The Effect of Bacteria vs Environment on Bone Diagenesis

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Abstract

The aim of this research was to determine the origins of the microbes that drive bone diagenesis. Studying the microstructural changes skeletal tissue can undergo after death could lead to more accurate ways of establishing postmortem intervals when dealing with skeletonised remains. Previous research has allowed the development of methods to determine the post mortem interval (PMI) during the decomposition of the soft tissue, but this estimation becomes less accurate as the PMI increases. This study focused on the physical changes that occurred, from the macroscopic level of weathering and surface modifications, to the histological changes to the microstructure of the hard tissue. Proteomic analysis to chart changes in the bone proteome over time was also conducted.

This research compared different tissue types and different deposition environments; three tissue types, defleshed bone, excised fleshed limbs, and whole rat; two deposition environments, buried in soil, exposed on the surface. Forty-six medium sized domestic rats (*Rattus rattus*) were used for this experiment, giving three repeats per condition plus control (day 0) analysis. This was a multi-analytical approach with a variety of techniques being implemented; soft tissue decomposition was recorded using the total body score (TBS) followed by the macroscopic analysis of bone surface weathering. Ultra-violet (UV) analysis was conducted alongside histological analysis using compound microscopy and digital microscopy. Further analysis of the bone was conducted using Confocal Laser Scanning Microscope (LSM). Bone proteomics were added as a result of collaboration with Dr Noemi Procopio at Northumbria University.

Overall, this experiment produced statistically significant results for the increasing presence of diagenetic changes to the bone with increasing PMI. P values of less than 0.05 were obtained between samples at 4 weeks PMI and 28 weeks PMI for the histological analysis, Confocal LSM and proteomic analysis, which confirm the increasing destruction of bone integrity with increasing PMI. It was also found that the earliest signs of microscopic foci of destruction (MFD) occurred prior to the skeletonisation of the remains, agreeing with previous research. It was found that the deposition environment did not play the significant role in bone diagenesis that was hypothesised. The same was true for the presence of the gut microbiome, which did accelerate the initial diagenesis of the bone within the first 8 weeks. Diagenesis appeared to slow after the first 8 weeks, and all samples showed little comparable differences in diagenetic changes by 28 weeks.

This research contributes to our understanding of bone diagenesis in forensic timescales. It gives us more information about the role of gut and soil bacteria in bone diagenesis and could aid the estimation of PMI in certain situations. However, more research is needed with longer timescales, and human subjects, to improve accuracy.

Key words:
Forensics, taphonomy, anthropology, bone, diagenesis, histology, autofluorescence, bacteria

Key phrases:
Forensic taphonomy, forensic anthropology, bone diagenesis, postmortem interval

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Copyright Statement

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Abbreviations

ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BI	Birefringence Index
HTA	Human Tissue Act
L	Longitudinal
LC	Liquid chromatography
LSM	Laser Scanning Microscope
MFD	Microscopical Foci of Destruction
MS	Mass Spectrometry
OHI	Oxford Histological Index
PCA	Principle Component Analysis
PMI	Post mortem Interval
SRIEC	School Research Integrity and Ethics Committee
T	Transverse
TBS	Total Body Score
UV	Ultra-violet

Chapter One:

1.1 Introduction

The purpose of this research was to determine the origin of the microbes that drive the decomposition of skeletal remains. It is believed that by studying the changes that bone can undergo after death, post mortem interval (PMI) estimations could be greatly improved (Ross & Cunningham, 2011; Hoke *et al*, 2013; Boaks *et al*, 2014). This could have huge implications in forensic science and police investigations, as well as having the potential to help in archaeological research.

During police investigations it can sometimes be necessary to analyse skeletal remains. This is most often undertaken by a forensic anthropologist, and during these anthropological investigations, the environment in which the remains have been left can have a major impact on the integrity of the bone (Cappella *et al*, 2018). This is due to microbial interactions which can cause measurable physical and chemical changes to the microstructure of the hard tissue. These changes are referred to as 'bone diagenesis' (Langley & Tersigni-Tarrant, 2017). Macroscopically, these changes can present as weathering, in the form of cracking, discolouration, and flaking to the external surface of the bone (Behrensmeyer, 1978; Pokines *et al*, 2018). Histologically, they can be seen as microbial infiltration. Known as microscopical foci of destruction (MFD), they present as tunnels and bores in the microstructure of the bone, obliterating the osteons and other features in their wake (White & Booth, 2014; Booth *et al*, 2016). Collagen, which makes up 90% of the organic matter within bone, has the ability to fluoresce under light from the ultra-violet (UV) spectrum. Due to this ability, the loss of collagen can also be measured as a reduction in fluorescence can be seen (Ramsthaler *et al*, 2009; Hoke *et al*, 2013). Proteomic analysis has recently been introduced as a potential source of information when analysing skeletal remains (Procopio *et al*, 2018). This analysis has mostly been utilised in archaeological contexts for detecting bone diagenesis (Buckley & Wadsworth, 2014). However, recent research conducted by Procopio *et al* (2017; 2018) has shown potential for PMI estimation.

Current literature focuses on two main themes; determining between archaeological bones, generally those more than 100 years old (Creamer & Buck, 2009), and those of forensic interest (Maggiano *et al*, 2006; Creamer & Buck, 2009; Capasso *et al*, 2017); and more sensitive methods for determining PMI (Ross & Cunningham, 2011; Wilson & Christensen, 2017; Cappella *et al*, 2018). Current research into PMI estimation centres around conflicting theories of the source of the microbes that drive bone diagenesis, with literature citing both the endogenous

microbiome of the gut (Jans *et al*, 2004; White & Booth, 2014), and the exogenous colonies found in soil (Hackett, 1981; Reiche *et al*, 2003).

The ability to accurately estimate PMI is of importance to police investigations as it could have the potential to help establish timelines, narrow down suspects, locate further evidence, and determine victim identity in the case of unidentified remains (Overholtzer, 2015; Pérez-Martínez *et al*, 2017). When skeletonised remains are found, the first thing to establish is whether they are of forensic interest or archaeological (Maggiano *et al*, 2006; Hoke *et al*, 2013; Capasso *et al*, 2017; Cappella *et al*, 2018). This can often be an ambiguous question as the cut-off limit can vary from region to region, for example Hoke *et al* (2013) writes that less than 50-60 years PMI is classed as forensically relevant, Ramsthaler *et al* (2009) states the limit as 30-50 years, while Creamer and Buck (2009) go further with a limit of 100 years. Once this has been established, it is necessary to narrow down the post mortem interval. Due to the complex process of decomposition, however, this has proven to be difficult to achieve. At best, forensic anthropologists can give a range of time; this can often be in the range of months in the case of fully skeletonised remains – the longer the PMI, the longer the range is (Langley & Tersigni-Tarrant, 2017).

Much research has already been conducted into ways to accurately estimate PMI but there is still much more to do (Ross & Cunningham, 2011; Hoke *et al*, 2013; Wilson & Christensen, 2017; Cappella *et al*, 2018). Decomposition is a complicated process and as such, it can be affected by many factors; this includes intrinsic factors such as the health and condition of the individual, and extrinsic factors, for example the burial environment, clothes, temperature (Langley & Tersigni-Tarrant, 2017). With this in mind, this project focused on two different conditions; buried in soil vs exposed on the surface, to determine how the different environments affect the decomposition process and level of bone diagenesis observed.

Further to this, the project's main aim was to determine the origins of the microbes that drive bone diagenesis. To answer this, various samples were left in the two conditions at HuddersFIELD, the University of Huddersfield's Outdoor Taphonomic Facility. These samples were divided into three categories; whole rats (*Rattus rattus*), the fleshed legs of rats, the defleshed long bones of rats. The purpose of these categories was to control the origin of potential microbes; the whole rats would contain endogenous bacteria from the body, including the gut bacteria; while the fleshed legs would contain only any bacteria present in the flesh (if any); and the defleshed bones would theoretically be free of endogenous bacteria associated with the flesh, so any bacteria seen could be assumed to be environmental bacteria (exogenous

bacteria). The long bones were extracted from the whole rats and fleshed legs and analysed alongside the defleshed bones to determine the presence of any diagenetic processes. The bones from the three categories were compared to determine the level of diagenetic alterations, and whether bone in one category/condition was more susceptible to diagenetic changes than the others.

A variety of techniques were used to analyse these samples; these ranged from macroscopic observations of the samples when they were recovered from HuddersFIELD, to the microscopic changes on the bone surface and within cross sections of the bones. To determine the PMI of human remains, forensic specialists can refer to the Total Body Score (TBS), a table of known, observable changes that occur to the body after death (Langley & Tersigni-Tarrant, 2017). This project used an adapted version of TBS to note changes that occurred to the soft tissues of the rat samples over time, which took into consideration differences such as fur and body size. Bone surface changes were analysed using a Keyence microscope, while histological changes to the bone were observed under compound microscopy, Keyence digital microscopy and Confocal laser scanning microscope (LSM). The Keyence was chosen due to its greater magnification ability and clarity of images while the Confocal LSM was chosen as a novel technique. The ability to conduct histological analysis with little preparation of the samples was an added factor, as was the Confocal LSM's potential to quantify collagen loss via fluorescence of the bones. With the help of Dr Procopio at Northumbria University, proteomic analysis was conducted on some samples. This was to determine whether any changes in protein content were affected by increasing PMI, different deposition environments, or tissue types.

Rats were used in this project as an alternative to human tissues. In the case of forensic relevance, it would have been preferable to obtain human subjects, however this was not an option due to restrictions in the UK (HTA, 2004). When it comes to the use of human tissue, there are certain factors to consider; the main two being consent, and the ethical implications of the research being conducted. The Human Tissue Act 2004, does not strictly prohibit the use of human remains for this type of research, therefore it could be possible were the remains donated specifically to this type of project, however the ethical considerations would also have to be met. Animals, such as pigs (*Sus scrofa domesticus*), are often used as analogues for humans, but this is not ideal. Differences in diet, disease, gut microbiome, bone microstructure (to name a few), can make them unreliable substitutes (Jans *et al*, 2004; Brönnimann *et al*, 2018). In fact, Jans *et al* (2004) found that animals bones are less likely to be attacked by microbes than human bone, and when they are attacked, fungal microbes are more likely to be

the cause than bacterial. A comparative atlas was used to compare rat bone to human bone, and to take into account any differences in the microstructure (Treuting *et al*, 2017).

1.2 Aims

The aims of the research are:

- To determine the origin of the microbes which drive bone diagenesis.
- To determine which techniques best identify bone diagenesis.
- To determine which criteria works best for quantifying bone diagenesis.
- To determine what impact the deposition environment has on bone diagenesis.

1.3 Research Questions

1. What is bone diagenesis?
2. How is it defined? (In terms of the following parameters: weathering patterns, discolouration, internal structure, tunnelling, bacterial infiltration, histological changes, collagen content using birefringence and UV fluorescence).
3. How do these parameters change with time?
4. Can changes in these parameters be used to determine post mortem interval?
5. Does autolysis (the cell lead decomposition) affect bone diagenesis? (Measured by set parameters using excised, fleshed legs).
6. Does the presence of internal gut bacteria influence bone diagenesis? (Measured by set parameters using whole rats).
7. Does the presence of soil influence bone diagenesis? (Measured using the parameters set above).

1.4 Hypotheses

Alternative hypotheses are presented, the null hypotheses are given in italics.

- There will be a statistically significant increase in bone diagenesis as measured by weathering patterns in the bones of the exposed, defleshed samples, compared to the whole and fleshed samples.
- *There will not be a statistically significant increase in bone diagenesis as measured by weathering patterns in the bones of the exposed, defleshed samples, compared to the whole and fleshed samples.*
- There will be a statistically significant increase in bone diagenesis as measured by discolouration in the bones of the buried, defleshed samples, compared to the whole and fleshed samples.

- *There will not be a statistically significant increase in bone diagenesis as measured by discolouration in the bones of the buried, defleshed samples, compared to the whole and fleshed samples.*
- There will be a statistically significant increase in bone diagenesis as measured by the integrity of the internal structure in the bones of the whole samples, compared to the fleshed and defleshed samples.
- *There will not be a statistically significant increase in bone diagenesis as measured by the integrity of the internal structure in the bones of the whole samples, compared to the fleshed and defleshed samples.*
- There will be a statistically significant increase in bone diagenesis as measured by tunnelling due to bacterial infiltration in the bones of the whole samples, compared to the fleshed and defleshed samples.
- *There will not be a statistically significant increase in bone diagenesis as measured by tunnelling due to bacterial infiltration in the bones of the whole samples, compared to the fleshed and defleshed samples.*
- There will be a statistically significant increase in bone diagenesis as measured by histological changes in the bones of the whole samples, compared to the fleshed and defleshed samples.
- *There will not be a statistically significant increase in bone diagenesis as measured by histological changes in the bones of the whole samples, compared to the fleshed and defleshed samples.*
- There will be a statistically significant increase in bone diagenesis as measured by the decrease in collagen content in the bones of the whole samples, compared to the fleshed and defleshed samples.
- *There will not be a statistically significant increase in bone diagenesis as measured by the decrease in collagen content in the bones of the whole samples, compared to the fleshed and defleshed samples.*
- There will be a statistically significant increase in bone diagenesis as measured by the change in bone proteome in the bones of the whole samples, compared to the fleshed and defleshed samples.
- *There will not be a statistically significant increase in bone diagenesis as measured by the change in bone proteome in the bones of the whole samples, compared to the fleshed and defleshed samples.*

General observations

- There will be a continuing, measurable increase in weathering in the bones of the exposed samples over the time period of 32 weeks.
- *There will not be a continuing, measurable increase in weathering in the bones of the exposed samples over the time period of 32 weeks.*
- There will be a continuing increase in discolouration in the bones of the buried samples over the time period of 32 weeks.
- *There will be a continuing increase in discolouration in the bones of the buried samples over the time period of 32 weeks.*
- There will be a continuing, measurable change in the integrity of the internal structure of the bones in all conditions over the time period of 32 weeks.
- *There will not be a continuing, measurable change in the integrity of the internal structure of the bones in all conditions over the time period of 32 weeks.*
- There will be a continuing, measurable increase in tunnelling due to bacterial infiltration in the bones in all conditions over the time period of 32 weeks.
- *There will not be a continuing, measurable increase in tunnelling due to bacterial infiltration in the bones in all conditions over the time period of 32 weeks.*
- There will be a continuing, measurable increase in histological changes in the bones in all conditions over the time period of 32 weeks.
- *There will not be a continuing, measurable increase in histological changes in the bones in all conditions over the time period of 32 weeks.*
- There will be a continuing, measurable decrease in collagen content in the bones in all conditions over the time period of 32 weeks.
- *There will not be a continuing, measurable decrease in collagen content in the bones in all conditions over the time period of 32 weeks.*

Condition/origin of bacteria

- There will be a measurable change in bone diagenesis in the bones of the fleshed samples due to autolysis.
- *There will not be a measurable change in bone diagenesis in the bones of the fleshed samples due to autolysis.*
- There will be a statistically significant increase in bone diagenesis in the bones of the whole samples due to the presence of gut bacteria, compared to the fleshed and defleshed samples.

- *There will not be a statistically significant increase in bone diagenesis in the bones of the whole samples due to the presence of gut bacteria, compared to the fleshed and defleshed samples.*
- There will be a measurable difference in bone diagenesis in the bones of the buried samples, compared to the exposed samples.
- *There will not be a measurable difference in bone diagenesis in the bones of the buried samples, compared to the exposed samples.*

This research has potential to impact forensic science as an understanding of the origins of bacteria which drive decomposition could help in the cases of clandestine graves (White & Booth, 2014). Not only could it help establish the PMI of the remains, it could also have the potential to determine whether the remains have previously been buried elsewhere due to the effect different environments could have on decomposition (Langley & Tersigni-Tarrant, 2017). Further to this, it could also inform the archaeological sciences in their understanding of bone degradation.

Chapter Two:

Taphonomy and Bone Diagenesis – Current Research and Theories

2.1 Introduction

It is well known that the soft tissues decompose after death. Not only has there been much research into this, but it is obvious due to the visible changes remains undergo (Wilson & Christensen, 2017). What is lesser known, particularly in non-research fields, is that the hard tissues also undergo decomposition (Ross & Cunningham, 2011). Unlike the soft tissues, these changes are not as obvious; they occur at a much slower rate and, with the exception of surface modifications as the result of weathering and/or soil erosion, they are not easily visible (Jans *et al*, 2004). These changes occur to the microstructure of the osteological tissue, and therefore cannot be seen without the aid of histological or analytical equipment (Turner-Walker & Syversen, 2002; Jans *et al*, 2004).

A greater understanding of bone diagenesis and the forms it can take could have important implications for the fields of forensic anthropology, and archaeology. For the former, it could lead to better techniques for estimating the PMI, while for the latter it could give an understanding of how long bones are likely to last in different burial contexts (Capella *et al*, 2018). More accurate techniques for establishing PMI is of great interest to many researchers and much has been written on this (Bell *et al*, 1996; Creamer & Buck, 2009; Ramsthaler *et al*, 2011; Wilson & Christensen, 2017; Capella *et al*, 2018; Sarabia *et al*, 2018). Due to the complex nature of decomposition, however, determining PMI accurately is somewhat elusive (Vass, 2011); currently forensic investigators can determine a range of time but this becomes less accurate the longer the post mortem period (Wilson & Christensen, 2017).

Bone diagenesis studies have mostly focused on archaeological samples (Reiche *et al*, 2003; Booth *et al*, 2016; Capella *et al*, 2018), therefore it had been assumed that diagenesis only occurred once remains had reached the skeletal phase; the final and longest stage of decomposition (Brönnimann *et al*, 2018). Recent studies, however, have led to a shift in this assumption (Bell *et al*, 1996; Boaks *et al*, 2014; White & Booth, 2014).

There are four ways to determine whether bone diagenesis has taken place. According to Dixon *et al* (2008) they are;

1. The destruction of the histological integrity,
2. Change in crystallinity,
3. Increase in porosity,

4. Loss of collagen

This research focuses on the loss of histological integrity and current literature cites the most likely causes of these changes to be microbial infiltration, either fungal (generally only seen in aquatic depositions), or bacterial (Hackett, 1981). There are two theories for the origins of these bacteria (White & Booth, 2014);

1. The endogenous bacteria of the gut microbiome
2. The exogenous bacteria from the burial environment, such as bacterial colonies within soil.

This review will discuss the taphonomic processes that lead to bone diagenesis, in particular the loss of histological integrity, and the key research that has been conducted in this area.

2.2 Process of Decomposition

The phrase 'taphonomy' comes from the Greek words, *Taphos* and *Nomos*, meaning grave law (Blau, 2014). It is the study of decomposition; a broad subject that covers the processes from the point of death through to the retrieval of the remains (Pokines & Symes, 2014). Decomposition begins soon after death and can be broken down into stages (Goff, 2009). While the order of each stage can be determined with some accuracy (Brooks, 2016), due to the potential effects of intrinsic and extrinsic factors, the rate at which each stage occurs cannot be (Gelderman *et al*, 2019).

Langley & Tersigni-Tarrant (2017) write that there are five stages of decomposition;

Table 2.1 Stages of decomposition.

Stage	Characteristics	Time Interval
Fresh	Algor mortis, livor mortis, rigor mortis	1 day – approx. 1 week
Discolouration	Marbling, abdomen slight green, skin slippage	Day 1 – weeks
Bloat (active decomposition)	Distention of tissues, overall colour changes, leaching of VFAs	Starts within 48 hours
Advanced decomposition (initial skeletonisation)	Purging of body fluids, exposure of skeletal elements	Starts at least 1 week after death
Skeletonisation	Exposure and drying of the entire skeleton	Several weeks – months after death

Source: Langley & Tersigni-Tarrant (2017). p.281Autolysis is the first stage to occur; this is the destruction of the cells due to swelling, which in turn is caused by the lack of oxygen being circulated (Pokines & Symes, 2014). Within the first 24 hours the *Mortis* Triad takes place;

1. Livor mortis – the settling of the blood to the lower parts of the body due to gravity, resulting in discolouration (Goff, 2009). This can be seen within less than an hour of death, and can become 'fixed' around 8 hours after (Pokines & Symes, 2014),
2. Rigor mortis – the stiffening of the muscles (Pokines & Symes, 2014). This occurs as a result of a decrease in ATP and pH, it can begin within 2 hours of death occurring, with the body entering full rigor at around 12 hours after death (Goff, 2009). It is generally accepted that this stiffness lasts for around 12 hours before starting to disappear, again over the course of 12 hours (Brooks, 2016), although extrinsic factors, such as temperature, can change the rate at which this occurs (Goff, 2009; Cockle & Bell, 2015).
3. Algor mortis – the cooling of the body temperature (Goff, 2009). This can be greatly affected by both intrinsic and extrinsic factors, such as mode of death, size of the individual, placement of the individual in relation to heat sources (Goff, 2008; Pokines & Symes, 2014).

