University of Alberta

Microbial Biodeterioration of Human Skeletal Material from Tell Leilan, Syria (2900 – 1900 BCE)

by

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Abstract

Human bone is considered one of the most direct and insightful sources of information on peoples of the past. As a result, curation protocols have been developed to ensure that the integrity of human skeletal collections is maintained. Although collections are generally considered safe when these protocols are followed, the results of this investigation show that the Tell Leilan skeletal collection from Syria (circa 2900 – 1900 BCE) was contaminated by microbial growth (also known as biodeterioration) during curation. This biodeterioration was evaluated by light microscopy (LM), by the application of a histological preservation index (HPI), and by scanning electron microscopy (SEM). All samples (n=192) were found to be biodeteriorated by LM and the HPI. SEM confirmed that the Tell Leilan skeletal material had been contaminated by a complex microbial aggregate known as a biofilm. *Amycolatopsis* sp. and *Penicillium chrysogenum*, along with species of *Aspergillus*, *Chaetomium*, and *Cladosporium* were isolated and cultured from several contaminated bones and were identified based on morphology and DNA sequences. The results of this research suggest that we must focus on new techniques to examine bone as well as on new conservation protocols designed to limit the growth of biofilms in human skeletal collections in the future.

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Chapter 1

Introduction

1.1 Introduction

Bone is one of the most often recovered biological materials at an archaeological site. By studying the macroscopic, microscopic, and chemical properties of bone from groups of individuals, information can be gleaned about ancient culture, demography, health, population movement, as well as the environment in which past populations lived. This information is reliable only when bone properties survive and are unchanged in the burial environment. Bone properties must also be preserved through long-term curation of archaeological collections, thus ensuring that the material is viable for future analyses, including those not yet developed. Thus, whether bone remains stable over time is determined by the context from which it derives and then by the conditions in which it is stored.

Research has shown that bone is subject to physical, chemical, and biological processes, all of which can contribute to its decay in the burial and curatorial environments (Cronyn, 1990; Grupe and Dreses-Werringloer, 1990; Henderson, 1987). Of these deterioration processes, those of biological origin are considered the most influential, but are the least understood (Grupe and Dreses-Werringloer, 1990). Microorganisms such as bacteria and fungi have been implicated in the biodeterioration of bone, and their destructive changes have been recognised in human skeletal collections worldwide (Colson et al., 1997; Grupe et al., 1993;

Hackett, 1981; Hanson and Buikstra, 1987; Jans et al., 2004; Maat, 1993; Schoeninger et al., 1989; Turner-Walker and Syversen, 2002)

Researchers recognise that microbial growth causes dramatic changes to the physical and chemical properties of bone. A growing body of research suggests that such change can lead to difficulties in isolating intact collagen and DNA as well as obtaining unaltered trace element profiles, resulting in the loss of valuable information about past peoples and their environments. To understand this loss, for almost 150 years researchers have examined aspects of bone biodeterioration. Using light microscopy (LM) (Hackett, 1981; Jans et al., 2004; Marchiafava et al., 1974; Wedl, 1864), scanning electron microscopy (SEM) (Jackes et al., 2001; Maat, 1993), and SEM in backscattered electron (BSE) mode (Bell, 1990; Guarino et al., 2006; Turner-Walker and Syversen, 2002) as well as several diagenetic parameters (Haynes et al., 2002; Turner-Walker et al., 2002), researchers have characterised microbial growth in bone. Recently, attempts have been made to determine how to select better samples for bioarchaeological investigations (Colson et al., 1997; Haynes et al., 2002).

The rate in which bone is biodeteriorated by microorganisms depends on several intrinsic and extrinsic factors, some of which are interrelated. Investigators have argued that the age and sex of the individual, as well as the presence of pathological conditions, have an effect on biodeterioration rates (Jans et al., 2004; Turner-Walker and Syversen, 2002). Moreover, factors such as temperature,

moisture, and lighting, as well as the nature of deposition, the sediment, and the dynamics of the microorganisms involved have been shown to determine the rate at which biodeterioration occurs (Bradshaw et al., 1994; Henderson, 1987). Unfortunately, the factors at work in the burial environment cannot be controlled. In the curation environment the situation is different and there is more control.

Attempts have been made to protect bone from environmental factors in the curation environment. Several protocols have been developed to be followed by individuals working in institutions (i.e., universities, museums, or other repositories) in which archaeological bone is stored (see Appelbaum, 1991; Canadian Conservation Institute, 1983; Cronyn, 1990; Department for Culture, Media and Sport, 2005; Gehlert, 1980; Odegaard and Cassman, 2007; Sease, 1994). These protocols focus on post-excavation procedures as well as on environmental controls. The manner in which bone is cleaned and stored has been shown to have dramatic effects on the survival of bone in curation (Buikstra and Ubelaker, 1994; Cassman and Odegaard, 2007; Gehlert, 1980; Odegaard and Cassman, 2007; Rose and Hawks, 1992). For example, the storing of wet bone, or failing to maintain consistent environmental conditions in curation, can lead to the growth of microorganisms (Cronyn, 1990; Department for Culture, Media and Sport, 2005; Sease, 1994).

Only in rare conditions, such as in extreme cold or when metals are near, can bone be considered out of the reach of pervasive microorganisms such as bacteria and

fungi (Janaway, 1996; Matheson and Brian, 2003; Schultz, 1997). Thus, most archaeological bone has been exposed to microorganisms, during either burial or during curation. Bone not only might have suffered from biodeterioration in burial, but also might have been colonised by soil microbes whose assimilative cells and spores resumed growth upon institutionalisation. In addition, new microorganisms may enter bone sometime during curation, leading to new microbial growth. Unfortunately, however, it is difficult to pinpoint the onset of the biodeterioration, and because of this, few researchers have recognised the possibility of microbial growth in the curation environment and tend to associate the destruction with burial (for exception see Pruvost et al., 2007).

1.2 Dissertation Development

As in other studies involving the examination of bone histology (see Jackes et al., 2001; Piepenbrink, 1986), this doctoral dissertation research began as an attempt to produce thin sections of the Tell Leilan skeletal material from Syria, dated to between 2900 and 1900 BCE, for bioarchaeological analyses. The site, located in what is referred to as ancient Mesopotamia (Fig. 1.1), has been excavated by Harvey Weiss and members of the Tell Leilan Archaeological Project for over 30 years (see Ristvet and Weiss, 2000; Weiss, 1985; Weiss and Courty, 1993; Weiss et al., 1993). Between 1979 and 1989, the skeletons of 59 individuals were discovered at the site. These skeletons were shipped to Yale University in New Haven, Connecticut and were later sent for study in 1991 to the University of

California, Berkeley. In 1992, the skeletal material was shipped to the University of Alberta, Edmonton where it continues to be stored.

Although the material was stored under typical institutional storage conditions (see the Canadian Conservation Institute, 1983) and bore no macroscopic indicators of microbial decay, the material was found to be severely biodeteriorated at a microscopic level[1]. Normally, the occurrence of small focal areas of destruction may be overlooked; however, the condition of the Leilan material was well beyond that of a few isolated pockets of biodeteriorated bone and the overwhelming changes caused by microbial growth could not be ignored.

Following consultation with several specialists at the University of Alberta, a collaborative research team was formed to study and understand the biodeterioration of the Tell Leilan skeletal material. Several research questions were developed:

1. How does the phenomenon of bone biodeterioration occur?
2. What do the destructive changes look like microscopically?

[1] Thirty-nine thin sections were prepared by Lovell in 1996 for histopathological analysis. These sections were re-examined and showed no signs of microbial destruction confirming that growth had taken place during storage at the University of Alberta.

3. Is there a pattern to this destruction?
4. Which microorganism(s) are involved?
5. What are the implications of this biodeterioration for archaeological bone collections?

1.3 Dissertation Contents

The following chapters examine these and other more specific research questions related to the biodeterioration of the Leilan skeletal material. **Chapter 2** is a review of the literature concerning the study of the biodeterioration of archaeological bone. In **Chapter 3**, the use of LM, histochemical staining techniques, and SEM and SEM-BSE to characterise the biodeterioration in the Leilan material is described. A histological preservation index, successfully applied by several researchers to other collections (Colson et al., 1997; Guarino et al., 2006; Haynes et al., 2002; Hedges et al., 1995; Jans, 2005; Trueman and Martill, 2002) was used to further understand the range of destruction in the Tell Leilan material. In **Chapter 4,** the use of SEM to further document the microbial growth in the material is described. In addition, potential causal organisms were isolated and identified from several Leilan skeletal elements using a combination of culture techniques and DNA sequencing. **Chapter 5** provides a summary of the research results and conclusions as well as a discussion of the future study of biodeteriorated bone and of the curation of institutionalised human skeletal collections.

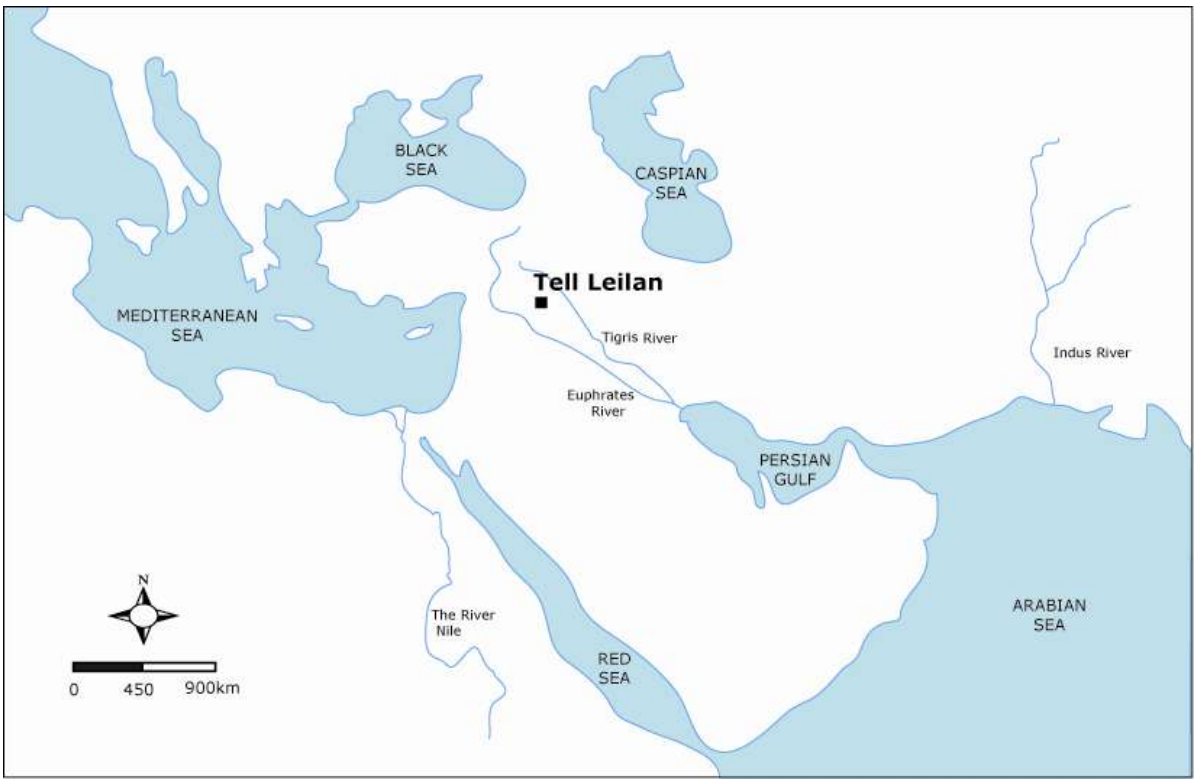

Fig. 1.1 Map showing the geographic location of Tell Leilan.

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Chapter 2

Microbial Biodeterioration of Archaeological Bone

2.1 Introduction

All archaeological bone is thought to be affected by microorganisms in some way, and as such is subject to biodeterioration. This process can begin in either the burial or curatorial environment, and proceeds at a rate that is dependent on several intrinsic and extrinsic factors. When affected, bone is altered at all levels i.e., macroscopic, microscopic, and chemical (see Colson et al., 1997; Grupe et al., 1993; Grupe and Piepenbrink, 1988; Iwaniec et al., 1998; Jackes et al., 2001; Jans et al., 2004; Palmer, 1987; Piepenbrink and Schutkowski, 1987; Pruvost et al., 2007; Schultz, 1997, 2003; Yoshino et al., 1991). A growing body of research suggests that microbial growth in bone has an effect on whether DNA, stable isotopes, and trace elements can be successfully extracted from contaminated specimens. Consequently, investigators have become increasingly interested in all aspects of microbial growth and behaviour and several international workshops have been held to consider their effects on bone (e.g., Bocherens and Denys, 1997; Fernandez-Jalvo et al., 2002; Lee-Thorpe and Sealy, 2008; Schwarcz et al., 1989).

For almost 150 years, researchers have focused on the causal organisms of biodeterioration (e.g., bacteria and fungi) (Child, 1995a; Grupe and Dreses-Werringloer, 1990; Marchiafava et al., 1974; Wedl, 1864), on the factors involved

in their growth in a variety of environments (Hedges, 2002; Henderson, 1987), and on the characterisation of their destructive patterns (Garland, 1987; Hackett, 1981). Recently, investigators have concentrated on methods to quantify damage caused by specific organisms and several techniques such as thin section analysis, scanning electron microscopy (SEM), and diagenetic parameters such as pore size distribution have been found to be particularly useful (see Bell, 1990; Hanson and Buikstra, 1987; Hedges and Millard, 1995; Hedges et al., 1995; Nielsen-Marsh et al., 2007; Pfeiffer and Varney, 2000, 2001; Turner-Walker and Jans, 2008).

This chapter is an overview of the literature concerning the microbial biodeterioration of archaeological bone and includes a general definition of biodeterioration and an examination of the factors that render bone an attractive substrate for microbial growth. A brief summary of the organisms thus far implicated in the process of biodeterioration is given as well as a description of their characteristic destructive tunnels. Furthermore, the factors affecting the biodeterioration of bone in both burial and curatorial environments and the protocols followed to avoid such destruction during curation are reviewed. An outline of the techniques employed to study the microbial decay of bone is also provided.

2.2 Defining Biodeterioration

The term taphonomy refers to the study of the events between the death and fossilisation of an organism (Efremov, 1940). Within the field of taphonomy,

diagenesis is the study of the physical, chemical, and biological pressures acting upon sediments in which the skeletal remains of an organism are deposited (Grupe et al., 1993; Grupe and Dreses-Werringloer, 1990). Although many physical and chemical pressures undoubtedly affect the diagenesis of bone, biological decay, or biodeterioration, is the most influential and is the least understood (Grupe and Dreses-Werringloer, 1990). Of the organisms affecting bone survival in archaeological contexts (e.g., animals, insects, microorganisms, and plants), microbes such as bacteria and fungi are considered leading agents in the biodeterioration of bone.

Hueck first defined biodeterioration as "any undesirable change in the properties of a material caused by the vital activities of organisms" (Hueck, 1968:9). Although in use since the late 1960s, several terms other than biodeterioration also refer to microbially-altered bone including bioerosion (Davis, 1997; Jans, 2008; Trueman and Martill, 2002); bone diagenesis (Colson et al., 1997; Guarino et al., 2006; Gutierrez, 2001; Hanson and Buikstra, 1987; Hedges, 2002; Hedges et al., 1995; Lee-Thorpe and Sealy, 2008; Nielsen-Marsh et al., 2007; Price et al., 1992); bone degradation (Nicholson, 1998); microbial decomposition or destruction of bone (Child, 1995b; Child and Pollard, 1990); and biogenous dead bone decomposition (Grupe and Piepenbrink, 1988; Piepenbrink, 1986).

In recent years, the number of examples of biodeteriorated bone has risen, because biodeteriorated bone is more readily recognized by new and more

invasive methods of examination. Biodeterioration has been detected in collections world-wide, dating from Neolithic to modern times in Europe (Child, 1995a, b; Colson et al., 1997; Garland, 1987; Grupe and Dreses-Werringloer, 1990; Grupe et al., 1993; Hackett, 1981; Hagelberg et al., 1991; Hedges and Millard, 1995; Jackes et al., 2001; Jans et al., 2004; Piepenbrink, 1986; Turner-Walker and Jans, 2008; Turner-Walker and Syversen, 2002), the Middle East (Maat, 1993; Piepenbrink and Schutkowski, 1987), Australia (Hackett, 1981), Indonesia (Hackett, 1981), Canada (Colson et al., 1997), and the United States (Gordon and Buikstra, 1981; Hackett, 1981; Hanson and Buikstra, 1987; Schoeninger et al., 1989). It is likely that microbial growth will continue to be recognised in archaeological collections at an increasing rate, as the techniques employed to examine curated skeletal material continue to improve.

2.3 Why Study Bone Biodeterioration?

Microbial growth leads to changes in both the macro- and microscopic properties of bone. Consequently, such growth affects the results of bioarchaeological studies and prevents us from learning information about past populations. For example, microbial growth limits the histological study of bone particularly when attempting to estimate age and diagnose palaeopathological conditions (Iwaniec et al., 1998; Jackes et al., 2001; Palmer, 1987; Piepenbrink and Schutkowski, 1987; Schultz, 1997, 2003). In addition, microbial growth has been shown to lead to alterations in the chemical composition of bone, thus hindering attempts at dating as well as elemental (Grupe and Piepenbrink, 1988, 1989; Jackes et al., 2001) and

DNA analyses (Colson et al., 1997; Haynes et al., 2002; Jans et al., 2004; Pruvost et al., 2007; Schoeninger et al., 1989; Trueman and Martill, 2002). Thus, an understanding of microbial growth in bone is important for the interpretation of most, if not all, anthropological questions derived from archaeological bone. For, once the process is understood, new criteria for selecting samples for such analyses can be suggested.

More importantly, however, prevention is the key. Knowledge of the process of bone biodeterioration can be used to prevent this process in archaeological skeletal collections in the future. Although seldom mentioned in the literature, knowledge of the causal organisms can be used against them. Their requirements for growth for example, can be withheld and their effects on bone can be minimised. Therefore, with this information, it becomes possible to create curation protocols to insure the survival of bone in curation. In addition, knowledge of bone biodeterioration may allow the re-creation of site formation processes in archaeological contexts (Lee-Thorp and Sealy, 2008; Nielsen-Marsh and Hedges, 2000; Turner-Walker and Jans, 2008). Thus, of the many reasons for studying bone biodeterioration, its prevention is the most important.

2.4 Bone as a Substrate

2.4.1 Bone Biology

Before discussing why skeletal material is vulnerable to microbial attack, it is necessary to review the biology of bone. Bone is a composite material that

includes both inorganic (mineral) and organic (mostly protein) components, but it is predominantly an inorganic substance (70%) composed of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]. This inorganic component is intimately associated with the organic component, which consists of collagen, non-collagenous proteins, and water. The ratio of the two components is not constant, however, since the relative amount of bone mineral increases with age.

At a macroscopic level, there are two types of bone whose structure varies depending on the location within the skeleton. Bone found on the interior of skeletal elements and near the growth plates of long bones is referred to as cancellous bone (Malluche and Faugere, 1986). Bone located on the surfaces of flat bones and in the shafts of long bones is referred to as compact bone. Both varieties of osseous tissue contain osteoblasts, osteoclasts, and osteocytes that are specialised cells in charge of the growth, destruction, and maintenance of bone respectively.

Although both cancellous and compact bone contain these bone cells, overall the histological structures within the two varieties are distinct (Fig. 2.1). Cancellous bone is characterised by interspaced lattice-shaped spicules (trabeculae) within which the three cell types are dispersed. In compact bone, however, the osteon, also referred to as the Haversian system, is the functional unit. During life, a Haversian canal, found at the centre of each osteon, contains blood vessels and other connective tissues (Malluche and Faugere, 1986). These canals are

interconnected perpendicularly by Volkmann's canals. Around the Haversian canal, concentric layers of lamellae are deposited and contain osteocytes within their lacunae (Ham and Cormack, 1979). Osteocytes communicate through the canaliculi that radiate from each of the lacunae.

There are two types of compact bone that can be identified at a microscopic level—woven and lamellar bone, both of which are remodelled during life (including in response to healing) (Schultz, 1997). Woven bone is comprised of primary osteons and is formed by an irregular arrangement of collagen bundles and bone cells (Malluche and Faugere, 1986). Although initially found in areas of new bone growth and bone repair, it is eventually replaced by mature (lamellar) bone. Lamellar bone is comprised of secondary osteons that form during the bone remodelling process. This type of bone contains a more organised arrangement of collagen bundles and bone cells rendering it more mechanically stable in comparison to woven bone. The secondary osteons within lamellar bone are surrounded by a highly mineralised layer referred to as the cement line. The layers of bone found between adjacent osteons are referred to as interstitial lamellae (Schultz, 1997).

