The Experimental Degradation of Archaeological Human Bone by Anaerobic Bacteria and the Implications for Recovery of Ancient DNA

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Abstract

Background/Aims

DNA recovery from human bone has been key to the developing science and technology of ancient DNA studies. The recovery of macromolecules from bone however, does not correlate well with recognisable parameters of preservation and predicting DNA recovery rates from ancient bone can be very difficult. The extent of degradation of buried bones often depends on environmental taphonomy and can vary from virtually none to complete and rapid destruction. Although soil or related microbes are undoubtedly responsible for the majority of this structural degradation over time, exceptionally little is known of the mechanisms or specific bacteria involved. Fungi were previously thought to be responsible for destructive processes (tunnelling) within bone but over the last 30 years the role of bacteria has been increasingly recognised. Our aim was to develop a less complex *in vitro* model of the destructive effects of microbes on bone which might allow a better understanding of the recovery of mitochondrial or pathogen DNA over time.

Materials and Methods

Bone sections from a human femur approximately 700 years old were prepared using a bone microtome and inoculated with anaerobic bacteria such as *Prevotella intermedia* in culture broth. Inoculated samples and controls (untreated sections) were incubated anaerobically at various temperatures and examined carefully with light and polarised microscopy.

Results

Despite some anticipated degradation in unexposed bone sections, more extensive degradation consistent with linear longitudinal and lamellate tunnelling was observed in sections examined 33 weeks or more after exposure to the anaerobes.

Conclusion

We have developed a novel methodology to investigate the destructive interaction of specific microbes with human bone.

Introduction

Bone is a composite of protein (collagen) and mineral (hydroxyapatite) capable of undergoing chemical and microbiological alteration. Diagenesis is the chemical changes that occur whilst bones are in soil [1]. However, the extent of diagenetic changes may vary dramatically depending on the duration of burial and conditions of the inhumation environment (humidity and temperature) [2]. Diagenesis can be determined using four main measurements; the destruction of histological integrity, change in crystallinity and porosity, and the loss of collagen [3]. Diagenetic degradation of bone may be due to hydrolysis of collagen, resulting in the loss of bone strength [4] which could be followed by an increase in pores due to the tunnelling of micro-organisms leading to further loss in mechanical strength. However, bones can vary greatly in their degree of diagenetic alteration. Lambert *et al.* [5] reported that different parts of the skeleton show a difference in resistance to destruction when in soil.

The most common mechanism of bone deterioration, which can occur rapidly after death, is probably destruction caused by microbes [6]. Microbial attack is probably the most important factor in the degradation of bone [7]. The results of studies by Hedges *et al.*[3] estimate that microbiological attack can be complete within 500 years of the bones being buried. However, these results are not conclusive due to the obvious restrictions of carrying out experimental research to prove this. The results of studies carried out by Yoshino *et al.* [8] demonstrated that histological changes of compact bone had occurred at least 5 years after death. Haversian canals were filled with bacteria formed in vacuoles ranging in diameter from 5 to 10 μm. When dissolution of the mineral phase in low pH occurs, any removed mineral would expose the collagen to extracellular microbial enzymes and therefore microbial attack [6]. A number of factors, such as temperature, time and environmental pH can all affect the rate at which collagen is lost. Bacteria produce an enzyme (collagenase) which digests bone collagen and creates a pathway for the invading organism. Dissolution as a result of microbial attack focuses in discrete zones of destruction, known as microscopic focal destruction (MFD). Organisms from the burial environments appear to penetrate the canal wall of buried bone and then attack the osteon tissue which results in MFD [2]. Microbes, for example, fungi and bacteria demineralise bone resulting in histological destruction of bone otherwise known as “tunnels” or “borings”. Marchiafava *et al.* [9] further demonstrated that biological alteration of bone was mostly caused by fungi and bacteria. Therefore organisms commonly found within the burial environment may produce and cause MFD in buried human bone.

