preserved. If bone is microbially attacked before the early fossilisation has occurred then the increased porosity of the bone may result in increased hydraulic conductivity, increased bone dissolution, leading to destruction, or simply a microbially attacked bone. Without microbial attack the hydraulic conductivity of the bone will be lower, enabling increased reprecipitation (rather than loss) of the mineral, hence increased crystallinity. Fossilisation may thus occur under the conditions where microbial attack is prohibited, and the mineral is stable in the soil, although it is not clear what changes a well-preserved bone into a fossilising one. More importantly, it is still not understood why or when microbial attack occurs. Although by definition 'fossilising' bone does not show evidence of microbial attack, it is apparent that bone does not exist with all other features of 'fossilisation' and extensive microbial attack. One explanation for this is that during fossilisation all the collagen is removed and thus there is nothing for the microbes to consume. 'Fossilisation' precludes microbial attack and this also appears to be the case for burning. Alternatively, it could be that microbial attack of fossil bone rapidly destroys it, therefore there is no evidence of partially microbially attacked fossil bone.

Equally, the incidence of microbial attack precludes the 'fossilisation' of bone. It may be assumed that certain soil conditions may promote each mode of diagenesis, however the fact that both processes can be seen at the same site, and under the same soil conditions would suggest that the diagenetic trajectory is determined in the initial stages of taphonomy, and not the long term conditions prevailing during deposition. One pivotal event in determining the diagenetic trajectory may be the season of burial. A body interred in a dry Mediterranean summer (leading to rapid desiccation) may not be amenable to microbial attack, whereas a winter burial may be more damp and predisposed to microbial attack. Thus the variation in the microbial attack, may be determined by the season of burial. If a body becomes defleshed before extensive microbial attack can occur then the result may be 'pre-fossilising' bone. Conversely, in Northern Europe microbial attack may be more prevalent in milder summers, and impeded during colder, drier winters. The data does not reveal a distinct pattern between the two areas of Europe, however the testing of modern experimental taphonomic assemblages may be a better method to elucidate a pattern if it exists.

Well-preserved bone may have the potential to take either diagenetic trajectory, although it is unfleshed and therefore unlikely to undergo microbial attack. The pre-fossilisation described may be a relatively stable end point of diagenesis, leading ultimately to a lithified bone. The conditions under which microbial attack occurs are not yet well enough understood, and more research is needed. Without understanding why it occurs, why it varies in its severity, and if it stops what is stopping it, the fate

of bone that has undergone (or is undergoing) this diagenetic mode will remain difficult to assess.

3.2.7 Acknowledgements

This work has been carried out as part of the Degradation of Bone as an Indicator in the Deterioration of the European Archaeological Property project funded by the EU (ENV4-CT98-0712). The authors would like to thank everyone who worked on this project providing data and discussion. Dave Dunbar and Steve Addison at the Central Analytical Services Unit (Newcastle University) are thanked for conducting Elemental Analysis, and Fourier Transform infrared spectroscopy.

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3.3 The Strange Case of Apigliano : Early 'Fossilisation' of Medieval Bone in Southern Italy

This paper is in press in *Archaeometry*

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3.3.1 Abstract

The preservation of the osteological material at the late Medieval site of Apigliano, in southern Italy, is characterised by bones with highly crystalline, and altered mineral phases. In addition to this, some material retains perfect histological preservation, with the exception of small microfissures present throughout this structure. Diagenetic porosity is indicative of collagen loss via chemical degradation. The levels of residual collagen in these bones are much lower than is predicted from simple models of gelatinization, and thus a more complex explanation for the state of preservation must be sought. Possible explanations for the rapid loss of bone collagen are considered, including a high temperature event, the acceleration of hydrolysis due to liming and extreme wetting and drying cycles.

Key Words

Bone, Diagenesis, Apigliano, Fossilisation, Collagen, Porosity

3.3.2 Introduction

The post-mortem changes that occur to bone over archaeological and geological time-scales are complex and unpredictable. Research, therefore tends to be based around simple conceptual and/or theoretical models to help understand the physical and chemical processes that occur both before and after deposition of the bone.

At one extreme bones can be fossilized (i.e. transformed to lithic pseudomorphs of the original bone), whilst those from permafrost have undergone little post-mortem alteration (Iacumin *et al.* 2000), highlighting the role of temperature in bone preservation. Bones and teeth from cave sites often contain significant amounts of well-preserved collagen after 80-120 thousand years (Bocherens *et al.*, 1997), perhaps due to the stable, cool, humid conditions. In contrast to this, the hot arid climate of the Nile Valley has yielded bones that contain high levels of acid soluble collagen (Iacumin *et al.*, 1996), which has not leached from the bone. All these extreme environments provide different insights into the processes of bone taphonomy.

As part of a study of bone preservation in Europe, a series of shallow burials were excavated at the deserted Medieval village of Apigliano in southern Italy. The bones from this site displayed many of the characteristics of fossil bones, in that they displayed excellent preservation at the microscopic scale, had highly altered mineral phases and lacked collagen, yet came from Medieval burials. The apparent 'fossilization' of Medieval bones prompted a more thorough investigation of the survival of bone on this site.

3.3.3 Material and Methods

3.3.3.1 Site Description

The deserted medieval village of Apigliano lies in the territory of Martano (province of Lecce, southern Puglia), see Figure 3.3-A. The site lies on a Terra Rossa plough soil in a flat, windswept, area of poor stony land with limestone bedrock breaking the surface in patches. The thickness of the soil above the bedrock is between 25-50cm, and the area is well drained. Excavations have been carried out for about a month each year, between 1997 and 2000. Excavations have shown the site to have developed from the eighth century A.D. as a Byzantine village or "chorion" and by the tenth century it probably covered about 2 hectares. By the later eleventh century it is likely to have fallen to the Normans, who conquered the whole of southern Italy. However, very little has yet been found dating from between the Norman invasion and the thirteenth century. Instead, excavations have brought to light a later medieval church and cemetery that appear to be of late thirteenth and early fourteenth century date. Some 53 tombs and 16 charnel pits have so far been excavated, and 82 individuals have been identified (Arthur, 1999).

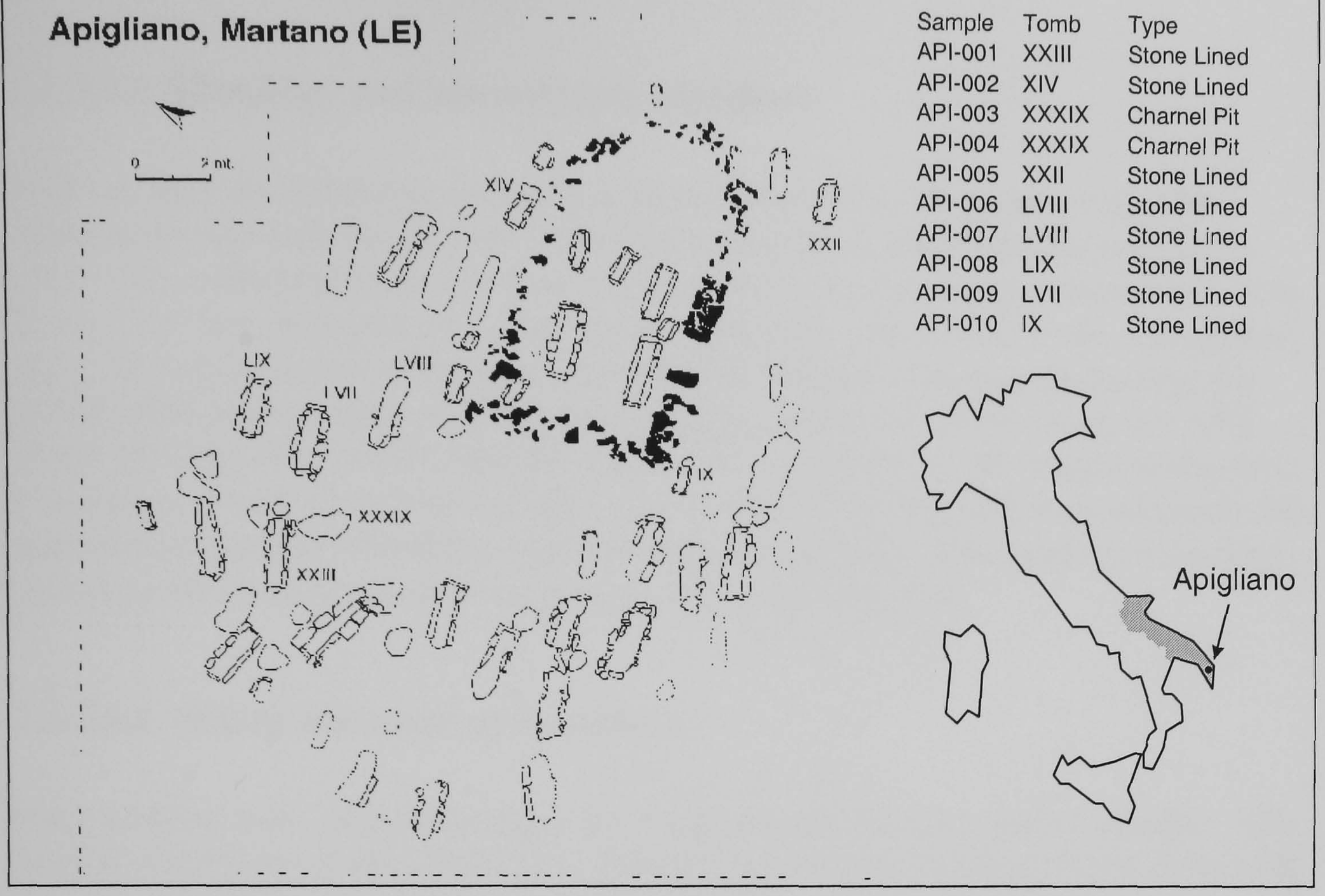

Figure 3.3-A. Site plan of Apigliano, showing tombs, and church, (Inset: location of Apigliano in Italy).

3.3.3.2 Materials

Archaeological human bone from Apigliano was excavated during the 1999-2000 seasons from the late thirteenth early to fourteenth century phases of the site. Figure 1 indicates which tombs the samples were taken from. All the samples analysed were taken from sections cut from mid shaft femurs, with the exception of API-007 which was taken from a femoral epiphysis, and API-003 which was taken from mid shaft tibia.

Modern cow tibia was obtained from a local butcher. The periosteum was removed with a scalpel and the marrow removed. The bone was de-fatted in acetone for 24hours. The de-fatted bone was then sawed in to chunks or freezer milled in to powder.

Hydrazine treated bone was prepared by the same method as Nielsen-Marsh (Nielsen-Marsh, 1997).

3.3.3.3 Methods

3.3.3.3.1 Histology and microscopic structure

Bone sections for histology were prepared as described by Hermann *et al.*, 1990. Samples of mid shaft femur were sawed from long bone, and embedded in epoxy resin. The embedded bone was then cut into 30µm sections using a microtome. The section was then mounted on a glass microscope slide and viewed under transmitted light. The preservation of the bones histological features was described using the Oxford Histological Index (Ox HI) score, ranging from 5 = >95% intact, 0 = <5% intact, (Hedges *et al.*, 1995; Millard, 2001). Microfissures (small cracks on the scale of an osteon) were also observed and quantified (the cracking index) by counting the approximate number of osteons with and without cracking. The number of cracked osteons is then expressed as a percentage (Jans *et al.* in press).

3.3.3.3.2 Whole bone nitrogen content

Bone samples were cleaned and ground in a pestle and mortar to a fine powder. The amount of nitrogen in the whole bone powder was measured using a Carlo Erba 1106 Elemental Analyser in accordance with the manufacturer's instructions. Analyses were carried out in duplicate.

3.3.3.3.3 % 'Collagen'

Bone shards (dimensions less than 0.5x0.5x0.5cm) of known weight (less than 0.06g) were demineralised in 2mls of 0.6M HCl overnight in 2 ml eppendorf tubes. The tubes were then centrifuged (at 6000 rpm for 5 minutes). The acid was decanted, and the remaining acid insoluble residue was washed by the addition of 2 ml of distilled water and a second centrifugation. The wash was repeated twice more. The acid insoluble fraction was then oven dried overnight at 65°C, and weighed. The carbon and nitrogen values of a sample of the acid insoluble fraction were then obtained using a Carlo Erba 1106 Elemental Analyser as above. Elemental analysis was carried out in duplicate. The % 'collagen' is calculated by multiplying the weight of the insoluble fraction (as a percentage) by the amount of nitrogen in the insoluble fraction (as a percentage), this is then divided by the amount of nitrogen in modern cow tibia bone powder (16.74%). The C:N ratio (as an atom percent) was calculated to assess if the insoluble fraction is collagen (Ambrose, 1990).

3.3.3.3.4 Crystallinity and Carbonate:Phosphate ratio

The crystallinity of the mineral fraction was measured using infrared spectroscopy. The crystallinity or infrared splitting factor (IRSF) is calculated using the splitting ratio of the phosphate *v*4 doublet at 567 and 605cm^{-1} (Weiner & Bar-Yosef, 1990). The infrared spectrum was also used to identify the presence of other mineral phases present, e.g., calcite, or silicates.

The carbonate:phosphate ratio (C/P) was also calculated from the infrared spectrum using the peaks at 1415cm^{-1} (CO_3^{2-}), and 1035cm^{-1} (PO_4^{3-}). This measurement can be interfered with by the presence of collagen, and is thus only used as semi-quantitative measure.

3.3.3.3.5 Mercury intrusion porosimetry

Chunks of bone (approximately 1g each) were dried in an oven at 85°C for at least 16 hours. The pore structure of the bone was then investigated using mercury intrusion (Nielsen Marsh & Hedges, 1999). The results of these analyses are reported as total intrusion of mercury, the skeletal and bulk density of the material, and a mercury intrusion pore size distribution (PSD) graph. The PSD graph is a plot of the amount of mercury intruded into the bone (volume/volume) against the nominal pore diameter.

3.3.3.3.6 Liming experiments

To each of 4 plastic tubes was added 10mls of 1M $Ca(OH)_2$. To the first of these a chunk (approximately 1g in weight) of modern bovine bone was added, to the other three tubes powdered bovine bone was added, in all cases approximately 1g of bone was used. These were then placed in a water bath and incubated at 37°C. The pH was measured, and powder samples removed at intervals of 1 day, 1 week, and 3 weeks. After removal from the water bath the % acid insoluble fraction was determined (see % 'collagen' above). The chunks were also removed after 3 weeks, washed and dried, and then underwent mercury intrusion porosimetry. A duplicate set of experiments was carried out, except the substrate was changed from $Ca(OH)_2$ to CaO.

3.3.3.3.7 Soil Analysis

For each bone sample a series of soil samples were taken; one from the immediate proximity of the bone, a feature soil sample from the grave cut, a third sample was taken from the natural soil of the site (this was not repeated for each bone), approximately 300-400g of soil was taken in all instances. These samples were characterised by a number of standard geological and chemical analytical techniques (Mattsson *et al.*, 1996 pp59-64, Jackson, 1965), including pH, exchangeable acidity, granulometry, total elemental composition, water-soluble anions and cations.

3.3.4 Results and Discussion

The soil is a typical terra rossa; calcareous, mainly silt with some sand. Quartz and silicate minerals with a high concentration of iron dominate. The pH (in water) is rather constant, in the region 7.5 – 7.8, with negligible exchangeable acidity. There are negligible amounts of soluble anions and cations in the soil (e.g. Na^+ 9-17ppm, Al^{3+} 5-8ppm, SO_4^- 3-30ppm) with the exception of high concentrations of calcium and (in some samples) chloride ions (Ca^+ 94-157 ppm, Cl^- 0-287 ppm). The soil samples are largely homogenous across the site, and thus are difficult to interpret in terms of particular modes of preservation for individual bones.

The preservation of bone at Apigliano can be divided into two categories, material with evidence of microbial attack (Ox HI <5) and samples without (Ox HI 5). Most notably the bones that score maximum on the Oxford histological index also have extensive cracking (the cracking index being an approximate % of osteons showing microfissures). There is also a qualitative difference in the pore size distributions of these two categories. Across the site, the amount of collagen is low. The C:N ratio of this collagen confirms that in all cases above 1% by weight the acid insoluble fraction is collagen. The infrared splitting factor (IRSF), values for this material are relatively high in comparison to values of other archaeological bones, and the carbonate:phosphate ratios are low indicating that the mineral is highly crystalline and has lost much of the endogenous carbonate (Table 3.3-i). There are also peaks in the infrared spectra of samples API-002, -004, -008 and -009, at around 631 cm^{-1}, 1032 cm^{-1}, and 1093 cm^{-1}, which may be indicative of other apatite phases, (e.g. β-tricalcium phosphate or whitlockite) in the mineral. Infrared spectra of samples API-002, -005, -006, -007 and -010 have evidence of calcite peaks. This would suggest that the mineral phase of this material has been highly altered.

3.3.4.1 Microbial attack

Theoretically, collagen will be lost from the bone by two methods, rapidly (over years to decades) through the action of microbes, and slowly (over hundreds to hundreds of thousands of years) via the chemical hydrolysis of peptide bonds (Collins *et al.*, 1995; Nielsen-Marsh *et al.*, 2000). Microbial attack affects both the collagen and mineral phases of the bone and the pore structure. This aspect of diagenesis could be considered the most variable, in that it is still unknown why microbial attack occurs in some bones, and not others, and why the extent of it can vary. Without this knowledge it becomes impossible to model the effects of microbes on the parameters measured. In the case of modelling collagen diagenesis in microbially attacked bone it is difficult, as in addition to the collagen lost via chemical hydrolysis, an unknown amount will be lost due to the direct action of the microbes. Samples API-005, API-006, and API-010, all show some evidence of microbial attack. In samples API-006 and API-010 there is evidence of extensive microbial attack shown by low OxHI scores, and peaks in the 0.1-10μm diameter region, of the pore size distribution (Figure 3.3-B.)

3.3.4.2 Chemical loss of collagen

For bone without evidence of microbial attack it is assumed that collagen is lost solely via the action of peptide bond hydrolysis. The pore size distribution of many of theses samples, as revealed by mercury intrusion porosimetry, shows a distinctive peak in the 0.01-0.1μm diameter range (Figure 3.3-B). This is consistent with the peak observed in bone treated with hydrazine hydrate to remove the protein (Figure 3.3-C). This feature has also been recreated by extensive boiling of bone in water, revealing a strong linear relationship between the pore volume in this diameter range, and the amount of nitrogen remaining in the bone (Roberts *et al.*, in press). This peak can be attributed to chemical hydrolysis of the collagen, rather than microbial loss. The Apigliano material with OxHI 5 appears to follow this trend, thus it would appear

that the cracking in these bones does not contribute greatly to the porosity of the material, as measured by mercury intrusion porosimetry.