2.4.2 Bone Vulnerability

Archaeological human bone is an ideal substrate for the growth of microorganisms because of its inherent properties. Structures such as Haversian and Volkmann's canals, lacunae, canaliculi, nutrient foramina, and trabecular

spaces, all of which are void in archaeological bone, serve as entry points for bacteria and fungi. These structures provide a protective space in which microorganisms can proliferate (Grupe et al., 1993; Jans, 2008; Lee-Thorpe and Sealy, 2008; Schultz, 1997). Most importantly, however, these cellular structures are highly interconnected and allow microorganisms to infiltrate bone deeply (Bell, 1990). Thus, bone is a likely candidate for microbial growth because of its complex network of cellular structures.

In addition, bone is vulnerable to microbial attack because of its composition at both inorganic and organic levels. Microorganisms need a variety of elements to survive including, but not limited to, potassium, phosphorus, calcium, and magnesium (Deacon, 1997; Kendrick, 2000; Turner-Walker, 2008), all of which are readily available within bone. Moreover, bone is inherently vulnerable to microorganismal attack because it contains collagen that can be broken down into the amino acids that are required in microbial metabolism. Certain microbes are capable of degrading collagen into its constituent parts. To do so, they must solubilise the barrier-like mineral inorganic component (Child, 1995a; Collins et al., 2002; Turner-Walker, 2008; Turner-Walker and Syversen, 2002) and then produce one of several enzymes (e.g., protease, collagenase) that can cleave the complex collagen molecule (Child et al., 1993; Turner-Walker, 2008). Several organisms isolated from archaeological bone have been found to be capable of producing such enzymes (see Child, 1995b, c; Child et al., 1993). Certain fungi, however, are capable of reducing the pH of bone leading to the removal of both

the collagen and mineral components through hydrolysis, thus bypassing the need for a destructive enzyme (Child, 1995b). It is possible, however, that a microorganism need not be capable of producing an enzyme on its own—as organisms have been shown to collaborate to digest a substrate, each carrying out a portion of the metabolic pathway (Costerton et al., 1994).

2.5 Microorganisms and their Patterns

That bacteria and fungi can colonise and digest bone has been known since the mid-1860s (Hackett, 1981; Marchiafava et al., 1974; Wedl, 1864). These bone-destroying microorganisms are thought to be absent by the time a bone has been unearthed (Jans, 2008). Their identity, however, can be determined by examining traces left behind in bone (Hackett, 1981). Evidence of microorganisms include, but are not limited to, gross changes such as staining caused by the accumulation of microbial pigments and the appearance of microscopic tunnels, which can be seen using techniques such as thin section analysis (Hackett, 1981). Although only rarely have the erosion troughs of both fungi and bacteria been found in the same bone (Jans et al., 2004), it is possible that several of these organisms work collectively in the digestion of bone.

2.5.1 Bacteria

Because bacteria are readily found in the air and one gram of soil may contain more than one million of the organisms (Clark, 1967), it is not surprising that bacteria have been found in archaeological bone. Most bacteria are very small,

single celled cocci and bacilli, which are in the order of a few micrometers (10^{-6}). In addition, some bacteria grow as fine filaments (i.e., actinobacteria), which look and behave like minute fungi. Most bacteria reproduce by binary fission and under optimal conditions a bacterial population can double within 10 minutes. Some bacteria, in times of stress, can produce resilient endospores that ensure their survival. Thus, following excavation, spores in archaeological bone can germinate in a curated environment if conditions become favourable.

Bacteria follow bone microstructure while invading bone—a pattern that is governed by bone properties such as mineral density and collagen content. Bacteria appear to more readily colonise proteinaceous substances than highly mineralised areas such as external and internal lamellae (Jackes et al., 2001; Jans, 2008; Turner-Walker and Jans, 2008). In compact bone, the external and internal lamellae are avoided initially and growth is directed towards the centre of a skeletal element. Similarly, in cancellous bone, the external lamellae are avoided and growth is centre-directed (Bell, 1990; Hackett, 1981; but see Hanson and Buikstra, 1987; Jans, 2005). Invaded areas will show various signs of infiltration including characteristic destructive tunnels, pigment accumulation, or both (Grupe et al., 1993; Hackett, 1981; Marchiafava et al., 1974).

Demineralisation is an early sign of bacterial activity in bone. This occurs because bacteria must first remove the mineral to access collagen (Turner-Walker, 2008). Demineralisation leads to the production of three distinct tunnel varieties i.e.,

budded, linear longitudinal, and lamellate (Garland, 1987; Hackett, 1981). These tunnels form through a combination of demineralisation and subsequent redeposition by microorganisms (Hackett, 1981). Bacterial tunnels are distinguished by their size, shape, and location (Hackett, 1981) (Fig. 2.2). They are similar, however, in that all varieties are infilled and present a hypermineralised rim that is formed during the redeposition process. Budded tunnels are described as irregular in shape and range from ~30 to 60 µm in diameter. Linear-longitudinal tunnels are considered the smallest of the bacterial erosion troughs (~5–10 µm) and are typically circular (Jans, 2008). Lamellate tunnels (~10–60 µm) follow bone's microanatomy and are the least common of the bacterial tunnels. Some researchers have noted that lamellate forms are typically associated with the budded variety and may represent an early manifestation of that form (Jans et al., 2004).

Bacterial activity, which causes the deposition of pigments and the formation of tunnels, has been noted at both a gross and microscopic level in the literature. Gross evidence, in the form of accumulations of pigments, has been observed in skeletal collections as coloured stains on the external surfaces of bone (Grupe and Dreses-Werringloer, 1990; Grupe et al., 1993; Marchiafava et al., 1974). At a microscopic level, evidence of bacterial activity (i.e., tunnels and accumulations of pigments) has been recorded in skeletal collections (Grupe et al., 1993; Hackett, 1981; Jans et al., 2004; Marchiafava et al., 1974). For example, Garland (1987) observed linear-longitudinal and lamellate tunnels as well as

accumulations of pigments in human bone from a variety of archaeological sites dating from 9000 BCE to the early 20th century. Garland (1987) noted that only a few of the bones bore gross indicators of biodeterioration.

2.5.2 Fungi

Fungi are considered one of the least explored groups of organisms on the planet (Webster and Weber, 2007). Larger than bacteria, fungi average ~5 µm in width but their length varies because of their branching filamentous nature (Kendrick, 2000). Fungi typically grow as hyphae (threadlike filaments), collectively referred to as a mycelium. Hyphae elongate at their tips and absorb nutrients from substrates through their cell walls. Fungi often produce spores when conditions become inhospitable (e.g., limited nutrients) (Deacon, 1997). These spores can later germinate when favourable conditions return (Kendrick, 2000). Throughout the growth period, fungi produce a variety of metabolites including antibiotics (Deacon, 1997; Piepenbrink, 1986) and pigments (Deacon, 1997), both of which can be visualised during the examination of bone. Other metabolites such as enzymes aid in the digestion of substrates such as bone.

Fungi produce changes in experimentally inoculated bone within 2–3 weeks (Davis, 1997; Marchiafava et al., 1974; Wedl, 1864). This is not surprising since fungal colonies can produce more than a kilometre of new hyphae in just 24 hours (Janaway, 1996). Unlike bacterial colonies, fungal filaments remain in contact with the burial environment, thus facilitating the transport of exogenous materials

into bone (Grupe et al., 1993). Inside bone, fungi dissolve the matrix through the release of protons and lytic enzymes, ultimately resulting in the formation of tunnels (Jans et al., 2004; Piepenbrink, 1986). According to Jans (2008), these tunnels are unlike those formed by bacteria because they do not follow bone microstructure and are typically seen on the inner and outer cortices of bone. Similar to bacteria, several fungal species (e.g., *Stachybotrys cylindrospora*, *Cephalotrichum stemonitis, Penicillium brevi-compactum*) are known to stain bone because of pigments they secrete (Grupe and Piepenbrink, 1988; Piepenbrink, 1986).

Although Wedl first identified fungal activity in bone in 1864, the resulting tunnels were not named (Type I Wedl tunnels) or fully characterised until 1981 by Hackett. Type I Wedl tunnels are the most common and are simple, large (10–15 µm in diameter), and branching (Fig. 2.2). Other varieties of tunnels have been recently recognised e.g., Type II Wedl and Hackett's tunnels (Davis, 1997; Trueman and Martill, 2002). Type II Wedl tunnels are less common and are smaller (5 µm in diameter) than Type I tunnels, and extend from osteonal canals (Trueman and Martill, 2002). The latest variety, Hackett's tunnels, were recognised in bird bone by Davis (1997) as large (50–250 µm in diameter) tunnels radiating from the external cortices of bone.

Although fungi were the first microorganisms found in association with biodeteriorated archaeological bone, their traces are rarely noted in the literature

in comparison to those of bacteria (Jans, 2008; Jans et al., 2004). Fungal hyphae, conidiophores, and spores have been identified in human skeletal collections (Jans, 2005; Maat, 1993). These bone specimens, however, lacked tunnels (e.g., Type I and II Wedl, and Hackett's tunnels) leading researchers to hypothesise that fungi may be living off other organisms within bone, and that bacteria are therefore more likely responsible for the majority of the destruction noted in skeletal collections (Grupe and Dreses-Werringloer, 1990; Jans, 2008). Wedl tunnels have been noted, however, in human vertebrae that had been exposed to *Mucor* by Marchiafava et al. (1974) and in human skeletal remains analysed by Garland (1987).

2.5.3 Biofilms: Complex Microbial Communities

Historically, microorganisms incapable of producing enzymes such as collagenase have been dismissed as not being involved in bone biodeterioration (Child, 1995a, b; Child et al., 1993; Child and Pollard, 1990). These organisms, however, were likely part of a larger microbial community, also known as a biofilm, which played a role in the ultimate breakdown of bone (Costerton et al., 1978). Costerton et al. (1978) first introduced the biofilm concept. A biofilm consists of microbial cells (e.g., bacteria, fungi) cemented together by extracellular polymeric substances such as polysaccharides, proteins, and nucleic acids (Blankenship and Mitchell, 2006; Davies, 2000; Flemming et al., 2000). Although composed primarily of microorganisms collaborating to digest a substrate, a biofilm will also contain organisms not directly involved in the decay of a substrate (Wimpenny,

2000). Taken as a whole, a biofilm provides an environment that protects organisms against pH changes, temperature variations, and toxins.

Although bacterial biofilms are usually discussed in the literature, fungi and other microorganisms can be present in both single and multispecies biofilms. Regardless of the type of organism present, biofilms must adhere to a substrate to thrive. Biofilms are found in a variety of substrates, most of which have some contact with water (Flemming et al., 2000; Wimpenny, 2000). Once attached, a biofilm proliferates across a substrate and eventually reaches maturity (Blankenship and Mitchell, 2006; Busscher and van der Mei, 2000; Davies, 2000; Kolenbrander et al., 2000). As the biofilm grows, organisms sequester organic and inorganic material and other substances (e.g., metals) from the external environment that will be incorporated into the film (Flemming et al., 2000). Eventually, however, because of either shear forces or limiting factors, portions of the biofilm degrade and recolonisation may follow (Wimpenny, 2000).

Biofilms have been isolated from a variety of substrates including marble, piping, and art (see Costerton et al., 1987; Doggett, 2000; Flemming et al., 2000; Saarela et al., 2004; Sanchez-Moral et al., 2003). Biofilms have also been recognised on teeth and on the bones of living patients suffering from infection (Gristina and Costerton, 1984; Kolenbrander, 2000; Kolenbrander et al., 2000; Sedghizadeh et al., 2008; Toshiyuki, 2005). Recently, Kaye and colleagues (2008) discussed possible biofilm growth in fossilised dinosaur bone. The authors noted that a

biofilm had coated the internal structures (including canals and lacunae) of the bone. Because the bone had been demineralised through microbial action, all that remained was the biofilm structure that took on much of the bone's original morphology (Kaye et al., 2008). These complex microbial aggregates, however, have not been identified as biofilms in archaeological human bone, even though it is likely that bone is subject to biofilm growth because of its ample surfaces, rich protein content, and moisture availability.

2.6 Factors Affecting the Biodeterioration of Bone

Although no single factor alone affects bone biodeterioration, several intrinsic parameters have been shown to increase the likelihood of an invasion, including sex, age of the individual at death, and the presence of pathological conditions (Gordon and Buikstra, 1981; Janaway, 1996; Matheson and Brian, 2003). In addition, several interrelated extrinsic factors such as temperature and moisture are known to affect microorganismal activity (Gordon and Buikstra, 1981; Hedges, 2002; Henderson, 1987; Matheson and Brian, 2003). These intrinsic and extrinsic factors are usually discussed in relation to burial conditions and not the curation environment (see Henderson, 1987; Matheson and Brian, 2003; Schultz, 2001; Weigelt, 1927). As will be shown, these factors are at work regardless of location and their importance may be decreased depending on the dynamics of the microorganisms themselves.

2.6.1 Age, Sex, and Pathology

Depending on age, sex, and health status, skeletal composition will vary. Differences in mineral content and collagen levels will have an effect on a skeleton's susceptibility to microorganismal attack both in the burial and curation environments. Age has been shown to have implications for biodeterioration by several investigators (Binford and Bertram, 1977; Gordon and Buikstra, 1981; Guarino et al., 2006; Guy et al., 1997; Haynes, 1981; Jans et al., 2004; Matheson and Brian, 2003; Micozzi, 1991; Walker et al., 1988; but see Turner-Walker and Syversen, 2002). The bones of younger individuals (which are more porous and have a higher collagen content) are considered more susceptible to attack by microorganisms. In addition, other factors such as sex and bone quality and quantity (e.g., osteoporosis) have been mentioned (Janaway, 1996; Micozzi, 1991; Turner-Walker and Syversen, 2002).

2.6.2 Nature of Deposition

The manner in which a body enters a burial environment and the condition in which a skeleton arrives into a curated one have dramatic effects on the agents of decay (Turner-Walker, 2008). Whether a skeleton is articulated, disarticulated, fleshed, or defleshed has an impact on microbial growth in the burial environment (Nielsen-Marsh et al., 2007). Bone from a fleshed articulated body is more prone to microorganismal activity because enteric organisms may have already demineralised portions of the skeleton located near the abdomen (Child, 1995b; Nielsen-Marsh et al., 2007). With the mineral component removed,

microorganisms have easier access to collagen, thereby speeding up biodeterioration. Without a soft-tissue covering, a skeleton is further subject to abrasion, cracking, polishing, and breakage, all of which create new entry points for bone-invading organisms in both burial and curatorial conditions.

2.6.3 Moisture

Moisture is one of the more important factors in microbial growth, both in the burial and curatorial environments (Goffer, 1980; Matheson and Brian, 2003; Turner-Walker, 2008; Valentin, 2003). The loss of water can weaken the bonds between the organic and inorganic components of bone (Haynes, 1981; Henderson, 1987; Stone et al., 1991; Turner-Walker, 2008) rendering it more susceptible to microbial decay. More importantly, however, moisture is necessary for the development of microorganisms and without it, many bacteria and fungi cannot grow (Janaway, 1996; Valentin, 2003). Therefore, it is not surprising that drier environments inhibit microbial attack of soft tissues and bone (Hedges, 2002). Excessive water, however, is also harmful to microorganisms, as waterlogged environments are anoxic, thus inhibiting aerobic microbial growth (Hedges, 2002; Henderson, 1987; Janaway, 1996; Matheson and Brian, 2003; Stone et al., 1991; Turner-Walker, 2008).

2.6.4 Temperature

Temperature affects the biodeterioration of bone in both the burial and curatorial environments. Its effect in the burial environment, however, depends on factors

such as latitude, season, and depth of burial (Turner-Walker, 2008). Although temperatures between 10 and 40° C are favourable for microorganisms, growth is optimal between 25 and 35° C (Janaway, 1996). While extreme (hot and cold) temperatures inhibit microbial growth (Hedges, 2002; Jans et al., 2004; Matheson and Brian, 2003; Micozzi, 1991; Valentin, 2003), certain microbes can produce spores that are temperature-resistant and can survive until more favourable conditions return (Yakovleva et al., 2006).

2.6.5 Sediment

Sediment has both direct and indirect effects on the survival of skeletal material (Turner-Walker, 2008). Compounds found within sediment can destroy microorganisms or inhibit their growth. For example, bone found in proximity to humic acids and elements such as copper, mercury, or lead may be shielded from the deleterious effects of microorganisms (Child, 1995c; Jans, 2005; Jans et al., 2004; Matheson and Brian, 2003). In addition, sediment pH has a dramatic affect on microbial growth and thus on bone survival. Severely alkaline or acidic sediments inhibit the activities of most microorganisms (Janaway, 1996). For example, microbial enzymes such as collagenase do not function at a pH of less than six (Jackes et al., 2001). Thus, the best sediment conditions for bone (neutral or slightly alkaline) are also those that are conducive to microbial growth which means that if bone does survive the burial environment, it is likely to have been affected by microorganisms.

2.6.6 Microorganism Dynamics

Researchers have argued that bone biodeterioration is slowed when bone is infiltrated by a variety of microorganisms because energy must be directed towards the control of other microorganisms instead of towards the digestion of a substrate (Child, 1995b, c; Child et al., 1993). Although it is true that inter-species dynamics do affect bone biodeterioration, researchers have ignored that the majority of microorganisms live and grow in complex microbial communities such as biofilms and that cooperation between species may actually increase the rate of biodeterioration in bone.

Multiple intra- and inter-species interactions occur in microbial aggregates such as biofilms. These interactions involve the sharing of multiple metabolic pathways, thus organisms not directly producing bone solubilising enzymes can still be involved in the process of biodeterioration (Bradshaw et al., 1994; Marsh and Bowden, 2000; Wimpenny, 2000). Other interactions buffer certain microorganisms against harsh conditions such as pH, temperature, humidity, and chemicals (Chen et al., 1996; Davies, 2000; Marsh and Bowden, 2000). Thus, one organism can modify the local habitat rendering it more hospitable for others (Kolenbrander et al., 2000; Marsh and Bowden, 2000). Hence, these interactions have a direct effect on future preservation strategies because of the increased challenge of preventing the formation and perpetuation of biofilms.

2.7 Preventing Biodeterioration in Skeletal Material

Although curation protocols are not necessarily specific for preventing microbial growth in human skeletal collections, they do attempt to render conditions inhospitable for most microorganisms. Overall, curation protocols aim to keep bone clean and dry through the implementation of various post-excavation procedures (cleaning) and through environmental control (lighting, temperature, and relative humidity) (see Appelbaum, 1991; Canadian Conservation Institute, 1983; Cronyn, 1990; Department for Culture, Media and Sport, 2005; Gehlert, 1980; Odegaard and Cassman, 2007; Rose and Hawks, 1992; Sease, 1994; Weintraub and Wolf, 1992).

2.7.1 Cleaning

It is widely accepted that bone should be cleaned before it is curated. Whether the cleaning involves light dusting or the removal of large amounts of dirt is situational. There are various techniques to clean bone that involve mechanical (brushes, bamboo skewers, metal probes), aqueous (water), and solvent means (Odegaard and Cassman, 2007). Although various techniques exist, if necessary, soft brushes are the preferred tools for cleaning bone as they add less potential for damage to bone (Gehlert, 1980) While water is often considered inert, it should be avoided as it introduces foreign chemicals into bone (Odegaard and Cassman, 2007). Whatever the condition of the skeletal material, it should be dry (not force-dried) before it is curated (Buikstra and Ubelaker, 1994; Gehlert, 1980).

2.7.2 Curation

The proper curation of bone is one of the most direct ways of preventing microbial growth in institutionalised archaeological skeletal collections. Protocols typically call for cleaned skeletal material to be labelled and placed in inert packaging materials (Cassman and Odeggard, 2007; Department for Culture, Media and Sport, 2005). Although specially designed storage boxes for skeletons are now available (Cassman and Odegaard, 2007), most institutions store skeletal material in wood, plastic, metal, cardboard, and fibreglass boxes—each of which have their advantages and disadvantages (Gehlert, 1980). Individual bones are placed in a variety of bags made of cloth, plastic, and paper (Buikstra and Ubelaker, 1994; Cassman and Odegaard, 2007). The following environmental controls are integral in the safe curation of bone to prevent microbial growth in institutions.