Putrefaction, also referred to as the bloating or active stage (Langley & Tersigni-Tarrant, 2017), occurs after. This is the destruction of the soft tissues by the host's own microbiome and is discussed in section 2.3.1.

Advanced decomposition occurs when skeletonisation begins (Pokines & Symes, 2014). The microbial activities of the intrinsic bacteria, as well as the activities of various insects that feed on the remains, result in the destruction of the soft tissues and exposure of the osteological material (Pokines & Symes, 2014; Langley & Tersigni-Tarrant, 2017).

The final, and longest stage, of decomposition is the skeletal stage (Langley & Tersigni-Tarrant, 2017). This is considered to have been reached when more than 50% of the bone tissue has been exposed (Langley & Tersigni-Tarrant, 2017).

Current PMI estimation techniques take these stages into account; it is clear from Table 2.1 that as the remains progress onto each stage, the potential time interval increases. The mortis triad; *algor mortis, rigor mortis, livor mortis*, can help in the first hours, or even days after death (Byers, 2017) but once this stage has passed, methods of PMI estimation become less accurate (Wilson & Christensen, 2017). The skeletonisation stage is prone to the most inaccuracy when determining the PMI interval. This is in part due to a lack of understanding of the influence the endogenous and exogenous microbes have on the skeletal tissue (White & Booth, 2014; Wilson & Christensen, 2017).

2.3 The Role of Bacteria

Bacteria plays a big role in the process of decomposition (Hyde *et al*, 2015), it is the driving force behind the putrefactive stage and leads to the eventual skeletonisation of the remains (Goff, 2009; Hyde *et al*, 2015). These bacteria come from a variety of sources; from the intrinsic bacteria in the body, to the extrinsic bacteria found in the environment, most often that found within the burial soil and/or carried to the remains via the insects that come to feed on the soft tissue (Goff, 2009).

2.3.1 The Microbiome of the Human Gut

The human body contains tens of trillions of bacterial cells (Ursell *et al*, 2012). While these are found throughout the body, the majority reside in the gut, helping to ensure a healthy digestive system (Lloyd-Price *et al*, 2016). Systems within the body help to keep these microbes in place, and their numbers limited in life (Goff, 2009) However, in death, they are able to migrate throughout the body as the process of decomposition occurs.

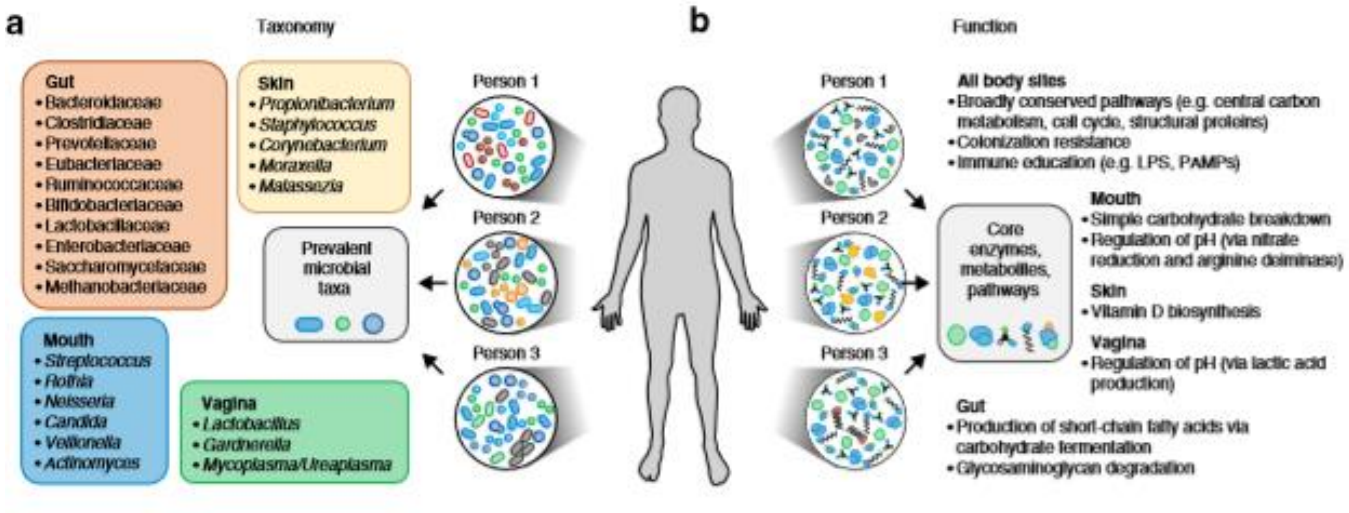


Fig 2.1 A healthy microbiome. Source: Lloyd-Price *et al* (2016)

These microbes, shown in Fig 2.1, waste no time digesting the soft tissues of the body after death, resulting in the putrefaction stage (DeBruyn & Hauther, 2017). This occurs first in the gut, where the greatest abundance of bacteria reside (Hyde *et al*, 2013). As the bacteria digest the tissues, gases are produced resulting in bloating of the abdomen and the purging of fluids from the body (Goff, 2009; Hyde *et al*, 2015). It is already understood that these microbes play a big role in the decomposition of the soft tissues, but the role they play in bone diagenesis is still a subject of debate (Bell *et al*, 1996; White & Booth, 2014).

Research undertaken by White & Booth (2014) aimed to show whether these endogenous microbes played a part in bone diagenesis. This study involved a comparison of stillborn piglets (*Sus scrofa*) to piglets of less than 6 months of age, using the theory that the stillborn piglets

would have no gut microbiome due to the sterile environment of the uterus (White & Booth, 2014). It has long been theorised that the uterus is absent of bacterial microbes during pregnancy and therefore babies are sterile until the moment of birth (Lim *et al*, 2019). The White & Booth (2014) study does appear to support the endogenous microbial theory, with the stillborn piglets showing no bacterial infiltration when compared with the older piglets. The older piglets did not display the expected diagenetic changes, with the authors theorising that they were most likely seeing an earlier form of microbial tunnelling (White & Booth, 2014). Given UK farming practices however, and the overuse of antibiotics (White, 2009; Sustain, 2019), one has to wonder whether the piglets used were not the most ideal candidates when looking for bacterial origins of bone diagenesis. It has been well established that antibiotics change the gut microbiome of the users; in this case the mother pigs, and the surviving piglets, therefore they could have the potential to affect any results seen (Ursell *et al*, 2012). Out of twelve piglets, only two had been stillborn; this is a small number of biological replicates and not enough to be statistically significant in supporting the endogenous microbiome theory.

Other literature has suggested support for the endogenous theory (Bell *et al*, 1996; Jans *et al*, 2004; Booth *et al*, 2016). Jans *et al* (2004) found that the bones from complete burials, were more likely to show bacterial infiltration than the bones from incomplete burials. This suggests that the microbiome of the gut could have influenced the diagenetic changes seen (Jans *et al*, 2004), however the incomplete burials were because of butchery, therefore the lack of bacterial infiltration may have been due to how the remains were treated prior to burial rather than the lack of putrefaction.

2.3.2 Bacterial Colonies in Soil

A body that has been buried will not decompose as quickly as one that has been left on the surface (Goff, 2009). The soil provides protection against factors such as temperature changes, predator access, insects; these have an impact on the speed of decomposition (Goff, 2009). However, the body is not fully protected from microbial infiltration; soil contains its own colonies of bacteria and fungi, which have the potential to feed upon the remains (Metcalf *et al*, 2016).

Due to the complexity of the burial environment, the effects soil colonies could have on remains is little known (White, 2009; Hyde *et al*, 2015). It is believed that the bacteria could feed upon the body from the outside, and the presence of fungi in grave sites does appear to support this (Tranchida *et al*, 2014). However, factors such as burial depth can impact decomposition; fewer microbes will be found in the soil the deeper the burial (Fierer *et al*, 2003).

Recent soil studies have turned their attention to the changes bacterial colonies in soil undergo during the process of decomposition (Finley *et al*, 2015; Hyde *et al*, 2015; Procopio *et al*, 2019). The bacteria from the putrefactive stage is released from the body, allowing it to colonise the soil and change the natural microbiome (Child, 1995; Goff, 2009). Literature suggests that this change is short lived, however, with the more competitive endogenous soil bacteria eventually destroying the invading bacteria (Child, 1995).

Child (1995) writes that the destruction of the bacteria from the remains leads to it having a limited effect on bone diagenesis and therefore, what is seen must be caused by infiltration of the soil colonies, rather than infiltration from the body itself (Child, 1995). The theory that soil microbial colonies affect bone diagenesis has been supported by other researchers (Hackett, 1981; Reiche *et al*, 2003; Lopez-Costas *et al*, 2016).

Reiche *et al* (2002) used a variety of analytical methods, including scanning electron microscopy and elemental analysis. Overall, they supported the exogenous microbial theory, having found that the interactions between the skeletal tissue and the soil affected the level of diagenetic changes seen (Reiche *et al*, 2002). However, although there were several analyses involved in this research, the fact that these are archaeological samples must be taken into consideration. The burial practices cannot be known with certainty and these may have influenced how the bone has degraded. Also, given that they are archaeological, the soft tissue decomposed many years ago and the soil has had time to interact with the hard tissue; even if the endogenous origins theory is the correct one, the microbial communities in soil have had several years to interact with the remaining skeletal tissue. Of course, this is only true if microbes are present in the soil at the depth of burial. Given that microbial communities are more prevalent closer to the surface (Fierer *et al*, 2003) and the average burial depth is 6 feet (ICCM, 2004), the question must be asked whether soil microbes are present? If these colonies cannot survive at that depth, then how does bone diagenesis occur, if not caused by the host's own microbiome?

2.4 The Structure of Bone

Osteological tissue consists of two types (Kendall *et al*, 2018);

1. Cortical bone – also known as 'compact bone', it is a dense, hard material found externally on the long bones and flat bones (White & Folkens, 2005),
2. Cancellous bone – also referred to as 'spongy bone' due to its honeycomb appearance, it is found at the ends of the long bones and the centre of the flat bones (White & Folkens, 2005).

Despite their different structural appearances, these two types of tissue have the same molecular compositions (White & Folkens, 2005).

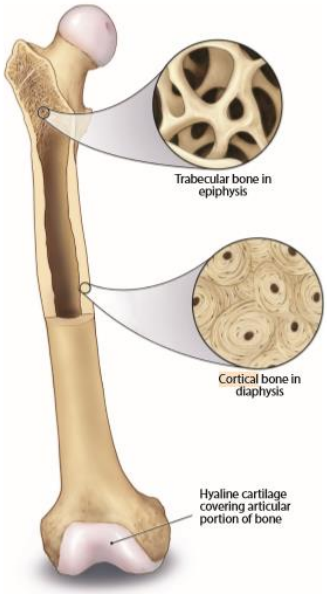


Fig 2.2
The gross anatomy of a long bone (femur). Source: Langley & Tersigni-Tarrant (2017), p.85

Bone diagenesis analysis primarily involves the cortical bone; this is usually taken from the mid-shafts of the long bones as there is a higher abundance of cortical bone tissue available (Turner-Walker & Syversen, 2002; Jans *et al*, 2004).

The internal structure of adult human bone is composed of *Osteons* (White & Folkens, 2005). A vascular canal (*Haversian canal*) takes its place at the centre, surrounded by lamellar bone (White & Folkens, 2005). This lamellar bone contains *osteocytes* which reside within lacunae; small cavities within the bone. Small canals (*canaliculi*) run between the lacunae, which allow the osteocytes to communicate with each other (Folkens & White, 2005). Collagen fibres make up the lamellar bone, and it is these bundles that give the bone it's strength (Byers, 2017). Bone diagenesis can affect the histological structure of the bone, including these collagen fibres (White & Booth, 2014; Assis *et al*, 2015; Delannoy *et al*, 2018).

2.4.1 *Rattus rattus*

Due to restrictions within the UK, this project used long bones from the domestic rat, rather than human bone. Rodent bones are very similar to human bone with just a few exceptions (Treuting *et al*, 2017).

Table 2.2 Comparison of human and rodent bone.

Feature	Rodent	Human
	Gross	
Vertebrae	Greater number of thoracic, lumbar, and coccygeal (tail) vertebrae than human	24 articulating cervical, thoracic, and lumbar vertebrae and 9 fused sacral and coccygeal vertebrae
Cervical	7	7
Thoracic	13; T2 large prominent spinous process, varies by strain	12
Lumbar	6	5
Sacral	4	5
Coccygeal	27–31	4

Source: Treuting *et al* (2017) p.70

Unlike in humans where the tibia and fibula are separate bones, the fibula is fused to the tibia in the rat (Treuting *et al*, 2017). The rat also has more vertebrae than humans due to the bones in their tails (Treuting *et al*, 2017).

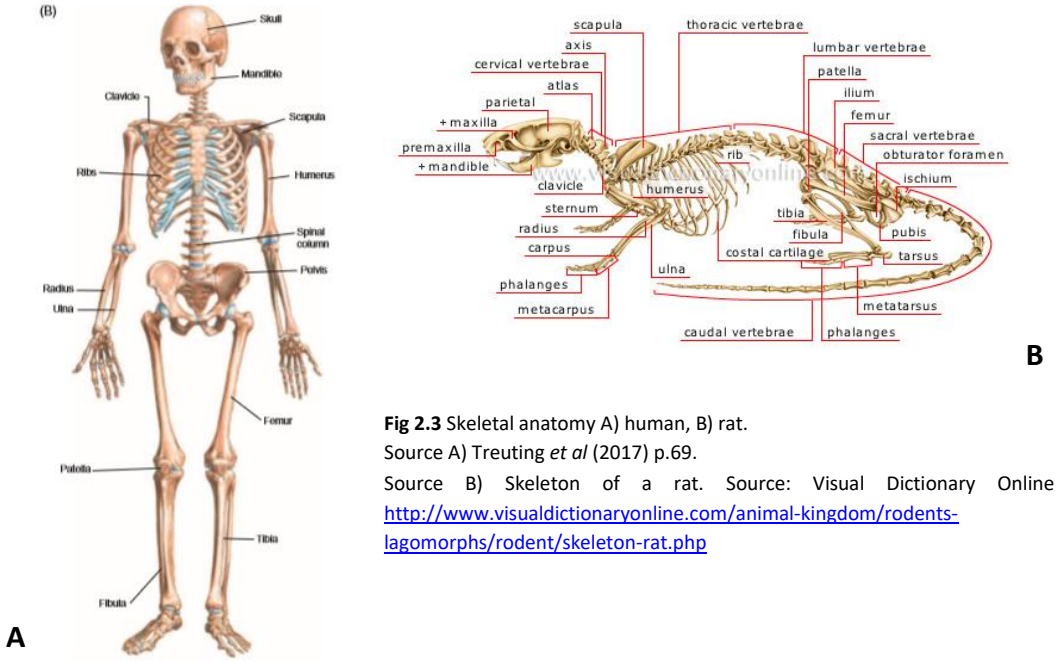
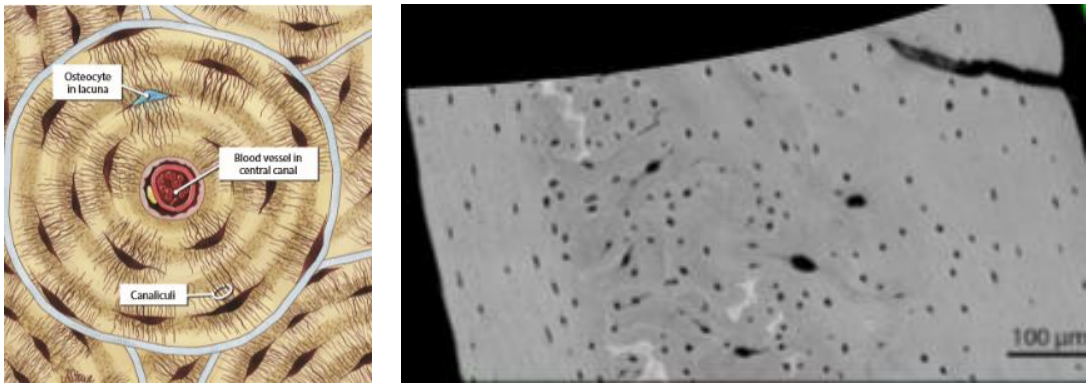


Fig 2.3 Skeletal anatomy A) human, B) rat.
Source A) Treuting *et al* (2017) p.69.
Source B) Skeleton of a rat. Source: Visual Dictionary Online http://www.visualdictionaryonline.com/animal-kingdom/rodents-lagomorphs/rodent/skeleton-rat.php

Unlike human bone, rodent bone does not contain osteons (Treuting *et al*, 2017). Instead, the bone is composed of two types; the central part is made up of disorganised bone, with osteocytes and vascular canals irregularly spaced within, while the outer sections are more organised with the osteocytes appearing more evenly spread, vascular canals are not often seen here (Bach-Gansmo *et al*, 2015).



A **B**

Fig 2.4 Bone cross sections A) Human. Source: Langley & Tersigni-Tarrant (2017) p.84. B) Rat. Source: Bach-Gansmo *et al* (2015)

Table 2.3 Comparison of human and rodent bone histology.

Feature	Rodent bone	Human bone
Cortical bone	Osteons absent; porosities develop with age	Osteons present
Cancellous bone	Secondary spongiosa present during first several months of life and decreases thereafter	Secondary spongiosa absent after skeletal maturity
Growth plate	Persists throughout life	Present only during growth and development
Articular cartilage	Thinner than human; variability in thickness of subchondral plate; no clear tidemark (in skeletally immature animals)	Thickness depends on joint; uniform subchondral plate; tidemark present
Bone marrow	Hematopoietic marrow in axial and appendicular skeleton throughout life	Hematopoietic marrow during development and in adult axial spine; fatty in adult appendicular skeleton
Woven bone	Present	Present
Lamellar bone	Present	Present
Osteoblasts	Present	Present
Osteoclasts	Present	Present
Brown fat	Present in adulthood largely in dorsal intrascapular region	Mostly involutes but can persist in adulthood in neck region

Source: Treuting *et al* (2017) p.70

2.5 Bone Diagenesis

Bone diagenesis is the term used to refer to the changes that bone can undergo after death (Hedges & Millard, 1995; Jans *et al*, 2004; Kendall *et al*, 2018). This is caused by many factors, such as chemical and mineral loss through the breakdown of the skeletal tissue (Collins *et al*, 2002) and the interaction between the skeletal tissue and the microbes nearby; potentially endogenous microbes or exogenous (White & Booth, 2014). Current literature cites both microbial origins as a potential cause of bone diagenesis (Reiche *et al*, 2003; Jans *et al*, 2004; White & Booth, 2014; Lopez-Costas *et al*, 2016) and is described below.

While this research focuses on microbial infiltrations, in the form of MFD, as a way of determining the presence of bone diagenesis, there are other ways to assess the level of preservation of skeletal material. Smith *et al* (2007) and Nielsen-Marsh *et al* (2007) noted that the porosity of the bone was of importance when determining how well preserved a sample was. This was a study conducted on archaeological samples, but the theory could be just as relevant in forensic samples. It was determined that different types of bone have unique pore structures. This could be utilised to assess the level of bone diagenesis that has taken place (Smith *et al*, 2007, Nielsen-Marsh *et al*, 2007). The results of their study indicated that on

average the pore size of the bone samples increased as the OHI category given decreased (Collins *et al*, 2007). Nielsen-Marsh *et al* (2007) notes that increased porosity can be a result of microbial infiltration.

2.5.1 Microbial Infiltration

The histological analysis of thin sections of bone can be used to determine the presence of microbial activity (Pokines & Symes, 2014). Much research has already been conducted with the focus being on thin sections (Hoke *et al*, 2011; White & Booth, 2014), but while there has been progress, there is still too much inconsistency in the results to produce a valid method of PMI estimation (Bell *et al*, 1996; Delannoy *et al*, 2018). The problem appears to stem from the complexity of decomposition; as with the soft tissues, many factors can affect the rate at which the bone degrades (Child, 1995; Delannoy *et al*, 2018). Intrinsic factors, such as the age, weight, health of the deceased can affect how decomposition occurs (Nicholson, 1996); Creamer & Buck (2009) found that the bones of juveniles are more susceptible to diagenetic effects. Extrinsic factors, such as mode of deposition, burial medium used, and climate, have been shown to have an effect also (Fernandez-Jalvo *et al*, 2010). To establish an accurate method of PMI estimation these factors, which can be many, all need to be considered.

The destruction of osteological tissue by microbial infiltration was first observed in 1864 by Carl Wedl when he noticed tunnelling within the microstructures of bones and teeth that had been left in well water (Hackett, 1981). He determined that these had been caused by fungal microbes within the water, and they later became known as *Wedl tunnels* (Hackett, 1981). Subsequent forms of tunnelling were identified by Hackett in 1981. These were given the title of *non-Wedl* tunnels and are thought to be caused by bacteria (Trueman & Martill, 2002).