The rate of protein loss (the largest part of this being collagen) is a function of time and temperature assuming that there is enough water for the reaction to proceed (Von Endt & Ortner, 1984). In API 001-004, 008, and 009, there is no histological evidence of microbial attack, although samples API-003 and 004 do have an increase in pore volume in the 0.1-10μm diameter range, which may indicate some microbial attack and thus heterogeneous preservation in the samples used for the two tests. If there is no histological attack the principal mode of collagen loss is via peptide bond hydrolysis (Child, 1995). Assuming that this reaction has been occurring at approximately neutral pH (dissolution of bone mineral will buffer against low pH, and the soil pH is 7.5-7.8) then knowing the amount of nitrogen remaining in the bone, and the age of the material, an approximation of the temperature that the bones must have been at can be made (see Appendix E).

According to this calculation API-008, which has the highest %N of the histologically perfect specimens without large increases in pore volume in the 0.1-10μm diameter region, would have to have been kept at a constant 43°C (or 41°C for a zero order reaction) for 700 years to reduce the amount of collagen to the observed level of ~30% of the original amount. Modern day mean monthly temperatures recorded by weather stations in this region report temperatures in the region of 16-17°C (Baker *et al.*, 1994). Accounting for the fact that the relationship between the rate and temperature is exponential, and thus during the summer the rate of the reaction will increase dramatically the effective temperature (integrated over seasons) (Wehmiller *et al.*, 2001) can be given as approximately 19-21°C using the maximum seasonal amplitude. Not withstanding a dramatic climate change in the region, the temperature required to reduce the amount of protein in these bones by simple chemical hydrolysis alone is almost twice what they are likely to have been exposed to.

In the absence of any evidence of microbial attack on these bones it would appear that the rate of collagen loss has been accelerated. There are a number of processes that could be responsible for this accelerated collagen loss, some of these will be discussed below taking into account the other diagenetic changes that have occurred in the bone, and the archaeological evidence.

Sample	Organic % 'Collagen'	'Collagen' C:N	Whole Bone % Nitrogen	Mineral Infrared Splitting factor	Carbonate Phosphate ratio	Other Mineral phases	Porosity Bulk Density (g/ml)	Skeletal Density (g/ml)	Total Intrusion (ml/ml)	Intrusion <0.1um (ml/ml)	Microscopic Ox HI	Crack Inde
Modern Bovine Bone	22.7	3.2	4.2	2.8	0.43		1.9	2.1	0.0958	0.0514	5	0
Deproteinated Bone	N/A	N/A	0.3	3.2	0.20		1.3	2.5	0.4622	0.4178	5	N/A
API-001	4.8	3.3	0.6	3.8	0.13		1.4	2.8	0.4826	0.3825	5	100
API-002	0.7	5.9	0.3	4.9	0.08	w,c	1.2	2.9	0.5914	0.4755	5	100
API-003	7.2	3.4	1.3	4.1	0.14		1.5	2.6	0.7221	0.3509	5	100
API-004	0.0	N/A	0.5	4.8	0.08	w	1.2	2.9	0.5649	0.3465	5	100
API-005	8.9	3.3	1.5	4.1	0.18	c	1.4	2.6	0.4181	0.3001	4	100
API-006	6.1	3.3	1.1	4	0.14	c	1.4	2.5	0.4010	0.1733	2	31
API-007	N/A	N/A	N/A	4	0.13	c	1.5	2.3	0.3249	0.2086		N/A
API-008	5.2	3.3	1.2	4.4	0.11	w	1.4	2.8	0.4647	0.3227	5	100
API-009	0.0	N/A	0.2	4.9	0.09	w	1.4	2.8	0.4867	0.3685	5	100
API-010	7.0	3.5	1.2	3.6	0.24	c	1.2	2.6	0.5075	0.1568	0	0

Table 3.3-i. Diagenetic Parameters for modern, deproteinated and archaeological bone from Apigliano. N/A = not measured, for other mineral phases; w = whitlockite or ß-tricalcium phosphate, c = calcite.

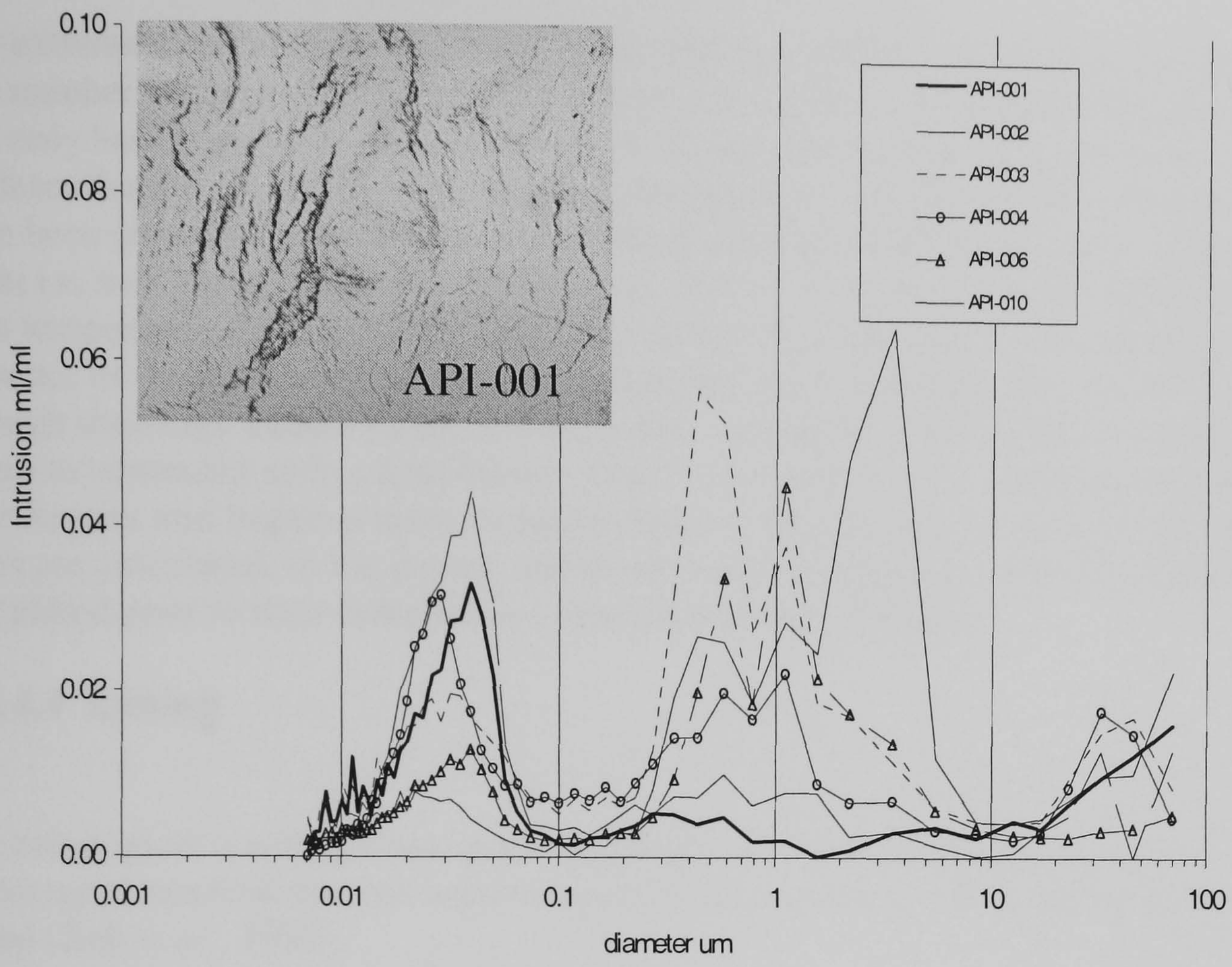

Figure 3.3-B. Pore size distribution graph of selected bones from Apigliano, (Inset: histological section of API-001 showing extensive cracking).

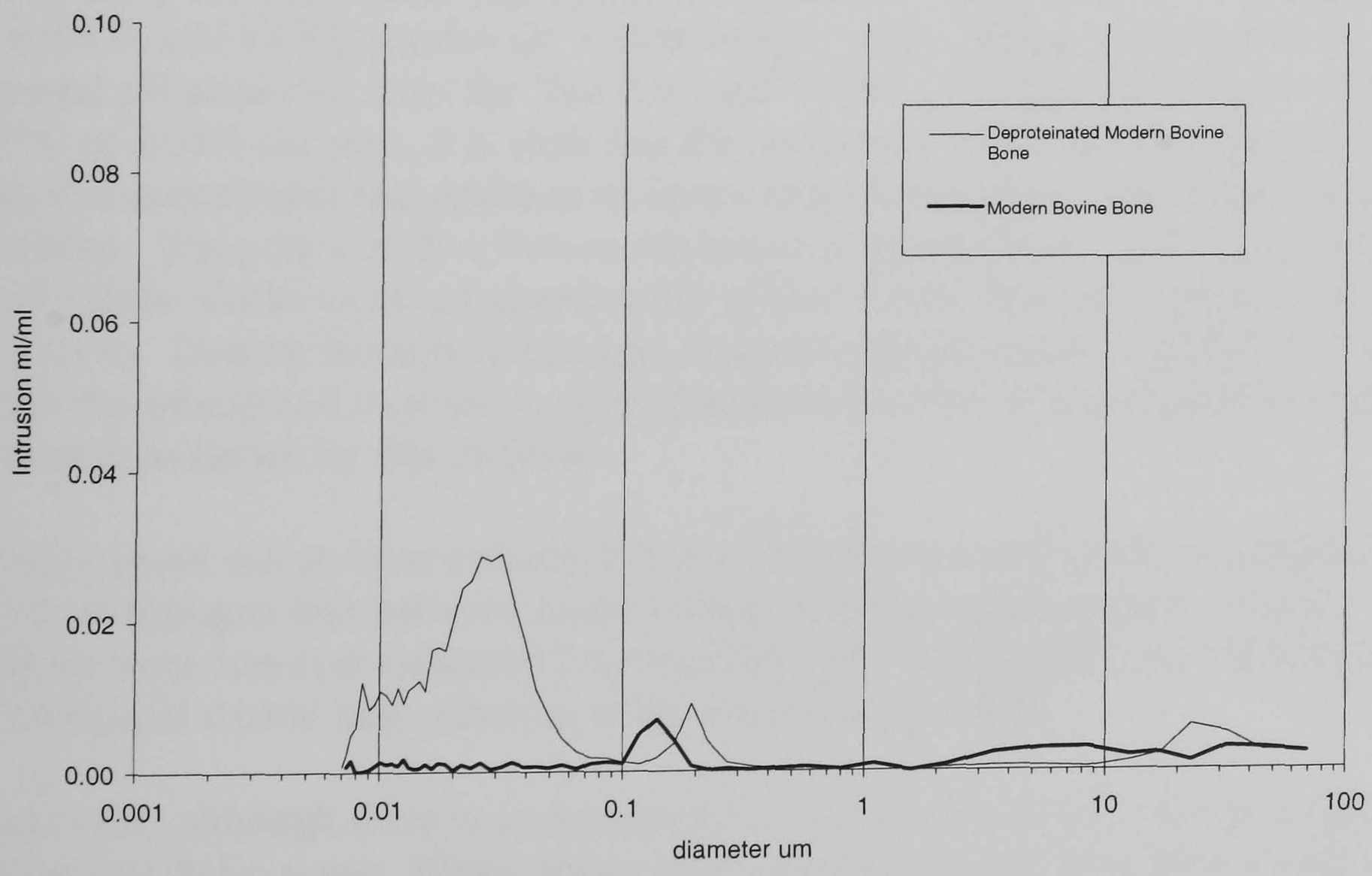

Figure 3.3-C. Pore size distribution graph of modern bovine bone, and modern hydrazine hydrate treated bone.

3.3.4.3 High Temperature

The extreme mineral changes evident in this material (IRSF 3.6-4.9) may be the result of a number of processes, and may be in part the cause for the rapid collagen loss. Not only has crystallinity increased (i.e., IRSF has increased), but there is also evidence for the presence of β-tricalcium phosphate or whitlockite, both of which have been observed in skeletal material before that has undergone a high temperature event i.e. burning or cremation (Stiner *et al.*, 1995). If the material had undergone a high temperature event for a sufficient period then it could possibly explain both the presence of the β-tricalcium phosphate phase, the rapid collagen loss, and the cracking through shrinkage caused by the loss of water. Archaeological evidence however, seems to contradict such a hypothesis. There is no evidence for cremating in Italy after Roman mid Imperial times in native Italian contexts, and the skeletons in most cases are articulated, in the graves, and those found in ossuaries were presumably articulated prior to their redeposition, thus cremation is unlikely.

3.3.4.4 Liming

The histological cracking observed at Apigliano is similar to that observed in osteological material that has been exhumed from a grave to which slaked lime was added (Bell *et al.*, 1996).

The results of the liming experiments indicate that that the pH of bone powders held in saturated solutions of $Ca(OH)_2$, or CaO remained relatively stable (pH 12.1-12.6), and that the alkaline environment significantly accelerated the rate of collagen gelatinisation. The rate of loss of the insoluble fraction in these experiments calculated by a simple linear regression, for $Ca(OH)_2$, and CaO, were 1.86% per day ($R^2 = 0.93$), and 1.64% per day ($R^2 = 0.91$) respectively. When compared to the rate at neutral pH predicted from the Von Endt and Ortner (Von Endt & Ortner, 1984) data at 37°C of 0.04% per year, it is clear that the reaction is accelerated at high pH values. Thus, it is conceivable that addition of lime could accelerate the rate of collagen loss from bone. The pore size distribution of chunks of bovine bone incubated at 37°C in lime for three weeks were not significantly different from those of modern bone (data not shown). Despite the anticipated loss of significant amounts of collagen from these chunks the anticipated increase in pore volume in the 0.01-0.1μm diameter range, has not been reproduced by this method.

A study carried out on bone exhumed in the UK observed no significant difference in the rate of nitrogen loss between limed coffins, and non-limed coffins. These skeletons were however exhumed less than 100 years after burial, and many of them still contained visible lime adhering to the bone (Jarvis, 1997)

Furthermore, although there is archaeological evidence for the use of lime in graves in Italy (Arthur & Lo scavo, 1994), where substantial patches of lime were found in the soil, at Apigliano there is no direct evidence for the use of lime in the graves or for the processing of lime nearby.

3.3.4.5 Weathering

The unusual state of preservation of the bone material at Apigliano may be due to extreme natural processes. The site is situated on a Terra Rossa soil which is well drained, and midday temperatures in the summer may reach in excess of 40°C. It is possible that the combination of extreme heat and a leaching soil may cause extreme wetting and drying cycles of the bone. This may cause swelling shrinkage, resulting in the fissuring observed, and increases in crystallinity via rapid dissolution/reprecipitation of the mineral. This severe recrystallisation may also play a role in the rapid collagen loss. The mineral/collagen interaction is a key relationship, and when this is disrupted, the rate of collagen degradation will be more rapid. Microcracking, rapid recrystallisation and collagen loss have all been recognized as features of a key stage in natural fossilization (Pfretzschner, 2000).

3.3.5 Conclusions

The preservation of osteological material at the Medieval village of Apigliano has been defined using a number of diagenetic parameters. The preservation of the material is unusual in that the histological structure of the bone is well preserved (i.e. there is no microbial attack), but the organic matter has been removed. A possible clue to the process driving the loss of collagen is the extensive microscopic cracking, in transverse section and pore structures indicative of chemical loss of the collagen. Across the site the crystallinity of the bones is very high, and contains unusual mineral phases. This type of unusual preservation is best described as akin to 'fossilisation', and may be indicative of an unusual mechanism of degradation at the site, however it is unclear what this mechanism might be. Liming, high temperature, and extreme environmental conditions have been proposed as possible mechanisms for the state of the Apigliano bones, however, there is no conclusive evidence for any one of them, indeed it may be a combination of factors that has produced the unusual condition of the bone.

This state of preservation of the OxHI 5 material from Apigliano has some features that are similar to that of fossil bone, in that the mineral is highly altered, the histological preservation is good, and there is poor organic preservation. The similarity is not complete however as Trueman describes the process of 'fossilisation' as also requiring the recrystallisation of the mineral to francolite, and the infilling of pore space with diagenetic minerals (Trueman, 1999). The alternative diagenetic pathway is that of mineral dissolution and destruction of the bone. The Apigliano style of preservation appears to fit neither model. The distinct diagenetic pore structure of the bone indicates that there has been no infilling with mineral nor is there evidence for francolite (in the 567/605 cm^{-1} peak ratio in the infrared spectra), at least not after 700 years of deposition. Equally there is little evidence for dissolution of the material, soil phosphate levels are low indicating that there is little leaching of phosphate to the soil, and there is no extensive increase in the porosity that would be expected if mineral were dissolving in the ground water.

3.3.6 Acknowledgements

This work has been carried out as part of the Degradation of Bone as an Indicator in the Deterioration of the European Archaeological Property project funded by the EU (ENV4-CT98-0712). We would also like to thank Dave Dunbar, and Steve Addison at the Central Analytical Services Unit (Newcastle University) for conducting Elemental Analysis, and Fourier Transform infrared spectroscopy. Brunella Bruno is acknowledged for excavating and sampling skeletal remains and Trevor Anderson (Canterbury Archaeological Trust) for anthropological examination of the skeletal material. The authors would also like to thank Dr. Eva Valsami-Jones for thoughtful discussions about mineralogy, and the two anonymous referees for their comments, from which this manuscript has benefited. The LDEO/IRI Data Library is acknowledged for facilitating access to climatic data.