Human skeletal collections should be stored away from direct sunlight, in a dry environment in which there are no abrupt swings in temperature and relative humidity (RH). Direct sunlight is both harmful to bone and the packaging materials in which it is stored (Canadian Conservation Institute, 1983; Cronyn, 1990; Department for Culture, Media and Sport, 2005; Weintraub and Wolf, 1992). To limit microbial growth, storage temperature should be kept below 25 °C (Appelbaum, 1991; Canadian Conservation Institute, 1983; Gehlert, 1980). Furthermore, as the RH increases, so does the possibility of microorganism growth. Most researchers advocate that RH should be checked between 35–70%

and should not exceed 85% (Canadian Conservation Institute, 1983; Gehlert, 1980; Sease, 1994). While a lower temperature and RH would appear to limit microbial growth—the conditions can result in cracking and flaking in bone (Department for Culture, Media and Sport, 2005; Sease, 1994).

2.8 Studying the Biodeterioration of Bone

Bone biodeterioration has been characterised at a gross level and quantified using gross preservation indices (GPI) (Haynes et al., 2002). Also, biodeteriorated bone has been examined at a histological level using light microscopy and a variety of histochemical techniques (Hackett, 1981; Schultz, 2001). In addition, SEM and SEM in backscatter mode (SEM-BSE) have been used to observe bone affected by microorganisms (Bell, 1990; Jackes et al., 2001; Maat, 1993; Turner-Walker and Jans, 2008). Recent advances have been made to quantify bone decay (including biodeterioration) using diagenetic parameters such as histological preservation indices (e.g., Haynes et al., 2002; Hedges et al., 1995; Jans et al., 2004), crystallinity (Hedges et al., 1995), pore size distribution (Gutierrez, 2001; Hedges et al., 1995), carbonate content (Nielsen-Marsh and Hedges, 2000), nitrogen (%N) and calcite content (%calcite) (Smith et al., 2002), as well as mercury intrusion porosimetry (Jans et al., 2004). Moreover, experimental studies carried out in both the laboratory and the field have been instrumental in identifying causal microbial species and the characteristic changes they produce in bone (Child, 1995 a, b; Hackett, 1981; Marchiafava et al., 1974).

2.8.1 Gross Examination

There are few visible signs of microbial contamination in bone—although the accumulation of microbial pigments, the most extreme of these changes—can be observed at a gross level. Several genera such as *Pseudomonas*, *Fusarium*, and *Actinomadura* can produce yellow, red, and brown pigmented stains respectively (Deacon, 1997; Grupe and Dreses-Werringloer, 1990; Grupe et al., 1993; Marchiafava et al., 1974). In addition, black and violet-blue pigments have been observed in human skeletal remains from Switzerland and West Germany (Piepenbrink, 1986). Visually these stains can be confused with colour changes to bone caused by mineral contamination from copper (Janaway, 1996; Morris, 1981), vivianite (Grupe and Dreses-Werringloer, 1990), manganese (Schultz, 2001), iron (Schultz, 2001), and the percolation of humic acids into bone (Jans, 2005). Thus, relying solely on gross changes in bone to understand microbial biodeterioration is limited.

Gross preservation is quantified using an index such as the gross preservation index (GPI) (see Haynes et al., 2002). A GPI, however, is merely a measure of all the visible changes to bone caused by its environment—only some of which may have been caused by microorganism activity. GPI values are typically compared to histological integrity to determine whether appearance alone can be used to suggest whether a bone is preserved, thus aiding in sample selection (Gordon and Buikstra, 1981; Haynes et al., 2002; Jans et al., 2002). These researchers, however, have found no correlation between gross and histological preservation.

Thus, although a bone may appear ‘well-preserved’, no histological structures may remain.

2.8.2 Thin Section Analysis

Thin section analysis has been used to study bone biodeterioration since the mid-1860s (Hackett, 1981; Hedges et al., 1995; Jans, 2005; Marchiafava et al., 1974; Pfeiffer and Varney, 2000; Piepenbrink, 1986; Wedl, 1864). It was not until 1981, however, that Hackett characterised the tunnels produced by specific organisms, and this helped investigators determine the nature of the involved organisms. Researchers investigating biodeterioration typically prepare undecalcified thin sections of bone. Although unembedded bone samples have been prepared (Frost, 1958; Maat et al., 2001), typically a small sample of bone is cut and embedded into a variety of media such as Biodur® (Piepenbrink, 1986; Schultz, 2001), epoxy resin (Schoeninger et al., 1989; Turner-Walker and Jans, 2008; Turner-Walker and Syversen, 2002), and methyl methacrylate (Bell, 1990; Hackett, 1981; Hanson and Buikstra, 1987). The embedded bone is cut using a variety of saws to produce either a thick or a thin section. Thick sections must be ground so that histological structures are visible and scratches are removed. A variety of grinding and polishing materials have been used including sand paper (Frost, 1958; Maat et al., 2001) and aluminum oxide powder (Fitzgerald and Saunders, 2006). Once a desired final thickness is reached the resulting thin section can be treated with various stains, mounted, and examined.

Thin sections are examined under normal, fluorescing, and polarised light, and stains such as toluidene blue and crystal violet can be applied to highlight particular structures and exogenous materials within bone (De Boer et al., 2010; Garland, 1987; Grupe and Dreses-Werringloer, 1990; Grupe et al., 1993; Guarino et al., 2006; Hackett, 1981). In addition to tunnels, microbial pigments can be recognised using thin section analysis (see Grupe and Dreses-Werringloer, 1990; Marchiafava et al., 1974; Piepenbrink, 1986). More complex examination of thin sections (e.g., preservation indices, SEM, SEM-BSE) can also provide an idea of the degree of preservation of a skeletal element/collection.

2.8.3 Scanning Electron Microscopy

SEM has been used to examine embedded and unembedded samples of microbially altered bone (Bell, 1990; Guarino et al., 2006; Jackes et al., 2001; Maat, 1993; Turner-Walker and Jans, 2008; Turner-Walker and Syversen, 2002). Bone samples have been fixed and dehydrated using glutaraldehyde, osmic acid, and acetone and coated with gold or carbon prior to examination (Maat, 1993; Piepenbrink, 1986; Piepenbrink and Schutkowski, 1987). Bone samples are bombarded by a beam of electrons that causes other electrons, either secondary (SE) or backscattered (BSE), to be ejected. The electrons are collected and converted into an image (Goldstein et al., 2003). Using SEM, researchers can obtain both structural and analytical information from biodeteriorated bone at a wide range of magnifications (10 to 500,000X). Although SE imaging provides a 3D representation of bone morphology, BSE imaging can reveal density

differences within biodeteriorated bone (Bell, 1990; Turner-Walker et al., 2002). Dark and light areas within polished thin sections have been interpreted as corresponding to de- and hypermineralised zones respectively (Bell, 1990; Turner-Walker and Jans, 2008). These colour variations represent different backscatter intensities related to atomic weight differences.

2.8.4 Diagenetic Parameters

Although not specific to bone biodeterioration, diagenetic parameters are used to quantify the amount of alteration in bone, including alteration caused by the activity of microorganisms. Whereas the process of alteration in bone is not entirely understood, the following parameters provide an idea of the chemical and microscopic changes that a bone has undergone (Hedges et al., 1995). Though a consensus has yet to be reached about which diagenetic parameter is most useful in the study of biodeteriorated bone, histological integrity and pore size distribution have been particularly useful (Haynes et al., 2002; Hedges et al., 1995; Pfeiffer and Varney, 2000; Jans, 2008; Smith et al., 2007) and will be discussed here.

2.8.4.1 Histological integrity - Hedges et al. (1995) developed the Oxford Histological Index (OHI) to quantify the amount of altered bone within a thin section. Other similar indices, such as the Histological Preservation Index (HPI), were developed not long after (Haynes et al., 2002). Although not specifically representing microbial biodeterioration, preservation indices have been used to

quantify the changes resulting from microbial decay. Using these indices, a thin section is assigned a score, typically from 1 to 5 (5 being the best preserved) based on its main histological appearance. Several researchers have found indices such as these useful in documenting the range of variability of histological preservation in a skeletal collection (Colson et al., 1997; Guarino et al., 2006; Gutierrez, 2001; Hanson and Buikstra, 1987; Haynes et al., 2002; Pfeiffer and Varney, 2000; Smith et al., 2007; Trueman and Martill, 2002). In addition, investigators have used such indices to identify bone samples appropriate for bioarchaeological studies including DNA isolation (Colson et al., 1997; Haynes et al., 2002).

2.8.4.2 Pore size distribution - Bone pore size distribution is altered in biodeteriorated bone by microbial action and chemical degradation in the burial environment. Although porosity changes have been measured using several criteria (e.g., Gutierrez, 2001; Hagelberg et al., 1991; Hedges et al., 1995), mercury intrusion porosimetry (HgIP), a method that involves forcing mercury into pore spaces, has been shown to have many advantages over other porosity measurement methods (see Jans, 2008; Jans et al., 2004; Nielsen-Marsh and Hedges, 1999; Turner-Walker et al., 2002). Whereas other diagenetic parameters cannot identify the changes caused by microbial decay, HgIP has shown that porosity changes in the range of 0.1 and 1 μm are caused by microorganismal activity (Turner-Walker et al., 2002). In addition, HgIP is a more rapid technique

in comparison to other diagenetic parameters and will more than likely prove useful in outlining microbial decay in skeletal collections in the future.

2.8.5 Experimental Studies

Biodeterioration experiments are extremely useful in creating a database of bone-inhabiting organisms and the specific tunnels produced by their activity. Researchers have tested the ability of microorganisms to produce enzymes such as collagenases and proteases (Child, 1995a, b; Child et al., 1993; Child and Pollard, 1990; Yakovleva et al., 2006). Other investigators have experimentally inoculated known bone-metabolising microorganisms into sterile bone (archaeological and modern) to observe the destruction that these organisms cause (Child and Pollard, 1990; Grupe et al., 1993; Jackes et al., 2001; Marchiafava et al., 1974; Piepenbrink, 1986; Yoshino et al., 1991). Similarly, experiments have focused on the effects that microbial activity can have on the trace elements, isotopes, and DNA sequences extracted from bone (Grupe and Piepenbrink, 1988, 1989; Grupe et al., 1993). Few of these studies, however, have contemplated the combined effects of microorganisms on bone (for exception see Grupe et al., 1993)

2.9 Conclusions and Future Directions

Over the years, researchers have examined biodeteriorated bone at both macroscopic and microscopic levels using a variety of techniques such as GPI, thin section analysis, HPI, SEM, SEM-BSE, and HgIP. Although progress has been made in the identification of causal organisms (e.g., Davis, 1997; Hackett,

1981; Trueman and Martill, 2002), the study of the microbial decay of bone is still in its infancy and there needs to be a shift in focus towards the prevention of microorganisms in archaeological skeletal collections.

Although biodeterioration has been approached by a variety of investigators using several techniques, a holistic approach is needed whereby researchers from various fields such as biological anthropology, chemistry, cultural heritage management, and microbiology collaborate to study this phenomenon. Whereas past research has focused on isolating and identifying causal microorganisms─ future research must go beyond documenting individual species to examining how groups of organisms (e.g., biofilms) cooperate in the alteration of bone. No doubt more techniques (perhaps those which are less invasive and far more superior) already in use by microbiologists will eventually be used to examine biodeteriorated archaeological bone (e.g., confocal laser microscopy, denaturing gradient gel electrophoresis) (see Wimpenny, 2000 for a review). Knowledge of bone biodeterioration will lead to an improved understanding of archaeological site formation processes and the conditions in which bone will preserve in a variety of contexts (including in curation). Ultimately, knowledge of bone biodeterioration is essential so that the growth of microorganisms in bone can be prevented in the future.

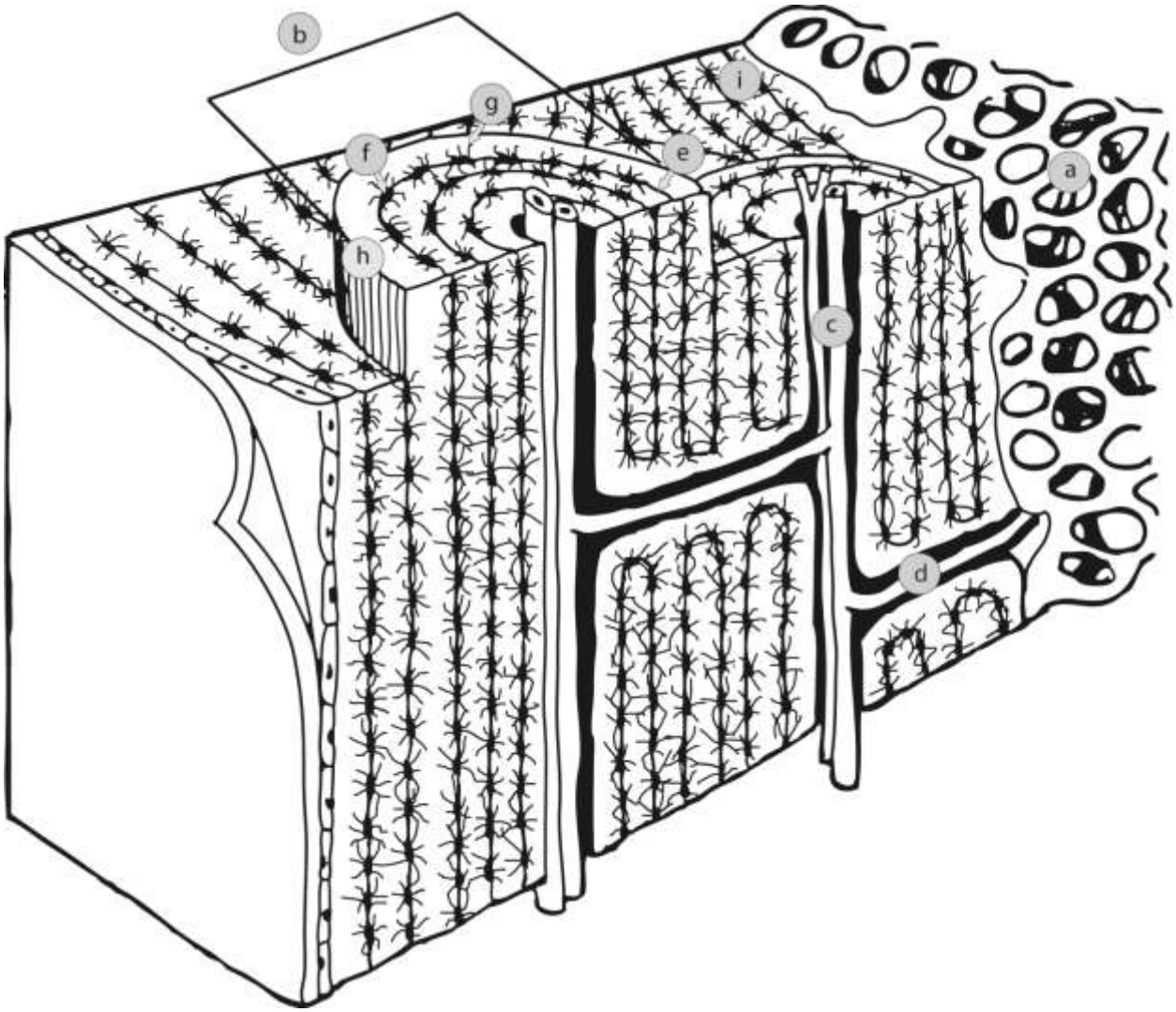

Fig. 2.1 Microscopic structure of bone showing: (a) cancellous bone, (b) an osteon, (c) a Haversian canal, (d) a Volkmann's canal, (e) a lamella, (f) a lacuna, (g) a canaliculus, (h) a cement line, and (i) interstitial lamellae.

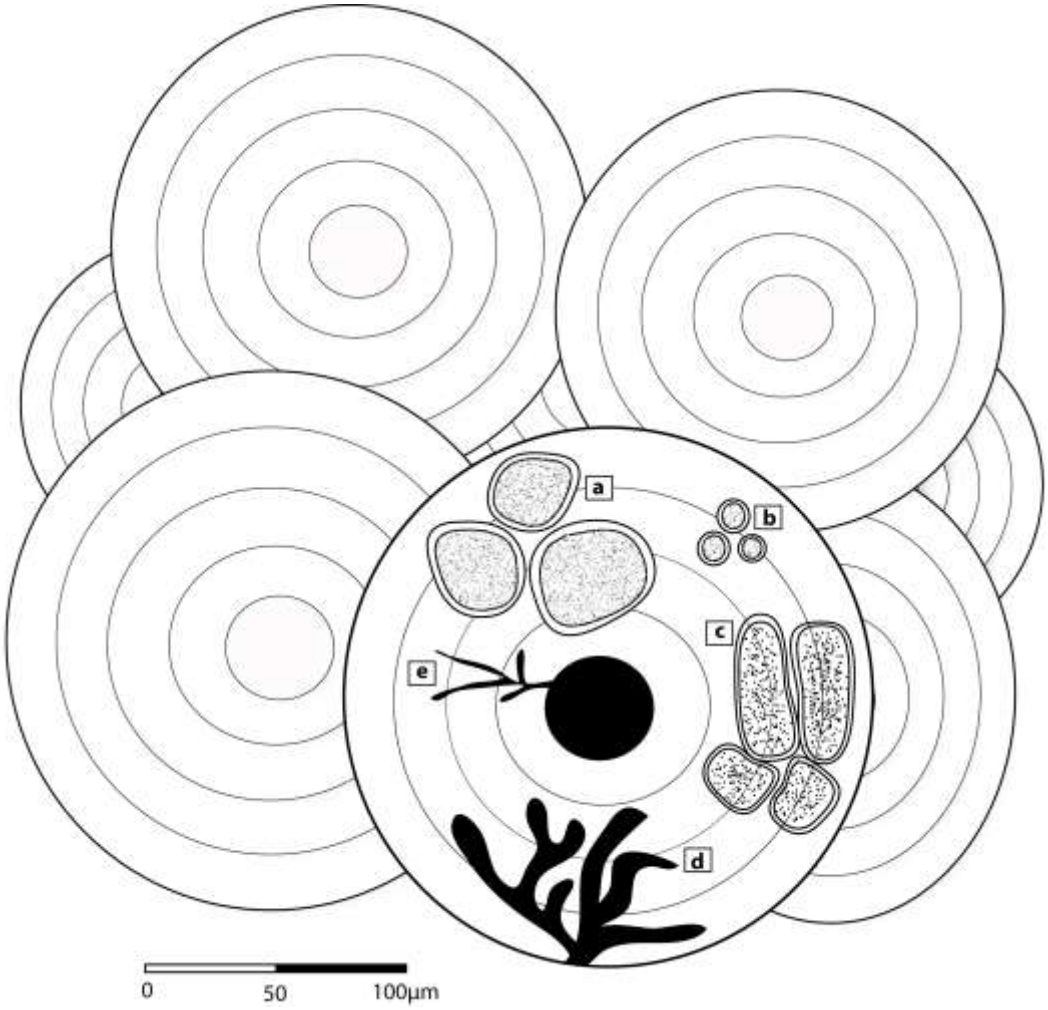

Fig. 2.2 Microbial tunnels in compact bone. An osteon containing: (a) budded, (b) linear-longitudinal, and (c) lamellate tunnels caused by bacteria as well as (d) Type I and (e) Type II Wedl tunnels created by fungi.

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Chapter 3

A Histological Study of Biodeteriorated Bone from Bronze Age Syria

3.1 Introduction

Extensive microbial decay, also known as biodeterioration, was recognised in thin sections prepared of the Tell Leilan human skeletal material (2900 – 1900 BCE) from ancient Mesopotamia. This biodeterioration was present even though the skeletal material was curated under typical institutional conditions and bore no macroscopic indicators of biodeterioration. This paper describes the results of an investigation into the histological characteristics of the biodeterioration of the Leilan skeletal material. The study was undertaken to describe in more detail the process of decay and to document the degree of destruction that the bone had undergone while in curation.

Although bone biodeterioration has been studied for almost 150 years (e.g., Roux, 1887; Wedl, 1864), the phenomenon is far from understood. This is not unexpected since bone subject to microbial decay typically appears normal to the unaided eye and is only somewhat discoloured even when severely altered. Distinctive microscopic changes have been recognized, however, such as the production of pigments of various colours (e.g., black, violet-blue, yellow) and, more commonly, tunnels that appear as small pores or borings throughout bone (Grupe and Dreses-Werringloer, 1990; Grupe et al., 1993; Hackett, 1981; Jans, 2008; Marchiafava et al., 1974).