Hackett [2] suggests that bacteria are the major cause of microscopic focal destruction (MFD) and not fungi as previously thought. Hackett [2] identified three types of MFD and classified them as “linear longitudinal”, “lamellate” and “budded” tunnelling. The three types were distinguished mainly according to their shape and size. In transverse section, linear longitudinal tunnels are seen as small round foci approximately 5-10μm in diameter [2]. The cement line stops the tunnels from spreading out of the osteon and therefore these are usually concentrated around single osteons. Budded tunnels are found mainly along the osteon canals and extend to fill the osteon. These tunnels are bigger in diameter than linear longitudinal tunnels at approximately 30μm or wider [2]. In transverse section, these tunnels may appear round and stippled. Lamellate foci are by far the largest in size ranging from 20-250μm [2]. They are rounded in transverse section and normally found towards the

surfaces of the cortex. Lamellate foci curve to the shape of the lamellae. In comparison, centrifugal (Wedl) tunnels produced by fungi [2] are uniform in diameter and range in size from 5-10μm. The outline of this type of tunnelling is well defined with circular markings (borings).

This study considers the effect that specific bacteria have on archaeological human bone over a minimum of an eight-month period. The anaerobe *Prevotella intermedia* is commonly found in soil and is also a human pathogen which is common in the gingival crevice and often isolated from cases of gingivitis. It was used in a study as Slots *et al.* [10] which established that this type of bacterium degraded the protein substrate found within bone. Even though the optimum growth for *Prevotella intermedia* is 37°C, the low temperature of 4°C was also chosen as it more closely resembles the ground temperature in which bones may be buried over time.

A single human femur bone was used in this study. According to Jans *et al.* [11], a femur will give a high frequency of tunnelling due to its size. She demonstrated that Wedl tunnelling was more commonly observed in animal bone whereas linear longitudinal and budded tunnelling were more common in human bone. Furthermore, in her study Jans *et al.* [11] showed that human bones were more prone to bacterial attack than animal bones.

The bone used in the present study, was degraded due to its age and the environmental conditions in which it was buried. It was removed from a 700 years old excavation site in Monks Road (Lincoln, United Kingdom) which had well preserved skeletal remains.

Materials and Methods

A human femur bone excavated from Lincoln, UK was used throughout this study. Bone samples were cut by hacksaw from the femur and thin sections prepared by individually placing them in an ice cube tray containing LR White resin, labelled and removed from the ice cube tray prior to being cut with a saw microtome. Each sample was cut into thin sections approximately 60μm – 100μm. Archaeological bone is usually cut at this thickness due to its fragile nature compared to fresh bone, which can generally be cut to 50μm. Archaeological bone should also be cut at a slower speed due to its fragile nature, a slower speed resulting in a smoother surface and also less force that bears on the object and the saw blade. Each sample was placed on a separate microscope slide, labelled and placed in a Petri dish for storage prior to inoculation. Samples were inoculated with cultures of either anaerobic bacteria under various experimental conditions and some samples left untreated. Samples were inoculated with approximately 10μl of a fully-grown culture of the reference strain *P. intermedia* NCTC1370 culture broth. All samples; including inoculated and untreated samples, were then placed into the appropriate pre-determined environment (anaerobic cabinet or anaerobic jar) at different temperatures (37°C and 4°C). According to Holt *et al.* [12] the optimum temperature for the *P. intermedia* bacteria to grow and develop is 37°C. The low temperature of 4°C was chosen in addition because it closely resembles that of soil temperature where human bones are usually buried.

After a minimum of 33-36 weeks, the bone samples were taken out of the anaerobic cabinet (37°C) and the anaerobic jar (4°C) for 'fixing' with approximately 10ml of 1% formaldehyde solution for approximately 48 hours. They were then rinsed off with distilled water, mounted onto a microscope slide using a drop of the mountant Euparal and observed using light and polarised microscopy. Analysis of

bone structure by light microscopy was used to determine the type of MFD present based on the morphological descriptions as given by Hackett [2]. The bone samples were not stained with methylene blue, as this technique has given irregular results previously [2].

Polarised microscopy was used to calculate the degree of diagenetic change in bone using the Oxford Histological Index (OHI), which was developed by Hedges *et al* [3].

Results

Light Microscopy

Analysis by light microscopy allowed observation of tunnelling just beneath the surface of the bone [13]. Examination of ultra-thin bone sections using light microscopy generally produces better resolution images [14] when bone sections were cut to a thickness of approximately 100µm. Of the samples that were analysed in the present study, most appear to be significantly more degraded than the samples that were left untreated. The untreated sample "BII" (Fig. 1) did not appear to show much evidence of microbial attack, as the bone was substantially intact where lacunae (the spherical features within the osteons) were observed under light microscopy.