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3.4 Bone Diagenesis and the survival of Osteocalcin in Archaeological Bone

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3.4.1 Abstract

It has been demonstrated that the protein osteocalcin can survive in bone in the archaeological record, and it has been postulated that it has the potential to survive for geological time. The precise mechanism for this longevity of survival is not yet fully understood, and has not been extensively studied in comparison to other diagenetic aspects of archaeological bone. We present here a study of more than 60 bones from various archaeological sites. The state of preservation of the bones has been assessed using a number of diagenetic parameters that measure the amount of 'collagen' in the bone, the mineral changes, the porosity, and the histological preservation of the material. In addition, the amount of osteocalcin has been assayed, using ELISA. The amount of osteocalcin surviving in the bones is discussed with reference to the diagenetic state of the bone as a whole. The results here indicate that microbial taphonomy, and mineral alteration of bone may have a profoundly damaging effect on the preservation of osteocalcin.

Keywords

Osteocalcin, Archaeological bone, Bone diagenesis, ELISA, Histological Index, Infrared Splitting Factor, Collagen.

3.4.2 Introduction

Fossil bone proteins have been widely studied in archaeology. The main uses of ancient bone proteins are as a substrate for radiocarbon dating (Hedges & Law 1989), and for palaeodietary studies (e.g. Ambrose & DeNiro 1986). Bone 'collagen' is the substrate of choice because; it is abundant (making up approximately 90% of bone protein), it is easily extracted from the bone (by simple acid demineralisation), and simple quality control procedures can be applied to ensure adequate purity. In temperate regions it has the potential to survive for in excess of 100 000 years (e.g. Bocherens 1999).

In heavily degraded bone the use of the 'collagen' fraction for both dating and stable isotopic studies is less reliable. Low 'collagen' yields are more susceptible to contamination, whilst selective preservation of the non-collagenous proteins (NCP's) over collagen, (Masters 1987), could alter the nature of the extracted 'collagen' fraction. The possibility of the preferential preservation of NCP's, notably osteocalcin (Oc), the second most abundant bone protein, has lead to investigations of this protein fraction as an alternative substrate to 'collagen' (Ajie *et al.*, 1991; Ajie *et al.*, 1992; Burky *et al.*, 1998). Osteocalcin forms 10-20% of the NCP fraction, and has a high affinity for apatite (Tuross, 1993); it has been suggested that Oc (or at least some regions of the molecule) has a greater potential to survive than bone collagen (Collins *et al.*, 2000), and can survive intact in the geochemical environment, (Ostrom *et al.*, 2000).

Successful fossil bone protein studies require the proteins to be, preserved in sufficient quantities for analysis, and normally require the proteins to be qualitatively well preserved. Following death bone will undergo a number of physical and chemical transformations, the changes that occur in the soil are termed diagenesis. The diagenetic changes that occur to bone will affect both the quality and quantity of the protein preserved. Many of the studies of Oc in fossil bone focus upon its potential utility as an alternative substrate to 'collagen' in 'collagen degraded bone', but if its pattern of diagenesis is similar to, or even linked to, 'collagen' then it will be of little potential value in archaeology.

The diagenesis of organic matter in bone is a complex phenomenon (Collins *et al.*, in press). Archaeological bone protein will degrade principally via two mechanisms; digestion by soil microbes and fungi, and by chemical alteration (principally driven by hydrolysis of peptide bonds). The precise nature of the preservation of Oc in archaeological bone is not fully understood, although it is known to bind to both collagen ($K_{assoc} \approx 10^{-5}$ mol $^{-1}$; Prigodich & Vesely, 1997) and mineral ($K_{assoc} \approx 10^{-7}$ mol $^{-1}$), the latter possibly inhibiting enzymolysis (Ajie *et al.*, 1992). Studies of early diagenetic changes in bone (the first 10 years of surface exposure) indicate Oc survival is linked to the presence of unchanged mineral crystals in the bone (Tuross *et al.*, 1989). Collins *et al.*, (2000) have similarly shown that the original 'packaging' of the Oc (i.e., the mineral-protein interaction) in bone is important to the long-term survival of the protein in heated bone powders, and demonstrated that more extreme changes in the mineral fraction of archaeological bone are coincident with lower levels of Oc survival.

It has been suggested that DNA may also survive in the archaeological record via the same method i.e., by binding to bone apatite (Tuross, 1993), although most studies of DNA diagenesis have considered the role of microbial taphonomy (Hagelberg *et al.*, 1991; Colson *et al.*, 1997), and not mineral changes. If the same mechanism is responsible for the survival of both biomolecules, then it would be expected that the patterns of preservation of the two molecules would be similar, particularly with respect to microbial taphonomy.

We have analysed more than 60 bovid bones from 14 archaeological sites to determine the state of preservation of the bone in general and to relate this to the amount of Oc remaining in the bone. The state of preservation of the bone is determined by measuring a number of 'diagenetic parameters' that quantify the changes in the organic and mineral fractions, the changes to the pore structure, and the amount of damage to the histological structure (caused by microbial and fungal attack) that has occurred to the bone. In addition to this, an enzyme-linked immunosorbent assay (ELISA) using polyclonal bovine osteocalcin (Boc) antibody has been carried out to determine the amount of Oc remaining in the bone.

3.4.3 Material and Methods

3.4.3.1 Materials

Modern bovine tibia was obtained from a local butcher. The periosteum and marrow were removed with a scalpel. The bone was then sawn into chunks and defatted for 24 hours in acetone. The chunks were then freezer milled under liquid nitrogen into a powder. This powder was used as the standard modern bovine bone for subsequent analysis, unless otherwise stated.

Aurochs (*Bos primigenius*) bone was used as an archaeological control specimen. This specimen was excavated from Norfolk, UK, and dated to ~130 kyrBP, by its stratigraphy.

Archaeological bone was excavated from a number of sites throughout Europe (Britain, the Netherlands, Italy, and Sweden). The age of the material ranged from c6000BP to c200BP it was from a variety of different burial contexts, and contained various skeletal elements (see Kars & Kars, 2001 for further information). All the material tested had been identified as *Bos*, cattle or cow.

In addition Bison (*Bison priscus*) samples were excavated from the Yukon area (USA), the age of the samples varies from 800 years BP to 53 000 years BP.

3.4.3.2 Diagenetic parameters

3.4.3.2.1 Histology and microscopic structure

Bone sections for histology were prepared as described by Hermann *et al.*, 1990. Samples were sawn from the bone, and embedded in epoxy resin. The embedded bone was then cut into 30μm sections using a microtome. The section was then mounted on a glass microscope slide and viewed under transmitted light. The preservation of the bones histological features was described using the Oxford Histological Index (Ox HI) score, ranging from 5 = >95% intact, 0 = <5% intact, (Hedges *et al.*, 1995; Millard, 2001). Microfissures (small cracks on the scale of an osteon) were also observed and quantified (the cracking index) by counting the approximate number of osteons with and without cracking. The number of cracked osteons is then expressed as a percentage (Jans *et al.*, in press). Histological analysis of samples B1-01-B1, B1-07-B1 and B1-07-B2 was made using polished thick sections of bone (approx 2cm thick) embedded in resin, histological index was scored as above, although cracking was not assessed.

3.4.3.2.2 Whole bone nitrogen content

Bone samples were cleaned, and ground in an agate pestle and mortar to a fine powder. The amount of nitrogen in the whole bone powder was measured using a Carlo Erba 1106 Elemental Analyser. Analyses were carried out in duplicate (mean values are given).

3.4.3.2.3 % 'Collagen'

Bone shards (dimensions less than 0.5x0.5x0.5cm) of known weight (less than 0.06g) were demineralised in 2mls of 0.6M HCl overnight in 2 ml eppendorf tubes. The tubes were then centrifuged (at 6000 rpm for 5 mins). The acid was decanted, and the remaining acid insoluble residue was washed by the addition of 2mls of distilled water and a second centrifugation. The wash was repeated twice more. The acid insoluble fraction was then oven dried in an oven overnight at 65°C, and weighed. The carbon and nitrogen values of a sample of the acid insoluble fraction were then obtained using a Carlo Erba 1106 Elemental Analyser as above. Elemental analysis was carried out in duplicate. The % 'collagen' is calculated by multiplying the weight of the insoluble fraction (as a percentage) by the amount of nitrogen in the insoluble fraction (as a percentage), this is then divided by the average amount of nitrogen in modern bone powder insoluble fraction (16.74%, n=20). The C:N ratio (as atom percent) was calculated to assess if the insoluble fraction is collagen (Ambrose, 1990).

3.4.3.2.4 Crystallinity and Carbonate:Phosphate ratio

The crystallinity of the mineral fraction was measured using infrared spectroscopy. The crystallinity index or infrared splitting factor (IRSF) is calculated using the splitting ratio of the phosphate $v4$ doublet at 567 and 605cm^{-1} (Weiner & Bar-Yosef,

1990). The infrared spectrum was also used to identify the presence of other mineral phases.

The carbonate:phosphate ratio (C/P) was also calculated from the infrared spectrum using the peaks at 1415cm^{-1} (CO_3^{2-}), and 1035cm^{-1} (PO_4^{3-}). This measurement can be compromised by collagen that also absorbs in this region, and is thus only used as semi-quantitative measure.

3.4.3.2.5 Mercury intrusion porosimetry

Chunks of bone (approximately 1g) were oven dried at 85°C for at least 16 hours. The pore structure of the bone was then investigated using mercury intrusion porosimetry (Nielsen Marsh & Hedges, 1999). The results of these analyses are reported as total intrusion of mercury, the skeletal and bulk density of the material, and a mercury intrusion pore size distribution (PSD) graph. The PSD graph is a plot of the amount of mercury intruded into the bone (volume/volume) against the nominal pore diameter. The PSD is divided into three approximate ranges of pore size diameters: 0.01-0.1μm, >0.1μm<10μm and >10μm-70μm.

3.4.3.2.6 Osteocalcin ELISA

Aliquots of bone powder were demineralised in 0.6M HCl (<60mg bone/2ml acid) at 4°C for 16 hours and neutralised with NaOH. Disodium Ethylenediaminetetraacetate (Na_2EDTA; 0.5M) was added to chelate any metal ions and the pH was adjusted (pH 7–9). The samples were centrifuged (14,500 g) and aliquots of the supernatant were used in all subsequent assays. Control samples, including blank extracts and coating buffer (PBS, 10mM PO_4^{3-} 0.87% NaCl pH 7.4, 0.5M Na_2EDTA) and doubling dilutions of modern bovine bone extracts were included in all the assays, in addition to the analytical samples (diluted 1:2 in coating buffer). Standard 96 well plates (Nunc; DK, Maxisorb™) were coated, in triplicate, with 100μl of each sample and incubated overnight at 4°C.

After the antigen was removed, the plates were blocked with 1% wt/vol yeast extract in PBS (10mM PO_4^{3-} ,0.87% NaCl pH 7.4) at 37°C for 2 hours. The plates were washed (x5 in PBS-tween; PBS containing 0.01% vol/vol tween-20) and 100μl of rabbit anti-Boc polyclonal antibody diluted 1/4000 in PBS-tween were added to each well for 1 hour at 37°C. Following washing, 100μl of biotinylated mouse anti-rabbit Ig antibody (Amersham L.S, UK), diluted 1/5000 in PBS-yeast were added to each well and the plates were incubated for 1 hour at 37°C. After a washing, 100μl of streptavidin horseradish peroxidase complex (Amersham L.S, UK) diluted 1/5000 in PBS-Yeast were added to each well and the plates were incubated for 1 hour at 37°C. After a final wash, 100μl of o-phenylenediamine dihydrochloride solution were added to each well and after 30min, 50μl of 3M HCl were added and the plates were read at a wavelength of 490nm.

The mean optical density was calculated for each sample per unit of bone. The amount of Oc (as % of modern signal) was calculated using the linear part of the standard curve obtained from the modern bovine bone dilution series. Analysis was

run in duplicate, the average and standard deviation is given in the figures. Absolute quantification of amounts of Oc in this assay design was difficult and the Oc values given here are only semi-quantitative. Negative control samples e.g. extraction blank, and coating buffer, could not be normalised ,as they contain no bone. Using 1.5 times the average coating buffer value (the highest negative control) as a negative result, values less than 23% of the modern bone value are considered negative results.

3.4.4 Results and Discussion

Diagenetic alteration of bone is quantified by differences between the diagenetic parameters of the archaeological bone and modern bone. The precise nature of the changes varies form site to site (and to a lesser extent between samples), but the following transformations are usually seen in bones with increasing diagenetic alteration. The proportion of bone protein declines, as evidenced by a reduction in the amount of nitrogen and 'collagen'. The bone crystallinity (ISRF) increases and the carbonate:phosphate ratio declines (both measured by FTIR) as bioapatite is transformed into more thermodynamically stable apatite. The extent of microbial and fungal attack varies, but can be semi-quantified by a decrease in the histological index (HI) from 5-0. The bone also becomes more porous; an increase in sub-collagen fibril (~0.01-0.1μm pore diameter) porosity is interpreted as (chemically driven) loss of collagen, while porosity increases in the > 0.1μm < 10μm pore diameter range are usually associated with microbial damage (see Chapter 3.1). The results of this data set are summarised below using principal components analysis (PCA), and bivariate plots of key parameters.

3.4.4.1 Principal components analysis

Principal components analysis was employed to simplify and detect any underlying structure in the data set. The following variables were analysed by PCA: IRSF, porosity in pores with diameter 0.01-0.1μm, porosity in pores of diameter >0.1μm<10μm, bulk density, skeletal density, Cracking index and Oc (as % of modern signal). In addition the histological index was included expressed as the mid range percentage of visible intact bone (see Millard, 2001), rather than the histological category score. Also a quality controlled % collagen was used where 'collagen' residues that had C:N ratios outside of the range 2.9-3.6 were considered not to be collagen and thus scored 0. Excluding bones that did not contain full data for all the variables 55 bones were used in the principal components analysis.

Figure 3.4-A summarises the data as a plot of PC2 vs. PC1 which describes ~71% of the total variation in the data. Similar analysis of a larger data set (of which this is a sub sample - See chapter 3.1) has revealed a similar pattern where PC1 shows the general diagenetic trend, e.g. a higher degree of mineral alteration is observed in bones with lower 'collagen' yields and *vice versa*, and PC2 can be interpreted as showing two distinct mechanisms of diagenesis, 'fossilisation' or burning and microbial attack. Although this data set is somewhat smaller and contains fewer 'fossilising' bones, these interpretations can also be applied to this data analysis. Table 3.4-i shows the correlations matrix used to construct Figure 3.4-A.

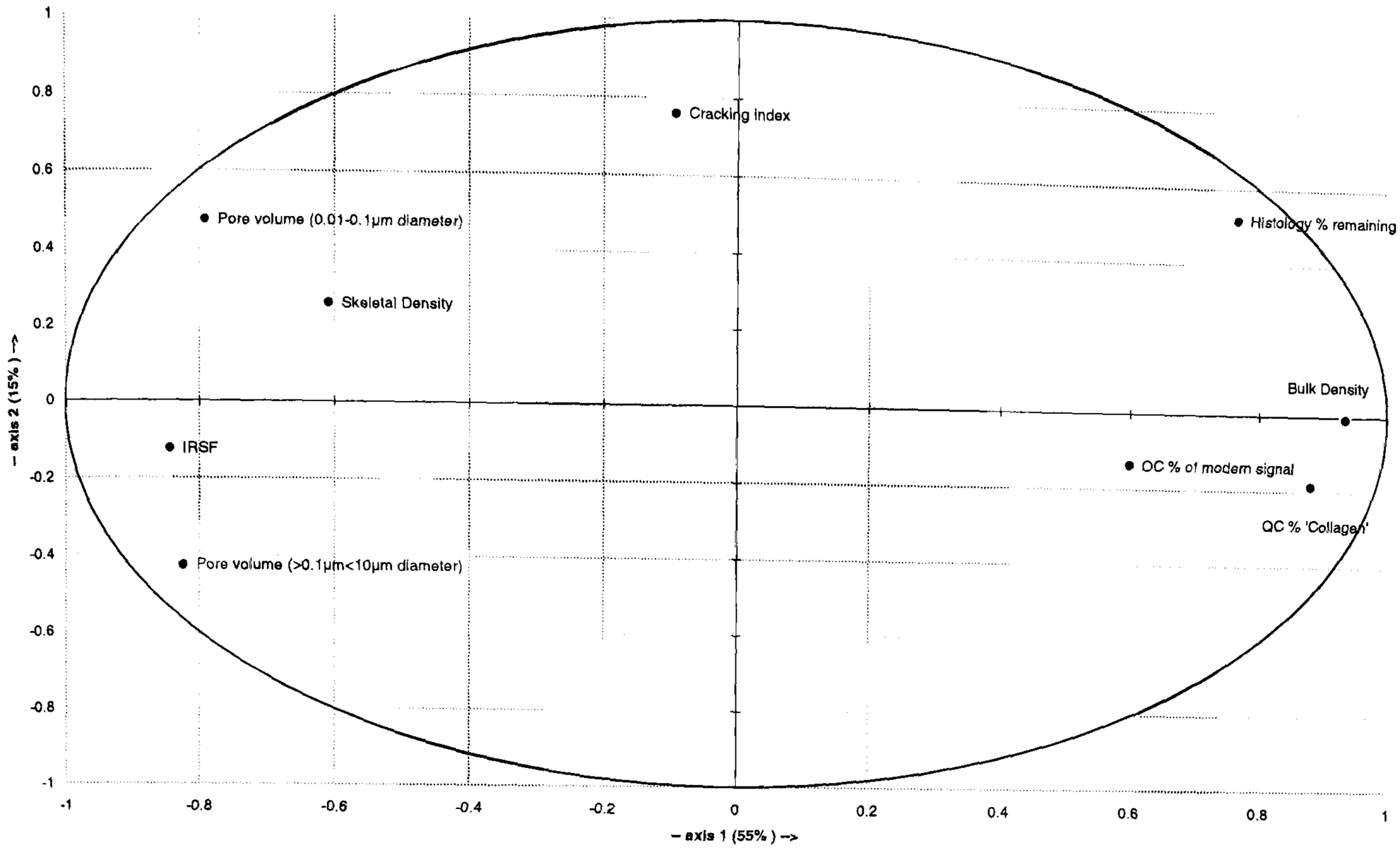

Figure 3.4-A. Correlations circle for principal components 1 and 2 for bovine bone.