The tunnels formed by microorganisms in archaeological bone develop through a sequence of demineralisation and redeposition, and the causative agent can be distinguished by the diameter, infill, and distribution of the tunnels (Fig. 3.1). Fungi create three types of infilled tunnels: Type I and II Wedl tunnels and Hackett's tunnels (Davis, 1997; Trueman and Martill, 2002). Type I Wedl tunnels range in diameter from 10–15 µm and appear as simple, branching networks, while the less common Type II tunnels have a diameter of ~5µm and extend from Haversian canals (Trueman and Martill, 2002). The most recently identified variety, Hackett's tunnels, was first recognised in bird bone by Davis (1997) as large (50–250 µm in diameter) tunnels radiating from the external cortices of bone. By contrast, bacteria create three types of empty tunnels, ranging in diameter from 5–60 µm, which follow bone microstructure and are surrounded by a rim of hypermineralised bone (Hackett, 1981; Jans, 2008). The most common of these three tunnels, budded tunnels, are irregular in shape and range in diameter between ~30–60 µm. Linear-longitudinal tunnels are the smallest of these bacterial erosion troughs (~5–10 µm in diameter) and are typically circular (Jans, 2008). Least common are the lamellate tunnels (~10–60 µm in diameter), which follow bone lamellae.

Both accumulations of pigments and tunnels in bone have been characterised at a microscopic level using light microscopy (LM) and histochemical techniques (Grupe et al., 1993; Hackett, 1981; Jans et al., 2004; Marchiafava et al., 1974). In addition, scanning electron microscopy (SEM) has been shown to be particularly

helpful in obtaining information on three-dimensional changes in the structure of biodeteriorated bone (Guarino et al., 2006; Maat, 1993), whereas SEM in backscattered electron (BSE) mode can reveal mineralisation changes brought on by microbial activity (Bell, 1990; Turner-Walker et al., 2002; Turner-Walker and Syversen, 2002). Moreover, researchers have found histological preservation indices useful in documenting the range of variability of histological preservation in skeletal collections (Colson et al., 1997; Hanson and Buikstra, 1987; Haynes et al., 2002; Hedges et al., 1995; Jans, 2005; Jans et al., 2004; Nielsen-Marsh and Hedges, 2000; Smith et al., 2007; Trueman and Martill, 2002).

In this paper, the biodeterioration of the Tell Leilan skeletal material from Syria (2900 – 1900 BCE) is characterised and evaluated using histology, histochemical techniques, and SEM and SEM-BSE. In addition, the histological integrity and variability of the skeletal material were checked using a preservation index modified from Haynes et al. (2002). Although these approaches already have been used to study biodeteriorated archaeological bone (see Bell, 1990; Hackett, 1981; Hanson and Buikstra, 1987; Haynes et al., 2002; Hedges et al., 1995; Jans et al., 2004; Maat, 1993; Marchiafava et al., 1974; Nielsen-Marsh and Hedges, 2000; Turner-Walker and Syversen, 2002), they have yet to be used to study bone that has deteriorated while in curation. Although the purpose of this paper is not to suggest alternative curation protocols for archaeological bone collections, the results of this study emphasise the need for more research into the proper curation of archaeological bone.

3.2 Materials and Methods

3.2.1 Site Description

Tell Leilan is a 90-hectare walled site in northeastern Syria (Fig. 3.2) that was occupied from the mid-sixth to early second millennium BCE (Weiss, 1985; Weiss and Courty, 1993; Weiss et al., 1993). It is situated on the left bank of the Wadi Jawah in a broad, undulating plain composed mostly of flood deposits and sands and is underlain by Pleistocene gravel and plateau basalts (Besonen and Cremaschi, 2002; Weiss, 1985; Weiss and Courty, 1993). The climate at Tell Leilan is semiarid with cool and wet winters, hot and dry summers, and an average annual rainfall of between 300 and 500 mm (Cullen et al., 2000).

3.2.2 Skeletal Material

The skeletons of 38 juveniles and 21 adults, dating to between 2900 and 1900 BCE, were recovered from intramural burial contexts between 1979 – 1989 in a range of alkaline deposits identified as light to brown clays and silty clay loams[1]. Following excavation, the skeletons were shipped to Yale University in New Haven, Connecticut and were later shipped for study in 1991 to the University of California, Berkeley. In 1992, the skeletal material was shipped to the University of Alberta (U of A) in Edmonton. Upon arrival at the U of A, the skeletal material was cleaned manually with bamboo sticks and brushes of varying firmness. Once skeletal elements were cleaned and inventoried, they were transferred from paper bags to 3 mil plastic bags. The skeletal material is presently curated in a centrally

[1] Sediment analysis carried out by Krista Gilliland, University of Alberta.

heated building that ranges in temperature and RH from 15 to 25° C and 15 to 50% respectively depending on the season[2].

In 1996, 39 thin sections of bone from the Tell Leilan collection were prepared for histopathological analysis (these were available for comparison with those prepared for this study). Two years later, Feasby (1998) was successful in isolating collagen from the Tell Leilan collection to examine stable carbon and nitrogen isotope ratios of 16 individuals. In addition, dental characteristics and bone health of the skeletal material have been studied (e.g., Haddow and Lovell, 2003; Lovell and Dawson, 2003; Lovell and Haddow, 2006; McKenzie, 1999).

3.2.3 Thin Section Preparation

One hundred and ninety-two bone fragments/bones from 59 individuals, both juveniles and adults, were thin sectioned in 2009. All available long bones and cranial material were sampled and several sections (including serial) were taken from a single element; samples were embedded in Buehler EpoThin® epoxy (resin and hardener). Undecalcified thin sections (cross and longitudinal sections) were cut using a low speed Buehler IsoMet® saw with a 5 inch diamond wafering blade. Sections were ground to ~100 µm on an Ecomet III® grinder/polisher using three Ultra-Prep© diamond grinding discs (15 µm, 9 µm, 6 µm) and a 3 µm

[2] Records provided by Museum and Collection Services, U of A (P. Mayne Correia, pers. comm.).

CARBIMET® disc. Several thin sections were stained with toluidine blue, a basic stain, to help identify exogenous organic compounds in the sampled bone (see Dore et al., 2001).

3.2.4 Scanning Electron Microscopy

Seven embedded samples were polished using a 6 µm Ultra-Prep© diamond grinding disc followed by a 3 µm CARBIMET® disc, and then gold coated using a Nanotech SEMPrep 2 DC sputter coater. SEM was carried out using a JEOL 6301F (field emission scanning electron microscope) at the Scanning Electron Microscope Laboratory in the Earth and Atmospheric Science Department at the U of A. Secondary electron images were obtained using an Everhart-Thornley detector and BSE micrographs were taken using a silicon diode detector. Samples were examined for areas of high (hypermineralised) and low (demineralised) mineral content; these areas appeared as bright and dark patches respectively owing to differences in atomic weight density in bone (cf. Bell, 1990; Turner-Walker and Jans, 2008).

3.2.5 Examination of Histological Integrity

Thin sections were examined under bright field LM from 2.5X to 63X magnification using a Zeiss AXIO Scope.A1 fitted with an Optronics MacroFire digital camera (LM-MFCCD), and under polarized light at 32X magnification using a Leitz Laborlux 11 Pol microscope. Samples were examined under polarized light in order to detect the presence of collagen (areas that are

birefringent owing to the orientation of collagen fibrils and the alignment of bone mineral) (Schoeninger et al., 1989; Schultz, 2003; Wolman and Kasten, 1986).

The histological integrity of the skeletal material was assessed using a modification of the HPI developed by Haynes et al. (2002). Modification created more defined parameters for each of the categories (including percentages) in order to minimise observer error. A score of 1 to 4 was assigned according to the main histological appearance of a section:

HPI 1: < 25% of structures are unaltered

HPI 2: ≥25% and <50% of structures are unaltered

HPI 3: ≥50% and <80% of structures are unaltered

HPI 4: ≥80% and ≤100% of structures are unaltered

All thin sections were assigned an HPI value. Pitre assigned HPI values to each section twice to test for intra-observer error. In addition, thin sections were scored by Mayne Correia to test for inter-observer error. Differences in HPI value designation between Pitre and Mayne Correia were discussed and a final consensus was reached.

3.3 Results

3.3.1 Characteristics of the Biodeterioration

Whereas thin sections of the skeletal material from 1996 (Fig. 3.3) are translucent and yellow and show little biodeterioration, the 266 thin sections prepared in 2009

presented the following biodeterioration characteristics which are summarised in Fig. 3.4. The lacunae and canaliculi in the midcortical regions of bone show the first signs of microbial decay. By LM, infiltrated areas appear brown and are opaque[3] (Fig. 3.5). When examined under polarized light, the infiltrated areas lack birefringence. In addition, infiltrated areas stain when treated with toluidine blue indicating that the brown opaque material is organic. Although the microorganisms initially avoid cement lines (Fig. 3.5) and the inner and outer cortices of bone, they form larger focal areas of destruction that eventually enter previously unoccupied areas. As the microbial invasion progresses, sections are brown and opaque by LM.

This brown opaque material eventually appears mottled (Fig. 3.6) and begins to break apart revealing the presence of tunnels (Fig. 3.7). These asymmetrically globular tunnels possess thick rims and range in diameter from 5 to 90 μm. The tunnels are filled with the brown opaque material that stains with toluidine indicating that it is organic. The Leilan tunnels eventually lose their brown opaque contents and appear translucent by LM, occasionally showing green and pink tints. Once emptied of their contents, the tunnels no longer stain blue with toluidine. The tunnels were attributed provisionally to bacterial attack and specifically as Hackett's (1981) lamellate variety. The end result is that the

[3] The appearance of this brown opaque material can be minimised temporarily using agents such as toluene and xylene.

Haversian canal is the only identifiable histological structure in sections made of the Leilan material; the rest is amorphous and unidentifiable. When examined under SEM-BSE, empty tunnels appear demineralised as they no longer contain bone and are surrounded by remnants of the mottled brown opaque material that is hypermineralised (Fig. 3.8).

3.3.2 Histological Integrity

HPI values are presented in Table 3.1. Values ranged from 1 to 4 and varied in serial sections in 26.2% (17/65) of cases by at least one category. Intra-observer error was great for Pitre (25.3% error) and there was considerable inter-observer error in assigned HPI values between Pitre and Mayne Correia (24.4%).

3.4 Discussion

3.4.1 Characterisation

These findings suggest that the brown opaque material is evidence of microbial growth in bone—even before tunnels are formed. In studying this material, it was possible to determine that microbial decay follows a pattern that begins in the lacunae and canaliculi of the midcortical region and spreads to the inner and outer cortices of bone. Under LM, the brown opaque material eventually degenerates to reveal the presence of tunnels. Based on the characteristics and distribution of the tunnels, the results obtained here suggest that bacteria, rather than fungi, were the major contributor to the destruction of the skeletal material. Furthermore, the use of the HPI has revealed that the histological integrity of the Leilan samples is low

and that preservation varies throughout bone. Testing of the HPI, however, has shown that the index is prone to both intra- and inter-observer.

Although the nature of the brown opaque material as observed by LM is unknown, toluidine blue indicates that it is organic in nature and not the product of air being trapped during the embedding process. Similar infiltrations observed in human skeletal material have been attributed to sediment contaminants such as humic substances; metals such as copper, and bronze (Jans, 2005; Morris, 1981); clothing dyes (Jans, 2005), and the accumulation of microbial pigments (Grupe and Dreses-Werringloer, 1990; Grupe et al., 1993; Marchiafava et al., 1974; Piepenbrink, 1986). Because this brown opaque material developed during curation, it cannot be the result of infiltrating contaminants from surrounding sediments or clothing, and therefore it is reasonable to assume that it is an accumulation of microbial pigment. Several microbial genera can produce visible accumulations of pigments (Deacon, 1997; Grupe et al., 1993; Piepenbrink, 1986), which may have penetrated the Leilan bone through diffusion.

Accumulations of microbial pigments in archaeological bone can be seen at a gross-level (Grupe et al., 1993; Jans, 2005), although some pigments may not be observed because they can wash out during burial (Grupe and Dreses-Werringloer, 1990). The Leilan material presented no extreme gross colour changes during curation at the U of A, and colour was not removed by leaching with water. Although the brown opaque material may to some extent be

comparable to the accumulation of microbial pigments noted by other researchers (Grupe and Piepenbrink, 1988; Jans, 2005), it probably includes not only pigments but also intact cells (either dormant, moribund or dead, or a combination), and associated debris, such as fragments of cell walls. The microbial nature of the material explains why it appears to degenerate. Further analyses of unembedded samples using SEM are required to examine this phenomenon in more detail.

Based on the distribution of the brown opaque material in the bone, the results of this study suggest that bone biodeterioration, particularly when caused by bacteria, is not random and that the process is guided by bone microstructure. The observation of this pattern is not unexpected, as the tunnels formed in bone by bacteria are known to be associated with bone microstructure (Hackett, 1981; Jans, 2008). In decomposing bone, bone structures such as lacunae and canaliculi are usually empty and provide avenues for microorganisms such as bacteria and fungi to ingress. In addition, soil contaminants, such as lime and iron oxide, have been shown to penetrate bone following these routes (Bell, 1990; Salomon and Haas, 1967; Turner-Walker et al., 2002). Accordingly, these results confirm other researchers' (see Bell, 1990; Garland, 1987; Turner-Walker and Jans, 2008) observations that lacunae and canaliculi are involved in bone biodeterioration.

Bacteria showed a predilection for colonising particular areas based on the degree of bone mineralisation and survival of collagen. Similar to other investigations

(e.g., Hanson and Buikstra, 1987; Jackes et al., 2001; but see Hagelberg et al., 1991, for an exception), the bacteria avoided more mineralised areas, such as cement lines, canal walls, and the internal and external cortices of bone, and concentrated in regions where they had better access to collagen. This pattern suggests that microorganisms follow the 'path of least resistance' when colonising bone (Turner-Walker and Jans, 2008), and explains why the microbial sequence begins in the less mineralised midcortical areas of bone and then gradually spread to more mineralised regions such as the circumferential and interstitial lamellae.

Whereas the pattern left behind by the invading microorganisms is clear, the nature of the tunnels is not. Although they appear to be of bacterial origin because of their diameter and distribution, their contents do not allow them to be easily classified following Hackett (1981). Early in the biodeterioration sequence, the Leilan tunnels are infilled with the brown opaque material. Although this infill should be present in bacterial tunnels, it is unlike the stippled infill of bacterial tunnels described by other investigators (Hackett, 1981; Jans, 2005, 2008; Jans et al., 2004). Later in the biodeterioration sequence, the Leilan tunnels are empty and appear translucent, characteristics that have been associated primarily with the tunnels produced by fungi (Hackett, 1981; Jans, 2008). Because the brown opaque material in the tunnels is microbial in nature, it would make sense that the tunnels would eventually empty, as the microorganisms themselves would decompose (Grupe and Dreses-Werringloer, 1990). That the Leilan tunnels are

both infilled and empty suggests that the presence or absence of infill should not be used to determine the cause of tunnels.

Although the Leilan tunnels were designated as the lamellate variety, their size range (from 5 to 90 μm) encompasses all three varieties of Hackett's (1981) bacterial tunnels. While Jans et al. (2004) have recently suggested that lamellate forms are an earlier manifestation of budded tunnels, few researchers have questioned Hackett's (1981) classification system. Expanding from Jans et al. (2004), it is possible that each of Hackett's tunnel-types are manifestations of earlier and later forms of bacterial attack and are not distinct varieties. In addition, it may seem as though there are distinct varieties of bacterial tunnels because they will look different, depending on the angle in which they are sectioned (cf. Cormack, 2001). Future research into this area should focus on whether there are distinct bacterial tunnel varieties.

3.4.2 Histological Integrity

The majority of the bones scored for histological preservation in this study are poorly preserved. With such low histological integrity, the skeletal material, particularly in the later stages of biodeterioration, is unfit for histological analyses (see also Iwaniec et al., 1998). The Leilan material is most likely also unfit for elemental and DNA isolation (see Colson and Bailey, 1997; Haynes et al., 2002; Schoeninger et al., 1989). It may be possible to isolate intact collagen for isotopic analysis as Pfeiffer and Varney (2000, 2001) found no association between

histological preservation and collagen integrity. Although Feasby (1998) was successful in isolating intact collagen from bones of the Leilan collection for stable isotope analysis, there were some problems possibly associated with sample deterioration. Future research should investigate if the intervening years (1998 – 2009) have affected whether intact collagen can be isolated. The histological integrity results presented here are similar to those of Jans et al. (2004), who noted that 91% of the skeletal material examined from 41 different archaeological sites was not well preserved, but this is a sharp contrast to results reported by other researchers. For example, Hanson and Buikstra (1987) reported that 23% of their skeletal samples were unsuitable for histomorphometric study. Similarly, Stout (1976) reported that 26% of his skeletal samples were destroyed and were not useful for histomorphometric study.

With respect to the distribution of the histological index categories, the pattern of biodeterioration observed in the Leilan bone samples typically proceeded to near completion. This differs from the bimodal distribution observed in other investigations, where thin sections were either highly altered or were unaffected (e.g., Haynes et al., 2002; Hedges et al., 1995; Jans et al., 2004). Other investigators have reported a more even distribution across the preservation categories, with most bones falling into the intermediate preservation phase (Haynes et al., 2002). Complementing the work by Haynes et al. (2002), study of the Leilan skeletal material provides an opportunity to understand the later phases of the decay process.

Variation in bone preservation, as seen in single thin sections and across serial sections, was not unexpected and has been reported in the literature (Guarino et al., 2006; Nicholson, 1998; Pfeiffer and Varney, 2000). Bone properties will vary depending on intrinsic factors such as age, sex, and pathological conditions (Guy et al., 1997; Janaway, 1996; O'Connor, 1987; Pruvost et al., 2007; Turner-Walker, 2008), and extrinsic factors such as variations in local conditions (i.e., pH, oxygen, and temperature). In addition, the variety and behaviour of the invading microorganisms will determine whether bone is homogenously attacked. The variation in preservation of the Leilan sections likely exacerbated the observer error in the assignment of HPI values.

The HPI is an established diagenetic parameter used to select samples for various archaeological investigations such as DNA analyses. But, although Haynes et al. (2002) report that the index is 100% repeatable and that error between scorers was limited to one category, this investigation was unable to reproduce results using the HPI, even when it was applied by experienced users. Indeed, an assignment difference of one category can mean up to a 50% disagreement for bone remaining intact between observers. Although an attempt was made in this study to create more defined HPI categories by introducing percentages and by removing one category, large discrepancies between scorers were still observed. Thus, in the future, a larger scale study involving more investigators, testing both intra- and inter-observer error, should be conducted to test the applicability of the HPI in assessing histological integrity.

3.4.3 Curation Concerns

The results of this investigation indicate that the bulk of the destruction in the Leilan skeletal material occurred during curation. There are two possibilities for the timing of the entry of the microorganisms. Microorganisms may have entered the Leilan skeletal material sometime during curation at the U of A. As the ability of microorganisms to withstand osmotic stress and periods of desiccation is well-documented (Chen and Alexander, 1973; Pitt, 1975), another possibility is that the skeletal material had already been colonised by soil microbes whose assimilative cells and spores were able to resume growth as conditions allowed. Neither scenario can be ruled out because several microorganisms that are capable of inhabiting bone are common in the soil and air (Valentin, 2003).

Contrary to the belief that deterioration processes are most dramatic during excavation (Cassman and Odegaard, 2007a), the results of this investigation show that the Leilan material experienced more deterioration during curation. Unfortunately, information concerning the treatment and curation of human collections to prevent microbial growth in the curation environment is both sparse and contradictory (cf. Appelbaum, 1991; Canadian Conservation Institute, 1983; Gehlert, 1980; Sease, 1994). Researchers tend to focus on microbial growth that occurs in the burial environment and on the deterioration that happens immediately following excavation (Cronyn, 1990). Although it has been suggested that microorganisms pose no threat to human skeletal collections in normal to dry institution conditions (Cronyn, 1990; Department for Culture,

Media and Sport, 2005), the agents of decay have been shown here to be just as active in curation as they are during burial.

Standard curatorial protocol recommends that objects be inspected regularly for insects and mould growth (Canadian Conservation Institute, 1983; Cassman and Odegaard, 2007a, b). Most protocols disregard human skeletal collections, and, more importantly, ignore the damage caused by microorganisms such as bacteria and fungi, which is, for the most part, invisible. The findings of this investigation indicate that plans for monitoring and treating human and animal skeletal remains as well as bone objects are a necessary part of curation and management.