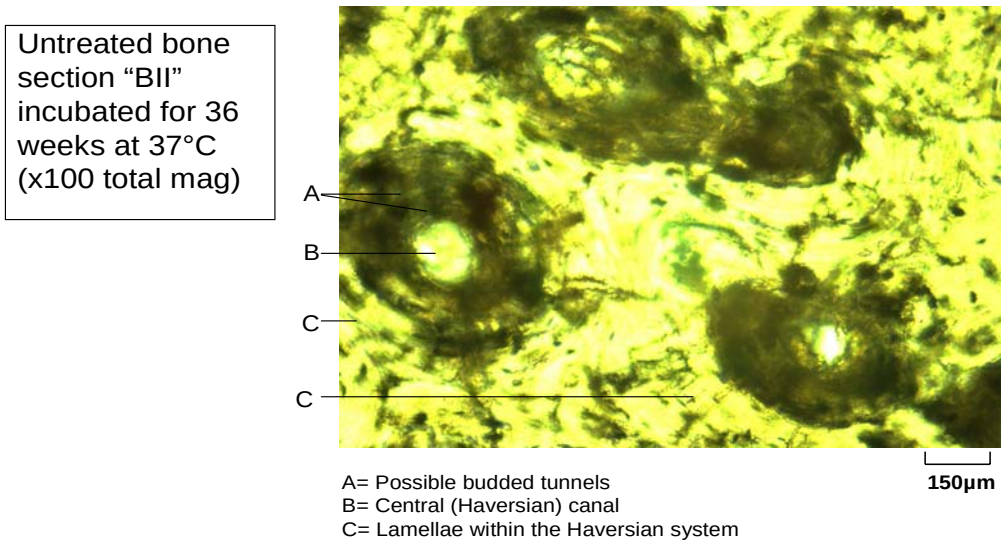

Figure 1: Untreated bone section "BII" incubated for 36 weeks at 37°C (x100 mag).

Even though there may be evidence of possible tunnelling in the untreated sample "BII", there is substantially more intact bone compared to MFD. The characteristic "fluffy" appearance of budded tunnelling appears to be evident as annotated in Figure 1. As the bones are over 700 years old and were excavated from a burial site in Lincoln they would have been subjected to microbial attack prior to excavation.

Sample “M” (Fig. 2) was inoculated with the bacterium *P. intermedia* and incubated at 37°C shows possible evidence of MFD in the form of linear longitudinal and lamellate tunnelling. When observed under light microscopy (low magnification) the MFD appear to have granulous or fibrillar content but when viewed at a higher magnification small pores can be observed [11]. There appears to be evidence of granulous content within the features characterised as possible linear longitudinal tunnelling (Fig.2)

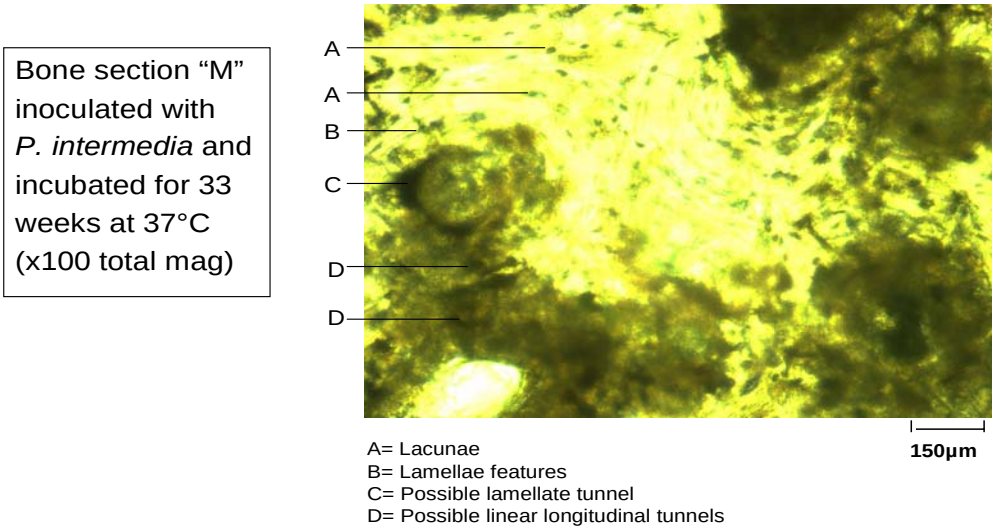

Figure 2: Bone section “M” inoculated with *P. intermedia* and incubated for 33 weeks at 37°C (x100 total magnification).