Table 2.4 The four types of tunnelling identified and their causes

Type	Cause
Wedl tunnel	Fungal microbes
Linear longitudinal tunnel	Bacterial microbes
Budded tunnelling	
Lamellate tunnels	

(Jans *et al*, 2004)Hackett (1981) referred to these tunnels as 'microscopic foci of destruction' (MFD) and much has been written about them (Dixon *et al*, 2008; White & Booth, 2014; Brönnimann *et al*, 2018; Morales *et al*, 2018), leading to the development of the *Oxford Histological Index* (OHI) (Hedges & Millard, 1995).

Table 2.5 Oxford Histological Index

Category	Approx % intact bone	Description
0	<5	No original features identifiable other than Haversian canals
1	<15	Small areas of well-preserved bone present, or some lamellar structure preserved by pattern of destructive foci
2	<50	Some well-preserved bone present between destroyed areas
3	>50	Larger areas of well-preserved bone present
4	>85	Bone is fairly well preserved with minor amounts of destroyed areas
5	>95	Very well preserved, similar to modern bone

(Errickson & Thompson, 2017)

This index has been used by many to determine the level of destruction seen in the skeletal tissue (Jans *et al*, 2004; Dixon *et al*, 2008; Assis *et al,* 2015; Morales *et al*, 2018), however it should perhaps be used with caution. Given that it was developed with archaeological bone in mind, it may be of little use in research that is attempting to determine the level of skeletal destruction in bones of forensic interest. Certainly, this could be the case with White & Booth (2014), who saw unexpected diagenetic changes in their piglet specimens. It must also be noted that this index does not distinguish between the different types of infiltration seen; only the level of destruction occurring (Brönnimann *et al*, 2018).

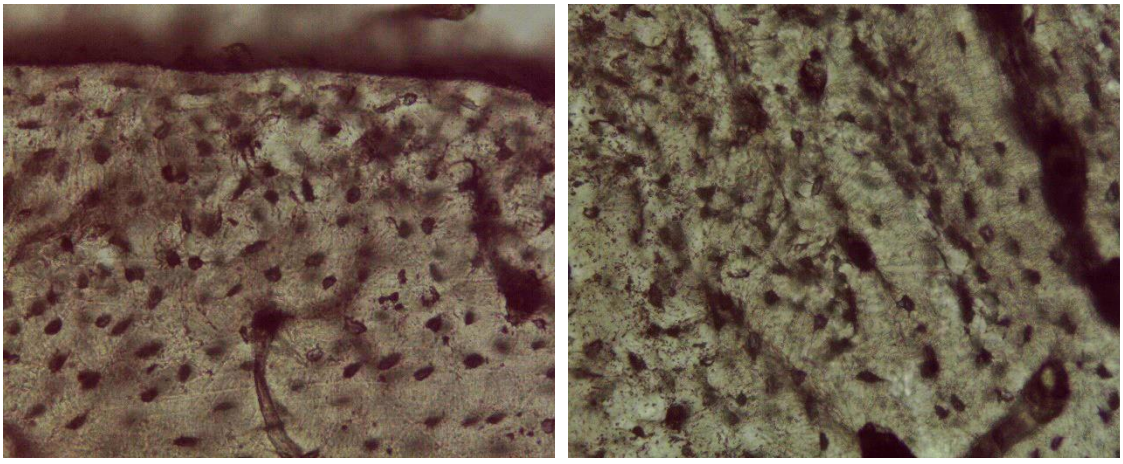


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Fig 2.5 Samples from HuddersFIELD. Samples were retrieved at 24 weeks PMI. x400 magnification. A) Exposed defleshed bone, B) Exposed whole rat Source: Author

2.5.2 Loss of Collagen

Collagen makes up 90% of the organic material in bone (Kendall *et al*, 2018). Literature states that this collagen can be lost through bone diagenesis (Dixon *et al*, 2008). The loss of collagen can be observed in several ways (Boaks *et al*, 2014; Mello *et al*, 2017). The most used method is analysing for the loss of birefringence (Brönnimann *et al*, 2018; Delannoy *et al*, 2018). This can

be done alongside light microscopy analysis and involves using cross polarised light to determine the level of birefringence seen in the sample.

Bone is a birefringent material due to the layout of the collagen fibres that make up the lamellar bone (Bell *et al*, 1996). As the collagen is destroyed by the process of diagenesis, this birefringence is lost (Delannoy *et al*, 2018); a method of measuring the loss in birefringence could directly measure the loss of collagen. Birefringence has been analysed by many researchers (Jans *et al*, 2002; Assis *et al*, 2015; Delannoy *et al*, 20018), however attempts at quantifying what is occurring has been limited. Jans *et al* (2002) determined the level of birefringence seen using a comparison method; fresh bone compared with the archaeological samples.

Current literature has also used the skeletal tissues autofluorescence to determine PMI (Hoke *et al*, 2011; Ramsthaler *et al*, 2011). This research has attempted to exploit the bones tendency to fluoresce when exposed to light of certain wavelengths; in most cases wavelengths within the ultra-violet (UV) spectrum (Hoke *et al*, 2011). It has been shown in some cases that the intensity of fluorescence, as well as the colour, could change over time (Hoke *et al*, 2013). While this may not be accurate enough to provide concrete PMI estimates for forensic investigations, it could one day have the potential to distinguish between archaeological bones and those of forensic interest (Ramsthaler *et al*, 2011).

2.5.3 Other Causes of Bone Diagenesis

While the focus of this research are the microbes that drive bone diagenesis, it must be noted that there are other factors which can also drive bone diagenesis.

Exposure to the natural elements can drive the degradation of bone; rainfall, sun exposure and temperature can lead to weathering, such as cracking and flaking. The extent of weathering can be determined using the Behrensmeyer weathering index (1978). Exposure to sunlight can lead to bleaching of the bones; extreme temperature changes can cause the surface of the bones to crack and flake over time. Using the Behrensmeyer weathering index (1978), it can be possible to determine the extent of weathering that has occurred. However, a study conducted by Fernandez-Jalvo *et al* (2010) found that most bone samples did not show signs of surface weathering, the exception were signs of corrosion at the sites where the bones touched the ground. Ross and Cunningham (2011) also found little evidence of surface weathering.

Collins *et al* (2002) writes that factors that can drive bone diagenesis include time, temperature and pH. High temperatures and/or extreme pH levels (acidic or alkali) can lead to the breakdown

and loss of proteins (Collins *et al*, 2002). A seven-year study conducted by Nicholson (1996) found little evidence of increased degradation occurring to samples at a higher pH.

Other analyses have been used to determine the presence of bone diagenesis. These include chemical methods (Knight & Lauder, 1967; Fernandez-Jalvo *et al*, 2010; López-Costas *et al*, 2016; Mello *et al,* 2017), which are not discussed as part of this project.

2.6 Summary

This chapter has given a brief review of the literature available on bone diagenesis. It has been shown that this process of decomposition is complicated and has many different pathways, with the histological integrity being at the forefront of research (White & Booth, 2014; Morales *et al*, 2018). It has been demonstrated that much has been written in this area, with many theories having been explored. However, there appears to be a lack of understanding in the origins of the microbes that drive diagenetic changes within skeletal material. White & Booth (2014) made progress in this area with their research, but little appears to have been carried out since. As they used stillborn piglets for their 'sterile' specimens, one cannot rule out the potential for contamination. While the theory still stands that the uterine environment is a sterile one (Lim *et al*, 2018), the potential for microbial presence cannot be eliminated. In the case of stillbirth, it has been shown that bacteria can be present within the amniotic fluid (Lim *et al*, 2018).

This is also the case for the literature in favour of the exogenous theory. Reiche *et al* (2003), for example, used archaeological bones as samples. As these bones will have been exposed to the gut microbiome during the initial soft tissue decomposition, it cannot be ruled out that the diagenetic changes seen are as a result of the putrefactive stage, rather than a result of the soil microbes.

This research has proposed something different. In an effort to determine the microbial origins, the questionable factors were removed as much as possible. This research used rat samples, with three different sample tissue types being chosen; whole rats to ensure the presence of the gut microbiome; excised, fleshed rat legs to allow for the effects of autolysis to be analysed; and defleshed bones from rats to remove all endogenous bacteria. Two environments were used; buried to allow the effects of soil microbes to be analysed; and exposed to show the absence of soil microbes.

Table 2.6 The microbes being included per condition. [Exc] Indicates microbes being excluded

	Exposed condition	Buried condition
Whole rats	Gut bacteria	Soil microbes Gut bacteria
	Exc *Soil microbes*	
Excised, fleshed legs	Autolytic effects	Soil microbes, Autolytic effects
	Exc *Soil microbes* *Gut bacteria*	Exc *Gut bacteria*
Defleshed bones	Environmental effects	Soil microbes
	Exc *Soil microbes* *Gut bacteria* *Autolytic effects*	Exc *Gut bacteria* *Autolytic effects*

There also appears to be some discrepancy with the way research in this area is presented, the histology, for example, is an observable analysis (White & Booth, 2014). While this is the nature of histological analysis, it would be beneficial to have some method of quantification in place. This appears to be the case not only for the tunnelling that can occur, but also for the autofluorescence and birefringence.

This project attempts to find a method of quantification for these analyses. Image software is used to determine a measurement of diagenetic alterations occurring in the histological contexts. The fluorescence of the samples are analysed further with the use of a Confocal LSM. This is an underutilised tool in bone diagenesis studies, and its ability to determine alterations in fluorescence are explored here.

Chapter Three:

Methods and Materials

This chapter sets out the methods that were followed in the course of this project, including problems that were encountered and how they were overcome.

It is noted that differences in the microstructure of rat bone could have the potential to affect the results seen in this study. These include, but are not limited to, the lack of haversian systems and presence of irregular vascular canals limiting the transmigration of the gut bacteria to the internal structure of the bone.

3.1 Sample Preparation

Forty-six medium sized (average weight = 151-250g) domestic rats (Rattus rattus) were used in this study. These were purchased from a reptile supplies store (https://www.reptilecentre.com/frozen-rats-medium-5-pack-151250g_p4023038.htm) and were not killed for the purpose of this research. The rats were flash frozen within 2-3 hours of death occurring and kept frozen until they were prepared for dissection. The rats arrived via 24-hour delivery packaged and still frozen. They were transferred to a freezer for storage until ready to be used (approximately 7 days). Prior to dissection, the required number of rats were defrosted at 4˚C for 24 hours. The rats were dissected over two days and kept refrigerated at 4˚C overnight[1].

An application for ethical approval was submitted to SRIEC and granted – grant number SAS-RIEC 18-2611-1.

Thirty-six rats were prepared as field samples; this was to allow for three repeats per condition and interval. A total of eight intervals were intended for this study, with samples being collected every four weeks. A further ten rats were put aside to be used as control/test samples.

There were two conditions; buried in soil and exposed on the surface of a plastic container. The tissue samples were divided into three categories; whole rats, the fleshed legs of rats, the defleshed long bones of rats.

[1] Time between death and deposition at HuddersFIELD was 10 days. It is assumed that freezing halts decomposition therefore PMI refers to time since deposition and does not consider time frozen.

Table 3.1 The conditions and categories used for this research. (n) indicates number of samples

Tissue samples		(n) Buried	(n) Exposed
Whole rats		8 (1 per condition)	8 (1 per condition)
Fleshed legs		24 (3x legs per condition)	24 (3x legs per condition)
Defleshed legs		24 (3x bones per condition)	24 (3x bones per condition)

Plastic boxes were used as this allowed some control over the deposition environment and easier control over scavenging animals. The boxes used measured 20x13.5x17 cm for the buried samples, as each repeat was buried separately, and 51x32x18 cm for the exposed samples. A different box was used for the buried samples to limit the need to disturb the burials until they were ready to be excavated. Holes were drilled into the bottom of all boxes to allow for drainage. Plastic lids were placed onto the buried sample boxes. *Godwins top soil* was used as a burial medium.

The whole rats were placed directly into their conditions as they required no dissections. Eight rats were placed into a large plastic container. Eight smaller 3 litre plastic containers were filled with 5 cm (approx 1.35 litres) of top soil. One whole rat was placed into each container and covered with a further 1.35 litres of top soil. Plastic lids were placed onto the boxes.

3.1.1 The Dissection

Examples of the boxes and their contents are shown in figs 3.1-3.6.

Fig 3.1 Small box with whole rats buried

Fig 3.2 Plastic box with exposed rats

All four limbs were removed from twelve rats. Twenty-four limbs were placed into a large plastic container. Eight smaller containers were prepared with 1.35 l of top soil. Three limbs were placed into each container and covered with a further 1.35 l of top soil. Plastic lids were placed onto the boxes.

Fig 3.3 Small box with fleshed legs buried

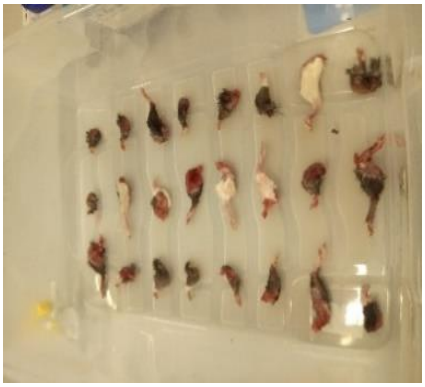


Fig 3.4 Large box containing exposed legs

All four long bones; femur and humerus, were removed from the remaining twelve rats using a size 10 scalpel. They were defleshed as much as possible without damaging the bone surface. Twenty-four long bones were placed into a large plastic container. Eight small containers were filled with 1.35 l of top soil, three long bones were placed into each box and covered with a further 1.35 l of top soil. Plastic lids were placed onto the boxes.

Fig 3.5 Small box with buried long bones

Fig 3.6 Large box with exposed bones

3.1.2 HuddersFIELD

On the afternoon of the 14th November 2018, the samples were transported to HuddersFIELD, the animal taphonomic facility used by the University of Huddersfield.

Elitech data loggers were used to record hourly temperatures of each condition[2]. They were placed into the three containers for weeks 4, 16, and 32 of the buried samples, and into all three boxes for the exposed samples.

The buried samples were placed behind the wall of a barn where they would be hidden from view and protected from the weather during the winter months. Large rocks and metal poles found in the field were placed on top of the boxes to prevent scavenger activity (see fig 3.7).

Fig 3.7
Small boxes in situ; back row whole rats, middle row fleshed, excised limbs, and front defleshed bone

The boxes containing the exposed samples were placed in a more open position within the field. The lids were removed to allow insects access. Metal wire cages were placed over the boxes to protect against scavenging animals (see fig 3.8).

Fig 3.8 Large boxes in situ; left to right, whole rat; fleshed, excised limbs; defleshed bone

3.2 Retrievals

Samples were taken every four weeks.

[2] The data loggers failed to record therefore average temperatures and rainfall were obtained from a local weather station.

Table 3.2 Dates samples were taken from HuddersFIELD

Collection number	Date
'Zero' - initial samples	14/11/18
1	14/12/18
2	08/01/19
3	05/02/19
4	05/03/19
5	02/04/19
6	30/04/19
7	28/05/19
8	25/06/19

Buried samples – as each set of samples were contained within their own boxes, one box each for the defleshed, fleshed, and whole, was collected each month. The lids were kept in place until they had been returned to the laboratory.

Exposed samples – photographs were taken of each box before retrieval of the samples. Three long bones, plus attached flesh in the cases of the fleshed and whole samples, were taken each time. They were placed into separate plastic bags.

3.2.1 Cleaning the bones

The bones all required cleaning prior to being frozen. The long bones from the fleshed and whole samples required dissecting. This was done in the following way;

Exposed bones	Cleaned using distilled water.
Exposed fleshed	Any flesh was dissected using a size 10 scalpel. The bones were rinsed in distilled water.
Exposed whole	The long bones were dissected using a size 10 scalpel then rinsed with distilled water.
Buried bones	The soil was sieved to find the bones. They were cleaned using distilled water.
Buried fleshed	The flesh was dissected from the bones using a size 10 scalpel. The bones were cleaned using distilled water.
Buried whole	The bones were dissected using a size 10 scalpel then cleaned with distilled water.

Photographs were taken of all samples throughout the cleaning process (see Chapter Four). The samples were placed into labelled plastic bags before freezing[3].

3.3 Histology and Analysis

Mid sections were taken for histological analysis. Fig 3.9 shows examples of defleshed long bones, the ends of the bones were not used for analysis.

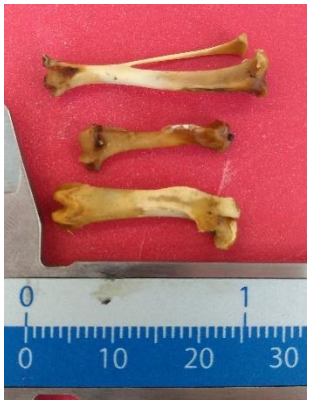


Fig 3.9 Examples of sections taken for analysis

Thin sections were prepared using the manual preparation technique adapted by Maat *et al* (2001). The technique was adapted slightly due to the small size of the samples.

Samples were cut into 3-4 mm thick pieces using a small hacksaw – fragile samples required thicker cuts. They were hand-sanded on wet 240 grit silicon-carbide sandpaper to remove most of the roughness. Once a sample thickness of 1 mm was achieved, the pieces were rinsed in distilled water to remove any excess grit. They were then further sanded on wet 1200 grit silicon-carbide sandpaper until the desired thickness was achieved. This was done by placing the sample onto a small piece of wet sandpaper. A further damp piece was placed on top of the sample, rough side down. This piece was moved in a circular motion to polish the sample. A small, clean paintbrush was used to turn the sample at regular intervals during this process to ensure an even sample. The sample was sanded until it started take on an opaque appearance.

Samples of various thickness were tested using a compound microscope; it was determined a thickness of 0.1 mm gave the best balance between clear images and keeping the samples whole. Fig 3.10 show examples of the thicker sample and the 'optimal' thickness – note the histological features are clearer in the thinner sample.

[3] It is noted that the freezing of the samples may have potentially affected results seen. As observed by Polkines *et al* (2018), freeze/thawing bone can lead to increased weathering effects, such as cracking, particularly superficial cracks to the surface and potential bone shrinkage.

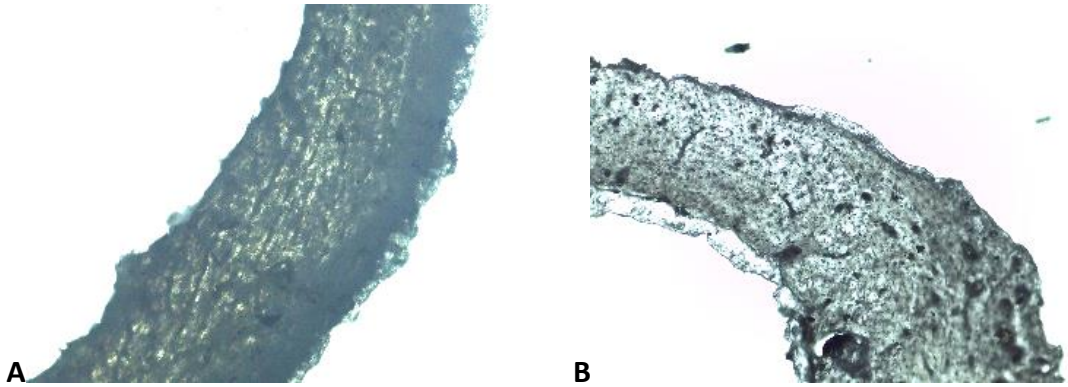


Fig 3.10 Trial samples A) 0.3mm thick sample; too dark to see histological features B) 0.1mm thick sample, histological features are clearly seen

Once the desired thickness was achieved, the sample was rinsed to remove any grit. This was a four-stage process, consisting of two small wells of distilled water mixed with a small amount of washing up liquid, and two further wells of distilled water. The washing up liquid allowed better removal of dirt as it stopped the grit from rising to the surface of the water (fig 3.11). Once rinsed, the sample was placed onto a clean, glass slide (fig 3.112).

Fig 3.11
Equipment used for manual preparation

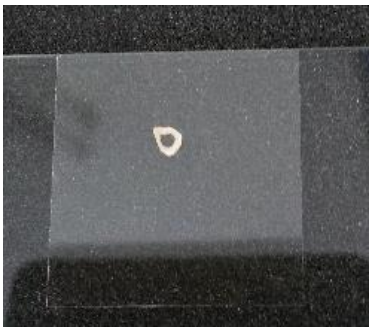


Fig 3.12
Thin sample on slide 0.1mm thick with glass coverslip

Desired thickness was confirmed using light microscopy. If the sample was too thick, it was further sanded using 1200 grit silicon-carbide sandpaper as described above. The desired thickness was achieved when it had reached 0.1mm, as determined using digital calipers.

3.3.1 Macroscopic Analysis

The samples were analysed in various ways to determine whether bone diagenesis was taking place. As fleshed and whole samples were used in this study, it was decided to take note of the decomposition of the soft tissues to determine whether the presence of the gut microbiome affected the rate of decomposition.