3.5 Conclusion

Thin section analysis and SEM revealed that microorganisms chose the 'path of least resistance' (Turner-Walker and Jans, 2008) in following bone properties during their invasion. SEM-BSE revealed that infiltrated areas had undergone extensive changes in mineralisation. The results presented here indicate that currently used classification systems to distinguish between the tunnels produced by bacteria and fungi need revisiting. Furthermore, the results of this investigation show that the HPI is not a reliable indicator of sample integrity owing to large observer error brought on by preservation differences in bone. Ultimately, the recognition that microbial growth developed during the curation of the Tell Leilan skeletal collection means that we must move towards monitoring skeletal

collections and improving their storage conditions, so that this decay is limited in other institutional collections in the future.

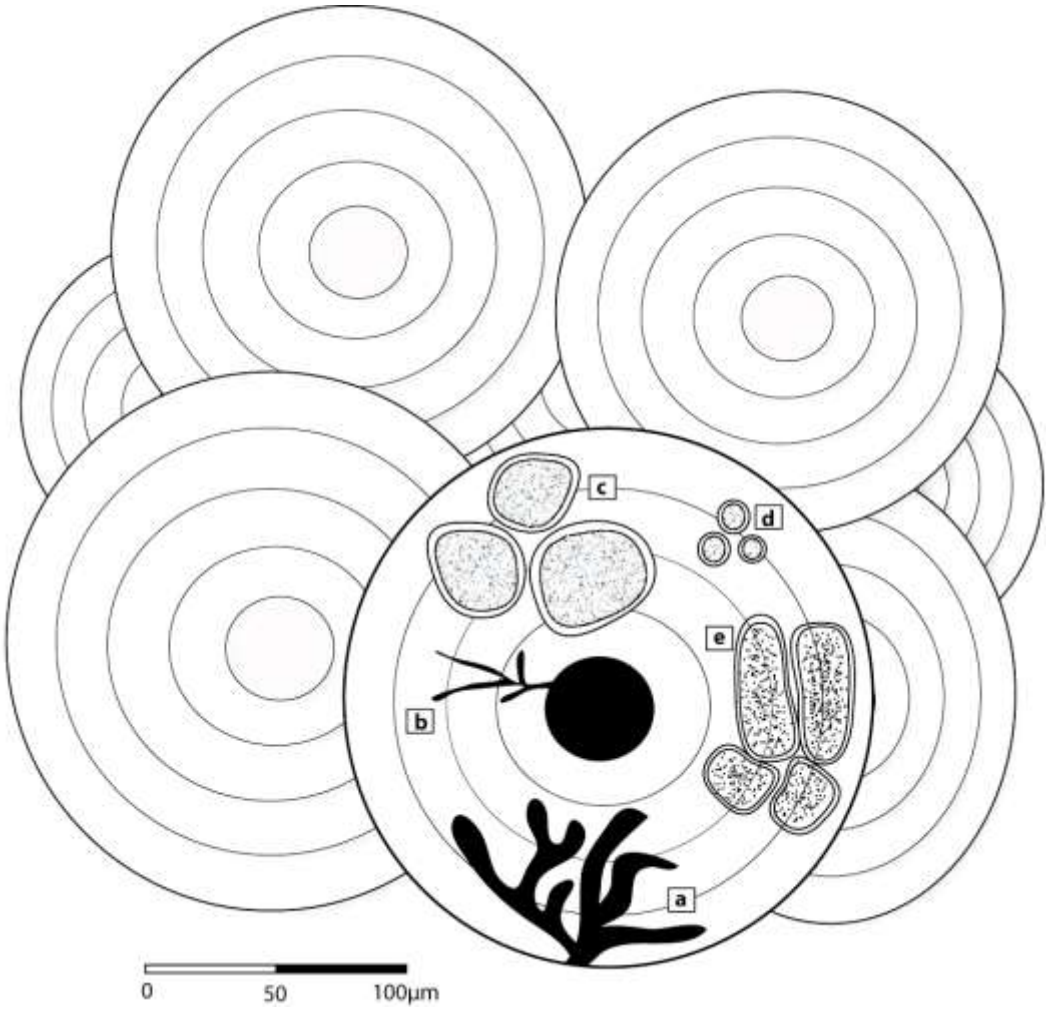

Fig. 3.1 Microbial tunnels in compact bone. An osteon containing: (a) Type I and (b) Type II Wedl tunnels caused by fungi as well as (c) budded, (d) linear-longitudinal, and (e) lamellate tunnels created by bacteria.

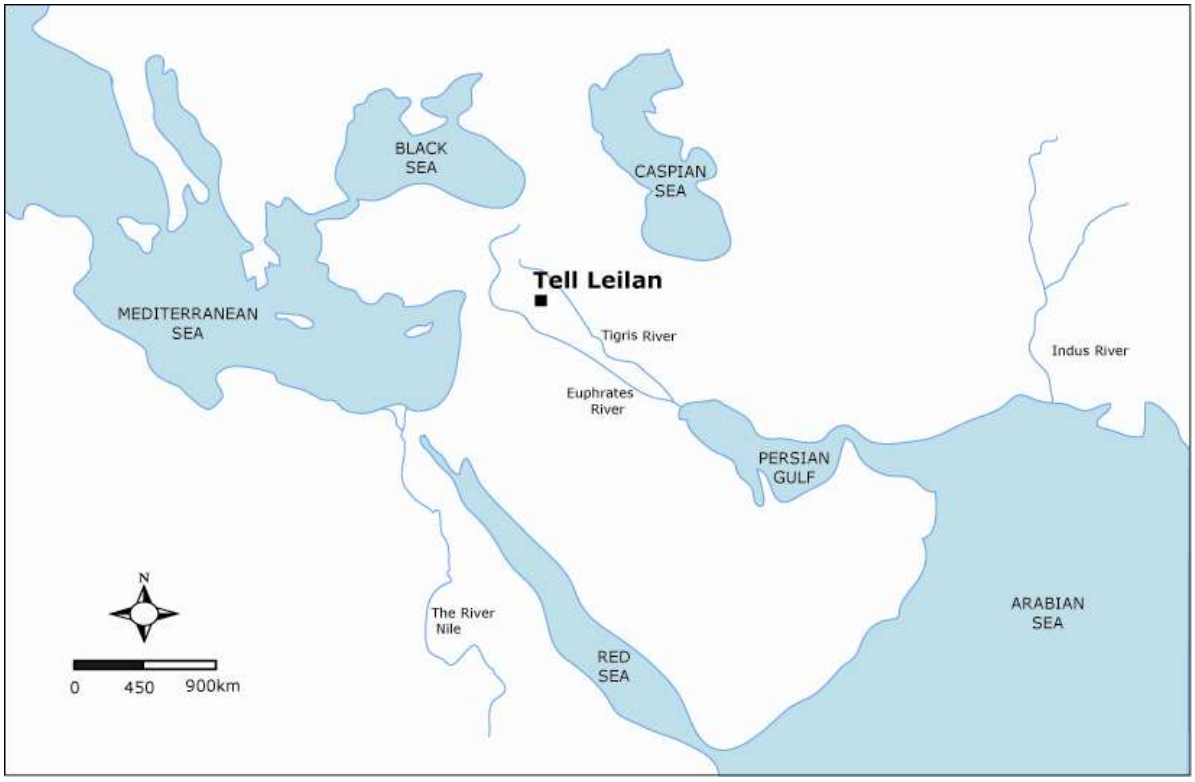

Fig. 3.2 Map showing the geographic location of Tell Leilan.

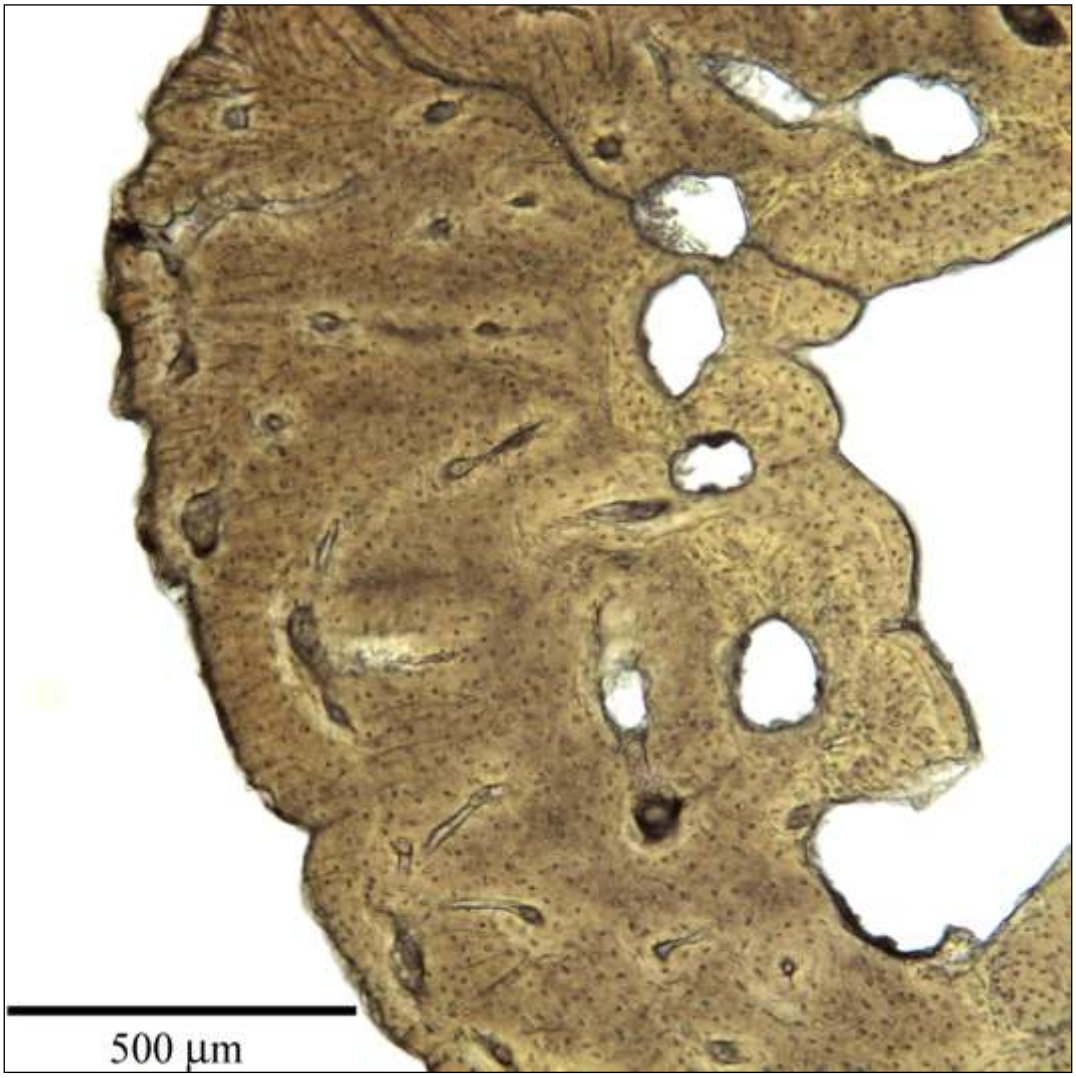

Fig. 3.3 Thin section showing that the Leilan skeletal material presented excellent structural preservation in 1996. Note how the bone is translucent and yellow. Section taken from the right radius of a neonate (L89 76E20 B1 167 Area 4).

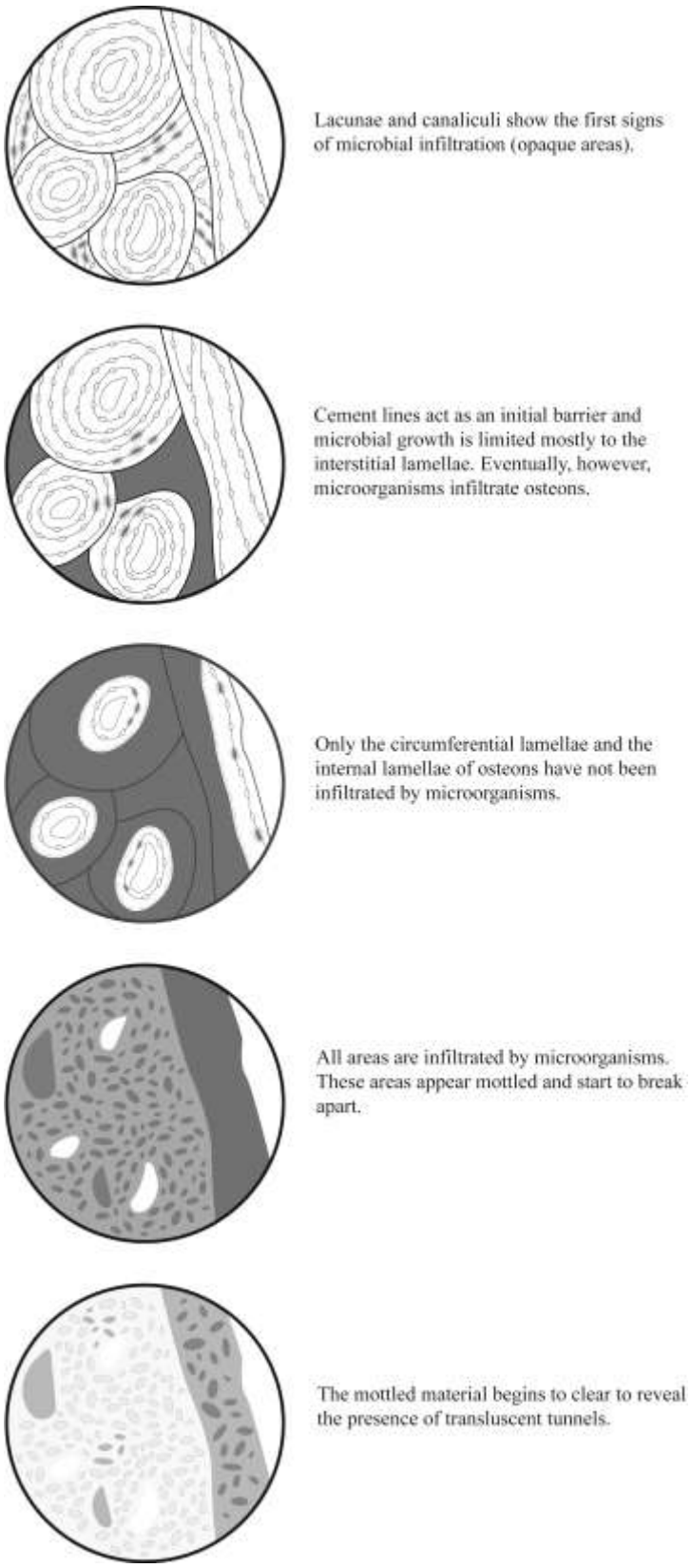

Fig. 3.4 Diagram showing the progression of biodeterioration in the Tell Leilan skeletal material from Syria (Image by Brian Levac).

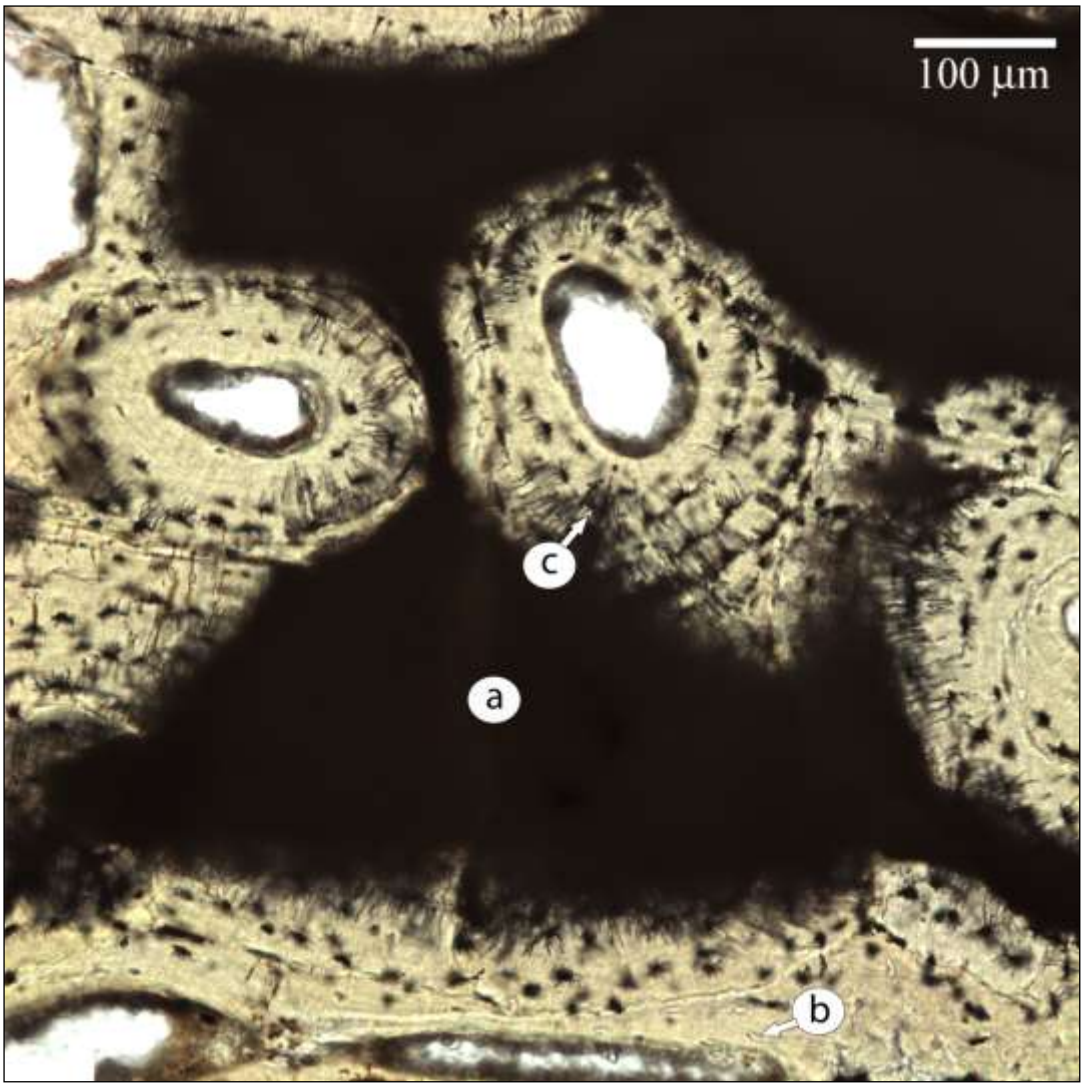

Fig. 3.5 Thin section showing the (a) dense brown opaque organic material, (b) empty lacunae and canaliculi in uninfiltrated areas, and (c) lacunae and canaliculi infiltrated by the opaque brown organic material. Thin section of the left radius of a 4–6 year old child from the Tell Leilan skeletal material (L87 76G20 58 Phase 4 B2).