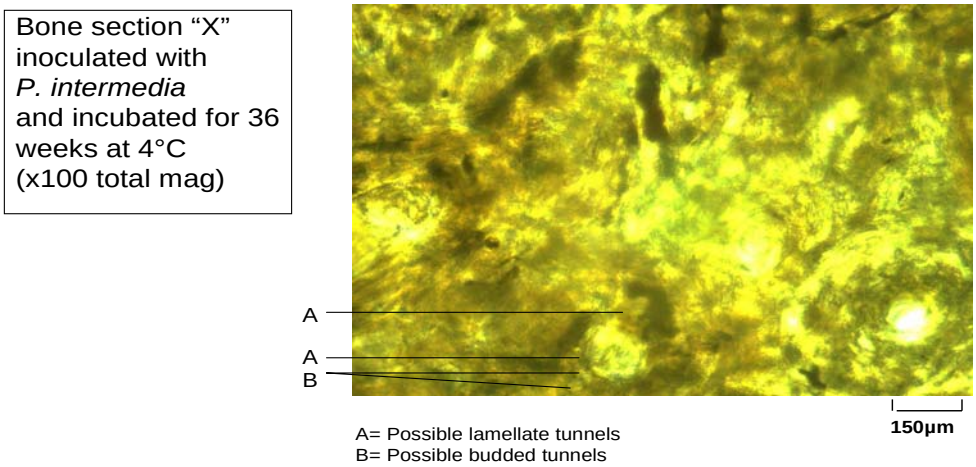

Figure 3: Bone section “X” inoculated with *P. intermedia* and incubated for 36 weeks at 4°C

Sample “X” (Fig. 3) inoculated with *P. intermedia* and incubated at 4°C does not appear to have much intact bone, which may suggest evidence of MFD. Possible lamellate and budded tunnels are evident. According to *Jans et al*. [11], lamellate and budded tunnelling are usually found in association with each other and that lamellate tunnelling is rarely found alone.

Polarised Light Microscopy

Polarised light microscopy involves examining thin transparent materials using polarised light. Contrast of the sample may be improved using this type of analysis and can also aid in microstructure determination. Polarised light microscopy can aid the identification of intact bone and also show any bone that has been destroyed by bacteria. The classic ‘Maltese’ cross indicates there is localised collagen and is therefore evidence of intact bone. Collagen is one of the few birefringent biomolecules and can therefore transmit polarised light depending on the quantity and orientation of the collagen fibres, which is shown as light and dark [15]. Any absence of the light and dark banding may therefore indicate loss of intact bone and therefore presence of MFD.

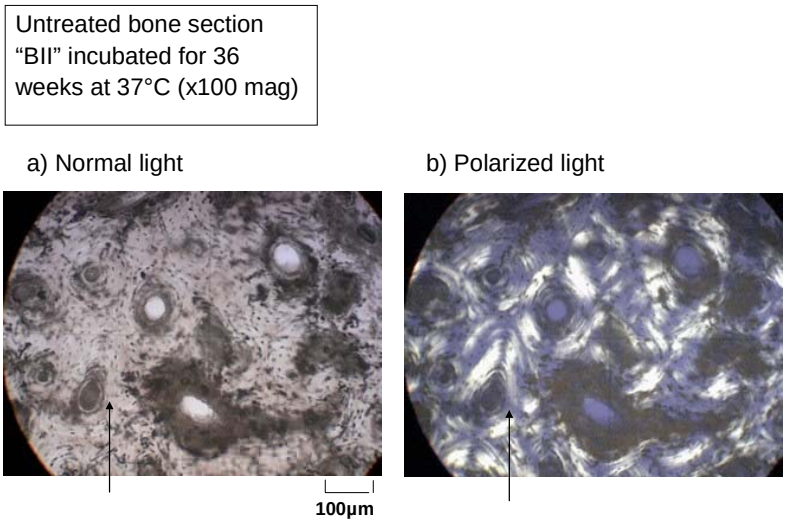

Figure 4a and b: Untreated bone section “BII” incubated for 36 weeks at 37°C (x100 mag); A: Intact bone.