	QC % 'Collagen'	IRSF	Pore volume (0.01-0.1µm diameter)	Pore volume (>0.1µm<10 µm diameter)	Bulk Density	Skeletal Density	Histology % remaining	OC % of modern signal
IRSF	-0.6499	-	-	-	-	-	-	-
Pore volume (0.01-0.1µm diameter)	-0.7834	0.6118	-	-	-	-	-	-
Pore volume (>0.1µm<10µm	-0.5752	0.6932	0.4097	-	-	-	-	-
Bulk Density	0.7978	-0.7807	-0.7178	-0.7922	-	-	-	-
Skeletal Density	-0.5155	0.4169	0.7100	0.4102	-0.4313	-	-	-
Histology % remaining	0.5852	-0.6260	-0.3117	-0.8912	0.7217	-0.2564	-	-
OC % of modern signal	0.5730	-0.4571	-0.4114	-0.3404	0.5189	-0.2046	0.3534	-
Cracking index	-0.1511	-0.0160	0.2714	-0.0973	-0.1866	-0.0032	0.1567	-0.1057

Table 3.4-i. Correlations matrix (r-values) for principle components analysis for bovine bones.

If the above interpretation is true for this sub set of data, Oc degradation correlates well with PC1 (R=0.60), but not with PC2 (R=0.14), indicating that the level of Oc is dependent on the general diagenetic state of the bone, but independent of the mechanism by which it has degraded. This interpretation may be contentious however, as the data set is dominated by a number of well preserved bones, and lacks enough examples of 'fossilising' and microbially attacked bones for the possible influence of these to be observed in the PCA. There is also variation in the level of Oc observed in diagenetically unaltered bone which may reflect either (i) variation in

the original amount of Oc in the bone (Oc content is observed to decrease with age, see Ingram *et al.*, 1994), (ii) alternative (unknown) mechanisms of Oc loss which do not affect the other diagenetic parameters, or most probably (iii) errors in the analysis, perhaps due to interference from bone collagen (see Tuross *et al.*, 1989). Higher relative standard deviations are observed in the Oc measurements in bones with higher 'collagen' yields, which are likely to be bones with higher amounts of Oc, this may reflect 'collagen' interference in the analysis.

Further investigation of the degradation of Oc is discussed in direct relation to the other diagenetic parameters.

3.4.4.2 Relationship between Oc and crystallinity (IRSF)

Collins *et al.*, 2000 observed that the Gla-rich α-helix of the osteocalcin molecule is well preserved only in bones with limited diagenetic alteration and make particular reference to the relationship between the IRSF of the sample and the preservation of this epitope. The amount of Oc detected in the bone here shows a broad trend with IRSF similar to that shown by Collins *et al.*, 2000, and concurs with the data of Tuross *et al.*, 1989 (see Figure 3.4-B). The IRSF measurement is a measure of the perfection, short-range order and size of the crystal. IRSF may increase, either by selective dissolution of the less perfect crystals, or dissolution and recrystallisation of imperfect crystals into more perfect ones. An increase in crystallinity and therefore crystal size and perfection will reduce both the overall surface area of, and the number of crystals thereby limiting the sites for adsorption of Oc. Desorbed Oc will suffer accelerated rates of Gla decarboxylation and proteolysis.

As crystallinity increases the maximum Oc yield decreases (Figure 3.4-B), bones with IRSF >3.1 do not contain significant amounts of Oc. The data here concurs in part with the broader assertion of Collins *et al.*, 2000, but suggests Oc survival may be related to the other diagenetic changes in the bone, not simply to changes in the mineral phase, as Collins *et al.*, 2000 propose for the Gla-rich α-helix.

3.4.4.3 Relationship between Oc and microbial alteration

An alternative mechanism of Oc (and collagen) loss is microbial attack. As Histological Index decreases (i.e., extent of microbial attack increases) the maximum yield of Oc decreases rapidly (Figure 3.4-C). This study shows that bones with HI less than 3 are unlikely to contain significant amounts of Oc. This result is surprising given that in such bones clear islands of histologically unaltered bone remain. Oc is concentrated within the osteon (Ingram *et al.*, 1994), but we do not anticipate that complete loss of Oc detection would occur in bones with HI of > 0, this may indicate a limitation of the assay but clearly merits further investigation.

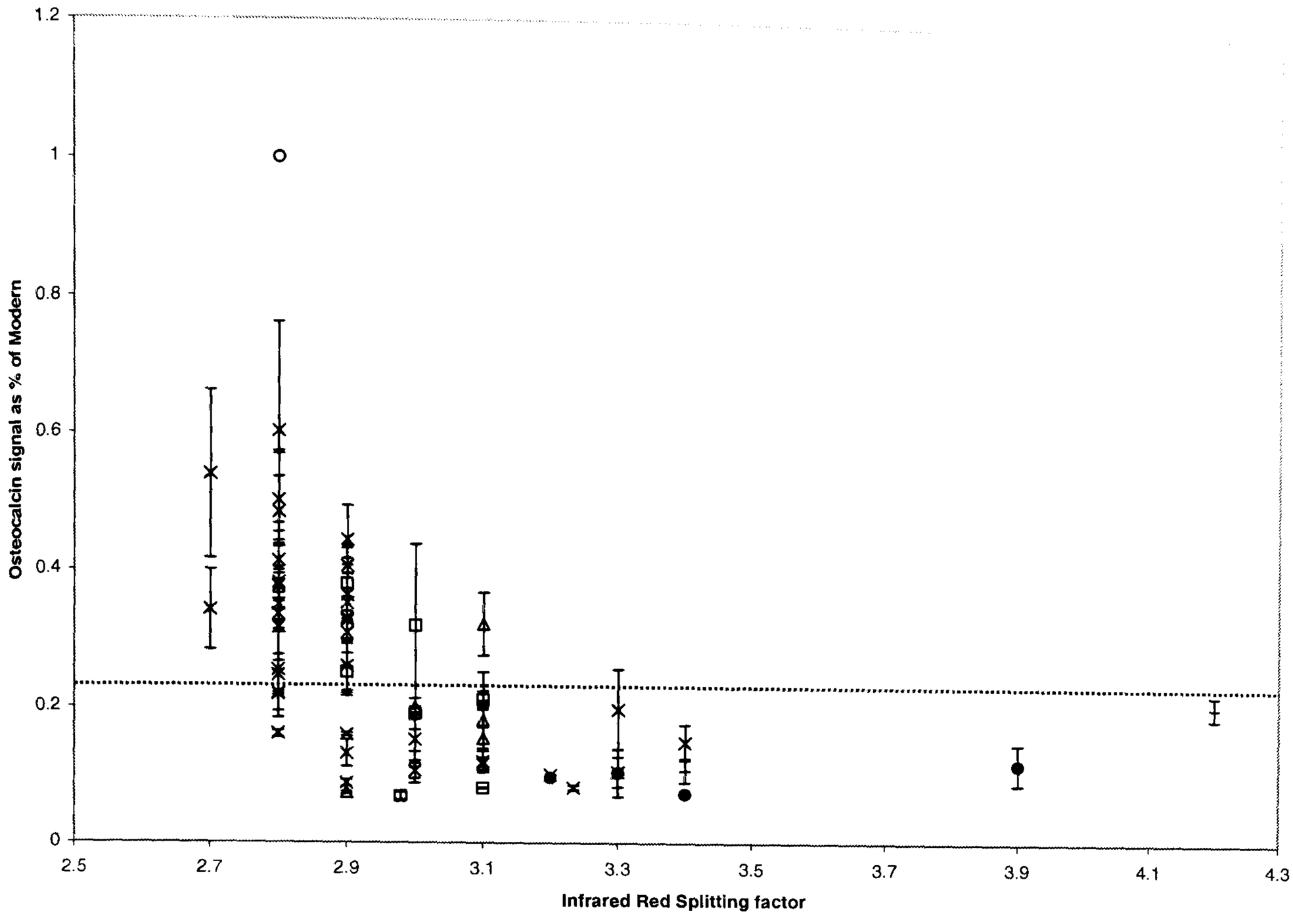

Figure 3.4-B. Amount of osteocalcin against splitting factor for bovine bone, with histological index (HI) indicated, X HI 5, □ HI 4, Δ HI 3, + HI 2, ■ HI 1, • HI 0, O Modern Bone. Error bars (1s.d) for amount of Oc are shown. Dotted line indicates negative control value.

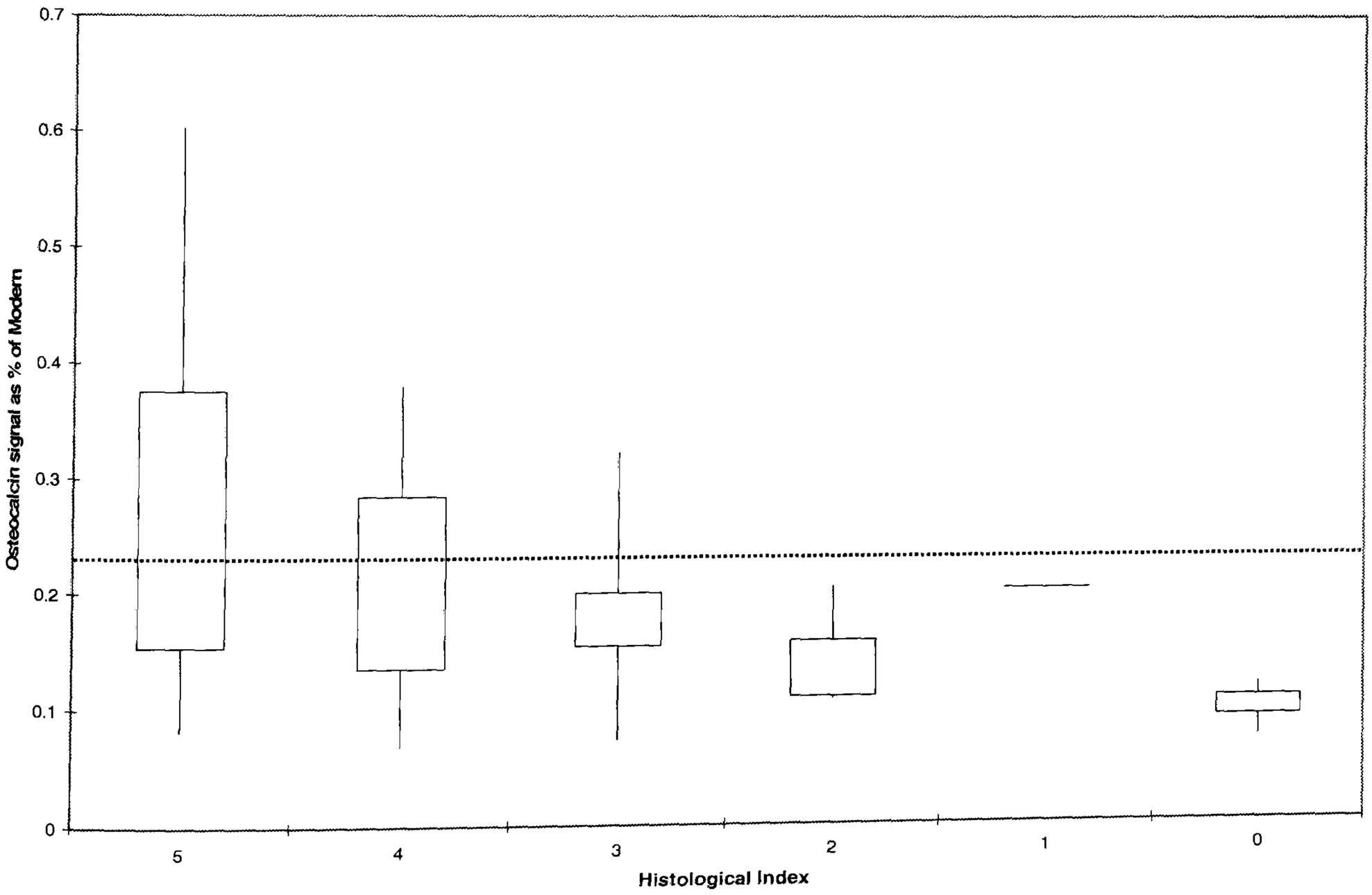

Figure 3.4-C. Range of average osteocalcin values for each histological category for bovine bone. Maximum, minimum and interquartile ranges are shown. Dotted line indicates negative control value. (HI 5, *n*=38; HI 4, *n*=7; HI 3, *n*=5; HI 2, *n*=3; HI 1, *n*=1; HI 0, *n*=4).

Further insight into the impact of microbial alteration on Oc loss can be made by examining the increase in the >0.1μm <10μm porosity associated with microbial alteration (Figure 3.4-D). The relationship between porosity increase and microbial damage is complicated by the fact that microbial alteration will have two effects, the removal of collagen and the remobilisation of mineral (and therefore increase in IRSF). In some instances (low pH and good drainage), remobilised phosphate will be removed from the bone, creating additional porosity. Porosity in the range >0.1μm<10μm represents the combined effects of these two processes. Despite this proviso, only when the >0.1μm <10μm porosity is less than 5% of the bone are significant levels of Oc measured. In this study 75% of the bones have less than 5% porosity in this diameter range, however in the larger study of bone diagenesis across Europe from which this is taken (see chapter 3.1) only 25% of the bones have 5% or less porosity in this diameter range.

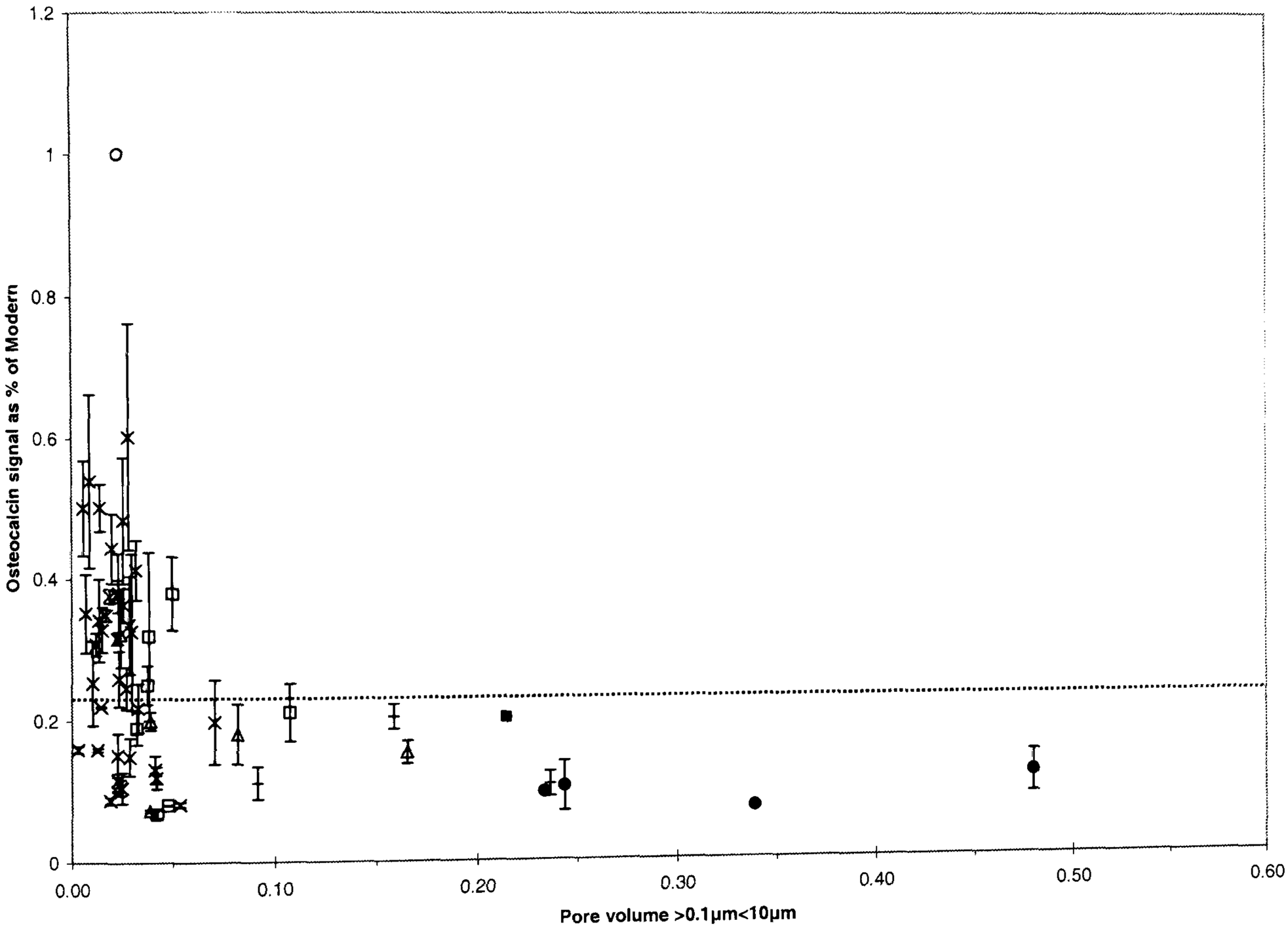

Figure 3.4-D. Amount of osteocalcin against pore volume in pores with diameter >0.1μm<10μm for bovine bone, with histological index (HI) indicated, X HI 5, □ HI 4, Δ HI 3, + HI 2, ■ HI 1, • HI 0, O Modern Bone. Error bars (1s.d) for amount of Oc are shown. Dotted line indicates negative control value.

3.4.4.4 Relationship between Oc and collagen

Initial studies of Oc in archaeological bone focused on the utility of Oc as an alternative substrate to collagen for radiocarbon and palaeodietary studies in collagen depleted bone, as it had been suggested that Oc may have greater longevity than the collagen fraction. It would be anticipated that Oc and collagen (both proteins) would share the same general modes of degradation and preservation, i.e. microbial

degradation and preservation via close mineral association, although the structure of collagen and its structural role in the bone as a whole may lead to it being more resistant. Assuming similar degradation pathways it would be anticipated that the amount of Oc and collagen would be correlated. Alternatively, preserved collagen may provide a mechanism for Oc survival. Prigodich & Vesely, 1997 report a strong Oc association constant for collagen (albeit 100 fold lower than for apatite), hence Oc may be preserved by close association with the collagen. Either mode would provide a strong correlation between Oc and collagen yield, however the causality of either mechanism would be difficult to prove.

In this study of archaeological bovid bones, low 'collagen' bones also have low amounts of Oc, concurring with the findings of Burky *et al.*, 1998, suggesting that the use of Oc as an alternative substrate to collagen may not be practical. In bones that have large amounts of collagen preserved the levels of Oc are variable (~10-60% of modern). This may be a feature of collagen interference in the assay, or indicate that collagen and Oc preservation are not necessarily linked. It should also be noted that this variation may reflect natural variation *in vivo*.