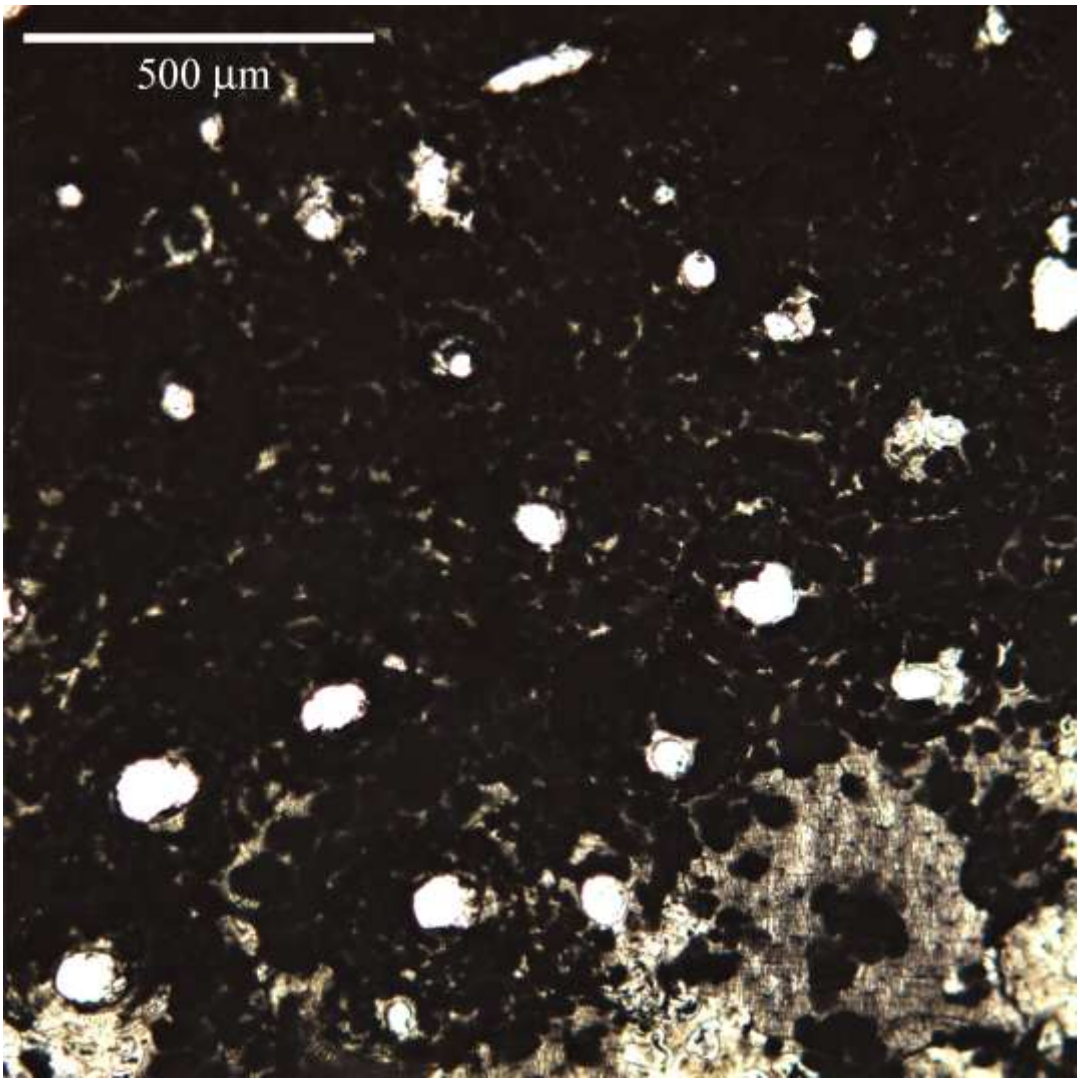

Fig. 3.6 Thin section showing how the brown opaque organic material appears mottled and breaks apart. Thin section of the right tibia of an adult from the Tell Leilan skeletal material (L80 OP2 36).

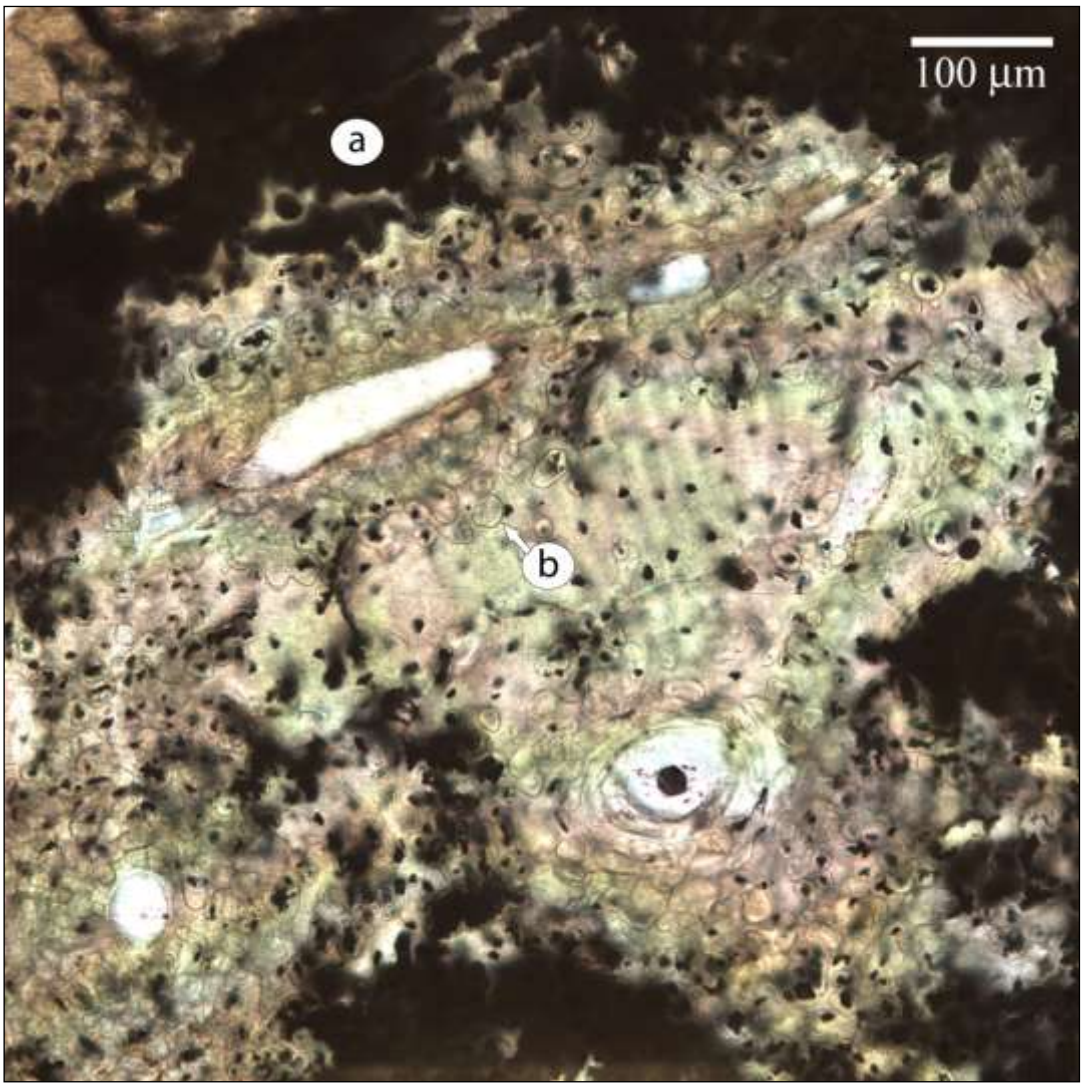

Fig. 3.7 Thin section showing how the brown opaque organic material begins to (a) mottle and break apart to reveal the appearance of (b) tunnels. Areas not covered by the brown opaque material take on pink and green tints. Thin section of the left fibula of a 2 year-old (+/- 8 months) child from the Tell Leilan skeletal material (L87 57FG05 89 Wall 5).

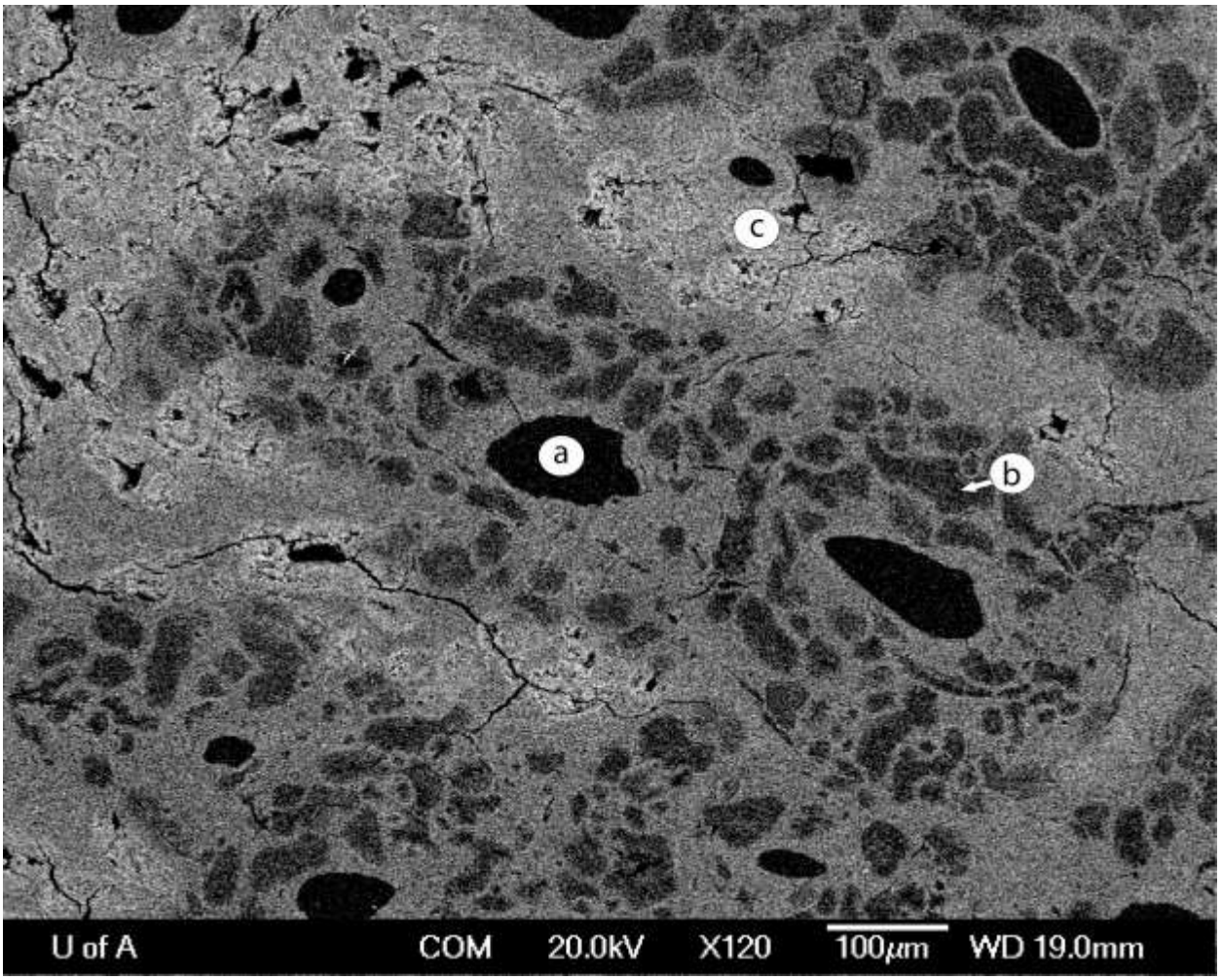

Fig. 3.8 SEM-BSE micrograph of biodeteriorated bone from the Leilan skeletal material where the (a) Haversian canals are black, (b) demineralised areas are lighter black, and (c) hypermineralised areas are white. From the left radius of an adult from the Tell Leilan skeletal material (L80 OP2 36).

Table 3.1 Histological Preservation Index (HPI) values assigned to bone samples from Tell Leilan.

HPI	% of bone structures unaltered	% of samples
1	<25%	67.3%
2	≥25% – <50%	22%
3	≥50% – <80%	8.3%
4	≥80% – ≤100%	2.3%

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Chapter 4

Biofilm Growth in Human Skeletal Material from Ancient Mesopotamia[1]

4.1 Introduction

Comparison of thin sections prepared in 1996 and 2009 of human skeletal material from the ancient Mesopotamian site of Tell Leilan (2900 – 1900 BCE) (Fig. 4.1) revealed that the skeletal material had been altered by microbial activity during curation. Thin sections made in 2009 showed that a brown opaque organic material had penetrated the Leilan bone. The appearance of this material bore a resemblance to a biofilm. Biofilms are complex aggregates of microorganisms surrounded by a blanket of extracellular polymeric substances (EPS) (Allison, 1998). Several groups of microorganisms participate in the formation of biofilms including bacteria, protozoa, fungi, and algae. Microorganisms embedded within a biofilm may work as part of a collective toward the decay of a substrate (Allison et al., 2000). Biofilms (single and multiple species) have been isolated from a variety of substrates including marble, piping, art, and perhaps dinosaur bone (see Costerton et al., 1987; Doggett, 2000; Flemming et al., 2000; Kaye et al., 2008; Saarela et al., 2004; Sanchez-Moral et al., 2003). Biofilms have also been

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recognised on teeth and on the bones of living patients suffering from infection (Gristina and Costerton, 1984; Kolenbrander, 2000; Kolenbrander et al., 2000; Sedghizadeh et al., 2008, 2009; Toshiyuki, 2005). Until now, these complex microbial aggregates have not been described as biofilms in archaeological human bone.

Researchers have long recognised the effects of microbial activity, also called biodeterioration, on archaeological bone (e.g., Marchiafava et al., 1974; Roux, 1887; Wedl, 1864) but their investigations have focused on single or groups of microbes rather than on complex microbial aggregates. The tendency for researchers to focus on a single biotic agent of decay has dramatic effects on future curatorial policies to control microbial growth in skeletal collections; aggregates of microbes are resistant to changes in temperature and relative humidity (RH), changes that normally affect single microbes (Marsh and Bowden, 2000). As a result, current curatorial policies for archaeological bone are ineffective in preventing the growth of biofilms.

In this paper, biofilm growth in the Leilan skeletal material was explored. The ultrastructural characteristics of the biofilm were described by scanning electron microscopy (SEM). Samples were prepared using ethanol and amyl acetate baths in combination with critical point drying (CPD) to preserve both the microorganisms and their secretions for examination (see Fratesi et al., 2004; Little et al., 1991; Maat, 1993; Piepenbrink, 1986; Piepenbrink and Schutkowski,

1987; Tsuneda et al., 1991). Microbes were isolated and cultured (from samples of the same bone that were examined with SEM) and identified based on morphology and the similarity of their 16S rRNA gene sequence to known organisms. Information gathered from this study can be used in the future to develop preventive strategies to avoid damage caused by biofilm growth in human skeletal collections.

4.2 Biofilms

4.2.1 Overview

The term biofilm was coined by Costerton et al. (1978) and refers to surface- and subsurface-associated microbial aggregates. Biofilms can be thick enough to be seen by the unaided eye, and may contain millions of microbial cells. A biofilm, however, is distinguished from other microbial aggregates because embedded microorganisms are surrounded by a self-produced matrix of EPS, which includes a mix of carbohydrates, proteins, and extracellular DNA (Allison, 1998; Blankenship and Mitchell, 2006; Davies, 2000; Flemming et al., 2000; Stoodley et al., 2002). This matrix is instrumental in biofilm maintenance as EPS materials provide the structural support and protection for the embedded microbial cells (Stoodley et al., 2002).

Investigators have outlined the physiological events involved in biofilm formation (Blankenship and Mitchell, 2006; Busscher and van der Mei, 2000; Costerton et al., 1987; Davies, 2000; Kolenbrander et al., 2000; Stoodley et al., 2002). The

initial phase of biofilm growth involves the attachment of an organism to a substrate, facilitated by the secretion of EPS (Allison, 1998). A mature biofilm is characterised by a complex network of pores and channels, and microorganisms within it are redistributed (Davies, 2000). Portions of the biofilm eventually slough off but repopulation and reestablishment of the biofilm can occur (Costerton et al., 1987; Stoodley et al., 2002). Consequently, localised areas within a biofilm can be at differing stages of development (Stoodley et al., 2002).

Generally, biofilms are highly structured communities and many members have specialised functions. Some may be parasites, and others scavengers or saprobes, in which case they actively digest the underlying materials (Costerton et al., 1987; Marsh and Bowden, 2000; Wimpenny, 2000). Organisms within a biofilm have been shown to work synergistically in the ultimate breakdown of a substrate (Costerton et al., 1987; Wimpenny, 2000). For example, Bradshaw et al. (1994) have outlined how oral bacteria degrade hog gastric mucin (protein secreted in the stomach) better when part of a biofilm.

Being part of a biofilm confers important advantages to a microorganism by interfering with the establishment of antagonistic organisms (Busscher and van der Mei, 2000; Costerton et al., 1987; Marsh and Bowden, 2000) and by improving their ability to cope with a wider variety of environmental conditions (Allison, 1998; Allison et al., 2000; Gilbert et al., 1997; Wimpenny, 2000) such as extremes in temperature, in pH, and in RH, as well as from the harmful effects of

chemicals (Davies, 2000). For example, several oral bacteria are sensitive to pH values below 5.0 and yet persist in aggregate form as dental plaque (Marsh and Bradshaw, 1999). Ultimately, the advantages conferred to community members also render biofilms more difficult to control (Doggett, 2000; Saarela et al., 2004; Sanchez-Moral et al., 2003).

Although biofilms have been recognised in patients suffering from bone and joint infections (Gristina and Costerton, 1984; Sedghizadeh et al., 2008, 2009; Toshiyuki, 2005), they have not been reported in archaeological human bone. Archaeological bone would act as an ideal substrate for biofilm growth because of its porous nature and overall composition. Structures such as lacunae, canaliculi, and Haversian and Volkmann's canals would act as conduits for microorganisms to ingress (Grupe et al., 1993; Jans, 2008; Lee-Thorpe and Sealy, 2008; Schultz, 1997). Furthermore, the relatively high level of protein in bone as well as water would predispose bone to biofilm formation

4.2.2 Examining Biofilms

Despite archaeological bone's suitability for biofilm growth, there is no mention of biofilms in the archaeological literature. The majority of published reports examining bone at a microorganism level use transmitted light microscopy and SEM (Jackes et al., 2001; Jans, 2005; Maat, 1993; Piepenbrink, 1986)—techniques in which a biofilm can easily be overlooked (cf. Kaye et al., 2008). Other investigations report on the use of backscattered electron microscopy

(SEM-BSE) to examine the effects of microbial growth in archaeological bone (Bell, 1990; Jackes et al., 2001; Turner-Walker and Jans, 2008). However, the technique does not lend itself to visualising microorganisms, but is useful for examining the changes in mineralisation in bone caused by microbial growth.

Microbiologists use several techniques to prepare samples for SEM so that biofilms can be observed (see Fratesi et al., 2004; Little et al., 1991; Tsuneda et al., 1991). These techniques involve the fixation and dehydration of a specimen using several sources such as air, ethanol, acetone, and critical point drying (CPD). Few researchers examining archaeological bone have made use of such techniques (for exception see Maat, 1993; Piepenbrink, 1986; Piepenbrink and Schutkowski, 1987). Specifically, these techniques reduce the destructive surface tension forces that occur at the air-water interface during air-drying and preserve both the microorganisms and their secretions for examination. One of these fixation and dehydration methods in particular, involving ethanol and amyl acetate baths in combination with CPD, was selected for the examination of the biofilm present in the Tell Leilan skeletal material from Syria. In addition, culture techniques and DNA sequences were used to get a better idea of the physical community structure of the Tell Leilan biofilm. The following is a summary of the results of this endeavour.

4.3 Materials and Methods

4.3.1 Site Description

Tell Leilan is a 90-hectare walled site in northeastern Syria (Fig. 4.1). Since 1979, excavations have shown that the site was one of the three largest cities on the Habur Plains during the mid-third millennium BCE, occupied from the mid-sixth to early second millennium (Weiss, 1985; Weiss and Courty, 1993; Weiss et al., 1993). The site is situated on the left bank of the Wadi Jawah in a broad, undulating plain of flood deposits and sands underlain by Pleistocene gravel and plateau basalts (Besonen and Cremaschi, 2002). The area receives between 300 and 500 mm of rain per annum and experiences cool and wet winters and hot and dry summers (Besonen and Cremaschi, 2002; Cullen et al., 2000; Weiss, 1985; Weiss and Courty, 1993).

4.3.2 Skeletal Material

The Tell Leilan skeletal material consists of 59 adult and juvenile skeletons discovered between 1979 – 1989 dating between 2900 and 1900 BCE. The skeletons were found in alkaline deposits while excavating building floors throughout the site. Following excavation, the skeletons were shipped to Yale University in New Haven, Connecticut and were later shipped for study in 1991 to the University of California, Berkeley. In 1992, the skeletal material was shipped to the University of Alberta (U of A), Edmonton. Upon arrival at the U of A, the skeletal material was cleaned manually with bamboo sticks and brushes of varying firmness. Once skeletal elements were cleaned and inventoried, they were

transferred from paper bags to 3 mil plastic bags. The skeletal material is presently curated in a centrally heated building that ranges in temperature and RH from 15 to 25° C and 15 to 50% respectively depending on season[2].

4.3.3 Scanning Electron Microscopy

A new fragment was sampled from the left proximal radius of a juvenile between the ages of 4–6 years (L87 76G20 58 Phase 4 B2) and was prepared for SEM following Tsuneda et al. (1991). The bone fragment was fixed in 3% glutaraldehyde for 8 hours at 4°C, washed in distilled water, postfixed in 2% tannic acid/guanidine hydrochloride solution for 3 hours, washed again in distilled water, and then postfixed in 2% osmium tetroxide (OsO_4). The fixed sample was dehydrated in an ethanol series (30%, 50%, 70%, 90%, 95%, 100%) followed by an amyl acetate series (50%, 100%). The sample was critical-point dried in a Balzers CPD 030 Critical Point Dryer for 1.5 to 2 hours, was coated with gold using a Nanotech SEMPrep 2 DC sputter coater, and was examined using a JEOL 6301F (field emission scanning electron microscope) fitted with a liquid nitrogen cooled lithium drifted silicon energy dispersive x-ray (EDX) detector with a Norvar window. Secondary electron (SE) micrographs were obtained using an Everhart-Thornley detector. In addition to the collection of SE micrographs, EDX was used to determine the nature of several crystalline inclusions within the

[2] Records provided by Museum and Collection Services, U of A (P. Mayne Correia, pers. comm.).

biofilm. SEM was carried out at the Earth and Atmospheric Science Department at the U of A.