Soft Tissue Decomposition

The whole and fleshed samples were observed for signs of decomposition. An adapted version of the Total Body Score (TBS) based on rabbit decomposition (Adlam & Simmons, 2007) was used. This was selected due to the similarities in rats and rabbits, such as the smaller body size and presence of fur. A copy of the TBS for rabbits (Adlam & Simmons, 2007) can be found in

Appendix Two. The TBS used for human remains (Langley & Tersigni-Tarrant, 2017) can also be found in Appendix One for comparison.

Fig 3.13 Examples of decomposition seen, A) 8 weeks PMI B) 16 weeks PMI C) 4 weeks PMI

The whole samples were observed in three areas; head and neck, abdomen, and limbs. These scores were then added to give the TBS. Results can be found section 4.4 to show the extent of decomposition observed.

As the fleshed samples were limbs only, they were observed only in this category. Results can be found in section 4.4. Fig 3.13 shows examples of the decomposition seen at various PMI's.

Surface Modifications

The samples were analysed for any surface modifications and signs of weathering. A *VHX-2000* Keyence microscope was used to determine what changes, if any, were occurring. Further images can be found in Appendix Three. Fig 3.14 shows examples of surface modifications observed.

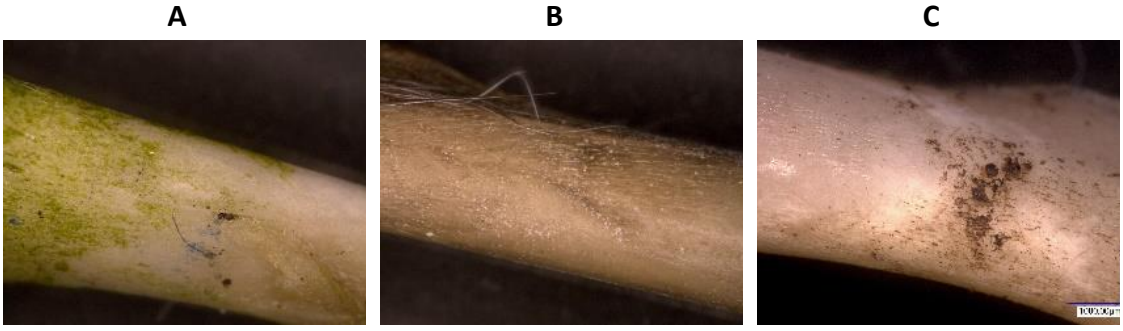


Fig 3.14 Examples of surface modifications seen, A) 16 weeks PMI B) 12 weeks PMI C) 12 weeks PMI

The buried samples were analysed for signs of soil erosion, flaking, discolouration. The exposed samples were analysed for signs of weathering. They were scored using the Behrensmeyer weathering index (1978) shown below (table 3.3):

Table 3.3 Behrensmeyer weathering index

Stage	Description
0	Bone is greasy and shows no signs of cracking or flaking; tissue may adhere to or cover the bone.
1	Bone exhibits cracking (longitudinal for long bone or mosaic for articular surfaces); residual soft tissue may be present.
2	Outer layer of cortex displays cracking and flaking; residual soft tissue may be present.
3	Outer layer of bone has roughened, fibrous patches where all cortical bone is missing; residual tissue is rarely seen.
4	Most of the outer bone surfaces is rough and fibrous, with no cortex remaining; cracks are open and have splintered.
5	Poor bone integrity, falling apart, extremely fragile, possibly spongy bone exposure, ill-defined bony shape.

Source: Langley, N.R. & Tersigni-Tarrant, M.A. (2017) p.285

Fig 3.15 gives examples of the weathering stages using the Behrensmeyer weathering index.

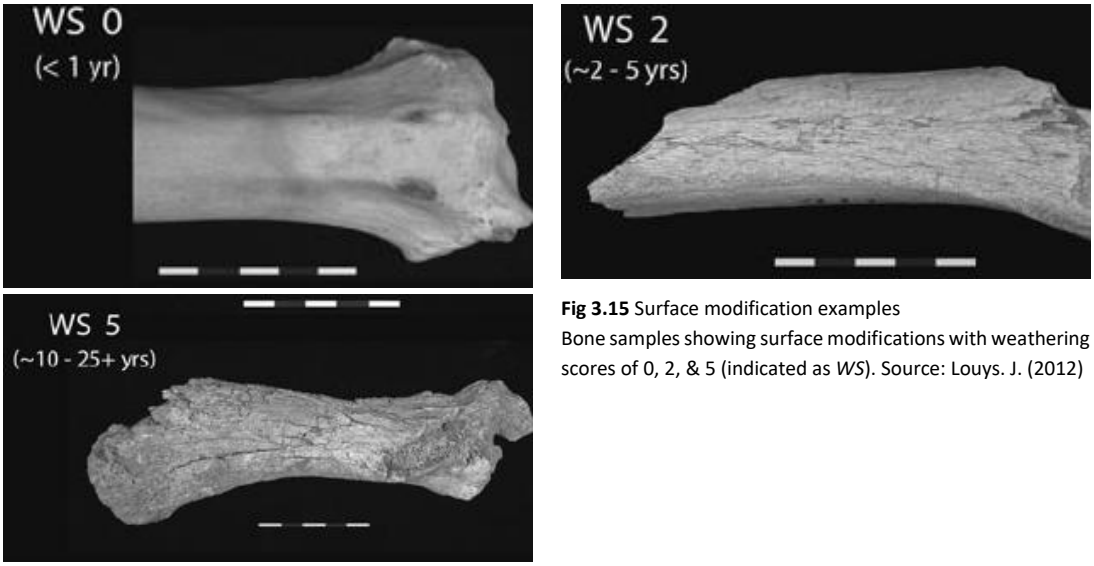
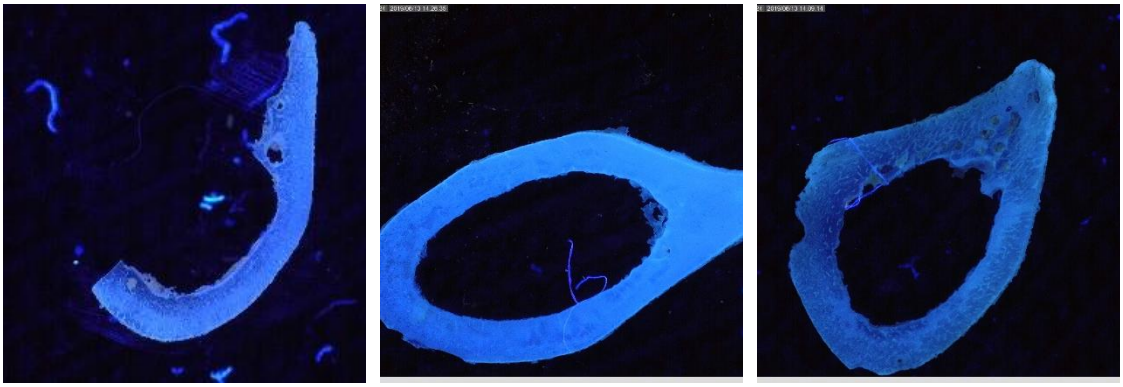


Fig 3.15 Surface modification examples
Bone samples showing surface modifications with weathering scores of 0, 2, & 5 (indicated as *WS*). Source: Louys. J. (2012)

Ultra-violet (UV) Fluorescence

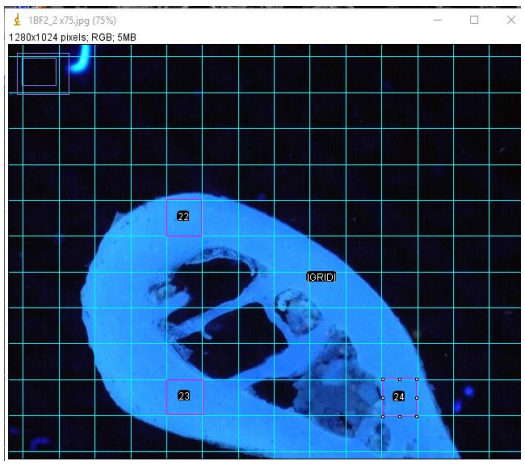
Studies conducted by Hoke *et al* (2011; 2013) and Yoshino *et al* (1991) showed the potential of measuring the level/intensity of autofluorescence in bone to determine PMI; due to the destruction of collagen within the bone structure. A *Dino-Lite* USB microscope with UV LEDs at a wavelength of 375 nm was used to assess fluorescence. Photographs of the autofluorescence were taken. These were compared with images of a control (fresh) sample to determine whether the samples' ability to fluoresce had been lost over time (fig 3.16).



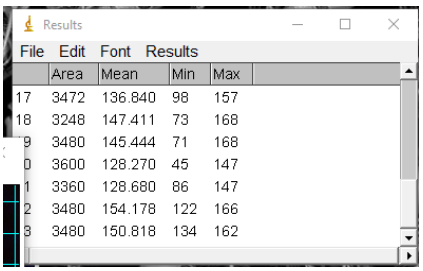
A **B** **C**

Fig 3.16 Examples of autofluorescence, A) fresh B) 8 weeks PMI C) 24 weeks PMI

ImageJ software was used to analyse fluorescence images. This was done by overlaying a grid at a specific size (3,500 pixels^2), three boxes were chosen at separate points on the sample and analysed using the *measure* function. The measurements were then used to calculate an average (fig 3.17).



A



B

Fig 3.17 ImageJ software. A) Sample with grid overlaid. B) Results table example.

3.3.2 Microscopic Analysis

The samples were analysed microscopically to check for any histological changes, such as tunnelling within the bones' microstructure. This was done in a variety of ways to maximise the chances of seeing any infiltrations. The following criteria were used to assess changes (table 3.4):

Oxford Histological Index (OHI)

Table 3.4 Oxford histological index

Category	Approx % intact bone	Description
0	<5	No original features identifiable other than Haversian canals
1	<15	Small areas of well-preserved bone present, or some lamellar structure preserved by pattern of destructive foci
2	<50	Some well-preserved bone present between destroyed areas
3	>50	Larger areas of well-preserved bone present
4	>85	Bone is fairly well preserved with minor amounts of destroyed areas
5	>95	Very well preserved, similar to modern bone

Source: Errickson & Thompson (2017)

Fig 3.18 gives examples of the OHI stages.

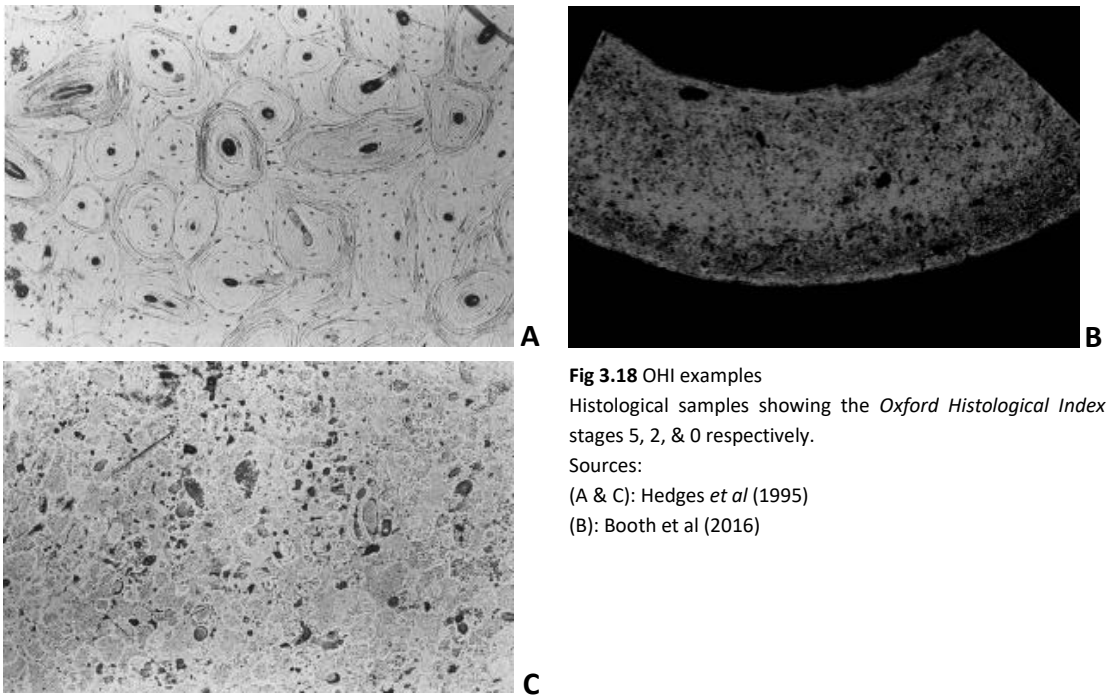


Fig 3.18 OHI examples
Histological samples showing the *Oxford Histological Index* stages 5, 2, & 0 respectively.
Sources:
(A & C): Hedges *et al* (1995)
(B): Booth et al (2016)

The type of tunnelling was also assessed where possible. Fig 3.19 is a schematic of each tunnelling type;

1. Wedl tunnelling – fungal caused
2. Linear longitudinal tunnel
3. Budded tunnelling — sometimes referred to as non-Wedl tunnelling
4. Lamellate tunnelling — Bacterial caused

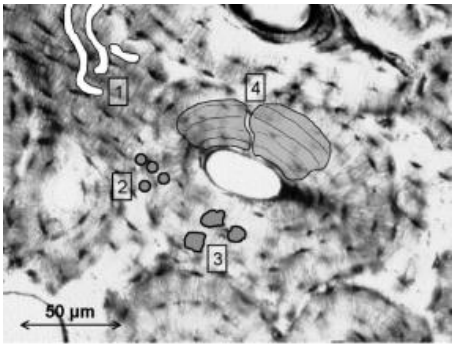


Fig 3.19 Types of tunnelling
Source: Jans *et al* (2004)

Osteocyte Count

Image software (*ImageJ*) was used to analyse the microscopy images. To do this a grid was overlaid onto the image being analysed at an area per point of 30,000 pixels^2 (fig 3.20). Three boxes were chosen from the grid; the number of osteocytes seen were counted within these boxes. These numbers were used to calculate an average number of osteocytes present per box.

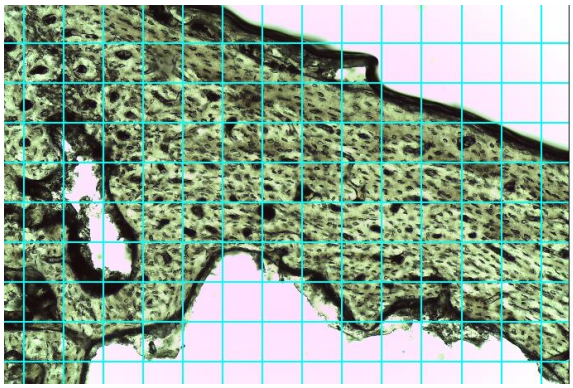


Fig 3.20 Osteocyte counting method. Fresh sample with grid overlaid on *ImageJ*

Compound Microscope

A *Leica ICC50HD* with a 10x eyepiece was used for this analysis to observe signs of diagenesis, such as tunnelling. The slide containing the sample was placed onto the stage. A lower magnification of 4x was used to establish where the sample was on the slide. Magnification was increased to 10x to capture images for quantitative analysis. 40x magnification was observed to verify the 10x images.

Birefringence:

Birefringence was analysed using light microscopy. This was done by placing a polarising light filter onto the microscope and turning it to achieve cross polarised light. This allowed the dark and light bands caused by the collagen content within the cross section of the bone to be seen. The birefringence of the field samples were compared to control (fresh) samples (fig 3.21).

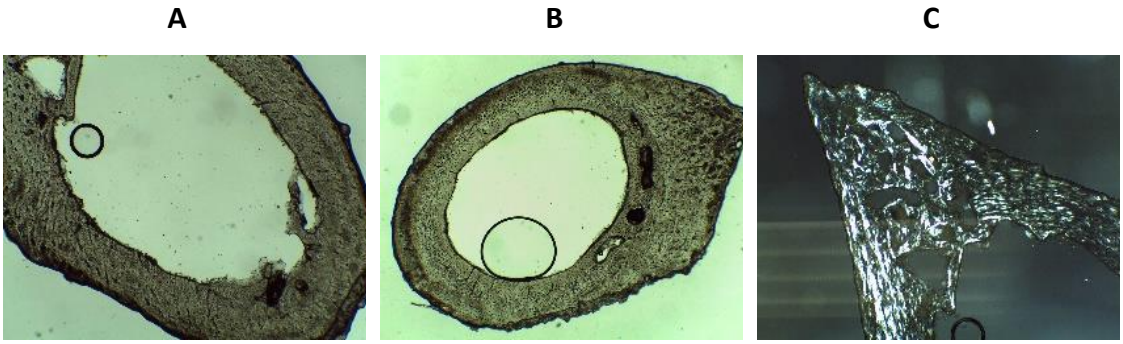


Fig 3.21 Histological sections, A) fresh B) 16 weeks PMI C) fresh birefringence

Keyence Microscopy

A Keyence microscope was used to analyse the samples at 500x and 1000x magnification to observe signs of diagenesis such as tunnelling and destruction of the microstructure of the sample (fig 3.22).

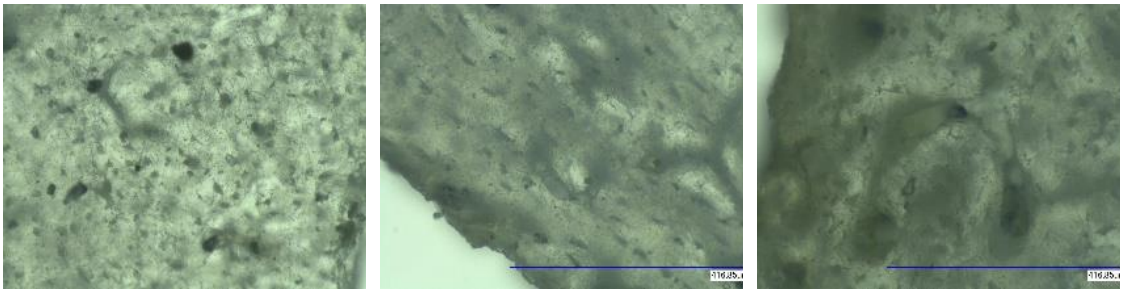


Fig 3.22 Histology sections 1000x magnification, A) fresh section B) 4 weeks PMI C) 16 weeks PMI

Confocal LSM

Confocal LSM was chosen because it allowed histological analysis and an examination of the fluorescence of the samples. Another advantage of the Confocal LSM was that it could analyse thicker samples; thicker samples of 1 mm thick were originally used. However, these proved too thick to allow for histological analysis, therefore this was changed to use the 0.1 mm thick samples (fig 3.23).

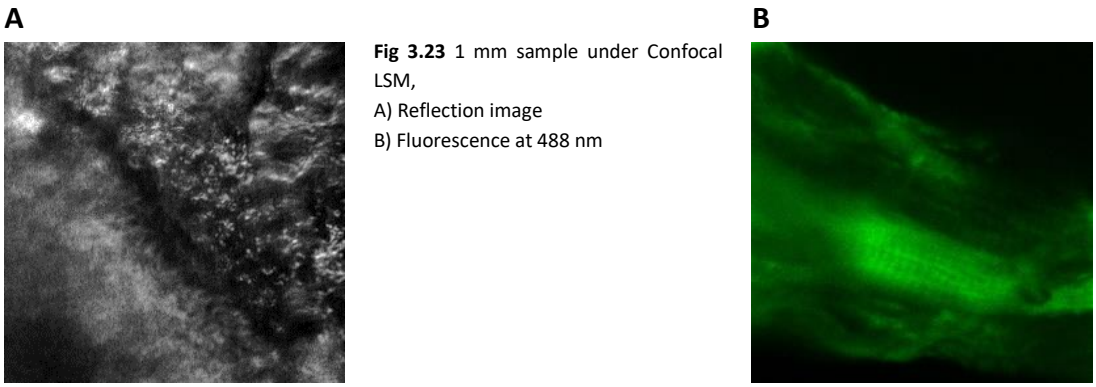


Fig 3.23 1 mm sample under Confocal LSM,
A) Reflection image
B) Fluorescence at 488 nm

The samples were examined on a *Zeiss LSM 880* Confocal microscope, using an excitation wavelength of 488nm as used by Capasso *et al* (2017). The detector was set to detect

fluorescence in the range of 508-695 nm. The placement of this fluorescence could be compared with a non-fluorescing image to determine where this was taking place (fig 3.24).