3.4.4.5 Changes in Oc level without histological alteration

The decrease in Histological Index is generally covariant with other changes in archaeological bone (i.e. increase in IRSF, decrease in collagen), and thus it is difficult to determine which change may have more influence on Oc degradation than the others. It is clear however that low amounts of Oc are detected in bones with HI 5, and thus in such cases microbial attack cannot be the mechanism of Oc loss (Figure 3.4-E).

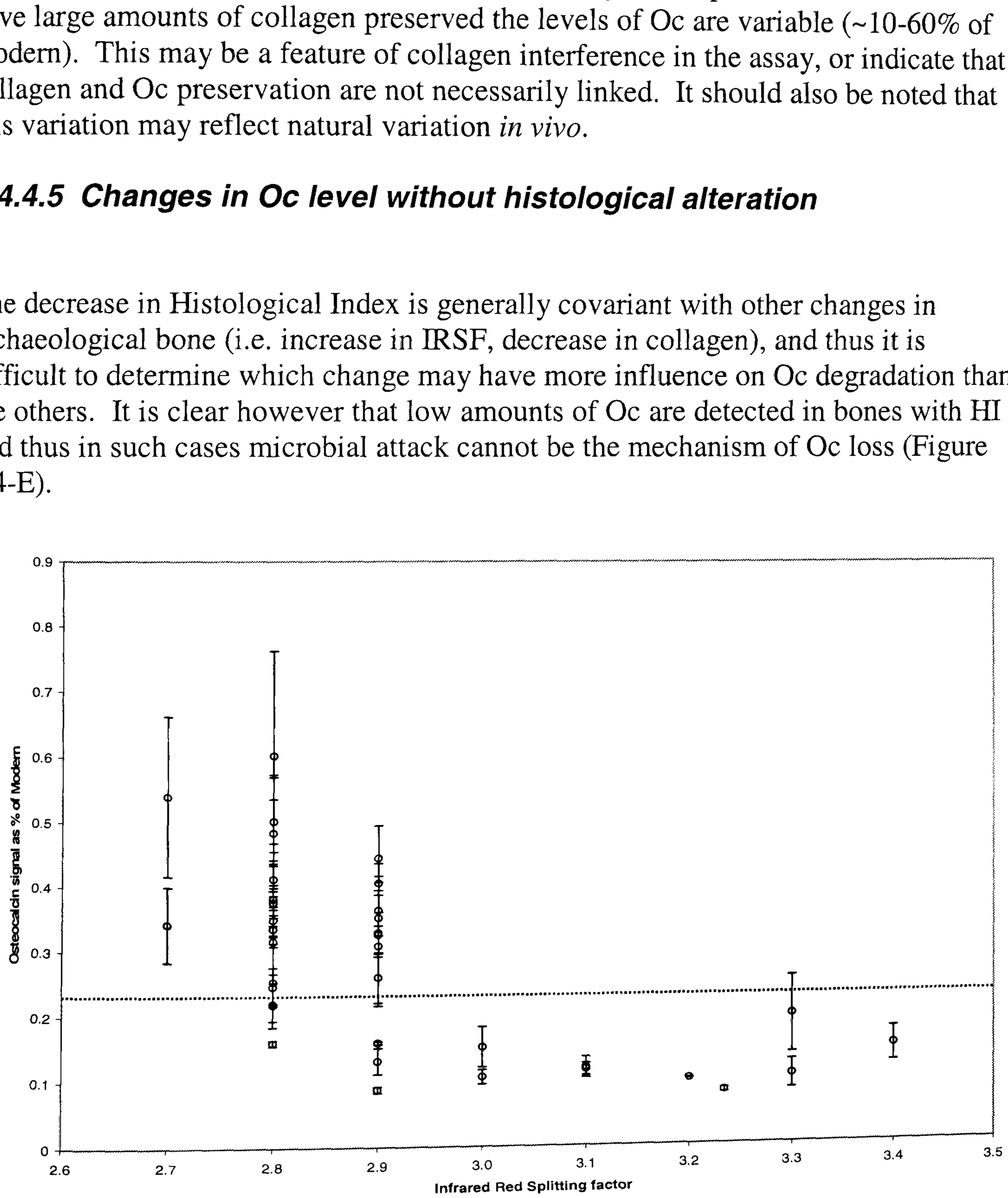

Figure 3.4-E. Amount of osteocalcin against splitting factor for bovid bones with HI 5 only. Error bars (1s.d) for amount of Oc are shown. Dotted line indicates negative control value.

There are some bones with HI 5 that have no collagen remaining which indicates that this rapid loss of collagen that is not microbially mediated. This type of preservation has been noted (normally associated with a high IRSF and Cracking index values) to be a special type of collagen loss, perhaps a rapid form of proto-fossilisation (Chapter 3.3). Of the 39 samples with HI 5, 5 have <6% 'collagen' and these have the 4 lowest Oc values. This may indicate that the rapid loss of collagen in this 'fossilisation' process is coincident with a rapid loss of Oc, i.e. that this process has a deleterious effect on both proteins studied here (and perhaps all protein in the bone).

For HI 5 bones that have >6% 'collagen' remaining increasing crystallinity appears to affect the amount of Oc that survives, such that bones with IRSF > 3.0 have low (less than the negative control) values for Oc, which is consistent with bone that has been microbially attacked. This indicates that crystallinity increase either via microbial attack, or through interaction with the soilwater may have an influence on the level Oc that survives in the bone.

The amount of Oc drops in bones with and without microbial attack due to increases in crystallinity, it also drops in bones that have lost 'collagen' due to either 'fossilisation' or microbial attack. This would explain why it is neutral to principal component 2.

3.4.4.6 Oc and DNA preservation?

The mechanisms for the survival of and degradation of Oc (binding to apatite, and microbial attack respectively) are similar to those suggested for DNA (Tuross, 1993, and Hagelberg *et al.*, 1991 respectively). When the ability to amplify ancient DNA was compared to the diagenetic state of the bone, specifically histological index, it was noted by Colson *et al.*, 1997 that in well preserved bones (HI 5) DNA could always be amplified whereas in poorly preserved bone (HI 0) it could never be amplified. In the intermediate categories the ability to amplify aDNA was less predictable. The pattern of Oc survival observed here is similar to that described above for DNA.

3.4.5 Conclusions

The bones analysed here follow (in general) expected diagenetic trajectories (see Hedges *et al.*, 1995; chapter 3.1) with decreasing amounts of protein, increasing crystallinity, and porosity, and decreasing histological index. The amount of Oc in the bones decreases with the general level of diagenetic change. In these samples (ranging in age from 400-53000 years BP), the changes are largely independent of the age of the material.

The amount of Oc decreases rapidly with increasing crystallinity of the bone mineral (such that bones with IRSF>3.1 are unlikely to contain significant amounts of Oc), concurring with previous findings (Collins *et al.*, 2000; Tuross *et al.*, 1989). The amount of Oc also decreases rapidly with decreasing histological index such that bones with HI<3 are unlikely to contain Oc (although there will be concomitant increase in splitting factor in such bones). Thus, Oc will only be preserved in otherwise well preserved bones. Collins *et al.*, 2000 suggest that, based on laboratory

heating experiments, small amounts of the α-helix may survive for long periods in the archaeological/geological record. Our results demonstrate, that in most cases where bone is diagenetically altered Oc will not survive, due to collagen and mineral alteration brought about via either microbial attack or 'fossilisation'. In bone that shows little diagenetic alteration, Oc can survive for >50 kya, although even in these bones there is variation in the levels of Oc detected (which may be due to 'collagen' interference in the assay). It could be that residual amounts of OC do survive for long periods in diagenetically altered bone, but are below the limit of detection of the assay employed here. Whilst residual amounts may be utilised in some techniques (e.g. MALDI-TOF, see Ostrom *et al.*, 2000), yields of Oc in collagen degraded bone are unlikely to be of practical use.

3.4.6 Acknowledgements

This work has been carried out as part of the Degradation of Bone as an Indicator in the Deterioration of the European Archaeological Property project, which is acknowledged for both funding and providing archaeological material (ENV4-CT98-0712). The authors would also like to thank Professor Peggy Ostrom and Dr. Gordon Turner-Walker for providing material for analysis.

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Part 4

Synthesis, conclusion, future work

4 Synthesis

Discussion of key points has already been made in the main body of the thesis. The aim of this chapter is to synthesise the findings of the different approaches in parts 2 and 3 into a coherent discussion of the thesis as a whole, with particular reference to biomolecular preservation.

4.1 Limits of biomolecular preservation in bone

The key theme of Part 2 of this thesis is the modelling of the theoretical limits of the survival of collagen and DNA in archaeological bone. The models make a number of conceptual simplifications, disregarding realistic modes of diagenesis (i.e. microbial attack) to investigate the limits of chemical degradation. It is assumed that the hydrolysis of these molecules will be slower than microbially mediated degradation, thus if the slowest mode can be accurately modelled then the limit of preservation will be attained. The measurement of diagenetic parameters of bones excavated from European Holocene sites detailed in Part 3 and the 'collagen' yields given in the Oxford radiocarbon database reveal that most bones do not fit the model because other diagenetic pathways are prevalent. Notably, bone without evidence of microbial attack can have very poor preservation of collagen, something not anticipated by the model. The assessment of osteocalcin preservation (a possible proxy for DNA preservation) allows insight into the preservation of other biomolecules. The results of the models are discussed with respect to the results gleaned from the measurements of archaeological bone, below.

4.1.1 Collagen

Understanding collagen degradation is important because, collagen imparts mechanical strength to the bone as a whole and is a source of much data for archaeological scientists. Loss of collagen results in increased porosity and decreased mechanical strength, consequently accelerating other diagenetic changes to the bone and potentially the destruction of the bone altogether. Understanding the modes and rates of collagen loss will provide valuable information about the limits of its preservation and thus the limits of this resource. It should be noted that there is an apparent increase in the C:N ratio of in bones with lower 'collagen' yields, highlighting that understanding the limits of preservation has implications for collagen quality as well as yield.

Collagen loss from archaeological bone is a complex phenomenon. It has been hypothesised that collagen is lost principally via two mechanisms, either rapidly via the action of microbes, or in the absence of microbial attack, more slowly through the action of chemical hydrolysis. Chapter 2.2 investigated the latter pathway by estimating rates of collagen loss from bone from high temperature experiments. This data was used to extrapolate the rate of collagen loss at lower temperature, i.e. those likely in archaeological sediments.

Predicted collagen yields were compared with measured yields for bone dated at the Oxford and Groningen radiocarbon laboratories. This revealed that the majority of bone has 30% or less of the predicted collagen yield (NB. if the rate used in chapter 2.2 is too high, then most bones would contain proportionally even less collagen than predicted). This distribution can be explained in a number of ways. The model may be fundamentally flawed, either because the rate of degradation or the temperatures of the bones are incorrectly estimated, or it may be that the rate of gelatinisation cannot be extrapolated to lower temperatures without incurring an unknown error.

The predictions will be wrong if predicted temperatures are systematically too cold. The estimated temperatures have been compared to published soil temperature data, whilst there may be large errors in individual cases and there is evidence of a systematic error of 1-2°C in the predicted effective temperatures in the 10-20°C range. The conclusion would remain the same that most bone contains less collagen than predicted, especially if the rate used in chapter 2.2 is too high. The rates of collagen loss from radiocarbon dated bone indicate that rates extrapolated from high temperature experiments will give absolute rates comparable to those observed in the radiocarbon dated bone. If the observed rates are used to constrain the high temperature estimates it is also apparent that the activation energy must be in the region of 150-180kJmol^{-1}. It thus appears that the survival of bone collagen is highly temperature dependent. If this is the case then the model would appear to be robust and other reasons for yields of collagen lower than those predicted must be sought.

The other plausible explanation for the distribution is that the model does not describe a significant diagenetic pathway for collagen, i.e. collagen rarely degrades in archaeological bone in the same manner as in laboratory experiments, and that other rapid modes of collagen degradation occur. Prior to the construction of the model, microbial degradation of the collagen was a well-known phenomenon, however, in the absence of this rapid degradation it was anticipated that non-microbially attacked bone would provide useful data with which to test the model. This data is not available from the radiocarbon data set, but predictions could be tested using the data measured in Part 3.

Microbial attack of bone results in destruction of the histological structure of the bone, which is semi-quantified by the histological index score. Bone with perfect histology will thus provide a useful test for the model. Such bone comes in two very different varieties, well-preserved bone, and 'fossilising' bone. Well-preserved bone described in Part 3 comes from young sites, no more than 1000 years old, indeed the oldest material tested is ~6000 years old, which at a nominal temperate effective burial temperature (~13°C) will only lead to approximately 10% of the original collagen being lost, that is, bones will be ~20% 'collagen' by weight. It would be difficult to perceive the effects of chemical collagen loss in young bone within the limits of error for the collagen extraction and the prediction. It may be possible to test the model more thoroughly with otherwise well preserved Pleistocene material, where significant amounts of collagen have been lost. More significantly, the levels of collagen in the 'fossilising' bone are far below those anticipated from the model. The loss of collagen in the fossilising bone appears to be chemically driven, i.e. without the aid of microbes, as there is no evidence of MFDs. The pore structure of 'fossilising' bone is similar to that of hydrazine hydrate treated bone, which has lost collagen through chemical hydrolysis, which is further evidence indicating a chemical

loss of collagen from fossilising bone. It should be noted that the chemical degradation of collagen will continue in microbially attacked bone (the modes need not be exclusive), but the extent of chemical loss is difficult to ascertain, as it is 'overprinted' by the more rapid microbial loss of collagen. The early fossilising process clearly promotes a rapid loss of collagen, more rapid than that anticipated by comparison to laboratory heating experiments of bone. One explanation for this may be that the stability of bone collagen is provided by its intimate association with the bone mineral. It is apparent that in archaeological bone the mineral phase can change (i.e. the crystallinity of the mineral increases); this change is quite marked in fossilising bone. It possible that this change in mineral is enough to disrupt the collagen/mineral association so that the collagen behaves as only partially mineralized, and thus the rate of hydrolysis is faster. This would result in the collagen yield being lower than that predicted by the model.

In summary, the model of collagen loss presented in Part 2 has demonstrated a plausible maximum limit to bone collagen survival based on hydrolysis rates from laboratory experiments, this limit is rarely exceeded in the archaeological record. It does not however represent a major diagenetic pathway for collagen, as collagen is typically lost more rapidly via microbial attack, or the 'fossilisation'.

The model presented in Chapter 2.2 also highlights the importance of microbial taphonomy of bone collagen. Significant amounts of collagen are lost from bone that has undergone microbial attack, whether this is the direct action of the microbes or associated diagenetic effects. It is estimated that microbial attack will be complete by ~500years *post mortem* (Hedges *et al.*, 1995). In the same period in temperate climates the amount of predicted chemical hydrolysis will be negligible; moreover under the same conditions collagen will last for tens of thousands of years, thus the rates of collagen loss by the two different modes are greatly contrasted.

4.1.2 Osteocalcin

The persistence of non-collagenous proteins over collagen in ancient bone is well documented (e.g. Masters, 1987), and the use of Osteocalcin (Oc) as an alternative substrate for isotopic analysis, and radiometric dating has also been investigated (e.g. Ajie *et al.*, 1992). The reason for the exceptional preservation of Oc is usually explained by its tight association to the bone mineral (see Tuross *et al.*, 1989), but the investigation of Oc survival has not been thoroughly integrated into a study of bone diagenesis.

Rates of Osteocalcin degradation have been modelled in a similar fashion to the collagen experiments described in chapter 2.2 (Collins *et al.*, 2000), demonstrating that Oc (or at least the Gla rich region) has the potential to last for perhaps millions of years. The fate of Oc in archaeological bone however appears to parallel that of collagen, in that whilst it may theoretically survive for a long time, taphonomic processes rapidly degrade it. It is apparent from the data in chapter 3.4 that the state of preservation of the bone as a whole and not the age is a key factor in Oc preservation. Significant amounts of Oc are only observed in well preserved bones, where minimal taphonomic change has occurred. The concomitant changes that occur during bone taphonomy make it difficult to assess whether any one change, i.e. mineral alteration, has a stronger effect on Oc preservation than any other. The

results here do not contradict the findings of Collins *et al.*, (2000) and Tuross *et al.*, (1989), that mineral preservation is a key factor in Oc survival. From the study made here it can be suggested that any form of diagenetic change will have associated deleterious effects on the amount of Oc remaining in archaeological bone and whilst other studies suggest that small residual levels may persist, the majority of the Oc will be lost from the bone should taphonomic changes occur.

It should be noted that Ajie *et al.*, 1992 suggest that γ-carboxyglutamic acid (Gla) is well preserved in archaeological bone (this is in agreement with the findings of Collins *et al.*, 2000), but that intact Oc may be present in only small amounts. Therefore, whilst Oc nominally survives, it does so as degraded peptide fragments. The use of polyclonal antibodies in this study will not reveal the state of preservation. The polyclonal antibody will recognise a number of different configurations and sequences (epitopes) in the protein; thus a bone containing degraded Oc may appear to contain as much Oc as one that contains well preserved Oc. The levels of Oc measured by the method here tend to reduce to a lower limit which may be indicative of a persistent residual Gla fraction; however this may simply be the background level of the assay. Alternative approaches such as the successful identification of Oc in Pleistocene bones by mass spectrometry (Ostrom, *et al.*, 2000) will hopefully provide more detailed information on the fate of this protein.

4.1.3 DNA

Limits of DNA preservation and authentication are important issues because of the technical difficulties and impact of the work. The model in Chapter 2.3 does not attempt to define an absolute limit for DNA survival but draws attention to the time-temperature factor in DNA survival (and the thermal history) as a method to rank sites in order of likely hood of DNA survival. Thermal history is important in Neanderthal sites, as the very cold Pleistocene in Northern Europe means that chronological age may have little to do with the theoretical limit of biomolecular survival.

The modelling and measurement of collagen and Oc survival have revealed that the molecules (in particular collagen) are not likely to survive to their theoretical potential as other prevalent degradation pathways will result in the rapid destruction of biomolecules in bone (see also Ovchinikov *et al.*, 2001 for comments on DNA). If the mode of DNA survival is similar to that of Oc then it is likely that DNA will only survive in well-preserved bone. Other studies have demonstrated that DNA survival is related to other diagenetic changes in the bone (Götherström *et al.*, in press; Poinar & Stankeiwicz, 1999). Although in the latter study the use of pyrolysis gas-chromatography mass spectrometry (PyGC-MS) may be a complex and expensive technique, where a simple acid demineralisation may suffice to assess collagen preservation. It is interesting to note that Oc survival may not be as intact Oc molecules but as peptide fragments. The parallel of this in DNA survival is already well understood, in that aDNA studies are normally only successful at analysing short fragments of DNA (~300 base pairs long). Like Oc, DNA may survive for long periods in archaeological bone but as short fragments that are of little or no benefit to aDNA studies.