4.3.4 Microbe Isolation and Identification

Samples, from six skeletal elements from burials located across the site and between excavation years, were assessed for their content of living microbes at the Department of Biological Sciences at the U of A. Each sample was rinsed in 95% ethanol, flamed briefly, wrapped in sterile foil, and crushed. Fragments and fine particulates were evenly distributed over three types of agar media: potato dextrose agar (PDA, Difco Bacto), oxytetracycline water media (16g selected agar, 1g Oxytetracycline in 40 mL 70% ethanol, add 4 mL/L media for 0.01%), and oatmeal agar (OA; 20g rolled oats, 20g selected agar, 1 L dH2O). Plates were incubated on the lab bench at room temperature, approximately 21–22° C. Fungal isolates were identified using microscopic morphology whereas bacterial isolates were identified using the DNA sequencing techniques described below.

4.3.5 DNA Sequencing

DNA extraction and PCR amplification of partial 16S rRNA genes were conducted according to Foght et al. (2004). Excess primers and nucleotides were degraded using ExoSap (USB) according to the manufacturer's protocol except that the incubation step at 37ºC was extended from the normal 15 to 30 minutes. Big Dye (ABI) dideoxy terminator sequencing was conducted at the U of A Department of Biological Sciences Molecular Biology Services Unit according to

the standard protocol, using ~80 ng PCR product per reaction as quantified using a Nanodrop spectrophotometer (Thermo). The sequencing primers used were PB36F, PB38R, 16S2R, and 16S5F (Cheng and Foght, 2007; Foght et al., 2004).

4.4 Results and Discussion

4.4.1 Scanning Electron Microscopy

SEM of the Leilan sample revealed a mixture of microbial cells surrounded by web-like EPS (Fig. 4.2). Both bacteria and fungi were embedded within the biofilm, either in isolation or in small microcolonies. Calcite ($CaCO_3$) crystals were scattered throughout the biofilm and were identified using EDX. The biofilm is established in all areas of the bone including within the medullary cavity, inside empty cellular structures and canals (e.g., lacunae and Haversian canals), as well as within the cortex. Whereas the fixation-dehydration technique used to prepare the Leilan sample is recognised as not creating artefacts, some of the following features of the biofilm may be overemphasized—see discussion within text (cf. Cohen, 1979; Fratesi et al., 2004; Little et al., 1991).

The EPS are the most notable component of the Tell Leilan biofilm. This is not surprising, as EPS have been found to make up as much as 90% of the organic matter in a biofilm (Allison, 1998; Flemming et al., 2000). The EPS appeared as a network of branching foamy strands that cement individual and groups of microbial cells together (Fig. 4.3) and fill in some bone pore spaces. In some locations of the bone the EPS are thick enough so that the individual foamy

strands were not visible and instead appeared as a mass that obscured the presence of bacteria and fungi (Fig. 4.3).

The appearance of EPS, however, can be distorted depending on the preparation technique used (e.g., air, ethanol, acetone, and CPD) (Fratesi et al., 2004; Little et al., 1991). In particular, the technique used to prepare the Leilan biofilm for SEM (i.e., ethanol and amyl acetate baths and CPD) has been shown to accentuate the internal fibrillar features of the EPS through the removal of the smooth matrix material (Fratesi et al., 2004). Similar looking EPS were observed by Costerton et al. (1978) in a sample prepared using the preparation technique used here. Thus, the Leilan EPS may appear as foamy strands because of the SEM preparation technique chosen. Ethanol and amyl acetate baths in combination with CPD, however, did leave some aspects of the EPS for viewing and the technique has been shown to be particularly good at preserving microorganisms for examination under SEM (see below).

The Tell Leilan microorganisms were embedded within the EPS. Because of their positioning, it was often difficult to determine their size and shape—two important characteristics used in distinguishing among different types of microorganisms. The sizes of the Leilan microorganisms were also distorted because of the nature of bone itself. As the Leilan bone is resource-limited in comparison to growth media, the growth of microbial structures within it was constrained and cells are diminished in size (see Constantinescu,1990)

It was possible, however, to identify some of the microorganisms that were embedded in the EPS. Both fungal spores (some germinating and traveling in Haversian canals, see Fig. 4.4) and hyphae were identifiable. While isolated fungal hyphae were observed, dense mycelia were not. Fungal spores were spherical with tuberculate walls (Fig. 4.5). Fungal spores ranged in size from 1.3to 2.7 µm in diameter. Although less numerous (perhaps obscured by the EPS) bacterial cells (cocci) were observed, some of which were seen in microcolonies while others flanked fungal hyphae (Fig. 4.6). These cocci were small in comparison to the fungal cells; all were less than 1 µm in diameter.

As with the EPS, the appearance of microbial cells can be distorted depending on the preparation technique used for SEM (see Fratesi et al., 2004; Little et al., 1991; Staugaard et al., 1990). For example, the cell wall structure of several conidia (asexual, non-motile spores of a fungus) varies depending upon the preparatory techniques for SEM (Staugaard et al., 1990). Although microbial cell-size in the Leilan material is likely diminished (see above), no microbial structures appear distorted because of preparatory techniques. In a few circumstances, however, some microbial structures (e.g., hyphae) were broken and collapsed. But, this may have more to do with the sampling of the bone before SEM. Thus, CPD following dehydration through an ethanol series and amyl acetate baths is a good technique to visualise microorganisms within biodeteriorated bone.

Several isolated crystals were observed scattered throughout the film which were identified as calcite ($CaCO_3$) by EDX. According to Herrmann and Newesely (1982) and Child (1995a), calcite crystals in bone are a by-product of surrounding soil conditions i.e., soil chemistry and pH. Because the Leilan biofilm is known to have proliferated during curation, the positioning of the calcite crystals deep within the film (Fig. 4.3) suggests that such crystals developed because of microbial activity. Bacteria within biofilms have been shown to produce insoluble salts, leading to the formation of such crystals (Costerton et al., 1987). Similar to the results presented here, other investigators (e.g., Jackes et al., 2001; Jans, 2005; Maat, 1993; Piepenbrink and Schutkowski, 1987) have noted calcite crystals in biodeteriorated bone from several archaeological collections.

4.4.2 Microbe Isolation and Identification

Several microorganisms were isolated, including the actinomycete, *Amycolatopsis* sp., and the fungi *Penicillium chrysogenum* and unidentified species of *Aspergillus*, *Chaetomium*, and *Cladosporium*. It is possible that the fungal spores observed by SEM are *P. chrysogenum*. The cocci, however, are unlikely *Amycolatopsis sp*. as they are typically filamentous. It is unlikely that the isolation of these species was a result of contamination because the samples were flamed prior to culturing. Furthermore, outgrowths of cells of both the *Amycolatopsis* species and *P. chrysogenum* were abundant and arose from many of the minute fragments. Several of these organisms have been isolated from biofilms on other substrates. For example, Doggett (2000) isolated *P. chrysogenum* from a

municipal water distribution system biofilm. Similarly, Saarela et al. (2004) isolated several species of *Amycolatopsis* from biofilms in Roman catacombs.

Because the bulk of the destruction to the Leilan material occurred during curation, it is assumed that the microorganisms isolated from the Leilan samples were involved in the ongoing decay of the material. It is possible, however, that other unculturable or at least non-isolated species might also be playing a significant role. Further laboratory studies involving inoculated deer bone are currently underway to confirm the role of the isolated species in the decay of the Leilan skeletal material.

It is significant in this argument to acknowledge that several of the organisms isolated from the Tell Leilan skeletal material are known bone-digesters. These organisms can solubilise bone through the production of enzymes such as collagenases and proteases (e.g., chymtripsin and pepsin) (Child, 1995b). Child (1995a) and Yakovleva et al. (2006) have shown that *P. chrysogenum* and species of *Cladosporium* can produce collagenase. In addition, according to Yakovleva et al. (2006), microorganisms such as *P. chrysogenum* can retain their collagenolytic capabilities for up to ten years. Also, fungal species isolated from the Leilan material such as *P. chrysogenum* (and perhaps actinomycetes) can digest bone through hydrolysis via hyphal secretion of acids (Child, 1995a); thus bi-passing the need for a complex enzyme. Although species of *Penicillium* and *Cladosporium* are known collagenase producers (Child, 1995a; Yakovleva et al.,

2006), it is unknown whether the rest of the organisms isolated from the samples can digest bone.

Nevertheless, in a biofilm, it is irrelevant whether an individual organism can produce an enzyme or digest bone through hydrolysis. Researchers have shown that a substrate can be digested easily through the combined efforts of several organisms, each carrying out a portion of the metabolic pathway (Costerton et al., 1994). Investigations have shown that a biofilm not only contains microorganisms that work as primary and secondary feeders, but also others that create products or environmental conditions on which other organisms rely (Marsh and Bowden, 2000; Wimpenny, 2000). Consequently, it is likely that several non-enzyme producing organisms have been dismissed as not playing a role in the decay of bone (see Child, 1995a, b; Child et al., 1993; Child and Pollard, 1990). Thus, to model successfully the process of bone biodeterioration, researchers must consider the collective effects of various individual organisms instead of examining them in isolation.

Although the isolated organisms may be those responsible for the biodeterioration of the Tell Leilan material, the timing of their infiltration is uncertain. Microorganisms may have entered the material sometime during curation. Another possible explanation is that the microorganisms came with the skeletal material from Syria, in the form of spores or quiescent vegetative cells that germinated, grew slowly, or resumed growth upon arrival in Edmonton. This

scenario is possible because several spores were noted when samples were examined under SEM. These spores would still be viable because, according to Yakovleva and colleagues (2006), spore viability can be retained for up to a decade. Neither scenario can be ruled out because the organisms isolated from the material are common in the soil and air, thus rendering both scenarios plausible.

4.4.3 Implications of Biofilm Growth for Archaeological Bone

Bone is affected by microbial growth whether it is contaminated by a single microbe or by a more complex microbial aggregate such as a biofilm. While it is not possible at present to distinguish between the effects of single microbes and microbial aggregates on archaeological bone, biodeteriorated bone is characterised by tunnels, pigment accumulations, and by changes in mineralisation, all of which affect the study of human skeletal collections (Bell, 1990; Grupe et al., 1993; Hackett, 1981; Marchiafava et al., 1974). For example, tunnels alter bone microstructure and prevent histological investigations of contaminated bone (Jackes et al., 2001; Palmer, 1987; Piepenbrink and Schutkowski, 1987; Schultz, 1997, 2003). Therefore, microbial growth in bone (including biofilms) has implications for researchers trying to answer anthropological questions using archaeological bone.

Although this is the first instance of a biofilm being described in archaeological human bone, it is likely that their effects have been documented previously under

the guise of ‘biodeterioration’ by researchers (Jackes et al., 2001; Jans, 2005; Jans et al., 2004; Maat, 1993). The discovery of a biofilm within the Tell Leilan skeletal material calls for a reinterpretation of published biodeterioration studies. For example, Maat (1993) reports how most of the endosteal surfaces of bones from Kuwait were covered with a thin patina of ‘dirt’. It is possible that this patina was evidence of a biofilm. In addition, what Jackes et al. (1991) identified as a mass of craters in bone inoculated with *Clostridium sporogens* may be EPS. An awareness of biofilm growth in the Leilan material will lead to the identification of more of these microbial aggregates in archaeological collections in the future.

Most importantly, however, the discovery of biofilm growth in the Tell Leilan material reveals that standard curation procedures for archaeological bone collections are inadequate. Skeletal collections are not static and require active environmental control, i.e., curation. Current curation standards cannot limit biofilm growth because they focus on factors that affect individual microorganisms such as temperature and RH (see Appelbaum, 1991; Canadian Conservation Institute, 1983; Gehlert, 1980; Sease, 1994). Thus, future research should focus on the use of biocides and environmental controls to limit biofilm growth so that better curation protocols for archaeological skeletal collections can be developed.

4.5 Conclusions and Future Considerations

In this paper, the characteristics of biofilm growth in archaeological human bone from the site of Tell Leilan, Syria are explored. The present study is the first of its kind to investigate biofilms in archaeological human bone. Culture techniques identified several species of bacteria and fungi that are likely involved in the ongoing decay of the Leilan material. Based on the results presented here, the fixation-dehydration technique presented by Tsuneda et al. (1991) is useful for visualising biofilm components in archaeological human skeletal material. The findings show that the Leilan biofilm is characterised by single cells and microcolonies embedded within EPS. EDX analyses revealed that calcite crystals were randomly distributed throughout the film. Both bacterial and fungal cellular structures were recognised and these structures provide us with an overall understanding of the community organisation of the Tell Leilan biofilm.

There are several implications of identifying a biofilm in the Tell Leilan skeletal material. Clearly, if a biofilm can exist in the Leilan bone it is possible that other skeletal collections are host to biofilm development. For this reason, we need to revisit prior interpretations of biodeterioration in the bioarchaeological literature and likely, other examples of biofilm growth in human skeletal collections will materialise. Moreover, the results of this investigation show that it is essential to explore procedures to limit or eliminate biofilm formation in archaeological bone in museum and research facilities. Because human skeletal material is not stable in curation, it must be subject to the same active rigorous environmental controls

as other artefacts. Having characterised the Leilan biofilm, we are now closer to developing such needed curation protocols for skeletal collections.

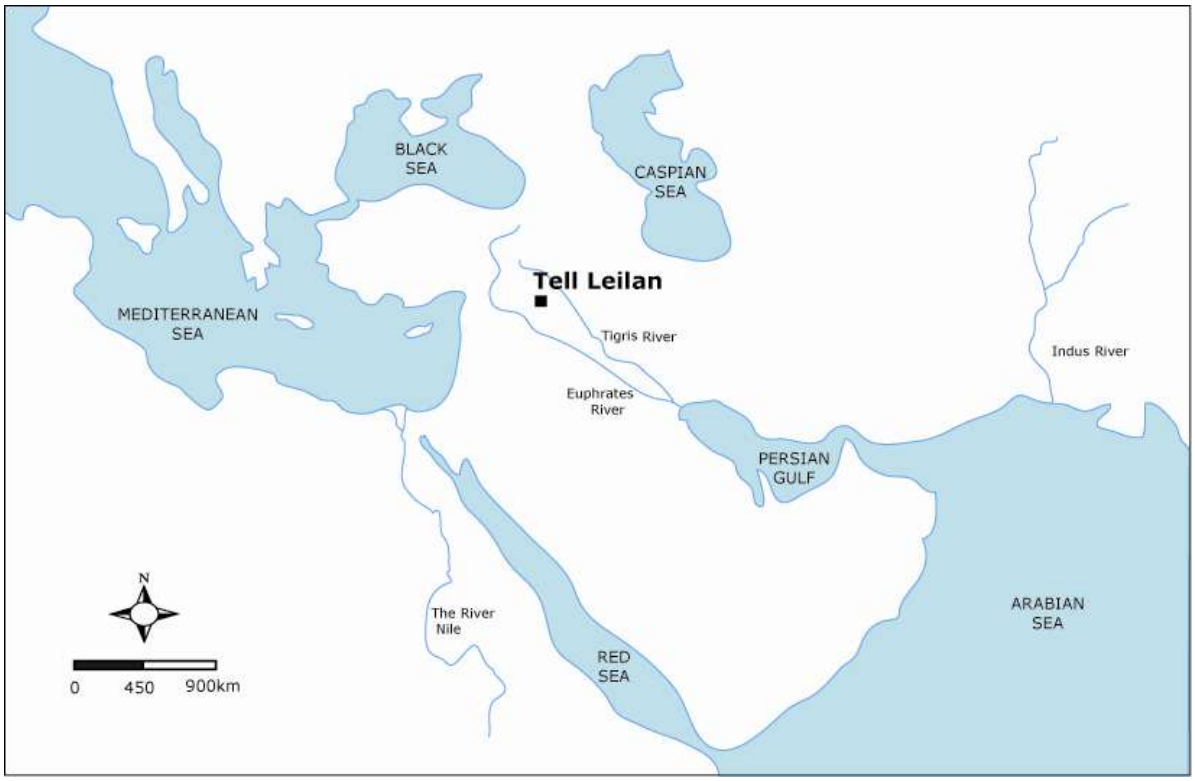

Fig. 4.1 Map showing the geographic location of Tell Leilan.

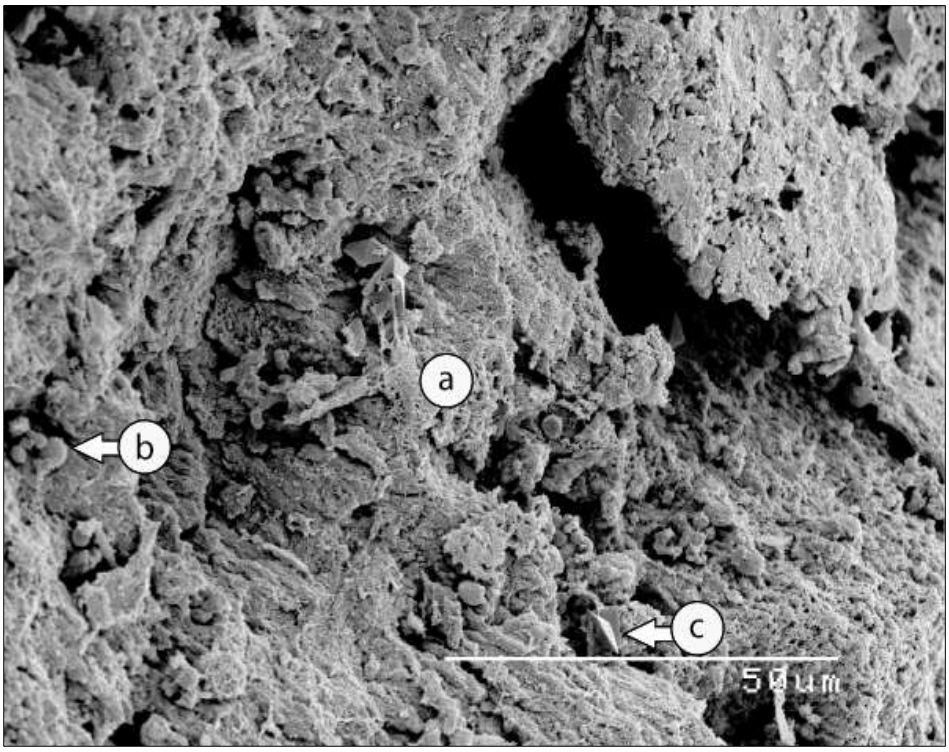

Fig. 4.2 Secondary electron micrograph of the Leilan biofilm showing: (a) extracellular polymeric substances, (b) microbial structures, and (c) calcium carbonate crystals.

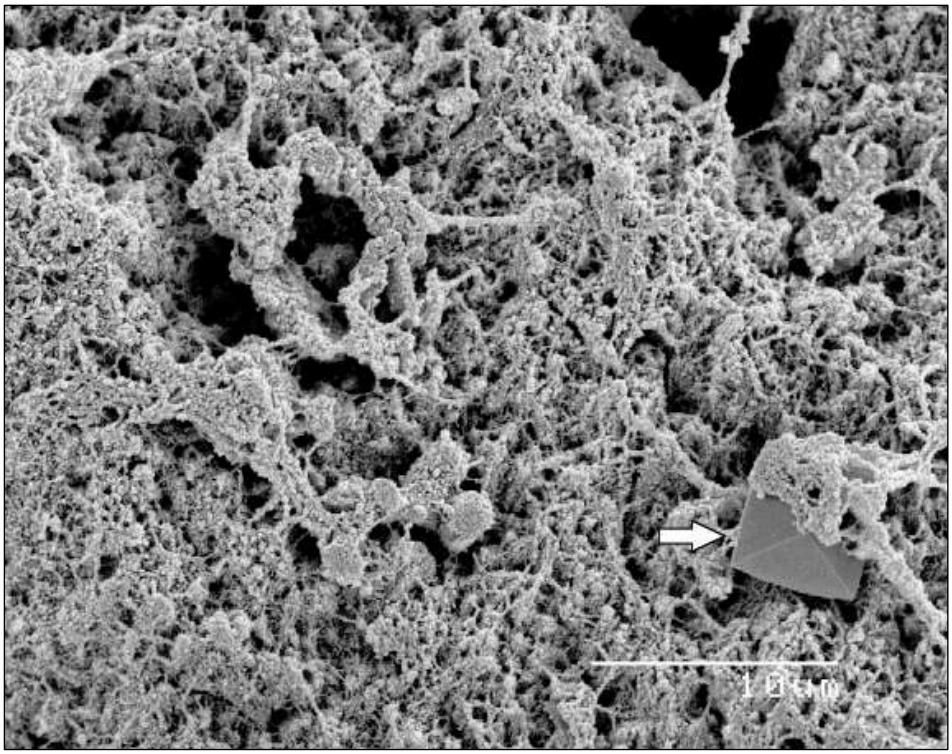

Fig. 4.3 Secondary electron micrograph of the Leilan biofilm showing the dense foamy appearance of the extracellular polymeric substances (EPS). The arrow points to the calcium carbonate crystal embedded within the EPS.