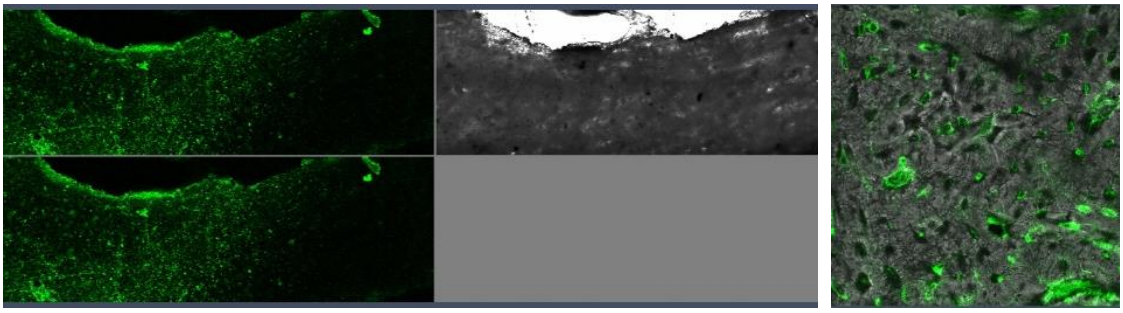


Fig 3.24 Confocal LSM, fresh sample, 0.1mm thickness

3.4 Proteomic Analysis

Samples from day 0, 12 weeks, and 24 weeks PMI were sent for proteomic analysis, in order to determine if there were changes in the bone proteome with time since burial. This was carried out by Dr Noemi Procopio at Northumbria University. Dr Procopio followed the protocol set out in the paper by Procopio & Buckley (2017). This paper discusses the use of two different agents for the extraction of proteins; in this experiment, formic acid was used. Three repeats were performed to allow for statistical analysis to be conducted. LC-MS/MS analysis was used to analyse the samples using a *Q Exactive Plus* orbitrap mass spectrometer with LC nano-flow *UltiMate 3000*.

Progenesis software was used to analyse the raw data gained from the LC-MS/MS. This allowed for comparisons to be made between the samples in a variety of ways; buried vs exposed; bone vs whole; fresh vs 12 weeks vs 24 weeks. *Progenesis* assigned a number to the proteins in the raw data, relative abundance was the term given to this number as it had no units. Principal component analysis (PCA) was used to show the data; this took all proteins into account and allowed the variability in the samples to be seen. Analysis of variance (ANOVA) was used to obtain a p-value for each protein, which was then corrected to a q-value.

3.5 Statistical Analysis

Three repeats were conducted of each method where possible to allow for statistical analysis to be conducted on results. Due to the small size and fragility of the samples, especially the later samples, three repeats were not always possible; however statistical analysis was conducted where it could be.

Due to the complex nature of the research, the results were presented in a variety of ways;

- The two deposition conditions were compared to determine the differences an exposed versus buried environment could make.
- Each condition was analysed as the experiment progressed to determine whether any changes were occurring at a uniform rate over time.
- The different sample types (defleshed bone, fleshed, whole) were compared to determine whether the presence or absence of gut bacteria affected the rate of bone diagenesis.
- To attempt to answer the gut vs soil theory, the buried, defleshed bones were compared to the exposed, whole specimens to determine if the presence of gut bacteria, or soil bacteria had affected the rate of bone diagenesis.

Chapter Four:

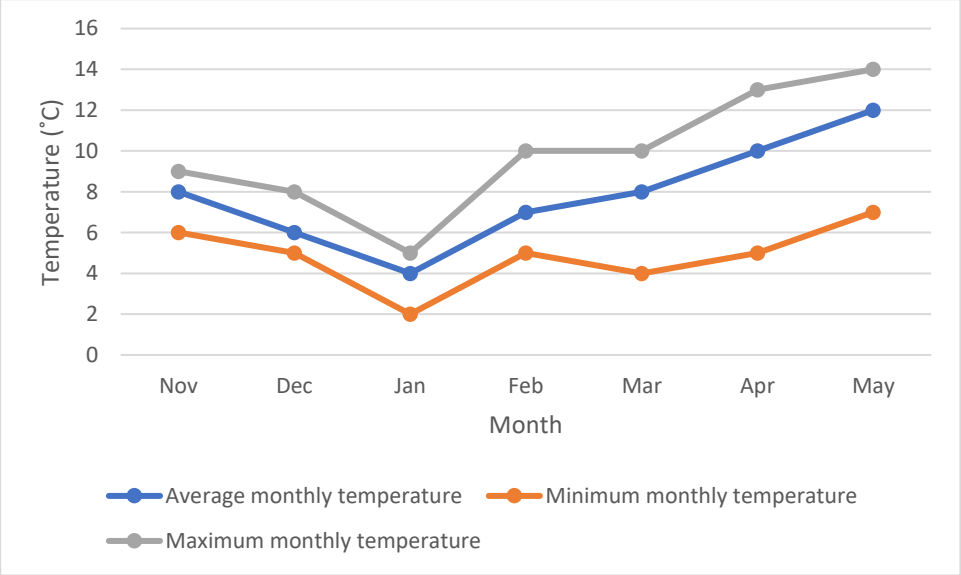
Results

4.1 Introduction

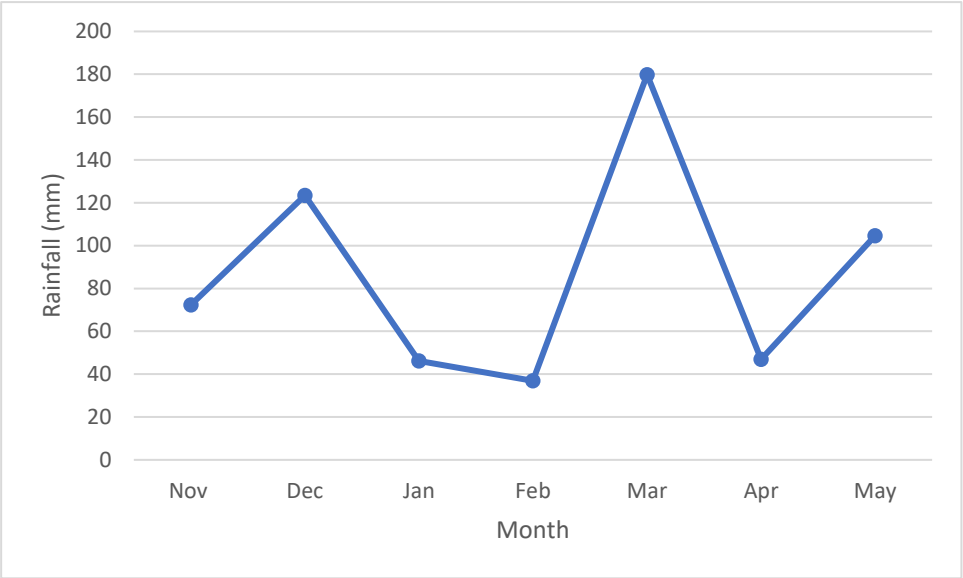
The results of this project are explained here. This was an extensive project incorporating multiple analytical techniques; therefore images are shown only to refer to the taphonomic and diagenetic changes that occurred over the course of the project. For further images, please refer to the Appendices at the end.

4.2 Temperature and Rainfall

Elitech data loggers were placed in various boxes to record the hourly temperature, however for reasons unknown, these did not record. Weather data was obtained from World Weather Online.



Graph 4.1 Average monthly temperature at the HuddersFIELD site during the period the samples were deposited. Source: WorldWeatherOnline (https://www.worldweatheronline.com/halifax-weather-averages/west-yorkshire/gb.aspx)



Graph 4.2 Average monthly rainfall at the HuddersFIELD site during the time the samples were deposited. Source: WorldWeatherOnline (https://www.worldweatheronline.com/halifax-weather-averages/west-yorkshire/gb.aspx)

4.3 Soft Tissue Decomposition

The specimens were deposited on the same day (14th November 2018). Samples from each condition were collected every 4 weeks for a total of 28 weeks. Table 4.1 gives images of the exposed samples at the deposition site; advancing stages of decomposition is PMI increases.

4.3.1 Exposed whole samples

The first samples were collected 4 weeks after deposition. It was clear to see that the exposed whole samples were undergoing a fairly advanced decomposition, with a TBS of 32 from the rat collected, considering the short time and cooler weather. There was a lot of entomological activity taking place on these samples, with some skeletonisation on the limbs. The remaining flesh was dry and brittle.

Entomological activity had ceased by 8 weeks PMI. Very little tissue remained on the limbs; they were easy to identify in the box, as were a couple of the rat skulls. By 16 weeks PMI, some of the long bones were beginning to disarticulate and they could be collected using tweezers due to very little adherent soft tissue.

By week 24 there was advanced skeletonisation, with even the tougher tissue on the tails starting to skeletonise. Structures such as the vertebrae, pelvis, and ribs were easily identifiable in the box. The paws remained covered in tissue. The same was seen for the week 28 samples, with little advancement in the amount of skeletonised remains seen (>75% skeletonisation).

4.3.2 Exposed fleshed, excised samples

Unlike the whole samples, the fleshed samples showed delayed decomposition, with no signs of insect activity at 4 weeks. The only visual indication something was occurring was the layer of yellowish fungus covering the surface. The tissue underneath was tough to cut through.

No entomological activity was seen until 12 weeks PMI. By 16 weeks PMI, the insects had disappeared, leaving very little soft tissue behind (> 50% skeletonisation). The long bones were easily collected using tweezers by week 24 due to advanced skeletonisation. As with the whole samples, the paws remained covered in soft tissue. Week 28 saw similar results, with the paws still covered in soft tissue; the long bones were easily identifiable and mostly disarticulated.

4.3.3 Buried whole samples

These samples did not reach skeletonisation. The soft tissue underwent decomposition, but this was very limited. At 4 weeks PMI, there were very little signs of decomposition occurring to the external features of the sample. The abdomen was visibly bloated by 8 weeks PMI, with rupture having occurred by the 12 week collection, making the soil very moist. Skin slippage also occurred at this time. Adipocere was seen at 16 weeks PMI, and by 20 weeks PMI it was throughout the sample, with the flesh becoming waxy and fragile. By the final collection at 28 weeks PMI, the flesh was very fragile and falling apart making extraction of the long bones easy.

4.3.4 Buried fleshed, excised samples

The fleshed samples followed a similar decomposition pattern as the whole samples with the exception of the wet, abdominal rupture. By 8 weeks PMI, some skeletonisation had occurred at the excision sites, however this was limited to just this area and did not progress over time. Adipocere started to develop at 16 weeks PMI, with the flesh becoming fragile and waxy by 24 weeks PMI. 28 weeks PMI saw the same results as the whole samples with the long bones being easily removed from the remaining tissue.

Table 4.1. Exposed whole and fleshed samples in situ at HuddersFIELD

Exposed	Whole bodies	Fleshed, excised limbs
Fresh	 Fresh, no signs of decomposition can be seen	 Fresh, no signs of decomposition can be seen

4 weeks Exposed bone	 Entomological activity. Skeletonisation can be seen	 No signs of entomology. Yellow coating on the surface of limbs
8 weeks Exposed bone	 Increased skeletonisation	 Yellow coating (suspected fungal) has increased on tissue surface
16 weeks Base of box shows layer of fluid from decomposition fluids and rain water	 Less entomological activity	 Exposed bone Skeletonisation starting to occur
24 weeks skull vertebrae	 Bones can be clearly seen and identified	 Bones can be clearly seen, still articulated

28 weeks	 More than 50% skeletonisation occurred, even paws and tails starting to be exposed	 Increased skeletonisation occurring

The decomposition process advanced with each collection date, with both the whole and fleshed samples showing advanced skeletonisation by 28 weeks PMI.

4.4 Total Body Score

The samples were visually examined upon recovery from HuddersFIELD. The fleshed and whole samples were given a score based on an adapted version of the Total Body Score (TBS) (Adlam & Simmons, 2007). For further information, see Appendix Two. The buried samples were difficult to assess due to the levels of damp soil adhered to the tissue, therefore a cautious score was made based on how easy the bones were to extract.

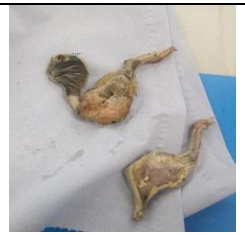
The TBS is split into sections for the whole samples (head & neck, abdomen, and limbs) and a total of these sections is given in table 4.2.

It is clear to see from Graph 4.3 that there was a delay in decomposition of the buried samples. This delay was seen in both whole and fleshed samples, indicating that it was the deposition environment that led to the difference, rather than the samples condition.

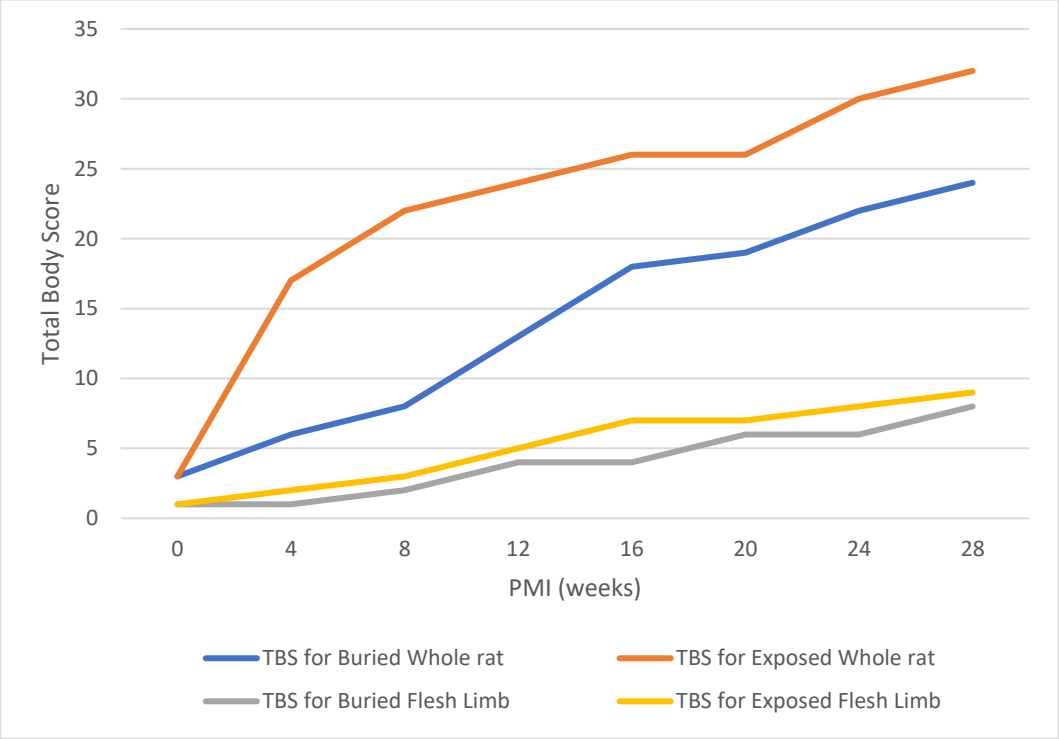
The whole samples decomposed in a quicker time than the fleshed samples, for both buried and exposed conditions, yet they both reached the same end point by 28 weeks (see Graphs 4.4 A & B).

Table 4.2. Condition of samples upon retrieval

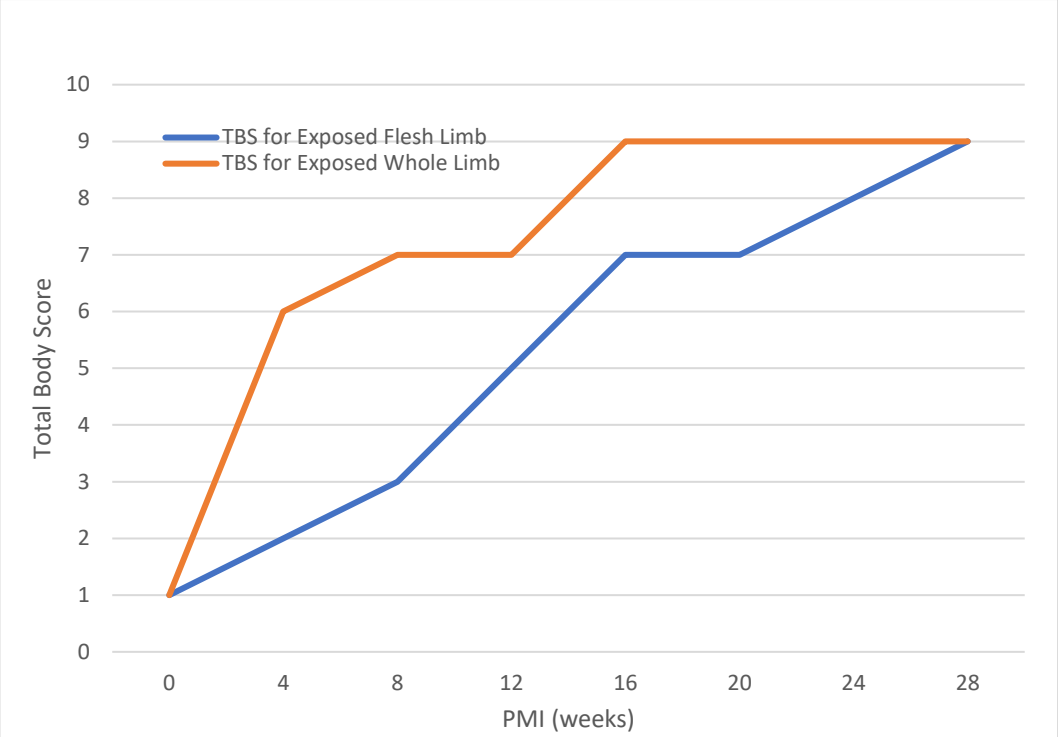
Fresh whole and fresh flesh	 HN:1, A:1, L:1 T:3	 L:1					
	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks	28 weeks
Buried fleshed, excised limbs	 L:1	 L:2	 L:4	 L:4	 L:6	 L:6	 L:8
Buried whole bodies	 HN:2, A:2, L:2 T:6	 HN:3, A:3, L:2 T:8	 HN:4, A:5, L:4 T:13	 HN:6, A:6, L:6 T:18	 HN:7, A:6, L:6 T:19	 HN:7, A:7, L:8 T:22	 HN:7, A:9, L:8 T:24

Exposed fleshed, excised limbs	 L:2	 L:3	 L:5	 L:7	 L:7	 L:8	 L:9
Exposed whole bodies	 HN:5, A:6, L:6 T:17	 HN:8, A:7, L:7 T:22	 HN:8, A:9, L:7 T:24	 HN:8, A:9, L:9 T:26	 HN:8, A:9, L:9 T:26	 HN:10, A:11, L:9 T:30	 HN:12, A:11, L:9 T:32

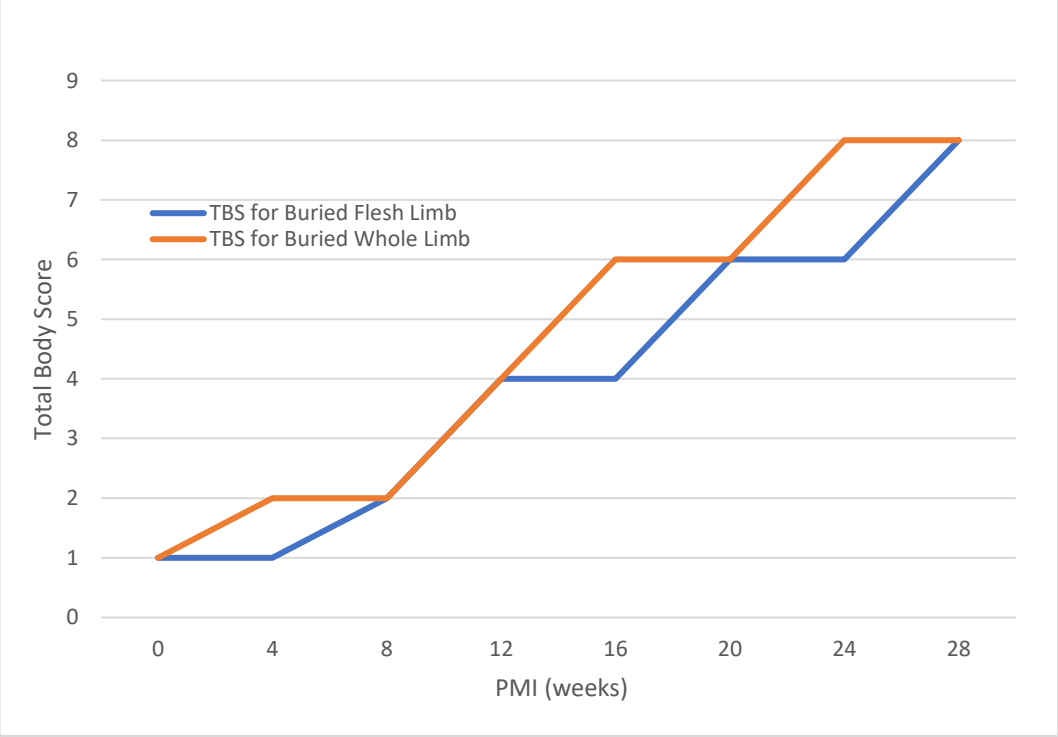
Fleshed and whole samples as they were recovered from the field. The decomposition process advanced with each collection. Head & Neck (HN), abdomen (A), limbs (L) scores are indicated below each image. The total (T) for the TBS is given.