Whilst the models described in Part 2 are useful for describing limits of preservation and comparing relative preservation between sites, it is important to note that in

archaeological bone these limits are seldom met as a number of diagenetic pathways are in operation, highlighting the need to measure diagenetic parameters to help describe and further understand these taphonomic pathways.

4.2 Conclusions

In laboratory experiments the rate of collagen loss from bone can be described as being very temperature sensitive, having a high activation energy in the region of 150-180kJmol^{-1}. Using these parameters it has been possible to estimate an upper bounding limit on collagen survival in archaeological bone. Using a similar approach it has been possible to rank Neanderthal cave sites in order of a theoretical level of DNA preservation. The efficacy of this model will only be borne out by future ancient DNA analysis.

It has become apparent, however, that biomolecules will rarely reach their theoretical survival limits in archaeological bone due to the taphonomic process, highlighting the need to assess the diagenetic state of the bone prior to further investigation, e.g. aDNA analysis. The analysis of over 200 bones from sites across Europe has revealed three discrete states of preservation in which bone is found at archaeological sites (complete destruction of the bone is another outcome). These states of preservation are defined by a group of characteristic diagenetic parameter values (see Table 4.2-i). The processes that cause these states, and the environments in which they occur are not yet fully understood, but possible explanations are discussed below.

4.2.1 Types of preservation

4.2.1.1 Well preserved bone.

Well preserved bone is similar to bone from a recently killed animal in terms of the diagenetic parameters measured here. In most cases archaeological bone will not retain the soft tissues associated with modern bone (the marrow and periosteum), and may be stained in colour, however with respect to other physico-chemical properties it will be comparable to modern bone. Well-preserved bone in general retains high collagen levels, low crystallinity and high carbonate phosphate ratio, and porosity values similar to modern, although most archaeological bone measured here does have slightly increased porosity in the larger pore diameter range. The histological preservation is perfect and there is no evidence of cracking.

State of preservation	% 'collagen'	C:N ratio of 'collagen'	%N of whole bone	Infrared Splitting Factor	Carbonate: Phosphate ratio	Bulk Density (gcm-3)	Skeletal Density (gcm-3)	Pore volume (0.01-0.1µm diameter)	Pore volume (>0.1µm<10µm diameter)	Pore volume (>10µm-70µm diameter)	Histological Index	Cracking Index (%)
Modern bovine bone	**22.7**	**3.2**	**4.2**	**2.8**	**0.43**	**1.9**	**2.1**	**0.0514**	**0.0235**	**0.0209**	**5**	**0**
Well Preserved	15+	3.2	2.8	3	0.4+	1.9	2.1	0.05-0.1	<0.1	0.02+	5	0
'Fossilising'	<5	3.2-30	<1	>3.9	0.1	1.0-1.5	2.5-3.0	0.3-0.4	<0.1	0.02+	5	100
Microbially Attacked	0-20	3.2-30	0-4	3-4	0.3-0.4	1.5-1.8	2.3-2.5	0.05-0.25	0.2-0.3	0.02+	0-4	0-100

Table 4.2-i Typical diagenetic parameter values for the different states of diagenetic bone.

4.2.1.2 'Fossilising' bone (the Apigliano style)

'Fossilising' bone is the subject of Chapter 3.3; it has a number of characteristics of bone in the early stages of fossilisation as described by Pfretzschner (2000). Like well-preserved bone the histological preservation of 'fossilising' bone is generally good (HI5), with the exception of a number of cracks (or microfissures) in the structure. The cracking index value is normally high 80-100%. Most of the other diagenetic parameters are highly altered: the collagen yield is <5% often 0% by weight, the splitting factor is often very high >4.0, the carbonate phosphate value low 0.1, and there is a large increase in the pore volume of pores with diameter 0.01-0.1μm. A slight increase in the pore volume in pores >10-70μm is also observed, but this is common to all diagenetically altered bone.

This form of preservation was not anticipated prior to the modelling of collagen diagenesis. It is apparent that the loss of mineralised collagen in these bones is more rapid than that predicted from the laboratory experiments, but is not microbially mediated. The bone samples from Apigliano were recovered from articulated human skeletons, and thus unusual preparation (e.g. cooking) is unlikely. One possible explanation was that the graves had been limed, however it was not possible to recreate a similar state of preservation using heat accelerated liming experiments in the laboratory. It is possible that this type of diagenetic pathway is the result of extreme diagenetic conditions during early taphonomy. The rapid collagen loss may be the result of early mineral diagenesis, sufficient to alter the collagen mineral interaction, resulting in the collagen being more labile and more rapidly lost than anticipated from the model in chapter 2.2.

The collagen/mineral stability may also help to slow mineral alteration, as it has been shown that DNA and gelatine can passivate the mineral surface and slow the rate of crystallinity increase (Okazaki *et al.*, 2001). Fossilising bones have low amounts of collagen and highly altered mineral phases. The latter resulting from interaction between the soilwater and the bone mineral crystals. In collagen depleted bones the mineral may be more exposed, increasing the interaction and thus the crystallinity. The characteristic pore structure of fossilising bones (increased porosity in the ~0.01-0.1μm diameter pores) may also play a role in the increased crystallinity. At Apigliano the soil is dry and the pores are only likely to become filled during periods of precipitation, i.e. a recharge regime (Hedges & Millard, 1995). Small diameter pores will be the first to fill with water, but also the last to dry out. Assuming sufficient precipitation to fill these small pores, water that enters these pores may potentially reach saturation, and then redeposit the mineral on drying. If there is no through flow of water there will be little overall dissolution of the bone, simply increased crystallinity. In contrast to microbially attacked bone (see below) where the pore sizes are larger which may influence a more dissolving, rather than reprecipitative regime.

Burnt bone also shows a similar pattern of diagenetic parameters to fossilising bone, i.e. low collagen, highly altered mineral, however the microcracking is not always associated with burnt bone, and the pore structure of burnt bone is different to that of 'fossilising' bone. Burnt bone has increased porosity in the pore size ~0.1-10μm diameter pores, not 0.01-0.1μm.

4.2.1.3 Microbial attack

Microbial attack is a ubiquitous diagenetic pathway, and is characterised by damage to the histological structure of the bone. There are a number associated effects that are apparently caused either by the direct action of the microbes and fungi, or by side effects of this process. One major effect of the microbial attack is the increase in porosity in the pore diameter range ~0.1-10μm. Within this range there are peaks that correspond with the approximate size of microbes observed in archaeological bone, 0.5-1μm (Jackes *et al.*, 2001). Other diagenetic parameter values are normally intermediate compared to the types of degradation described above, and vary greatly. Collagen values can vary from 0-20% by weight, the maximum observed for each histological category tends to decrease with histological index score, but yields can vary greatly. A similar pattern is observed in crystallinity index and carbonate phosphate ratio.

4.2.1.4 Destruction

By definition bone that is completely destroyed cannot have measured diagenetic parameter values, but it should be noted that this is the most likely long-term taphonomic outcome for most bones and is a genuine taphonomic end point. It is likely that the above mentioned states of preservation are only transitory and that ultimately the bones will be destroyed (see section 4.2.3).

4.2.2 Processes, environments and taphonomic events

Having identified and described four major states of bone preservation, it is useful to try to identify the processes that create these changes and the types of environment where they happen. It is apparent from the data in Chapter 3.2 that the chemical and physical properties of the soil do not give clear insight in to the diagenetic state of the bone, i.e. there is not clear correlation between soil properties and state of preservation of the bone. For example well preserved bone is observed at Millennium Bridge, Santa Lucia alle Malve and Nijmegen, three very different sites. Indeed Nijmegen is a case in point in that all three states of preservation are observed at this multi-period site. Moreover, the good preservation at Millennium Bridge may be due to the waterlogged condition of the site, and the fact that the bones are butchered animals (circumventing possible microbial attack). Whereas, at Santa Lucia alle Malve the well preserved bone tends to have some calcite infilling, which is apparently halting otherwise microbial decay at the site. These are plausible explanations for the good preservation at these sites. At Nijmegen the well-preserved bone is from infants, whilst other bones from the same period are adult and microbially attacked. Assuming that endogenous gut flora plays a significant role in the initiation of bone microbial attack, this could explain the disparity between adult (microbially attacked) and neonatal/infants (well-preserved) from the same period (Child 1995; Bell *et al.*, 1996). The long-term burial conditions at Nijmegen do not appear different enough to cause such disparity in the preservation of the bone, indicating that pre-burial factors may be important in determining the ultimate preservation of the bone. For bone to be maintained in a well-preserved state it

requires the exclusion of microbes, and benign conditions that do not cause the 'fossilisation' of bone.

It is observed that the microbial attack and fossilisation can occur at the same site of the same period. The depositional conditions should be nominally the same for such burials, further evidence that pre-burial factors are important in determining the taphonomic trajectory of a bone. One explanation would be that the seasonality of the burial might determine a fossilising or microbial trajectory. Burial under dry, waterlogged or cold conditions may stifle early bacterial growth in the corpse, resulting in a lack of microbial growth. If the flesh is removed without extensive microbial growth then the bone is unlikely to become microbially attacked. There are then three possible outcomes for the bone: 'fossilisation', well preserved, or destruction. Perhaps in acid soil, destruction is the likely outcome; a more benign soil, perhaps fossilisation, or maybe under exceptional circumstances good preservation. If microbial attack occurs early in a warm wet corpse, the bone will present evidence of microbial attack on excavation, or be destroyed.

4.2.3 Projections

In theory there is really only one outcome of bone diagenesis, and that is the complete destruction of the bone as we understand it, even heavily lithified fossils will be interacting with their environment. The states of preservation described above are somewhat artificial as they are determined by the point of excavation, had the bone been left in the soil, diagenetic changes would be ongoing. The timescales of these events are difficult to envisage as they are presumably ongoing processes and thus the bone analysed is not necessarily an 'end product' of a process. The state of the bone observed is an artefact of the moment it is excavated. Well-preserved bone is probably well preserved because it is held in benign conditions, thus assuming these do not change, the bone will remain well preserved. However, apparently well preserved bone may be undergoing diagenesis very slowly, and thus imperceptibly, moreover it can be assumed that all bone passes through a well-preserved phase, albeit briefly. 'Fossilising' bone has only residual organic matter remaining, so the only real changes are to the mineral phase and structure of the bone. There are thus two likely outcomes; dissolution, or in mineral benign soils further recrystallisation, until the bone becomes fully fossilised. In microbially attacked bone assuming that all the microbial attack has finished, then the again the main changes are going to be dissolution or recrystallisation of the mineral. Microbially attacked bone may have greater hydraulic conductivity than 'fossilising' bone, so may be more prone to dissolve, this may explain the dearth of microbially attacked bone in fossil bone assemblages.

4.3 Future work

Bone taphonomy in the most holistic terms is the passage of bone from the point of death of an organism to excavation by a scientist. Scientists are often interested in the bone as a source of data, and not directly in the diagenesis of the material. Describing the diagenetic state of the bone (or inversely the state of preservation) can provide information on the quality of the data provided by the bone, e.g. whether it is likely to contain aDNA, or the quality of the collagen for isotopic studies used in investigations of palaeodiet or ^{14}C dating. It can also be used to infer the events that may have occurred to the bone during its passage from organism to laboratory. Despite the utility of diagenetic studies they have been somewhat overlooked by workers outside of the field, although with increasing demands for isotopic data and aDNA, the measurement of simple diagenetic parameters as screening tools may help focus such studies saving valuable time and material. Furthermore with an increasing amount of data being generated diagenesis may become more predictable and thus specialist knowledge may even be used prior to the use of any physical measurement further helping to focus research projects involving bone.

One of the most obvious conclusions that can be drawn from this work is that bone diagenesis is still at the moment a very unpredictable phenomenon. This is because the state of the bone measured in the laboratory is the culmination of a number of factors that have occurred to the bone between death and analysis.

Three different states of archaeological bone have been described above, although disappointingly this study has not revealed clearly the mechanisms by which they occur; therein lies the next challenge.

The different states of archaeological bone are the result of different taphonomic histories. This study has focused on the role of soil properties in influencing diagenesis of bone, but there is no clear influence from the soil on the prevalent taphonomic state of the bone. There could be two reasons for this; firstly, it could be that soil conditions *do* influence patterns of bone diagenesis, but that this study has simply not revealed this. This could be a result of measuring bone or soil parameters that do not reveal such a pattern, or an artefact of the sample set. Secondly, it could be that soil conditions *do not* significantly affect the diagenetic state of the bone, and the diagenetic trajectory is determined by other factors prior to or at the point of burial. If the latter is true, future studies should concentrate on the early taphonomy of bone. One key factor is determining the commencement of microbial attack, a ubiquitous, rapid and devastating mode of diagenesis. Measuring how quickly and under what circumstances microbial attack occurs in experimental burials and surface kills will prove useful in determining if the microbial attack-'fossilisation' split does occur prior to burial. Such studies may also reveal how much the physico-chemical properties of the bone change prior to burial, as opposed to the net result of pre and post burial factors that are measured here.

The measurement of simple diagenetic parameters is an invaluable tool for analysing large sample numbers because the methods are simple and inexpensive. This approach has revealed the types of bone preservation described above, but has delivered little insight into the actual processes that produce these states. Future

studies may need to rely on more sophisticated techniques to reveal the progress of the changes observed at the microscopic scale. For example in the case of microbial attack it is observed that bone mineral is altered at the edge of damaged areas, although it may be relatively unchanged in other areas. This distinction is lost in a bulk mineral crystallinity measurement. The use of scanning microscale XRD and FTIR measurements will help reveal these changes to bone mineral at the microstructural level (Wess *et al.*, 2001; Miller *et al.*, 2001; Mendelsohn *et al.*, 2000). The use of Micro FTIR techniques has revealed that the crystallinity of bone changes across the osteon, which may have implications for the progress of taphonomic changes; there may also be subtle differences between species and with age. Changes at this scale may be crucial to understanding the broader scale changes observed using the diagenetic parameter approach. It is also possible that the measurement of alternative parameters may reveal more diagenetic states of bone, or at least detail within those described here.

4.4 References

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Appendices

Appendix A: Calculation of copy number as a function of time

Let $mtDNA_t$ be the estimated copy number at time t, and let l_{bp} be the total amplified length of a fragment. Then the estimated copy number remaining at time t is given by

$$mtDNA_t = mtDNA_{t_0} \times \exp[-l_{bp} kt]$$

and the expected time to the last remaining fragment is given by t when $mtDNA_t = 1$, i.e.

$$t = \frac{1}{l_{bp} k} \ln(mtDNA_{t_0})$$

where $mtDNA_{t_0}$ is the initial copy number.

An estimated initial copy number from 1g bone of 2.5×10^{11} has been used in the calculations.

Appendix B: Calculation of DNA Thermal Age

The thermal age of a fossil is calculated as follows, for a 60kyrBP fossil bone from Lake Mungo (Station 1, 1.5m depth), using temperature data for the year 1982/1983.

The modern day effective temperature T_{eff} is calculated using a simple sinusoidal model of soil temperature variation throughout the year, based upon soil temperatures measured at 1.5m in the years 1982-3, with mean 20.1°C and amplitude 2.5°C (Ambrose 1984).

Changes in rate of reaction are calculated using the formula

$$k = Ae^{-Ea/RT}$$

The rate of depurination (k yr^{-1}) is estimated at pH 7.4 from data given in Lindahl and Nyberg (1972)
where the pre-exponential constant, A = 1.45 x 10^{11} y^{-1} ,
and the activation energy, E_a = 127 kJ mol^{-1}

As the rate of rate of depurination is exponentially related to the reciprocal of temperature, the rate increases more during warmer periods than it decreases during colder periods, thus the amplitude of the sine wave is an important factor. The average rate of depurination is calculated for 1 year (k_{avg}) accounting for the exponential increase in rate. This rate of reaction is approximately equivalent to the sample being held at a constant 20.4°C, the *effective* temperature. This temperature is used for the last 16kyr of burial. The rate of reaction before 16kyrBP, i.e. the first 44kyr of burial is calculated assuming a constant temperature 9°C cooler than the present day arithmetic mean temperature (Miller *et al.*, 1997). The increase due to temperature variation is not calculated here, as this is accounted for in the palaeoclimatic data.
The relative amount of DNA depurination at each site has been normalised to the equivalent amount of damage to the DNA as if the sample were held at a constant 10°C, and reported as the thermal age. Therefore, in the above example, for the last 16kyr of burial the rate of reaction at 20.4°C is approximately 6.71 times faster than that at 10°C, every year at this temperature is equivalent to 6.71 years at 10°C. For this period the sample accumulates

16 kyr x 6.71 thermal years = 107 $_{kyr\ @10°C}$

For the previous 44kyrBP at 11.1°C the rate of reaction is 1.23 times as fast as that at 10°C,

44kyr x 1.23 thermal years = 54 $_{kyr\ @10°C}$

Thus the total thermal age of a sample under such conditions would be 161 $_{kyr\ @10°C}$.

Appendix C: Bone diagenesis data

Measured Bone diagenetic parameters. – indicates not determined, for Histological Index and Cracking Index –1 indicates an undeterminable score. Histological Index and Cracking index were measured by Miranda Jans, all other data was produced by myself and Dr Christina Nielsen-Marsh.