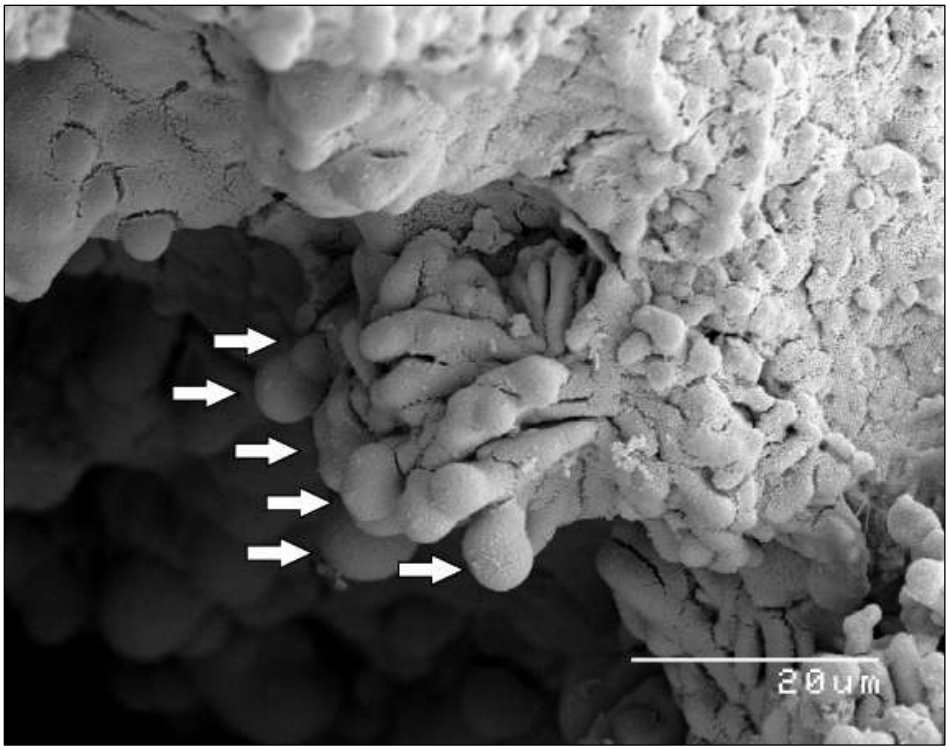

Fig. 4.4 Secondary electron micrograph of the conidial heads of fungi developing within a Haversian canal in the Leilan sample.

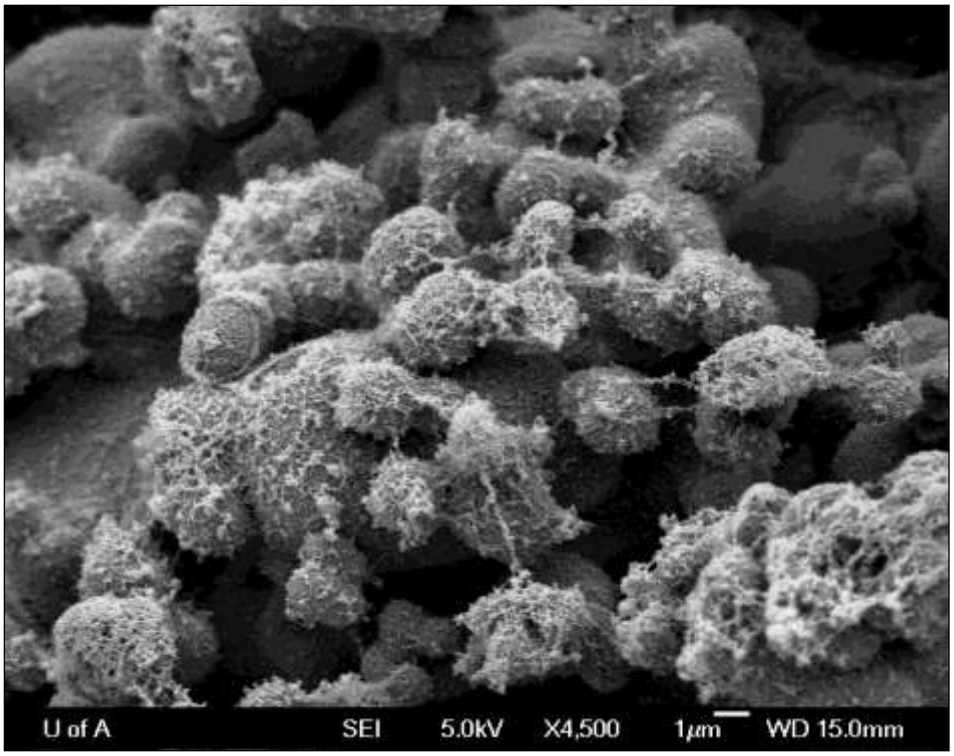

Fig. 4.5 Secondary electron micrograph of the Leilan sample showing numerous fungal spores covered by the foamy strands of extracellular polymeric substances.

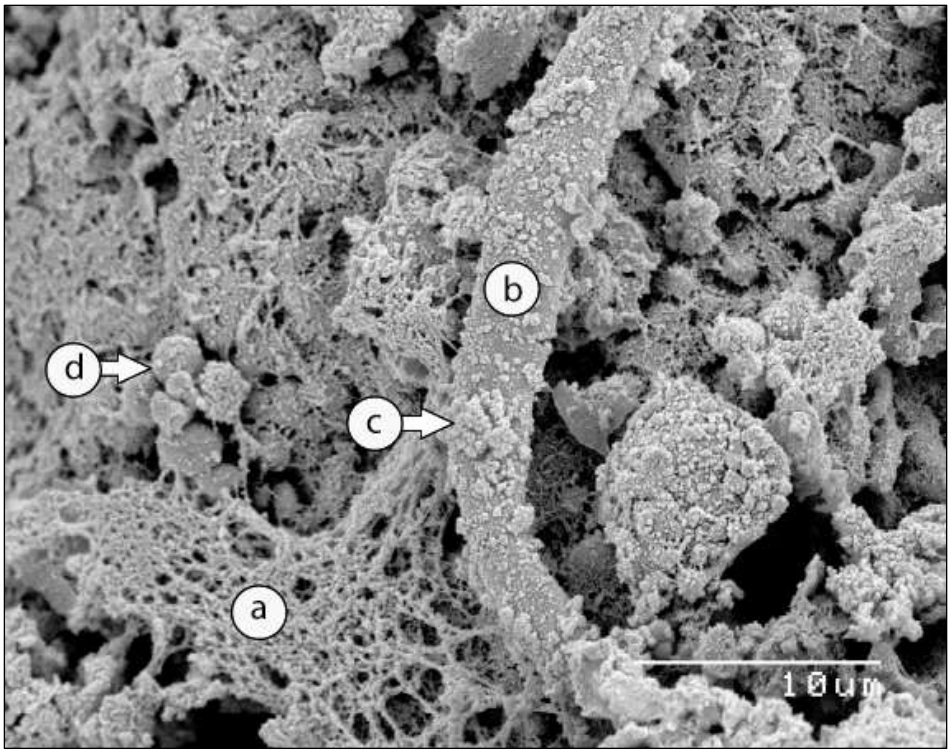

Fig. 4.6 Secondary electron micrograph of the Leilan specimen showing: (a) extracellular polymeric substances, (b) a fungal hypha, (c) microcolonies of cocci, and (d) fungal spores.

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Chapter 5

General Discussion and Conclusion

5.1 Introduction

The results of my dissertation have raised three important concerns about the storage of human skeletal material and the study of biodeteriorated bone. First, bone cannot be assumed to be stable during curation as it is vulnerable to attack by complex microbial aggregates such as biofilms. Second, biofilms go unnoticed because of the techniques commonly used to examine bone. Third, standard methods, such as identification of tunnel morphology (Davis, 1997; Hackett, 1981; Wedl, 1864) and the use of preservation indices (Hanson and Buikstra, 1987; Haynes et al., 2002; Hedges et al., 1995; Jans et al., 2004) are inadequate for characterising biodeteriorated bone. As will be discussed, these discoveries have important implications for future conservation efforts for curated skeletal material as well as for the study of biodeteriorated bone.

5.2 Major Research Findings

5.2.1 The Problem Facing Conservation

By far, the most important realisation to arise from this research is that bone is not stable in curation. This realisation, however, is not necessarily a novel one as conservators and other specialists have developed specific protocols for bone to ensure its safe storage (Canadian Conservation Institute, 1983; Cassman and Odegaard, 2007a, b; Department for Culture, Media and Sport, 2005; Gehlert,

1980; Odegaard and Cassman, 2007; Rose and Hawks, 1992). Although such protocols exist, the Department for Culture, Media and Sport (2005) in the United Kingdom continues to maintain that bone does not require very closely controlled environmental conditions. The results presented here, however, show that such claims should be questioned and that a definitive protocol standard for bone should be developed, one that considers the complex nature of bone and the forces that microorganisms exert on it.

That the biodeterioration of collections in curation has largely been ignored (for exceptions see Bowron, 2003; Cassman and Odegaard, 2007a, b; Sease, 1994) is not unexpected as it is difficult to pinpoint the onset of decay. Researchers have assumed that biodeterioration happens within the burial environment where bone is exposed to microbe-rich sediments (see Cronyn, 1990). Because of this focus, few have realised that biodeterioration is a problem in curation. Pruvost et al. (2007) and Bowron (2003), however, have reported on the biodeterioration of institutionalised collections of bone. Their results, as well the results presented here, show definitively that microbial growth is a threat in curation. We can now move toward understanding how bone, once removed from a burial context, adapts to a new curation environment (Jans et al., 2002; Sease, 1994) and focus on how to alter this new environment so that the growth of microbial structures is prevented.

According to current protocols, researchers should examine collections several times a year for signs of pest activity (e.g., droppings, casings) (Canadian Conservation Institute, 1983; Cassman and Odegaard, 2007a, b; Jessup, 1992). Researchers who follow such protocols will likely overlook biodeterioration, as microbial damage is mostly invisible. Thus, we need to develop new guidelines for monitoring skeletal collections for microbial damage. Unfortunately, however, even if researchers do observe microbial growth in a collection, there are no guidelines, at present, for what should be done in the case of microbial infestation. Until such preventive and treatment guidelines are developed, collections will continue to become contaminated and those that are already contaminated will worsen. As a result, individuals and groups may begin to question the value of curating archaeological bone since our methods for conserving them for future research are at present inadequate.

The results of this investigation show that human skeletal collections are susceptible to biofilm growth regardless of how they are treated post excavation. At the University of Alberta (U of A), the Leilan skeletal material was treated and stored following several protocols (see Appelbaum, 1991; Canadian Conservation Institute, 1983; Gehlert, 1980; Sease, 1994). Although these conditions should have prevented microbial growth they could not prevent the growth of the biofilm—as microorganisms within the biofilm were able to cope with a wider variety of environmental conditions, including those that normally limit microbial growth (Allison et al., 2000; Davies, 2000; Gilbert et al., 1997; Wimpenny, 2000).

Thus, the results of this research indicate that traditional conservation efforts (e.g., Canadian Conservation Institute, 1983; Cassman and Odegaard, 2007a, b; Department for Culture, Media and Sport , 2005; Gehlert, 1980; Odegaard and Cassman, 2007; Rose and Hawks, 1992) for bone are ineffective in the prevention of such complex microbial aggregates.

Although we do not know how the Leilan skeletal material was treated before it arrived at the U of A, we do know that, upon arrival, the material was relatively biodeterioration free between 1992 and 1996. Thus, something changed in the material after 1996—and this change is precisely what we need to understand to establish the conditions that were conducive to biofilm growth. Unfortunately, however, it is unlikely that we will be able to pinpoint the exact trigger(s) of the biofilm growth, as several factors (e.g., storage container, environment) are likely involved. The transfer of the skeletal material from paper to plastic bags in 1992 may have been a contributing factor to the growth of the biofilm. Although the bone was characteristically dry, having been excavated from an arid environment and never washed, moist conditions within the plastic bags may have provided the water needed for the assimilative cells and spores of fixed soil microbes to resume growth. This hypothesis, however, does not explain why there were no histological changes in the collection between 1992 and 1996—only between 1996 and 2009.

5.2.2 Studying Biodeteriorated Bone

The results of this research suggest that the methods used to study biodeteriorated bone affect how the process of biodeterioration is viewed. Specifically, the methods we use to study biodeteriorated bone are inherently flawed. Thus, any attempts at characterising biodeteriorated bone as well as determining what organisms are involved in the process are, and have been, ineffective in the past.

In Chapters 3 and 4, it was shown that biofilms are detectable only at certain levels of examination. In particular, the findings show that a biofilm is invisible at a macroscopic level as well as at a microscopic level when embedded ground bone is examined with scanning electron microscopy (SEM). It has yet to be determined, however, whether the brown opaque material that is visible under bright field light microscopy (LM) is incontrovertible evidence of a biofilm, and whether bone infiltrated by single microbes instead of an aggregate would not exhibit the brown material. Overall, the findings suggest that biofilms (including the microorganisms and the extracellular polymeric substances they secrete) are best observed when bone samples are fixed and dehydrated and examined using SEM.

That biofilms are recognisable only at certain levels of examination (i.e., SEM with fixation and dehydration) has important implications for past studies as well as for future conservation efforts. The results of this investigation suggest that researchers and conservators who examine and monitor skeletal collections have

overlooked biofilm growth. Using bright field LM, for example, a researcher may easily mistake the brown opaque organic material for accumulations of microbial pigments, clothing dyes, or sediment components (see Jackes et al., 2001; Jans, 2005; Jans et al., 2004; Maat, 1993). Similarly, a conservator who monitors visually skeletal collections would be largely unaware of biofilm growth as these aggregates are invisible at a macroscopic level. Thus, if we continue to examine and monitor bone in such a way, biofilm growth will continue to be overlooked in institutionalised skeletal collections.

Although SEM (when unembedded samples were treated with ethanol and amyl acetate baths as well as CPD) was useful in identifying the presence of the biofilm in bone, the technique was of no use in determining how the biofilm progresses within bone. Bright field LM, on the other hand, may be useful for this purpose only if the brown opaque material noted in Chapter 3 is truly indicative of biofilm growth. Once proven, the results of this research will have provided corroborating evidence (see Bell, 1990; Garland, 1987; Turner-Walker and Jans, 2008) to indicate that microorganisms within a biofilm use bone structures to infiltrate skeletal material and that the invasion is guided by bone mineralisation (see Hanson and Buikstra, 1987; Jackes et al., 2001; Turner-Walker and Jans, 2008).

Because biodeterioration is guided by bone mineralisation, traditional means of examining bone integrity such as preservation indices are ineffective (see Chapter 3, this thesis). Regional differences in bone preservation result in high intra- and

inter-observer error in assigned histological preservation index (HPI) values. Contra Haynes et al. (2002), the results of this research show that preservation indices such as the HPI are not useful for quantifying the destruction in biodeteriorated bone; therefore they probably are not useful for selecting samples for bioarchaeological studies involving DNA, isotope, and trace element analysis.

In addition, the results of this investigation have shown that, depending on the technique used, different organisms may be deemed responsible for the biodeterioration of bone. When used in isolation the methods described in Chapters 3 and 4 identify different culprits for the biodeterioration of the Leilan skeletal material, but when used in combination they show that both bacteria and fungi are involved. Thus, although bacteria may be causing visible damage to the bone in the form of microscopic tunnels, fungi, as part of the biofilm are also somehow involved in the process. By using combinations of techniques, it was possible to determine that several microorganisms may have been involved, not just those producing tunnels.

The results of this work show that a sole reliance on tunnel morphology will result in a distorted view of microorganismal involvement. The tunnel classification schemes described in Chapters 2 and 3 overlook the fact that complex microbial communities such as biofilms may be involved in the biodeterioration of bone. In such communities, although one particular organism may be forming the tunnels, other organisms may be cooperating in creating enzymes or in rendering the

environment more hospitable (Wimpenny, 2000). Because this is the first time that a biofilm has been recognised in archaeological human bone, its effects on tunnel formation are currently unknown. Thus, tunnel classification schemes should be used with caution when a biofilm is suspected.

Moreover, researchers using culture techniques to isolate microorganisms from biodeteriorated bone have been underestimating the cooperative capabilities of microorganisms. These researchers have dismissed the involvement of several non-enzyme producing microorganisms in bone decay (Child, 1995a, b; Child et al., 1993; Child and Pollard, 1990). Child (1995a), for example, limited his study to bacterial and fungal isolates that tested positive for collagenase. The results presented here, however, show that some of the isolated microorganisms from the Leilan samples may be incapable of producing such enzymes, but may be intricately involved in the biodeterioration of the collection as part of the biofilm.

5.3 Future Research

The results of this study call for future research into several areas. Most importantly, future research must focus on conservation methods that limit biofilm growth in collections. In addition, future investigations must focus on how to monitor collections for biofilm growth and to develop treatment protocols for contaminated collections. Research using experimentally inoculated deer bone is currently underway to determine the best curation protocols for skeletal material. In the end, however, if we cannot properly conserve entire skeletal collections

(because of cost considerations for example) we may have to look at the possibility of storing properly only samples for future investigations.

To develop such needed monitoring and curating protocols, researchers must focus on understanding the process of biofilm development in bone. Future researchers must use all available techniques, and must not rely on traditional techniques that may overlook or distort particular aspects of the process of decay. In this study, combinations of thin section analysis, SEM, and culture techniques were useful in identifying and describing biofilm growth in the Leilan skeletal material and will be helpful in studying other skeletal collections in the future. Once our techniques are checked, we must re-examine previously studied examples of biodeteriorated bone and look for evidence of biofilm growth.

In addition, standard methods used to describe biodeteriorated bone must be re-examined as they are not particularly useful in documenting bone contaminated by a biofilm. Thus, future research should focus on finding other ways to characterise bone as tunnel morphology and enzyme isolation overlook that microorganisms may cooperate in the biodeterioration of bone. In addition, we must conduct a larger study to examine the use of preservation indices such as the HPI for determining the extent of destruction in skeletal samples. Because the HPI may not be practical for quantifying bone destruction, we should look for other ways to select samples for bioarchaeological investigations.

Furthermore, future research into this area should involve an examination into the nature of the brown opaque material observed in thin sections in Chapter 3. We must determine whether the brown material observed is indicative of biofilm growth and whether the material can be used as a screening method to determine if a skeletal collection is contaminated. Experimental studies involving deer bone are currently underway to clarify this hypothesis. Such research is important so that biofilms are not attributed in the future to sediment contaminants such as humic substances, copper, and bronze, to clothing dyes, and to the accumulation of microbial pigments.

5.4 Final Remarks

The results of this research provide the first detailed evidence that human skeletal collections can become contaminated with microbial growth in curation. Thus, contrary to the belief of some conservators, human skeletal collections do require active monitoring in curation. In addition, the present study is the first of its kind to investigate biofilm growth in archaeological human bone. That microbial growth was found in a curated skeletal collection in the form of a biofilm has implications for the future development of conservation protocols. Having characterised the Leilan biofilm, we are now closer to developing such needed protocols.

To prevent biofilm growth in human skeletal collections we need to improve the manner in which we study biodeteriorated bone. At present, our methods prevent

us from recognising and characterising biofilm growth in bone. Thus, new methods and feasible techniques are required to first recognise biofilms so that we can eventually understand how and under what conditions they grow. Ultimately, further research in this area will improve the potential for applying new methods to understand biofilm growth in bone and will help in the development of curation protocols to prevent such growth in skeletal collections in the future.

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