The untreated bone sample (Fig. 4a and b) appears to be substantially intact where MFD may be evident in the darkened areas observed in both normal and polarised light. The histological assessment indicates that there is more than 85% intact bone present.

The bone samples inoculated with *P. intermedia* (Fig. 5a and b) indicates that at least 67% intact bone was present. However, there also appeared to be evidence of MFD (possible linear longitudinal and lamellate tunnelling). In contrast to the sample inoculated with *P. intermedia*, there is reduced birefringence (Fig. 5b), which could further indicate that the bone has been highly altered by microbial attack.

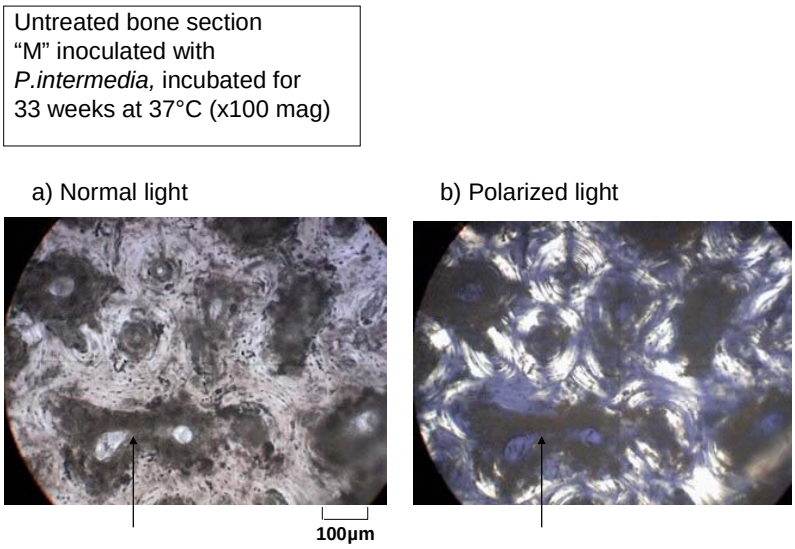

Figure 5a and b: Untreated bone section "M" inoculated with *P. intermedia* incubated for 33 weeks at 37°C (x100 magn).

Oxford Histological Index

The Oxford Histological Index (HI) as described by Hedges *et al.* [3] can be used to calculate the degree of diagenetic change. Histological examination of bone involves the examination of the samples in reflected light prior to recording the histological index of the bone samples [16]. The HI system gives a value of '0' to '5' depending on the amount of intact bone. A value of '0' indicates that there is <5 % original bone features evident. Whereas a value '5' indicates that there is >95% original bone features evident suggesting the bone is substantially intact. Table 1 provides details of the diagenetic changes for each value ranging from '0' to '5' as formulated by Hedges *et al.* [3].

Index	**Approximate % of intact bone**	**Description**
0	<5	No original features identified, apart from Haversian canals
1	<15	Small areas of well preserved bone present, some lamellar structure preserved by pattern of destructive foci
2	<33	Clear lamellate structure preserved between destructive foci
3	>67	Clear preservation of some osteocytes lacunae
4	>85	Small amounts of destructing foci but well preserved
5	>95	Well preserved and can't really distinguish from fresh bone

Table 1: Oxford Histological Index values summarising the degree of diagenetic changes in bone [3].

The estimated histological index (HI) values for this present study were recorded reflecting the amount of bone altered due to diagenetic changes. The HI distribution is

shown in Table 2. The HI values given for bone samples inoculated with *P. intermedia* are quite varied however it is important to note that these are only estimations due to the extent of destruction changes that can occur throughout the entire bone sample [3].

Bone sample	**Description and incubation temperature (°C)**	**HI value**
BII	Untreated at 37	4
M	Exposed to *P. intermedia* at 37	3
X	Exposed to *P. intermedia* at 4	1

Table 2: Histological index values given to bone samples analysed according to the degree of diagenetic change as produced by Hedges *et al.* [3].

Discussion

The primary aim of this study was to observe the tunnelling effects produced by the experimental inoculation of specific bacteria on human archaeological bone. Microbes, mainly fungi and bacteria, demineralise bone resulting in histological destruction artefacts known as “tunnels” or “borings”. The microscopic focal destruction (MFD) can be distinguished histologically by their morphology; size, shape, and presence of a hypermineralised rim and lamellate content [11]. We analysed our samples to determine any evidence of microbial attack using light microscopy (LM) and polarised light microscopy (PLM) and assessed the morphological characteristics and degree of diagenetic changes within the samples.