Table A 1. Diagenetic Parameters for Modern and Deproteinated Bone, and Key Sites

Bone Code	% 'collagen'	C:N ratio of 'collagen'	%N of whole bone	Infrared Splitting Factor	Carbonate: Phosphate ratio	Bulk Density (gcm-3)	Skeletal Density (gcm-3)	Pore volume (0.01-0.1µm diameter)	Pore volume (>0.1µm<10µm diameter)	Pore volume (>10µm-70µm diameter)	Histological Index	Cracking Index (%)
Laboratory Samples												
Modern bovine bone	**22.7**	**3.2**	**4.2 0.3**	**2.8 3.2**	**0.43**	**1.9**	**2.1**	**0.0514**	**0.0235**	**0.0209**	**5**	**0**
Modern deproteinated bone	**0.0**	**-**			**-**	**1.3**	**2.5**	**0.4178**	**0.0255**	**0.0190**	**5**	**-**
GB-MIL-AB-001	20.4	3.2	3.8	2.8	0.43	1.9	2.2	0.030	0.023	0.042	5	0
GB-MIL-AB-002	14.4	3.3	3.1	2.9	0.43	2.0	2.2	0.031	0.034	0.011	4	9
GB-MIL-AB-003	11.7	3.4	2.7	3.3	0.36	1.8	2.2	0.054	0.071	0.052	5	14
GB-MIL-AB-004	21.7	3.2	3.8	3.0	0.43	1.9	2.1	0.018	0.024	0.034	5	14
GB-MIL-AB-005	21.2	3.2	3.6	3.4	0.32	1.8	2.1	0.025	0.029	0.059	5	0
GB-MIL-AB-006A	21.0	3.2	3.9	2.8	0.48	1.9	2.1	0.044	0.015	0.036	5	18
GB-MIL-AB-006B	19.0	3.2	3.4	2.8	0.46	2.0	2.1	0.018	0.023	0.024	5	0

GB-MIL-AB-007	20.4	3.2	3.9	2.8	0.44	2.0	2.1	0.024	0.019	0.024	5	16
GB-MIL-AB-008	21.9	3.2	3.9	2.9	0.42	1.9	2.1	0.026	0.015	0.040	5	6
GB-MIL-AB-009	22.0	3.2	4.1	2.8	0.45	1.9	2.1	0.036	0.033	0.008	5	8
GB-MIL-AB-010	21.9	3.2	4.0	2.9	0.40	1.9	2.1	0.056	0.030	0.010	5	18
GB-MIL-AB-011	21.6	3.2	3.9	2.8	0.45	1.8	2.0	0.039	0.029	0.020	5	91
GB-MIL-AB-012	18.5	3.2	3.4	2.8	0.49	2.1	2.2	0.010	0.028	0.000	5	0
GB-MIL-AB-013	22.4	3.2	4.0	2.8	0.45	2.0	2.1	0.028	0.015	0.002	5	0
GB-MIL-AB-014A	23.1	3.2	4.1	2.9	0.45	2.0	2.1	0.039	0.012	0.004	5	9
GB-MIL-AB-014B	21.2	3.2	3.8	2.8	0.41	2.0	2.1	0.027	0.026	0.005	5	0
GB-MIL-AB-014C	21.8	3.2	3.6	2.9	0.34	1.9	2.1	0.068	0.023	0.017	5	37
GB-MIL-AB-014D	19.7	3.2	3.6	2.8	0.46	1.8	2.0	0.036	0.032	0.030	5	19
GB-MIL-AB-014E	19.0	3.2	3.4	2.7	0.49	2.0	2.2	0.019	0.016	0.041	5	0
GB-MIL-AB-014F	21.6	3.2	4.0	2.9	0.43	1.8	2.1	0.038	0.050	0.021	4	76
GB-MIL-AB-016	21.0	3.2	4.0	2.8	0.48	1.9	2.0	0.019	0.017	0.030	5	0
GB-MIL-AB-017	14.5	3.2	3.2	2.9	0.44	2.0	2.2	0.021	0.038	0.020	4	4
GB-MIL-AB-018	20.8	3.3	4.1	2.8	0.42	1.9	2.0	0.015	0.027	0.020	5	8
GB-MIL-AB-019	21.7	3.2	4.1	2.9	0.48	1.9	2.2	0.039	0.017	0.031	3	0
GB-MIL-AB-020	14.8	3.3	3.0	2.8	0.47	2.1	2.2	0.021	0.017	0.002	5	18
IT-API-HB-001	4.8	3.3	0.6	3.8	0.13	1.4	2.8	0.382	0.047	0.053	5	84
IT-API-HB-002	0.7	5.9	0.3	4.9	0.08	1.2	2.9	0.476	0.081	0.035	5	100
IT-API-HB-003	7.2	3.4	1.3	4.1	0.14	1.5	2.6	0.351	0.312	0.059	5	100
IT-API-HB-004	0.0	-	0.5	4.8	0.08	1.2	2.9	0.346	0.167	0.051	5	100
IT-API-HB-005	8.9	3.3	1.5	4.1	0.18	1.4	2.6	0.300	0.059	0.059	4	100
IT-API-HB-006	6.1	3.3	1.1	4.0	0.14	1.4	2.5	0.173	0.208	0.020	2	31
IT-API-HB-007	15.0	3.3	0.8	4.0	0.13	1.5	2.3	0.209	0.045	0.072	-1	-1
IT-API-HB-008	5.2	3.3	1.2	4.4	0.11	1.4	2.8	0.323	0.076	0.066	5	100
IT-API-HB-009	0.0	-	0.2	4.9	0.09	1.4	2.8	0.369	0.083	0.035	5	100
IT-API-HB-010	7.0	3.5	1.2	3.6	0.24	1.2	2.6	0.157	0.292	0.059	0	0
IT-SLM-HB-001	20.2	3.3	3.3	3.3	1.14	2.1	2.6	0.133	0.055	-0.001	4	18

IT-SLM-HB-002	18.2	3.3	3.1	3.3	0.86	1.4	1.8	0.065	0.128	-0.001	3	0
IT-SLM-HB-003	20.9	3.2	3.7	3.0	0.68	2.0	2.2	0.024	0.021	0.040	5	46
IT-SLM-HB-004	20.8	3.3	3.9	3.3	1.03	1.6	2.0	0.050	0.034	0.070	5	7
IT-SLM-HB-005	20.4	3.3	3.4	3.5	0.85	1.8	2.4	0.059	0.097	0.081	4	17
IT-SLM-HB-006	15.2	3.3	2.3	3.8	0.28	1.5	2.2	0.070	0.194	0.048	2	11
IT-SLM-HB-007	13.1	3.3	2.6	3.2	0.29	1.6	2.2	0.077	0.163	0.028	3	9
IT-SLM-HB-008	9.5	3.4	1.9	3.8	0.21	1.4	2.3	0.089	0.266	0.020	2	0
IT-SLM-HB-009	21.2	3.2	3.9	2.9	0.53	1.9	2.1	0.023	0.032	0.028	5	0
IT-SLM-HB-010	13.1	3.3	2.8	3.0	0.34	1.6	2.2	0.045	0.159	0.017	3	0
IT-SLM-HB-011	22.2	3.2	3.8	2.9	0.66	1.8	2.1	0.031	0.065	0.027	4	0
IT-SLM-HB-012	13.9	3.3	2.0	3.7	0.23	1.5	2.3	0.080	0.223	0.031	2	53
IT-SLM-HB-013	9.0	3.3	1.8	3.2	0.31	0.8	1.1	0.039	0.167	0.023	1	0
IT-SLM-HB-014	7.8	3.3	2.0	3.5	0.31	1.4	2.3	0.070	0.240	0.077	2	0
SE-AHU-HB-021	12.8	3.3	3.6	3.2	0.27	1.4	2.9	0.256	0.147	0.073	2	8
SE-AHU-HB-027	19.7	3.3	3.4	3.4	0.20	-	-	-	-	-	2	3
SE-AHU-HB-028	23.0	3.2	4.1	2.9	0.41	-	-	-	-	-	5	0
SE-AHU-HB-029	6.8	3.3	1.8	3.3	0.28	1.5	2.6	0.253	0.145	0.023	0	0
SE-AHU-HB-032	8.5	3.4	1.7	3.4	0.20	-	-	-	-	-	1	3
SE-AHU-HB-033	7.8	3.4	2.0	3.3	0.23	1.3	2.5	0.192	0.144	0.059	1	0
SE-AHU-HB-034	7.8	3.5	2.5	3.3	0.23	-	-	-	-	-	2	0
SE-AHU-HB-036	8.4	3.3	1.8	3.2	0.22	-	-	-	-	-	2	0
SE-AHU-HB-041A	4.9	3.5	1.6	3.1	0.26	1.5	2.3	0.161	0.126	0.050	1	0
SE-AHU-HB-043	15.9	3.2	1.9	3.2	0.26	1.6	2.3	0.117	0.109	0.051	2	3
SE-AHU-HB-045	5.8	3.4	1.7	3.3	0.25	-	-	-	-	-	1	3
SE-AHU-HB-050	4.8	3.4	1.6	3.2	0.26	-	-	-	-	-	1	0
SE-AHU-HB-052	13.2	3.3	2.2	3.3	0.23	-	-	-	-	-	2	2
SE-AHU-HB-053	16.3	3.2	3.5	3.1	0.29	-	-	-	-	-	2	3
SE-AHU-HB-054	21.3	3.2	2.7	3.2	0.22	-	-	-	-	-	3	0
SE-AHU-HB-060	7.1	3.5	1.5	3.3	0.23	-	-	-	-	-	1	0
SE-AHU-HB-061	13.2	3.3	1.5	3.2	0.21	1.5	2.4	0.209	0.130	0.044	2	6

SE-AHU-HB-062	13.8	3.3	2.8	3.0	0.31	-	-	-	-	-	3	9
SE-AHU-HB-063	4.9	3.5	1.4	3.6	0.13	-	-	-	-	-	1	0
SE-AHU-HB-067	10.9	3.3	2.6	3.3	0.25	1.6	2.3	0.133	0.069	0.058	2	0
SE-AHU-HB-069	6.8	3.5	1.5	3.3	0.21	-	-	-	-	-	1	0
SE-AHU-HB-070	19.2	3.2	3.7	2.9	0.37	1.6	2.2	0.149	0.062	0.054	3	0
SE-AHU-HB-072	11.9	3.3	2.8	3.3	0.26	-	-	-	-	-	1	0
SE-AHU-HB-075	13.4	3.3	2.4	3.2	0.29	1.6	2.4	0.173	0.107	0.043	2	0
SE-AHU-HB-078	17.8	3.2	2.1	3.1	0.29	1.5	2.3	0.218	0.121	-0.001	2	3
SE-AHU-HB-082	10.1	3.3	2.3	3.3	0.24	0.8	0.9	0.099	0.058	0.016	0	0
SE-AHU-HB-086	8.4	3.4	2.1	3.2	0.25	-	-	-	-	-	1	0
SE-AHU-HB-094	11.5	3.3	2.6	3.2	0.29	-	-	-	-	-	3	0
SE-AHU-HB-098	3.7	3.5	1.4	3.3	0.21	-	-	-	-	-	1	0
SE-AHU-HB-099	8.6	3.4	1.7	3.2	0.21	1.4	2.5	0.176	0.227	0.045	1	0
SE-AHU-HB-101	19.0	3.2	2.9	2.9	0.34	1.6	2.2	0.071	0.059	0.044	4	0
SE-VUO-AB-001	3.8	4.2	1.0	3.6	0.15	0.9	1.6	-0.002	0.361	0.064	0	0
SE-VUO-AB-002	12.2	3.6	2.1	4.1	0.11	1.3	2.0	0.069	0.185	0.010	1	0
SE-VUO-AB-003	0.0	-	0.0	5.7	0.04	2.1	3.0	0.084	0.161	0.010	5	0
SE-VUO-AB-004	0.0	-	0.0	4.7	0.08	2.3	2.9	0.026	0.057	0.048	5	20
SE-VUO-AB-005	0.0	-	0.2	5.5	0.03	2.3	3.1	0.040	0.184	0.019	5	27
SE-VUO-AB-012	2.2	4.3	0.5	3.9	0.12	0.9	1.5	-0.004	0.328	0.031	0	0
SE-VUO-AB-016	6.9	4.2	2.5	4.1	0.05	1.6	2.3	0.140	0.125	0.010	0	30
SE-VUO-AB-024	6.6	3.8	2.9	3.6	0.16	1.8	2.5	0.087	0.143	0.012	1	51
SE-VUO-AB-027	3.6	3.9	0.8	3.9	0.12	0.8	2.1	0.153	0.435	0.072	0	0
NL-NIJ-HB-001	25.2	3.2	4.0	3.0	0.35	1.8	2.1	0.029	0.020	0.074	5	0
NL-NIJ-HB-002	14.5	3.2	1.8	3.2	0.23	1.3	2.4	0.171	0.196	0.048	2	10
NL-NIJ-HB-003	7.3	3.4	1.2	3.4	0.17	1.2	2.5	0.189	0.266	0.054	1	3
NL-NIJ-HB-004	16.8	3.2	2.8	3.3	0.20	1.4	2.3	0.137	0.191	0.069	3	5
NL-NIJ-HB-005	7.3	3.4	1.3	3.2	0.23	1.2	2.4	0.234	0.205	0.051	1	0
NL-NIJ-HB-006	0.5	3.2	3.2	3.2	0.24	1.8	2.2	0.070	0.065	0.023	4	3

NL-NIJ-HB-008	3.2	3.8	3.4	3.3	0.23	1.1	2.8	0.491	0.063	0.040	5	59
NL-NIJ-HB-009	24.3	3.2	4.0	2.9	0.46	1.7	2.2	0.110	0.004	0.041	5	0
NL-NIJ-HB-012	5.2	4.2	0.3	3.6	0.15	1.1	2.8	0.505	0.045	0.039	5	85
NL-NIJ-HB-018	30.4	3.3	3.0	3.2	0.26	1.6	2.3	0.145	0.098	0.035	3	21

Other sites by country.

Table A 2. Diagenetic parameters for bone excavated from Britain

Bone Code	% 'collagen'	C:N ratio of 'collagen'	%N of whole bone	Infrared Splitting Factor	Carbonate: Phosphate ratio	Bulk Density (gcm-3)	Skeletal Density (gcm-3)	Pore volume (0.01-0.1µm diameter)	Pore volume (>0.1µm<10µm diameter)	Pore volume (>10µm-70µm diameter)	Histological Index	Cracking Index (%)
GB-BIF-AB-001A	0.3	6.2	0.1	3.6	0.23	1.3	2.7	0.406	0.058	0.053	3	30
GB-BIF-AB-001B	0.3	11.1	0.1	3.6	0.24	1.2	2.8	0.393	0.071	0.093	1	54
GB-BIF-AB-002	3.5	3.5	1.1	3.1	0.34	1.6	2.5	0.107	0.227	0.014	2	47
GB-BIF-AB-003	0.0	95.6	0.2	3.2	0.30	1.5	2.6	0.258	0.122	0.025	2	16
GB-BIF-AB-004	0.4	5.5	0.3	3.3	0.26	1.5	2.6	0.317	0.062	0.041	4	50
GB-BIF-AB-006	3.7	3.5	0.9	3.3	0.33	1.4	2.5	0.141	0.249	0.023	2	10
GB-BIF-AB-007	0.1	9.3	0.1	3.1	0.31	1.4	2.6	0.369	0.056	0.012	5	11
GB-BIF-HB-005	0.0	-	0.1	3.3	0.21	1.0	3.2	0.488	0.062	0.101	5	88
GB-KIT-AB-001	0.1	8.1	0.1	3.9	0.32	1.2	2.5	0.123	0.231	0.105	2	48
GB-KIT-AB-004	3.2	3.3	0.5	3.5	0.31	1.2	2.3	0.193	0.212	0.048	0	0
GB-KIT-AB-005	2.9	3.5	0.6	3.3	0.31	-	-	-	-	-	1	0
GB-KIT-AB-008	-	-	0.5	3.3	0.29	-	-	-	-	-	1	38
GB-KIT-AB-009	0.2	5.8	0.0	3.3	0.29	1.8	2.4	0.146	0.048	0.032	3	5

GB-KIT-AB-010	0.6	4.6	0.2	3.3	0.29	1.2	1.7	0.052	0.168	0.059	2	0
GB-MOO-AB-002	20.0	3.2	3.5	2.8	0.44	2.0	2.2	0.041	0.006	0.026	5	11
GB-MOO-AB-003	19.3	3.2	3.5	3.0	0.34	1.9	2.1	0.041	0.032	0.022	4	0
GB-MOO-AB-004	17.9	3.2	3.3	2.9	0.39	2.0	2.1	0.024	0.013	0.011	5	0
GB-MOO-AB-005	9.8	3.2	1.7	3.1	0.29	1.8	2.3	0.104	0.103	0.006	2	0
GB-MOO-AB-006	17.3	3.2	2.2	3.1	0.26	1.9	2.0	0.022	0.024	0.012	3	0
GB-MOO-AB-007	5.1	3.4	1.5	2.9	0.37	1.8	2.2	0.085	0.019	0.038	5	10
GB-MOO-AB-008	8.1	3.3	1.2	3.4	0.23	1.5	2.4	0.069	0.236	0.046	2	0
GB-MOO-AB-009	10.0	3.2	1.6	3.1	0.28	1.8	2.2	0.086	0.082	0.006	3	0
GB-TOR-HB-001	5.5	3.4	1.1	3.2	0.26	1.1	2.4	0.147	0.321	0.037	0	0
GB-TOR-HB-002	5.0	3.3	1.1	3.1	0.33	1.3	2.4	0.168	0.281	0.022	0	0
GB-TOR-HB-003	4.7	3.4	1.2	3.2	0.28	1.1	2.4	0.159	0.317	0.029	0	0

Table A 3. Diagenetic parameters for bone excavated from Italy

Bone Code	% 'collagen'	C:N ratio of 'collagen'	%N of whole bone	Infrared Splitting Factor	Carbonate: Phosphate ratio	Bulk Density (gcm-3)	Skeletal Density (gcm-3)	Pore volume (0.01-0.1µm diameter)	Pore volume (>0.1µm<10µm diameter)	Pore volume (>10µm-70µm diameter)	Histological Index	Cracking Index (%)
IT-FIP-HB-001	4.3	3.3	0.0	3.8	0.17	1.5	2.8	0.354	0.088	0.032	5	100
IT-FIP-HB-002	0.0	-	0.0	3.6	0.23	1.8	2.9	0.294	0.055	0.061	5	100
IT-HIP-HB-001	2.1	3.4	0.5	3.8	0.17	1.2	2.4	0.329	0.059	0.079	4	56
IT-HIP-HB-003	6.3	3.4	0.8	3.7	0.19	1.2	2.4	0.211	0.137	0.100	3	64