Graph 4.3. Total body scores for comparison between the buried and exposed whole rat and excised, fleshed limbs. TBS for the whole samples were based on three measurements therefore they were higher than the excised, fleshed limbs. It can see been that the exposed samples (whole and fleshed) decomposed more quickly than their buried counterparts.



A Comparison of the TBS from the limbs of the exposed whole rat samples and the exposed excised, fleshed samples. The whole samples decomposed more quickly than the fleshed samples initially, but they did reach the same TBS by 28 weeks.



B Comparison of the TBS from the limbs of the buried whole rat samples and the exposed excised, fleshed samples. It can be seen the whole samples decomposed more quickly than the fleshed samples, however they did reach the same TBS scores at variance PMIs.

Graph 4.4 Comparisons of the TBS of limbs from the whole and fleshed samples over time A) Exposed samples, B) Buried samples

4.5 Weathering and Surface Modifications

The bones from all conditions were analysed using Keyence microscopy for any signs of degradation. The exposed samples were scored using the Behrensmeyer (1978) weathering index (see Table 3.3). The buried samples were scored using the same method to indicate any surface modifications seen; however it should be noted that Behrensmeyer (1978) only intended for this index to be used on exposed bones.

As Table 4.3 shows, the samples did not succumb to extensive surface degradation, only one buried sample at 24 weeks PMI was given a score of 2 due to extensive flaking (fig 4.1).

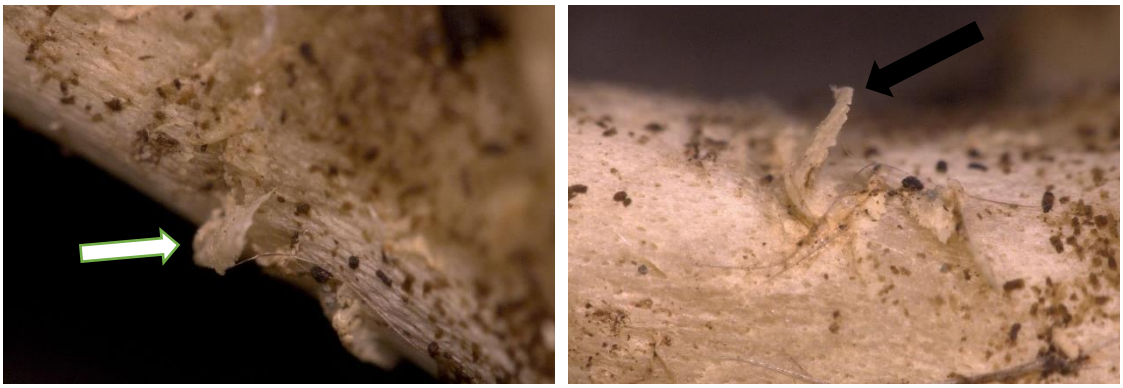


Fig 4.1. Buried bone showing category 2 weathering at 24 weeks PMI. Arrows indicate the flakes seen. Source: Author

Most samples had achieved a score on the weathering index of 1 by the end of the experiment, but overall 28 weeks PMI was not a long enough interval to show significant changes (table 4.3).

Table 4.3. Weathering scores

Condition	Time (weeks)	A	B	C
Fresh	0	0	0	0
Buried bone	4	0	0	Missing
	8	0	0	0
	12	0	0	0
	16	1	0	1
	20	1	1	1
	24	1	2	1
	28	1	0	Missing
Buried fleshed, excised limbs	4	0	0	0
	8	0	0	0
	12	0	0	0
	16	1	1	1
	20	1	1	1
	24	0	1	1
	28	1	1	1
	4	0	0	0

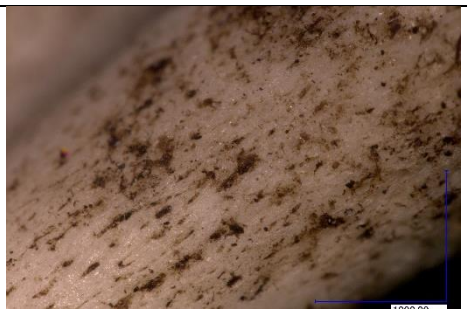
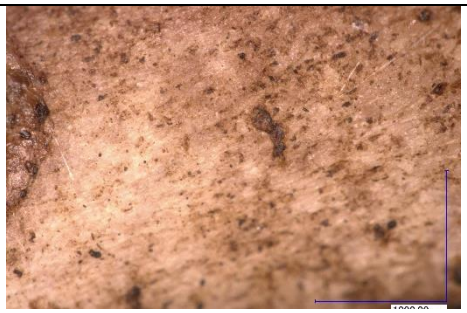
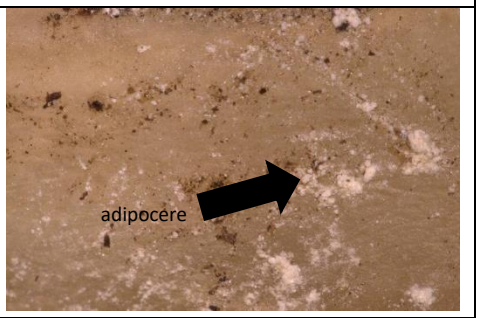
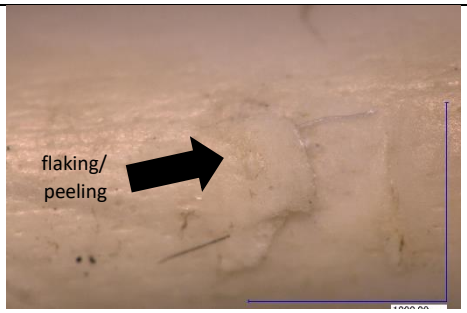
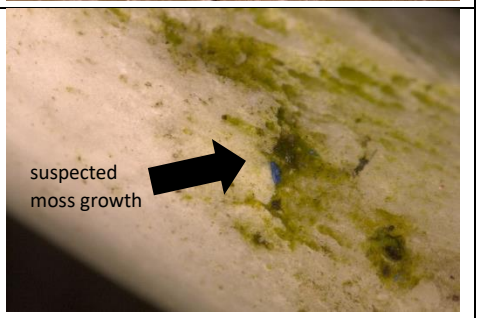
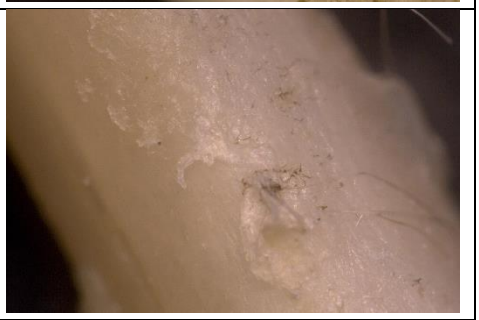
Buried whole bodies	8	0	0	0
	12	0	0	0
	16	0	0	0
	20	1	0	0
	24	1	0	1
	28	1	0	0
Exposed bone	4	0	0	0
	8	0	0	0
	12	0	0	0
	16	0	0	0
	20	1	1	1
	24	1	1	1
	28	1	1	1
Exposed fleshed, excised limbs	4	0	0	0
	8	0	0	0
	12	0	0	0
	16	0	0	0
	20	1	0	1
	24	1	0	1
	28	0	1	0
Exposed whole bodies	4	0	0	0
	8	0	0	0
	12	0	0	0
	16	1	1	1
	20	1	1	1
	24	1	1	1
	28	1	1	0

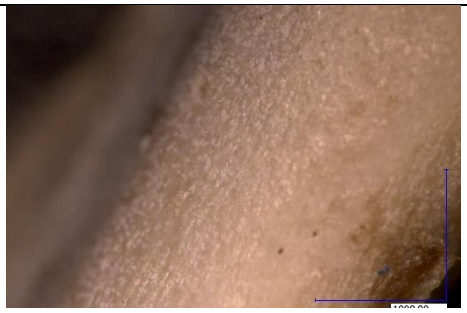
The samples did not succumb to extensive weathering and/or surface modifications. While many of the samples were scored 1 on the Behrensmeyer weathering index, many were still in good condition by 28 weeks PMI and only one samples was given a score of 2.The surface weathering seen included;

- pitting to the ends of the bones
- flaking and cracks
- soil adhesion
- Spots potentially due to bacteria or fungi
- Adipocere
- Blue flakes from painted panels stored nearby

Table 4.4 shows some examples of the types of changes seen throughout the experiment. This is not an exhaustive index and further images can be found in Appendix 3.

Table 4.4. Sample images from 4 and 28 weeks showing the changes observed

	4 weeks PMI	28 weeks PMI
Buried bone		
Buried fleshed, excised legs		
Buried whole bodies		
Exposed bone		
Exposed fleshed, excised legs		

Exposed whole bodies		

Many of the samples were still showing little signs of weathering/surface modifications throughout the project. Adipocere was observed on the buried whole and fleshed samples as the experiment progressed with the whole rat samples showing the most extensive adipocere formation (fig 4.2).

Fig 4.2. Buried whole body sample showing adipocere on the surface at 28 weeks PMI

Green growths were observed on the exposed samples, in particular the defleshed bone samples (fig 4.3). This was most likely due to the samples being placed into plastic boxes for the duration of the experiment. The boxes were intended to prevent predator disturbances but, due to the lack of drainage, may have allowed the samples to become wetter than they would have had they been left on the soil surface.

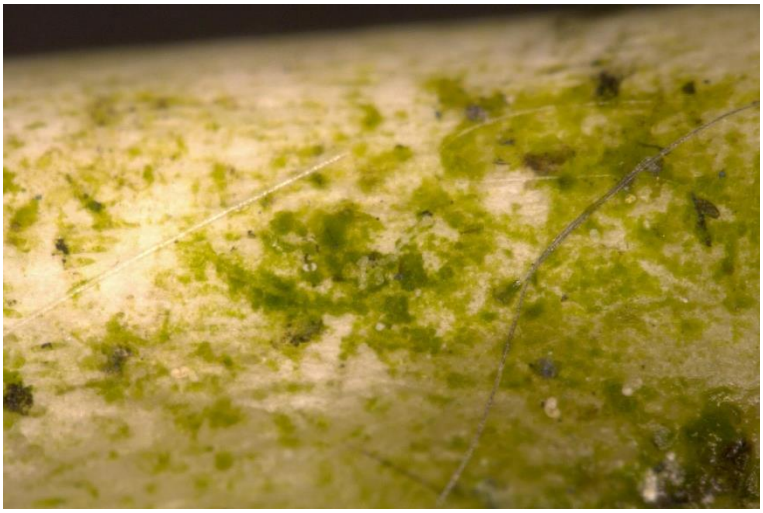


Fig 4.3. Exposed defleshed bone showing possible moss growth at 28 weeks PMI

Many of the bones exhibited signs of cracks, flakes and pitting as can be seen below in figs 4.4-4.9;

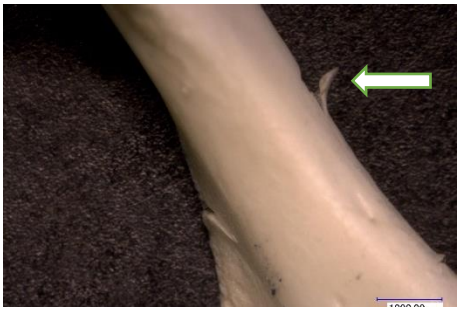


Fig 4.4 Exposed fleshed, excised sample at 8 weeks PMI exhibiting cracks

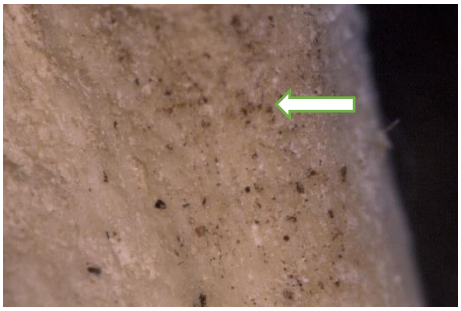


Fig 4.5 Buried whole body sample at 24 weeks PMI showing pitting, possibly due to soil erosion

Fig 4.6 Buried defleshed bone at 12 weeks PMI with pitting to proximal end

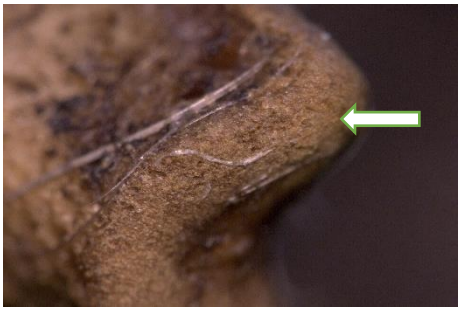


Fig 4.7 Exposed whole body sample at 24 weeks PMI with increased porosity

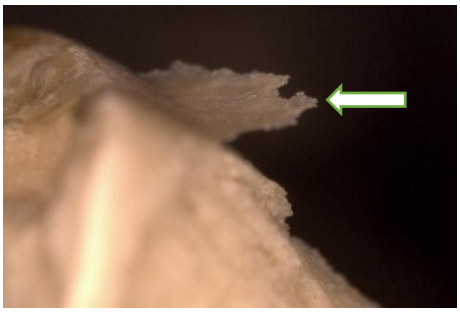


Fig 4.8 Exposed fleshed, excised sample at 12 weeks PMI with flaking to the proximal end

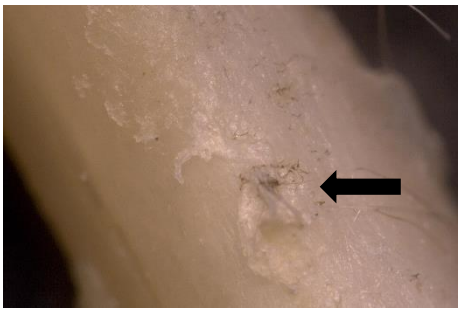
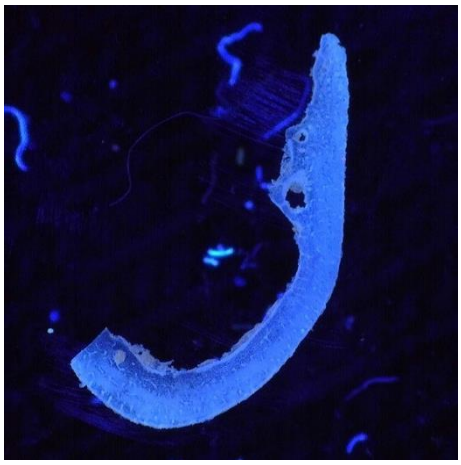


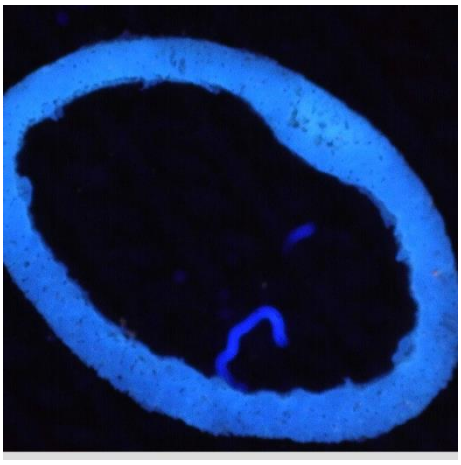
Fig 4.9 Exposed fleshed, excised sample at 28 weeks PMI showing signs of flaking

4.6 UV fluorescence

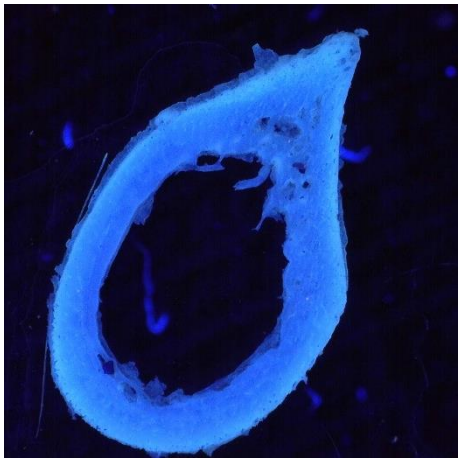
The bones were analysed using a *Dino-Lite* USB microscope with UV (375nm) LEDS to determine whether there was any loss in autofluorescence due to collagen degradation. Saukko & Knight (2015) discuss the 'sandwich' effect, whereby bone will slowly lose its ability to fluoresce, starting at the outer and inner rims and working its way across the entire cross section of the bone. The images were taken at a x75 magnification and examined visually for any emerging pattern.



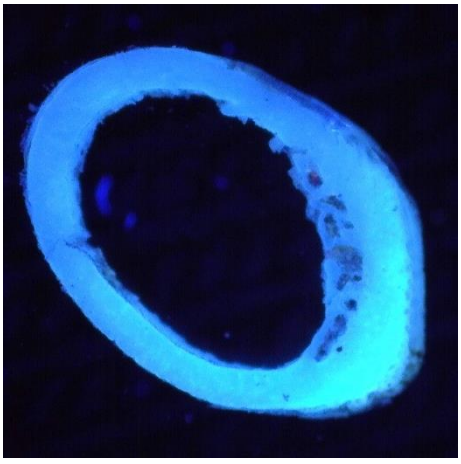
A. Fresh



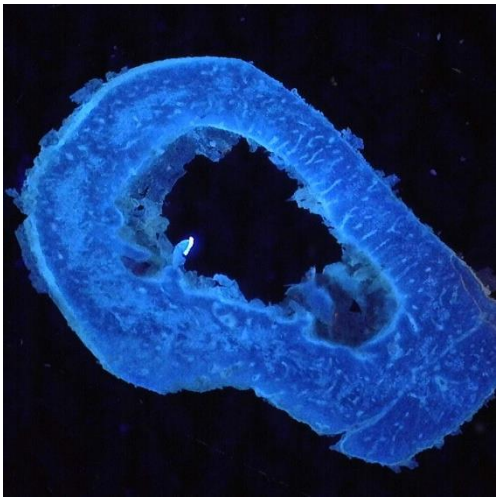
B. 4 weeks



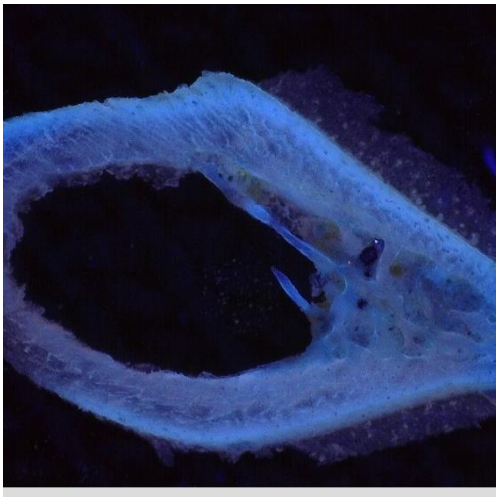
C. 8 weeks



D. 16 weeks



E. 24 weeks



F. 28 weeks

Fig 4.10 UV fluorescence of HuddersFIELD samples (A-F)

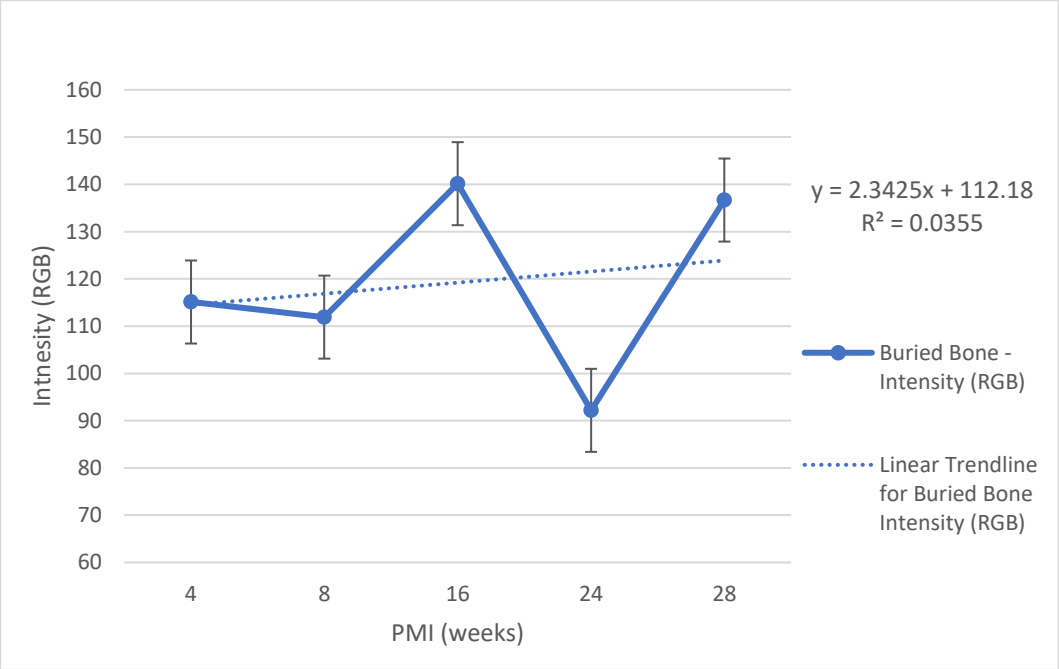
As the images in fig 4.10 show, there was a visual difference between the samples at different PMI's, however this was not consistent, and the sandwich effect was not clearly seen.