There appears to be more evidence of MFD in the samples inoculated with *P. intermedia* (Fig. 2 and 3) compared to those left untreated (Fig. 1). However, some diagenetic changes were observed in the untreated samples, which could be due to the age of the femur bone and the environment in which it was buried prior to excavation. Over time bone can increase in porosity, which may result in archaeological bone being more affected by other diagenetic processes [11], such as the increase in the loss of collagen as a result of microbial attack. However, as bone already has natural pores in the form of Haversian canals, it creates a natural pathway for invading organisms [11]. This helps to further explain why there was some evidence of possible (budded) tunnelling in the untreated samples. The optimum growth for *P. intermedia* is 37°C, however the lower temperature tested also appeared to have a destructive effect but this could not be conclusively determined as only one sample (Fig. 3) was incubated at the lower temperature.

The results of this preliminary study show that the anaerobe *P. intermedia* appears to play an unexpected role in the ‘tunnelling’ of archaeological human bone. Theoretically, anaerobic bacteria specifically *Clostridium histolyticum* produce collagenase [17], which results in the breakdown of bone collagen and creates a pathway for the invading organism causing different types of tunnelling. The bone samples inoculated with *P. intermedia* showed evidence of two different types of tunnelling; lamellate and linear longitudinal tunnelling, and lamellate and budded tunnelling respectively. According to Jans *et al.* [11] one bone sample can be affected by more than one type of tunnelling at the same time. However most MFD may also be observed alone apart from lamellate tunnelling, which is rarely found alone.

The results shown in Figure 3 appear to support studies by Jans *et al.* [11], which suggest that budded and lamellate tunnelling are always found in association with

each other. However, the results of Figure 3 appear to contradict this exact suggestion where lamellate tunnelling is found in association with linear longitudinal tunnelling as observed in this study. Under light microscopy, the distinctive shape of the lamellate foci conforming to the shape of the lamellae appears to be evident. However, there does not appear to be evidence of cuffing (mineral redeposition) around the foci, which is said to occur frequently [2]. Although the foci appear to expand and cross the osteon lamellate pattern [2].

The results of this preliminary study correlate with the findings of a study by Jans *et al.* [11], which "shows that bacterial attack may be responsible for the majority of biological alteration in archaeological bone" namely linear longitudinal and budded tunnelling. However, assessment based on the results of the same study suggest that lamellate tunnelling is present in very few samples [11]. In comparison, our preliminary study appeared to show evidence of possible lamellate tunnelling in the two bone samples inoculated with *P. intermedia*. A study using far more samples may result in substantially different conclusions compared to that determined in this small preliminary study.

Analysis of the bone samples using polarised light microscopy (PLM) appears to show that the bone sample inoculated with *P.k. intermedia* (Fig. 5a and b) has more degradation than the untreated sample (Fig. 4a and b). The degree of diagenetic changes within the bone samples were estimated according to the Oxford Histological Index (Table 1) as developed by Hedges *et al*. [3]. The untreated bone sample appeared to have at least 85% intact bone compared to the inoculated sample only showing evidence of approximately 67% intact bone. In comparison to the untreated sample (Fig. 5b) the reduction of birefringence as seen in the bone sample inoculated with *P. intermedia* (Fig. 6b) can indicate loss or deterioration of collagen due to microbial attack. However it could also indicate loss of orientation of the hydroxyapatite crystals within bone [15] possibly as a result of the loss of collagen.

Although this is a small study, both types of analyses (LM and PLM) appeared to show substantially more evidence of MFD in the bone samples inoculated with *P. intermedia* than un-inoculated controls.

Conclusions

P. intermedia appeared to have an unexpected destructive effect on human bone, based on the results of this preliminary study. This was evident in the presence of microscopic focal destruction (MFD) where possible linear longitudinal and lamellate tunnelling were mainly observed. The anaerobic organisms investigated appear to be effective over just months and not years as shown in this study. However, as this was a small preliminary study, more conclusive results could not be determined due to the limited amount of bone samples analysed but this type of experimental approach may help to predict the recovery of macromolecules from ancient bone.

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