IT-HIP-HB-005	0.7	4.2	0.0	3.8	0.12	1.1	3.0	0.478	0.069	0.063	5	100
IT-HIP-HB-008	0.0	-	0.0	4.1	0.12	1.3	2.6	0.429	0.039	0.030	5	73
IT-HIP-HB-010	0.0	-	0.0	4.0	0.13	1.3	2.8	0.446	0.028	0.041	5	100
IT-LMG-HB-001	7.0	3.4	1.4	4.0	0.30	1.3	2.5	0.176	0.159	0.069	1	0
IT-LMG-HB-002	5.8	3.4	1.1	3.4	0.20	1.3	2.6	0.146	0.275	0.054	1	0
IT-LMG-HB-003	6.7	3.4	1.2	3.6	0.25	1.3	2.5	0.125	0.272	0.038	0	0
IT-MES-HB-001	0.0	-	0.2	6.1	0.06	2.6	3.1	0.051	0.057	0.048	5	0
IT-MES-HB-002	9.3	3.2	1.0	3.6	0.44	1.6	2.5	0.122	0.146	0.032	2	27
IT-MES-HB-003	8.4	3.3	1.2	3.7	0.34	1.5	2.9	0.208	0.228	0.022	2	90
IT-SEG-HB-001	10.8	3.3	2.0	3.7	0.27	1.5	2.4	0.101	0.230	0.018	1	0
IT-SEG-HB-002	10.3	3.2	1.5	3.7	0.22	1.4	2.4	0.232	0.163	0.016	2	82
IT-SEG-HB-003	9.3	3.3	2.0	3.3	0.31	1.4	2.2	0.063	0.200	0.034	2	5
IT-SFS-HB-001	19.7	3.4	4.5	3.0	0.35	1.9	2.1	0.016	0.011	0.044	5	0
IT-SPB-HB-001	11.1	3.3	1.8	3.8	0.12	1.5	2.4	0.168	0.121	0.060	2	33
IT-SPB-HB-002	0.0	-	0.5	4.3	0.08	1.2	2.9	0.385	0.117	0.061	5	100
IT-SUP-AB-001	2.8	3.4	0.5	3.2	0.32	1.3	2.1	0.113	0.233	0.009	0	3
IT-SUP-AB-002	11.8	3.3	1.1	3.2	0.34	1.8	2.4	0.110	0.135	0.009	2	0
IT-SUP-AB-003	3.0	3.5	0.5	3.3	0.28	1.4	2.5	0.156	0.243	0.033	0	0

Table A 4. Diagenetic parameters for bone excavated from the Netherlands

Bone Code	% 'collagen'	C:N ratio of 'collagen'	%N of whole bone	Infrared Splitting Factor	Carbonate: Phosphate ratio	Bulk Density (gcm-3)	Skeletal Density (gcm-3)	Pore volume (0.01-0.1μm diameter)	Pore volume (>0.1μm<10μm diameter)	Pore volume (>10μm-70μm diameter)	Histological Index	Cracking Index (%)
NL-AAR-AB-001	16.6	3.2	3.3	2.8	0.47	2.0	2.1	0.017	0.003	0.001	5	5
NL-AAR-AB-002	15.3	3.2	2.9	2.8	0.44	1.9	2.1	0.018	0.036	0.020	5	15
NL-AAR-AB-003	10.7	3.4	2.4	2.9	0.45	1.7	1.9	0.032	0.039	0.029	3	11
NL-AAR-HB-004	16.9	3.3	1.9	3.1	0.33	1.8	2.1	0.037	0.054	0.031	3	16
NL-BOG-HB-001	0.2	6.6	0.1	3.7	0.22	1.2	1.9	0.093	0.163	0.082	1	0
NL-BOG-HB-002	0.1	12.6	0.1	3.4	0.26	1.6	2.6	0.323	0.044	0.015	5	42
NL-BOG-HB-003	0.1	13.0	0.1	4.0	0.25	1.3	2.1	0.242	0.083	0.052	5	20
NL-BOG-HB-004	0.0	12.6	0.1	3.4	0.27	1.5	2.2	0.267	0.006	0.034	5	3
NL-BOR-AB-002	7.6	3.4	1.0	3.5	0.17	1.2	1.7	0.003	0.208	0.063	1	5
NL-BOR-AB-002A	8.3	3.3	1.3	3.7	0.18	1.4	2.4	0.123	0.235	0.044	1	0
NL-BOR-HB-001	17.9	3.2	3.1	3.7	0.27	1.7	2.2	0.066	0.063	0.067	4	30
NL-GIL-HB-001	1.7	5.1	0.5	4.0	0.12	1.2	1.7	-0.001	0.283	0.031	1	0
NL-GIL-HB-002	2.3	6.3	0.6	4.1	0.10	0.8	1.2	0.004	0.254	0.088	1	10
NL-GIL-HB-003	4.1	5.1	0.9	3.6	0.14	1.3	2.5	0.369	0.020	0.063	5	100
NL-GIL-HB-004A	0.3	6.8	0.8	3.6	0.13	1.4	3.9	-0.006	0.288	0.098	2	0
NL-GIL-HB-004B	11.6	3.4	1.4	3.9	0.10	0.7	1.2	0.252	0.217	0.125	2	100
NL-GIL-HB-005	2.1	7.1	0.1	-	-	-	-	-	-	-	5	100
NL-GIL-HB-006	2.1	7.6	0.3	-	-	0.7	2.1	0.264	0.228	0.120	2	10
NL-GIL-HB-009	1.5	6.3	0.5	3.9	0.11	0.7	1.4	0.047	0.299	0.117	1	10
NL-GIL-HB-011	2.0	5.8	0.6	3.9	0.11	0.7	1.8	0.102	0.305	0.140	2	39

NL-RAA-AB-001	4.3	5.4	0.7	3.8	0.08	0.9	2.2	0.109	0.168	0.213	1	0
NL-RAA-AB-002	2.7	3.9	0.4	3.9	0.08	1.0	1.5	0.004	0.191	0.081	1	0
NL-RAA-AB-003	1.4	5.1	0.3	4.0	0.08	0.9	1.3	-0.001	0.125	0.122	0	0
NL-UBE-AB-001	5.5	3.4	0.8	3.7	0.17	1.2	2.5	0.231	0.223	0.055	3	72
NL-UBE-AB-002	15.2	3.2	2.3	-	0.19	2.1	2.3	0.044	0.010	0.007	5	0
NL-UBE-AB-003	5.0	3.4	0.7	3.5	0.20	1.0	1.6	0.173	0.156	0.027	1	0
NL-UBE-AB-004	4.8	3.4	0.4	3.4	0.20	1.4	2.3	0.289	0.030	0.049	5	62
NL-UBE-AB-005	1.2	5.0	0.6	3.6	0.18	1.2	1.7	0.077	0.191	0.021	0	0
NL-UBE-AB-006	4.8	3.4	0.8	3.5	0.19	1.0	2.2	0.143	0.307	0.079	1	20
NL-VOR-AB-001	2.6	3.9	0.9	3.0	0.33	1.7	2.2	0.167	0.042	0.006	4	39
NL-VOR-AB-002	13.2	3.2	2.4	3.0	0.39	1.8	2.2	0.068	0.092	0.009	2	0
NL-VOR-AB-003	0.2	6.6	0.2	3.2	0.32	1.4	2.3	0.265	0.081	0.034	5	35
NL-VOR-AB-004	1.0	4.4	0.3	3.2	0.29	1.2	2.1	0.215	0.054	0.078	5	10
NL-VOR-AB-005	10.1	3.2	1.2	3.0	0.33	1.4	2.3	0.149	0.198	0.027	2	0
NL-YPE-HB-001	0.9	13.7	0.3	3.5	0.22	0.9	1.5	0.122	0.180	0.116	3	43
NL-YPE-HB-002	7.8	4.5	0.8	3.4	0.27	1.0	2.6	0.215	0.351	0.041	3	22
NL-YPE-HB-003	1.7	5.9	0.5	3.5	0.22	1.1	2.5	0.279	0.178	0.074	3	53
NL-YPE-HB-004	1.3	26.1	0.3	3.4	0.25	1.0	2.5	0.357	0.369	0.067	0	26
NL-YPE-HB-005	1.0	23.5	0.3	3.4	0.24	0.7	1.9	0.163	0.322	0.105	0	14
NL-YPE-HB-007	0.8	26.0	0.4	3.7	0.20	0.8	1.3	0.089	0.084	0.177	4	63
NL-YPE-HB-008	1.3	12.5	1.0	3.4	0.24	1.4	2.1	0.118	0.126	0.042	1	41
NL-YPE-HB-010	2.0	6.7	0.4	3.6	0.21	1.1	2.5	0.299	0.168	0.084	3	79
NL-YPE-HB-011	1.5	27.6	0.2	3.8	0.19	-	-	-	-	-	-1	-1
NL-YPE-HB-012	1.3	23.9	0.3	3.5	0.24	-	-	-	-	-	-1	-1
NL-YPE-HB-014	0.9	16.9	0.3	3.5	0.24	1.5	2.3	0.141	0.180	0.030	3	29
NL-YPE-HB-015	3.8	5.4	1.3	3.4	0.23	1.3	2.1	0.161	0.176	0.047	3	27
NL-YPE-HB-016	2.1	7.7	0.6	3.4	0.21	1.0	2.1	0.260	0.183	0.064	3	40

NL-YPE-HB-017	4.1	4.2	0.6	3.3	0.25	1.4	1.7	0.377	0.179	0.029	4	76
NL-YPE-HB-018	3.5	5.4	0.5	3.4	0.23	1.2	2.4	0.141	0.332	0.034	1	21

Table A 5. Diagenetic parameters for bone excavated from Sweden

Bone Code	% 'collagen'	C:N ratio of 'collagen'	%N of whole bone	Infrared Splitting Factor	Carbonate: Phosphate ratio	Bulk Density (gcm-3)	Skeletal Density (gcm-3)	Pore volume (0.01-0.1µm diameter)	Pore volume (>0.1µm<10µm diameter)	Pore volume (>10µm-70µm diameter)	Histological Index	Cracking Index (%)
SE-CAR-AB-005	0.0	-	0.0	3.9	0.08	1.1	2.5	0.292	0.480	-0.001	0	5
SE-CAR-HB-001	0.4	4.4	0.1	3.1	0.09	-	-	-	-	-	5	50
SE-CAR-HB-002	11.9	3.6	3.5	3.4	0.18	1.4	3.0	0.146	0.153	0.132	1	0
SE-CAR-HB-003	1.1	6.6	0.6	3.6	0.15	1.0	2.7	0.402	0.243	0.184	5	100
SE-CAR-HB-004	21.0	3.2	3.2	3.4	0.17	1.1	2.3	0.239	0.164	0.142	4	67
SE-HAG-HB-001	6.5	3.8	1.0	3.9	0.11	0.9	2.6	0.165	0.420	0.058	1	79
SE-HAG-HB-002	2.7	4.6	0.4	3.5	0.16	1.0	2.3	0.190	0.221	0.115	2	34
SE-HAG-HB-004	8.7	3.9	1.9	3.6	0.15	1.2	2.2	0.083	0.020	0.008	3	47
SE-HAG-HB-005	17.8	3.4	3.9	3.6	0.16	1.6	2.1	0.049	0.130	0.007	4	64
SE-HAG-HB-006	26.2	3.3	3.8	3.5	0.18	1.5	2.1	0.098	0.100	0.009	5	53
SE-HAG-HB-007	7.9	4.4	1.7	3.6	0.14	0.9	2.2	0.060	0.060	0.006	2	44
SE-HAG-HB-008	18.1	3.5	2.4	3.4	0.18	1.4	2.1	0.093	0.093	0.009	5	66
SE-HAG-HB-009	1.8	4.9	0.8	3.7	0.08	-	-	-	-	-	3	-1
SE-HUS-AB-007	7.0	3.5	1.5	3.1	0.25	1.7	2.6	0.066	0.246	0.015	1	0
SE-HUS-AB-008	8.0	3.4	1.8	3.0	0.29	2.1	2.3	0.039	0.036	0.006	3	0
SE-HUS-AB-009	15.9	3.3	3.0	2.7	0.41	2.1	2.2	0.028	0.014	0.006	5	0
SE-NOR-AB-507	19.6	3.3	3.7	2.9	0.32	1.9	2.3	0.078	0.041	0.033	5	0

SE-NOR-AB-514	19.1	3.4	3.7	3.1	0.32	1.8	2.2	0.070	0.042	0.060	5	0
SE-NOR-AB-650	9.4	3.6	0.8	3.1	0.25	1.4	2.3	0.257	0.048	0.071	4	93
SE-NOR-AB-651	23.1	3.2	4.3	3.0	0.32	1.8	2.3	0.094	0.023	0.044	5	4
SE-NOR-AB-652	20.5	3.2	3.6	2.8	0.37	1.9	2.4	0.117	0.021	0.023	5	35
SE-ULL-AB-007	9.0	3.5	1.3	3.1	0.25	1.5	2.3	0.107	0.214	0.009	1	0
SE-ULL-AB-008	11.3	3.3	1.8	3.2	0.25	1.6	2.3	0.081	0.181	0.007	2	0
SE-VAL-HB-001	25.1	3.3	3.4	3.4	0.15	1.6	2.3	0.081	0.186	0.037	3	43
SE-VAL-HB-003	11.2	3.7	2.6	3.4	0.18	1.3	2.2	0.221	0.097	0.133	5	95
SE-VAL-HB-004	12.3	3.4	2.3	3.3	0.21	1.0	2.0	0.094	0.358	0.047	2	0
SE-VAL-HB-007	23.6	3.3	3.6	4.0	0.12	1.6	2.1	0.056	0.035	0.023	5	29

Appendix D: Brief Descriptions of Key sites

Sweden

Vuollerim (SE-VUO)

The site of Vuollerim is situated in northern Sweden (arctic climate), consisting of a number of well preserved huts along a forest (pine) river bed. The animal bone analysed here was excavated from the area inside and just outside one of the huts and near a large refuse pit, most bone material at the site appears to be domestic waste, some of it burnt. The site is dated to ~ 4000BC. The soil is a well sorted fine sand, pH 5.6-6.8.

Åhus (SE-AHU)

Human bone was sampled from the late medieval town of Åhus, situated on the south coast of Sweden. The samples were taken from the monastery cemetery which was in use between *c*1200-1550AD. The site has never since been built on, and is currently used as pasture and garden.

The site contained 265 mainly single burials of men, women and children (the children were either buried together, or with an adult). They are in four stratigraphic layers. All the burials except one have evidence for narrow wooden coffins (wood stained soil, iron nails). The burials are facing east-west. The site is described as dry with the water table ~1m below the deepest burial. The burials were approximately 1.3-2.2m below the ground. The soil is calcareous and lies on a sand moraine. The pH of the soil varies from 6.9-7.8, and has a high phosphate level, possibly due to bone dissolution.

Italy

Apigliano (IT-API)

The deserted medieval village of Apigliano lies in the territory of Martano (province of Lecce, southern Puglia). The site lies on a Terra Rossa plough soil in a flat, windswept, area of poor stony land with limestone bedrock breaking the surface in patches. The thickness of the soil above the bedrock is between 25-50cm, and the area is well drained. Excavations have been carried out for about a month each year, between 1997 and 2000. Excavations have shown the site to have developed from the eighth century A.D. as a Byzantine village or "chorion" and by the tenth century it probably covered about 2 hectares. By the later eleventh century it is likely to have fallen to the Normans, who conquered the whole of southern Italy. However, very little has yet been found dating from between the Norman invasion and the thirteenth century. Instead, excavations have brought to light a later medieval church and cemetery that appear to be of late thirteenth and early fourteenth century date. Some

53 tombs and 16 charnel pits have so far been excavated, and 82 individuals have been identified (Arthur, 1999). Burials at the site consist mainly of rectangular stone-lined tombs, with vertical side slabs and horizontal cover slabs, few burials were made directly in to the soil. The stone tombs look to have been periodically re-used, with the current burial removed to a charnel pit to make way for the recently deceased.

Santa Lucia Alle Malve (IT-SLM)

The Santa Lucia alle Malve site is a medieval cemetery situated on an exposed limestone platform above the monastery of Santa Lucia Vecchia which is cut into the rock below. The cemetery is likely to date from the 7^{th}-8^{th} centuryAD.

The cemetery has been excavated twice (in the 1940s and 1970s) before the 1999 excavations. The recent surveys have identified 120 burials cut into the limestone (there is no top soil), 50 of which have been excavated. The graves tend to be trapezoidal or rectangular pits with limestone slab covers and are no more than 40cm deep.

Britain

Millennium Bridge (GB-MIL)

The Millennium Bridge site is situated on the waterfront of the River Thames in the St Paul's Cathedral area in the City of London. The excavations were conducted as rescue archaeology preceding the building of a new footbridge across the river.

The site is likely to have been in use since the Roman period (albeit underwater), however the material used in this study is from a number of different contexts from the medieval period covering the 12^{th} - 15^{th} centuries AD. The material at the site (including the bone) appears to come from a foreshore dump, and was ~5m below the current ground surface.

The main soil type is a swampy anthropogenic soil. The water table fluctuates at the site due to the tidal nature of the river, and the site is approximately 20m from the water front.

Netherlands

Nijmegen (NL-NIJ)

The excavations undertaken at Nijmegen were in the current town of Nijmegen and excavation conditions were less than ideal (mechanical excavation), making the interpretation of the site difficult, particularly the relationship between inter-cutting graves. There are three phases at the site; a Roman phase ~350-650AD (Samples 8, 12 and 16), and two post Medieval phases ~1500-1700AD (samples 1,5,6,9 and 18), and ~ 1600-1800AD (samples 2-4).

The site itself is situated on a sand moraine, with a top soil thickness in excess of 0.5m. The soil samples taken from the site were described as 'rather homogenous' in terms of their chemistry.

Appendix E: Calculation of amount of protein remaining in archaeological bone

The amount of protein left in a bone (assuming a first order reaction) can be calculated thus.

$$\text{Amount of Protein (\%)} = \frac{1}{e^{(\text{Rate of reaction x Time})}} \quad (1)$$

Hence:

$$\text{Rate of reaction} = \frac{\ln(1/\text{Amount of Protein})}{\text{Time}} \quad (2)$$

the rate of the reaction can also be calculated by:

$$\ln k = \ln A - (Ea/RT) \quad (3)$$

based on the work of Von Endt and Ortner (1984) both Activation energy (*Ea*) and Frequency factor (A) can be calculated. R is the Universal Gas Constant.

Ea = 183.057 kJmol-1
A = 9.20626×10^{19}
R = 8.314
T = temperature

Substituting equation 2 into 3

$$T = \frac{Ea}{R(\ln A - \ln k)} \quad (4)$$