Images were analysed for level of intensity using *ImageJ*, and Table 4.5 was constructed to show the results.

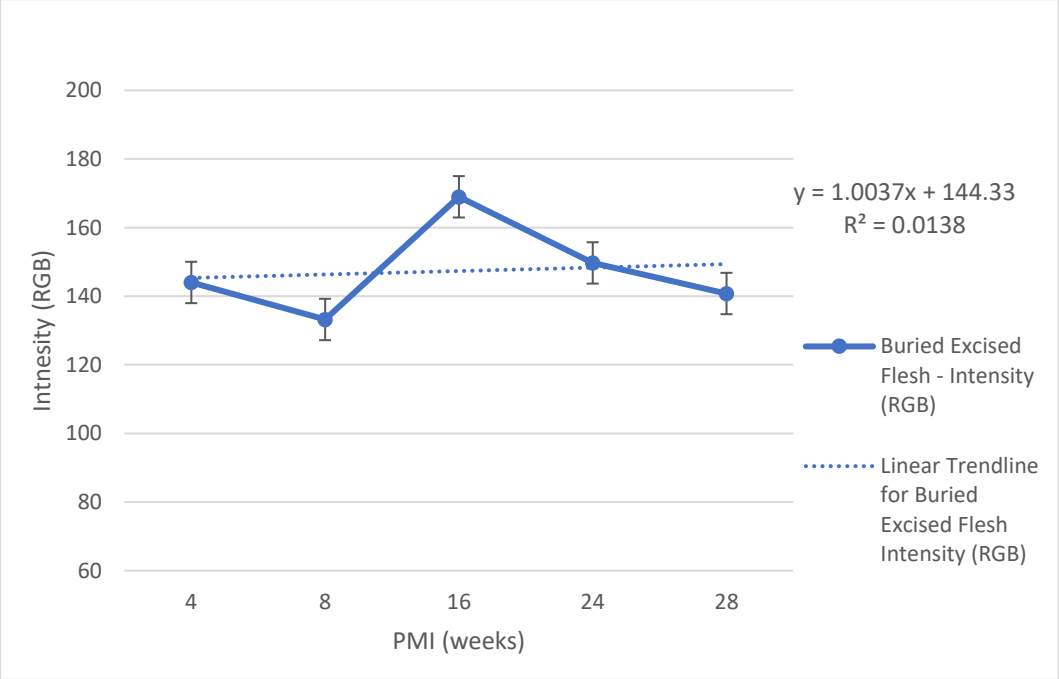
Table 4.5 Averages of the intensities obtained from ImageJ

Condition	Time (weeks)	A			B		
		Mean (RGB)	Min (RGB)	Max (RGB)	Mean (RGB)	Min (RGB)	Max (RGB)
Fresh	0	134.994	42	173	132.813	101	153
Buried bone	4	123.813	88	152	106.392	42	138
	8	115.194	88	144	108.692	76	142
	16	135.591	87	154	144.710	94	163
	24	99.175	68	136	85.210	47	147
	28	128.678	77	162	144.692	91	165
Buried fleshed, excised limbs	4	136.988	85	157	151.035	123	164
	8	140.655	54	193	125.780	56	179
	16	172.898	109	193	165.066	143	180
	24	159.981	119	181	139.420	104	154
	28	133.076	73	163	148.501	106	165
Buried whole bodies	4	140.765	98	155	144.578	98	160
	8	147.966	125	159	134.578	94	161
	16	163.534	99	190	166.695	114	188
	24	127.247	82	184	169.314	115	192
	28	157.639	75	176	124.822	82	158
Exposed bone	4	125.101	43	155	137.922	81	159
	8	134.517	68	158	141.920	104	170
	16	125.450	52	178	139.547	98	170
	24	99.233	56	158	73.957	41	122
	28	128.355	83	156	116.773	72	145
Exposed fleshed, excised limbs	4	146.401	66	170	132.251	79	164
	8	157.414	117	189	140.534	98	168
	16	163.909	111	182	148.694	116	174
	24	78.042	45	150	143.489	90	170
	28	145.359	123	161	108.806	60	151
Exposed whole bodies	4	136.358	90	161	101.909	56	130
	8	120.184	75	156	163.677	129	179
	16	154.738	118	175	151.878	105	173
	24	105.922	57	167	127.632	85	161
	28	147.324	106	169	149.309	103	172

The mean was used to determine changes within the samples; to calculate graphs. Maximum fluorescence and minimum fluorescence are given to show the differences observed within the same samples (fluorescence was not consistent throughout).Graphs 4.9 show the averages obtained from the *ImageJ* analysis for each condition and tissue type. These show inconsistencies within the samples; R^2 values (<0.3) were calculated to show that the variances cannot be explained from this data set, indicating that there was no correlation between fluorescence loss and time[4].

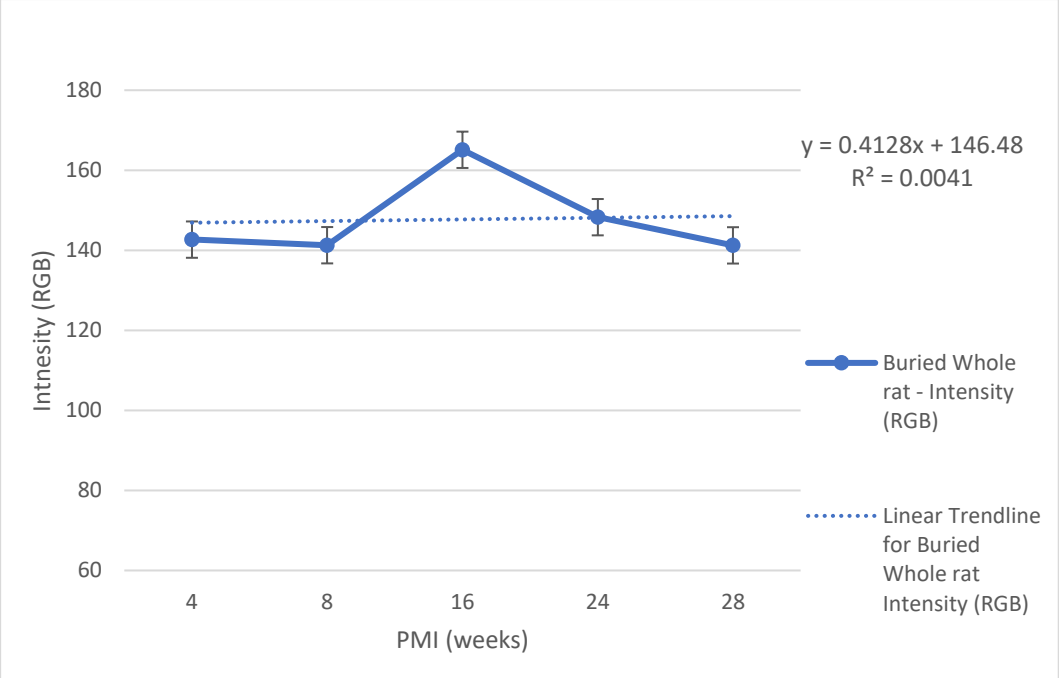


A The average intensity calculated from ImageJ for the buried bone samples over time with error bars

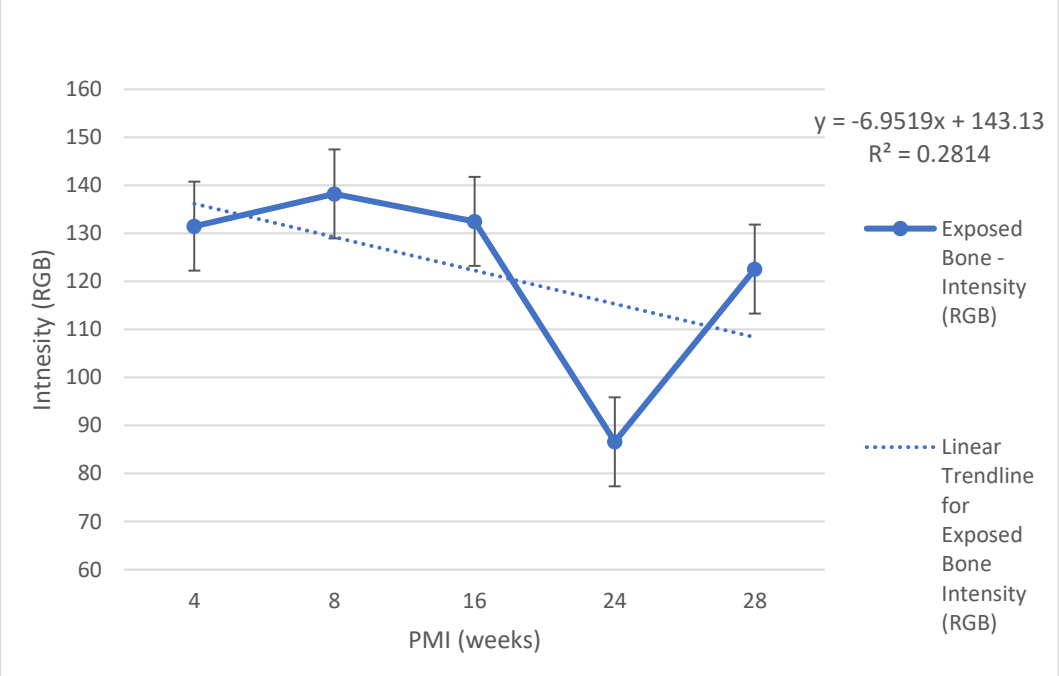


B The average intensity calculated from ImageJ for the buried excised, fleshed samples over time with error bars

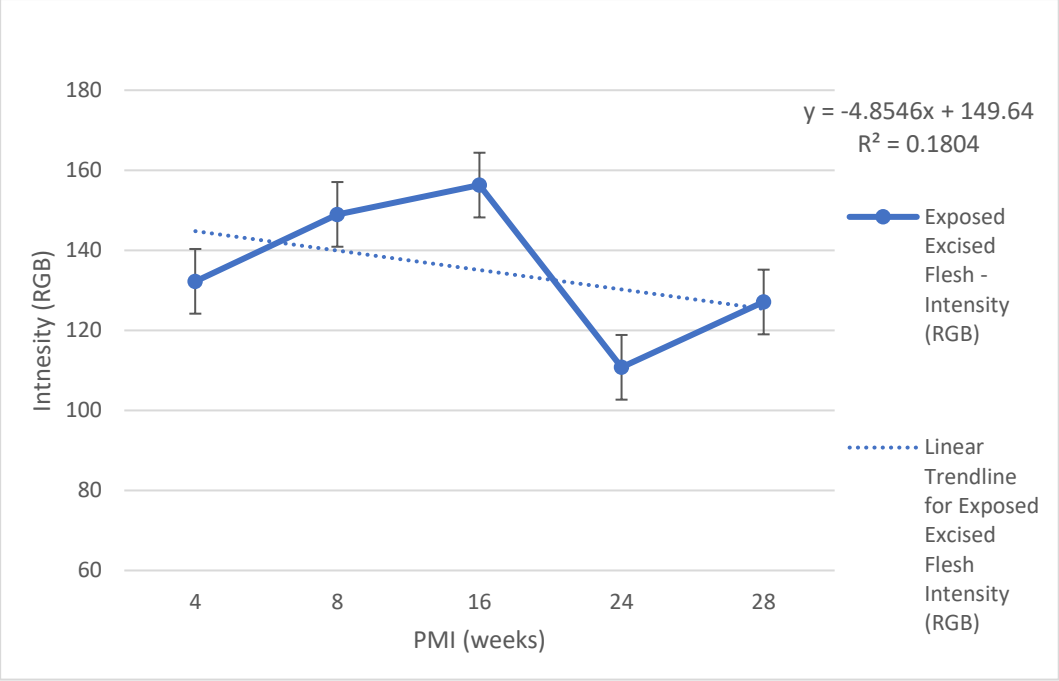
[4] R^2 values were classified in the following way; >0.3 noteworthy, >0.5 large, >0.7 very large (McCormick *et al*, 2015)



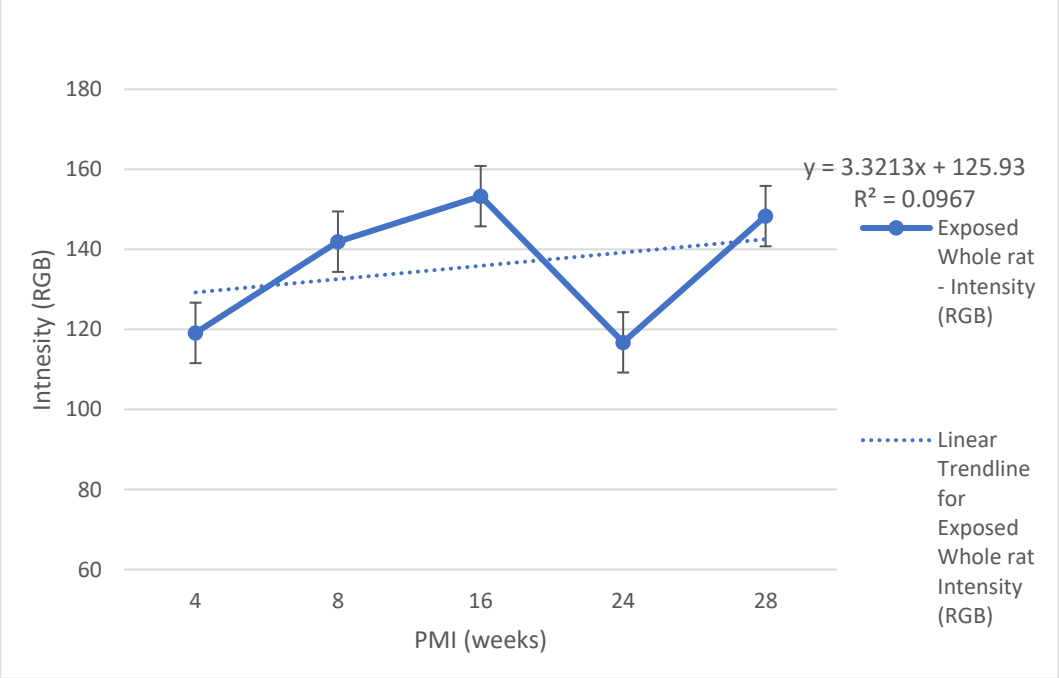
C The average intensity calculated from ImageJ for the buried whole samples over time with error bars



D The average intensity calculated from ImageJ for the exposed bone samples over time with error bars



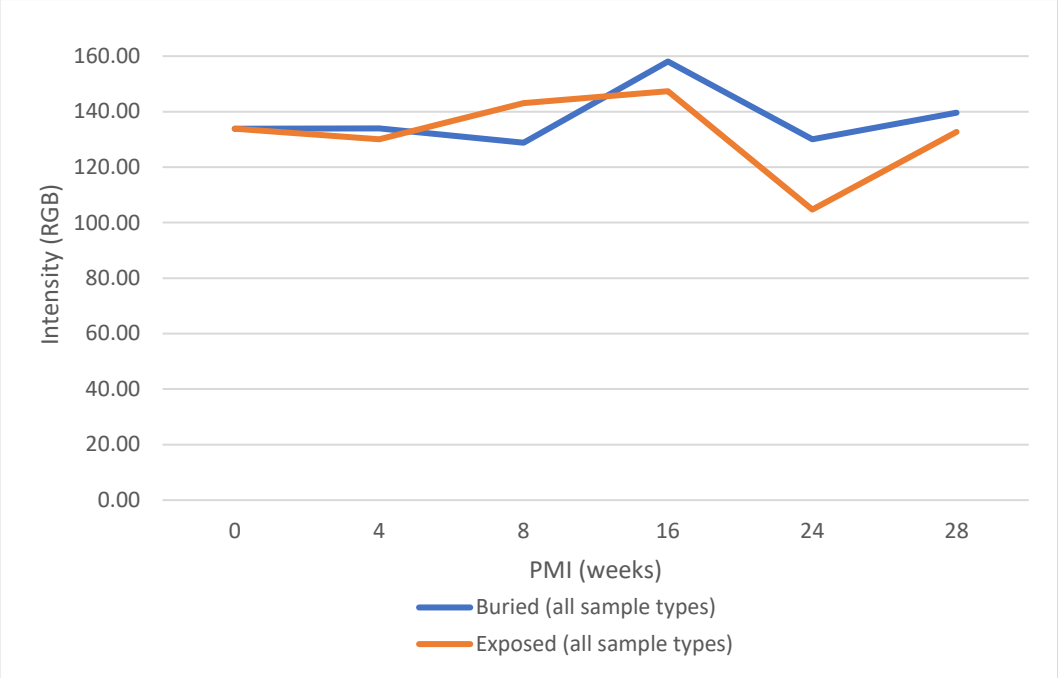
E The average intensity calculated from ImageJ for the exposed excised, fleshed samples over time with error bars



F The average intensity calculated from ImageJ for the exposed whole samples over time with error bars

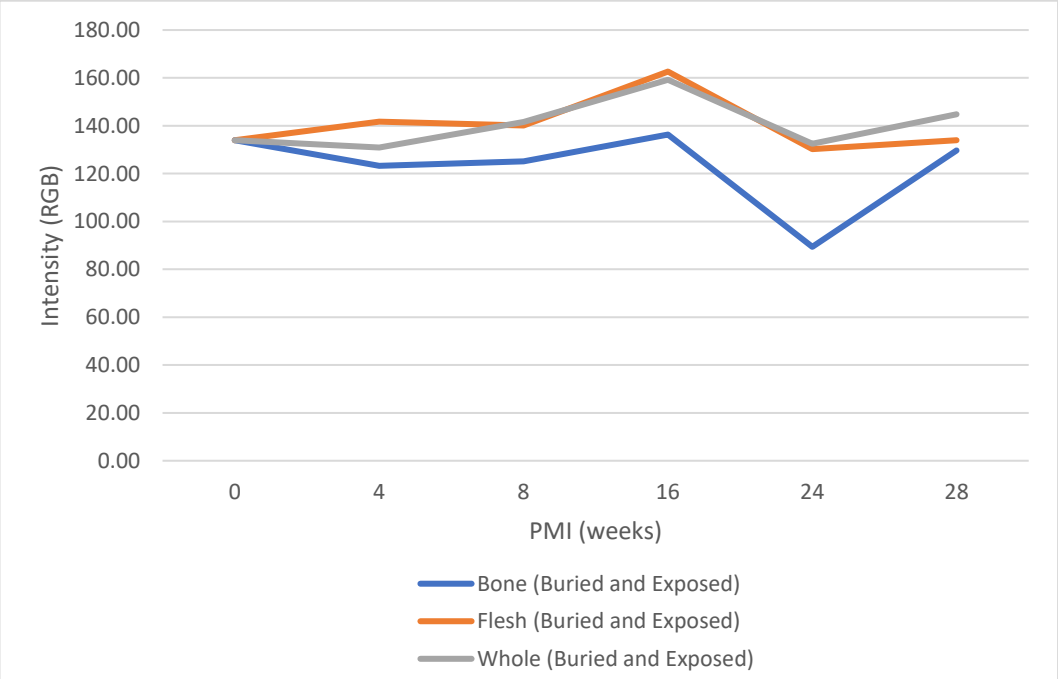
Graph 4.5 The average intensity (RGB) calculated using ImageJ for each sample/condition over time (with error bars). The inconsistencies encountered with the UV fluorescence can be seen.

Comparisons were made between the two deposition environments (graph 4.6); buried and exposed, to determine whether these influenced fluorescence loss. This showed there was an inconsistency in some samples, particularly between weeks 8 and 24; this appeared to have a greater effect in the exposed samples.



Graph 4.6 Comparison of the average intensity calculated with ImageJ for the deposition environments over time. Inconsistencies in the levels of fluorescence can be seen; the exposed samples showed the greatest loss in fluorescence by the end of the experimentation.

Further comparisons were made between the three tissue types to determine whether any pattern emerged (graph 4.7). As with the deposition environments, there appeared to be inconsistency in the samples.



Graph 4.7 A comparison of the average intensity calculated with ImageJ for the three tissue types over time. Inconsistencies in the levels of fluorescence are shown; the defleshed bone samples (all) showed the greatest overall loss in fluorescence.

To determine whether the measuring procedure was causing the issues with inconsistencies, further measurements were taken. This involved using the *freehand* tool in *ImageJ* to draw

around the sample, as close to the edges as possible (fig 4.11). The intensity of the pixels were then calculated.

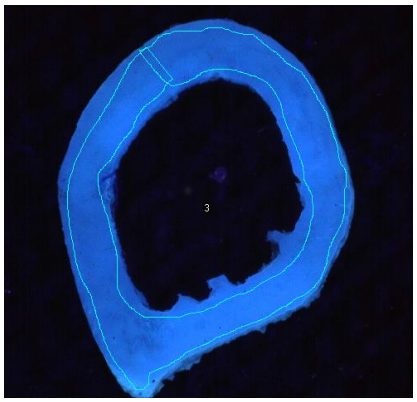
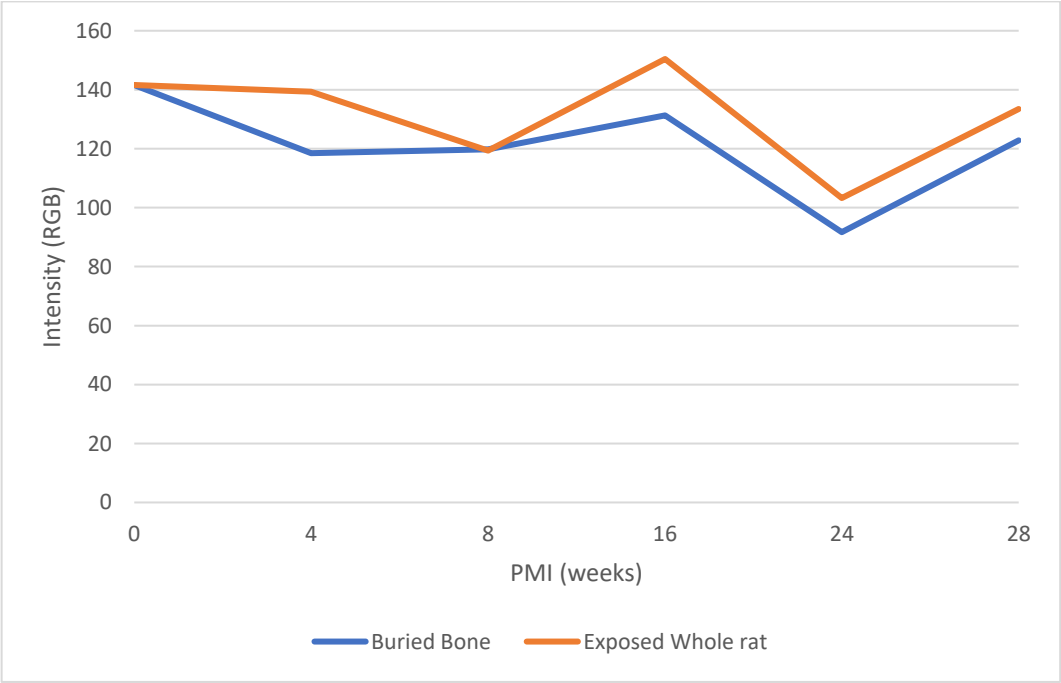


Fig 4.11 Secondary fluorescence measurement. The light lines show the freehand drawing tool used to obtain a measurement.

This method was carried out on the buried bone and exposed whole rat samples only as it made very little difference to the overall results being seen (graph 4.8).



Graph 4.8 A comparison of the average intensity calculated with ImageJ for the deposition environments using an alternative method of measurement (as described above). As with the grid method, inconsistencies were found in the samples.

4.7 Histology

As discussed in previous chapters, skeletal material can undergo microbial infiltration. The rate of destruction caused by these microbes increases over time. Hackett (1981) categorised these MFD into four categories; Wedl, linear longitudinal, lamellate, and budded. When analysing the samples from this study, efforts were made to identify which of these four MFD were present,

however due to the preparation method used[5], this was not always possible. Therefore, the microbial infiltrations seen were placed into two categories; Wedl and non-Wedl. There were times when unexpected changes were observed as described below.

Both compound microscopy and digital microscopy was used to determine any diagenetic alterations occurring. It was found, however, that due to the preparation method used, the digital microscope was not ideal for obtaining clear images, particularly as the PMI increased (fig 4.12).

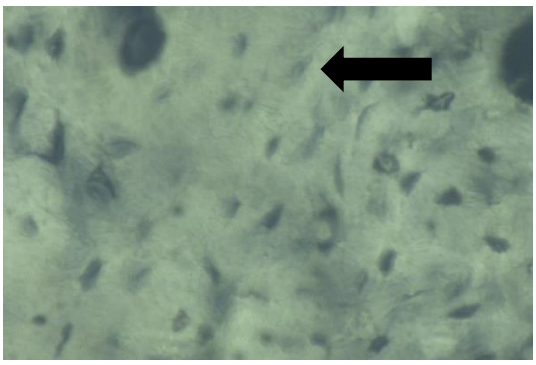


Fig 4.12 Exposed whole, 16 weeks PMI. Keyence microscope (1000x). Arrow shows where microscope struggled to focus due to thick sample

Amalgamations

Dark shadows were seen early on; these were quite extensive in places (figs 4.13 & 4.14). They did not take the expected form of tunnelling, making them difficult to identify. As they closely resembled the amalgamations described in White & Booth (2014), they were given this category in the results table.

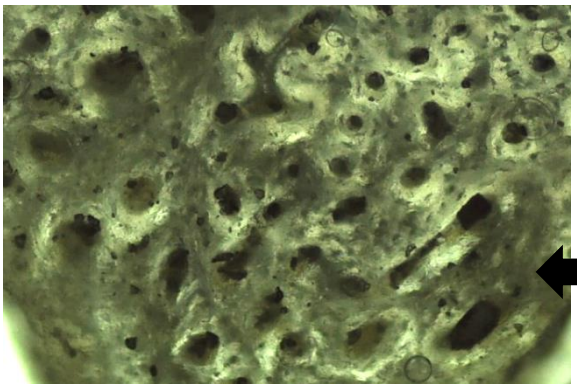


Fig 4.13 Amalgamations seen, buried bone, week 4 (100x)

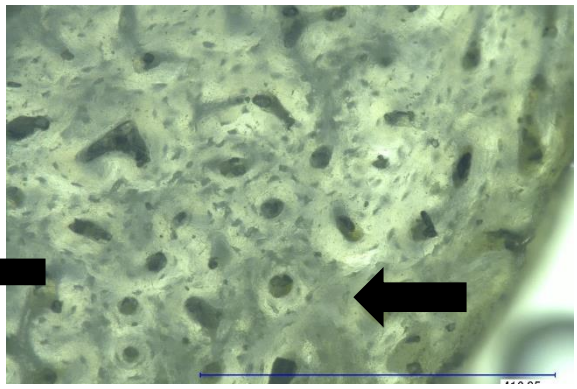


Fig 4.14 Amalgamations seen using Keyence microscopy, buried bone, week 4 (500x)

[5] Preparation method used sandpaper which was abrasive. Potential for damage to the samples meant the MFD seen could not be further categorised with confidence.

Diagenetic Osteocyte Lacunae

Larger than expected and sometimes misshapen osteocyte lacunae were also seen in many samples (figs 4.15 & 4.16). Again, these did not fall under the categories of MFD given, however due to the nature of them, it was clear to see that some form of degradation was taking place. Following White & Booth (2014), these were termed diagenetic osteocyte lacunae.

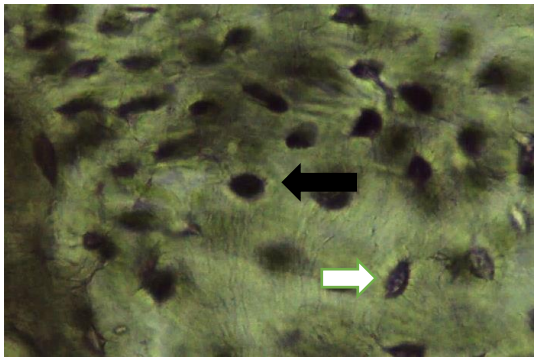


Fig 4.15 Abnormal osteocyte lacunae, exposed whole rat, week 16 (400x).
Black arrow – diagenetic, white arrow - normal

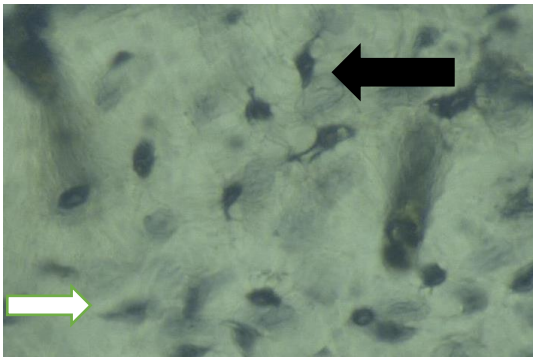


Fig 4.16 Abnormal osteocute lacunae, buried whole rat, 24 weeks (1000x) Keyence microscope
Black arrow – diagenetic, white arrow - normal

Fig 4.17 show examples of diagenetic osteocyte lacunae from White & Booth (2014).

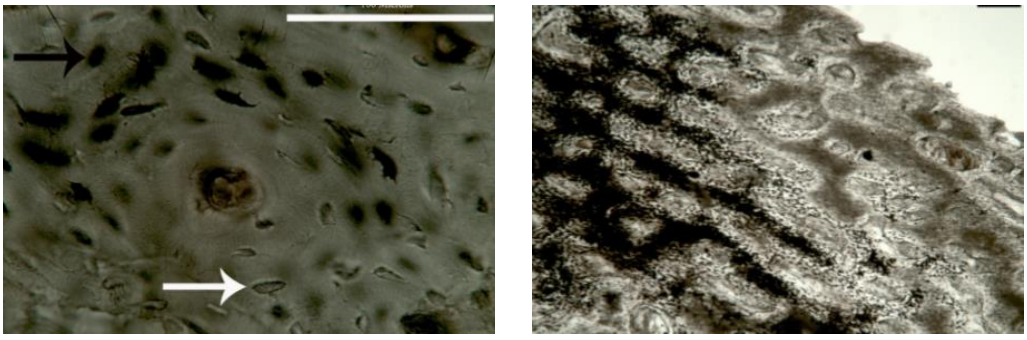


Fig 4.17 Examples of diagenetic osteocytes and amalgamations. Source: White & Booth (2014)

Wedl Type 2

Another type of MFD seen that has not been described often was *Wedl type 2* tunnelling. This takes places around the osteocytes and affects the canaliculi, making the tunnels larger than usual (fig 4.18).

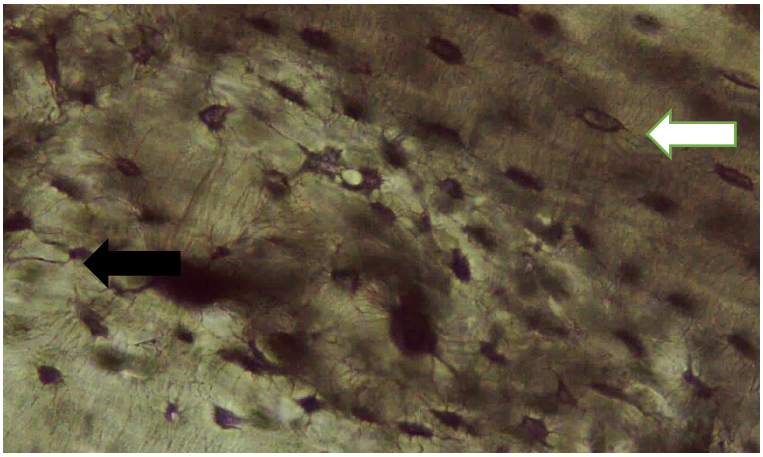


Fig 4.18 Wedl type 2 – enlarged canaliculi (exposed whole rat, week 16)
Black arrow – enlarged canaliculi, white arrow - normal

4.7.1 Diagenetic Features

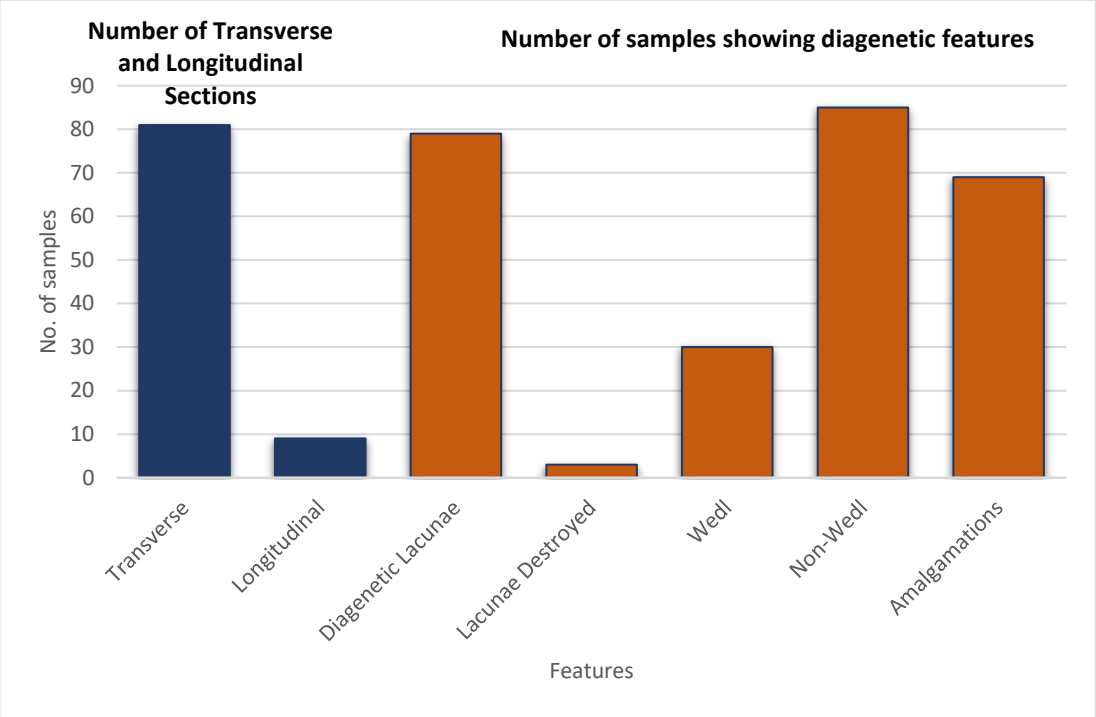
Table 4.6 A complete inventory of samples and features seen over time

PMI	Condition	Tissue Type	Sample	Section	Diagenetic Lacunae	Wedl Tunnels	Non-Wedl Tunnels	Amalgamations	Birefringence	OHI Score
0 weeks	Fresh	Bone	A	T					Normal	5
			B	L					Normal	5
4 Weeks	Buried	Bone	A	T	✓		✓	✓	Reduced	4
			B	L	✓		✓		Obliterated	3
			C	T	✓		✓	✓	Significant loss	3
		Fleshed limb	A	T	✓		✓	✓	Reduced	4
			B	T				✓	Reduced	3
			C	T	✓		✓	✓	Obliterated	3
		Whole rat	A	T	✓	✓		✓	Significant loss	2
			B	T	✓	✓	✓	✓	Localised loss	2
			C	T			✓	✓	Significant loss	3
	Exposed	Bone	A	T	✓	✓		✓	Significant loss	3
			B	T	✓		✓	✓	Significant loss	4
			C	T	✓				Localised loss	4
		Fleshed limb	A	T	✓		✓	✓	Significant loss	3
			B	T	✓		✓	✓	Significant loss	3
			C	T	✓				Significant loss	4
		Whole rat	A	T	✓		✓	✓	Significant loss	3
			B	T	✓		✓	✓	Localised loss	3
			C	T	✓	✓	✓		Significant loss	3
8 Weeks	Buried	Bone	A	T	✓		✓	✓	Localised loss	3
			B	T	✓		✓		Localised loss	3
			C	T			✓		Localised loss	4
		Fleshed limb	A	T	✓		✓	✓	Significant loss	3
			B	T	✓	✓	✓	✓	Reduced	3
			C	T	✓		✓	✓	Localised loss	2
		Whole rat	A	T	✓	✓	✓		Significant loss	3
			B	T	✓		✓		Significant loss	3
			C	T	✓		✓	✓	Significant loss	3

	Exposed	Bone	A	T	✓		✓	✓	Significant loss	3
			B	T	✓		✓	✓	Localised loss	2
			C	T	✓		✓		Significant loss	3
		Fleshed limb	A	T	✓		✓	✓	Significant loss	3
			B	T	✓		✓	✓	Localised loss	3
			C	T	✓	✓	✓	✓	Significant loss	3
		Whole rat	A	L	✓		✓		Localised loss	2
			B	T	✓		✓	✓	Localised loss	3
			C	T	✓		✓		Significant loss	3
16 Weeks	Buried	Bone	A	T	✓	✓	✓	✓	Significant loss	2
			B	T	✓	✓	✓	✓	Significant loss	2
			C	T	✓		✓	✓	Reduced	2
		Fleshed limb	A	T	✓	✓	✓	✓	Significant loss	2
			B	T	✓		✓	✓	Obliterated	1
			C	T	✓		✓	✓	Significant loss	2
		Whole rat	A	T	✓		✓	✓	Significant loss	2
			B	T	✓	✓	✓	✓	Significant loss	2
			C	T			✓	✓	Localised loss	2
	Exposed	Bone	A	T	✓	✓	✓	✓	Significant loss	2
			B	T	✓		✓	✓	Significant loss	3
			C	T	✓		✓	✓	Significant loss	2
		Fleshed limb	A	T	✓		✓	✓	Obliterated	1
			B	T	✓		✓	✓	Significant loss	2
			C	T	✓		✓	✓	Obliterated	2
		Whole rat	A	T	✓	✓	✓	✓	Reduced	3
			B	T	✓	✓	✓	✓	Obliterated	3
			C	T	✓		✓	✓	Significant loss	1
24 Weeks	Buried	Bone	A	T	✓	✓	✓	✓	Significant loss	1
			B	T	✓		✓	✓	Reduced	2
			C	T	✓	✓	✓	✓	Significant loss	1
		Fleshed limb	A	T	✓		✓		Significant loss	2
			B	T	✓		✓	✓	Obliterated	1
			C	T	✓		✓	✓	Reduced	1

		Whole rat	A	T	✓	✓	✓		Significant loss	1
			B	T	✓		✓	✓	Reduced	1
			C	T		✓	✓		Significant loss	1
	Exposed	Bone	A	L	✓	✓	✓	✓	Obliterated	2
			B	T	✓	✓	✓		Significant loss	1
			C	T	✓	✓	✓	✓	Significant loss	2
		Fleshed limb	A	L	✓		✓		Significant loss	2
			B	T	✓		✓	✓	Significant loss	1
			C	T	✓		✓	✓	Significant loss	1
		Whole rat	A	L			✓		Normal	3
			B	T	✓	✓	✓	✓	Significant loss	1
			C	T	✓	✓	✓		Reduced	3
28 Weeks	Buried	Bone	A	T	✓	✓	✓	✓	Localised loss	1
			B	T	✓		✓	✓	Significant loss	2
			C	T	✓		✓	✓	Significant loss	1
		Fleshed limb	A	L			✓		Significant loss	1
			B	T	✓		✓	✓	Normal	0
			C	T	✓		✓	✓	Localised loss	0
		Whole rat	A	T	✓		✓		Significant loss	1
			B	T	✓	✓	✓	✓	Obliterated	1
			C	T	✓	✓	✓		Significant loss	0
	Exposed	Bone	A	T	**0**		✓	✓	Obliterated	0
			B	T	✓	✓	✓	✓	Significant loss	0
			C	L			✓	✓	Significant loss	1
		Fleshed limb	A	T	**0**		✓	✓	Obliterated	0
			B	T	✓	✓	✓	✓	Significant loss	1
			C	L	✓		✓	✓	Significant loss	1
		Whole rat	A	L	**0**	✓	✓		Obliterated	0
			B	T	✓		✓	✓	Significant loss	0
			C	T	✓	✓	✓	✓	Significant loss	1

Diagenetic alterations were seen as early as 4 weeks PMI and steadily increased as time progressed with some samples scoring 0 on the OHI at 28 weeks PMI. Non-Wedl tunnels were seen frequently with 85 out of the 90 field samples showing Wedl tunnels and bores. However, despite research showing that animal bones are more prevalent to Wedl tunnelling (Jans *et al*, 2004; Brönnimann *et al*, 2018), they were not seen as often as expected with only 30 out of 90 samples showing this type of infiltration (graph 4.9).



Graph 4.9 A histogram to show the types and number of features seen in all samples. An overview of the samples obtained, and features seen throughout the experimentation.

Images of all samples can be found in Appendix five.

4.7.2 Birefringence

As well as determining what diagenetic features were present in the samples, birefringence was also observed. The level seen was recorded over time and categorised. Originally the Birefringence Index (BI) was used to determine the level seen; this used a scale of 1-0 with 1 being normal, 0.5 reduced, and 0 obliterated. However, this did not seem enough to account for what was being seen in the samples, therefore two further categories were added to give the following (table 4.7);

Table 4.7 Grades given for BI

Grade	Description
1	Normal birefringence seen
0.75	Localised loss seen
0.5	Reduced
0.25	Significant loss (localised sections may still appear normal)

0	Obliterated

Fig 4.19 gives examples of each category in the adapted BI. It can be seen that each image gets progressively darker with more birefringence lost until the final stage where it is completely obliterated.

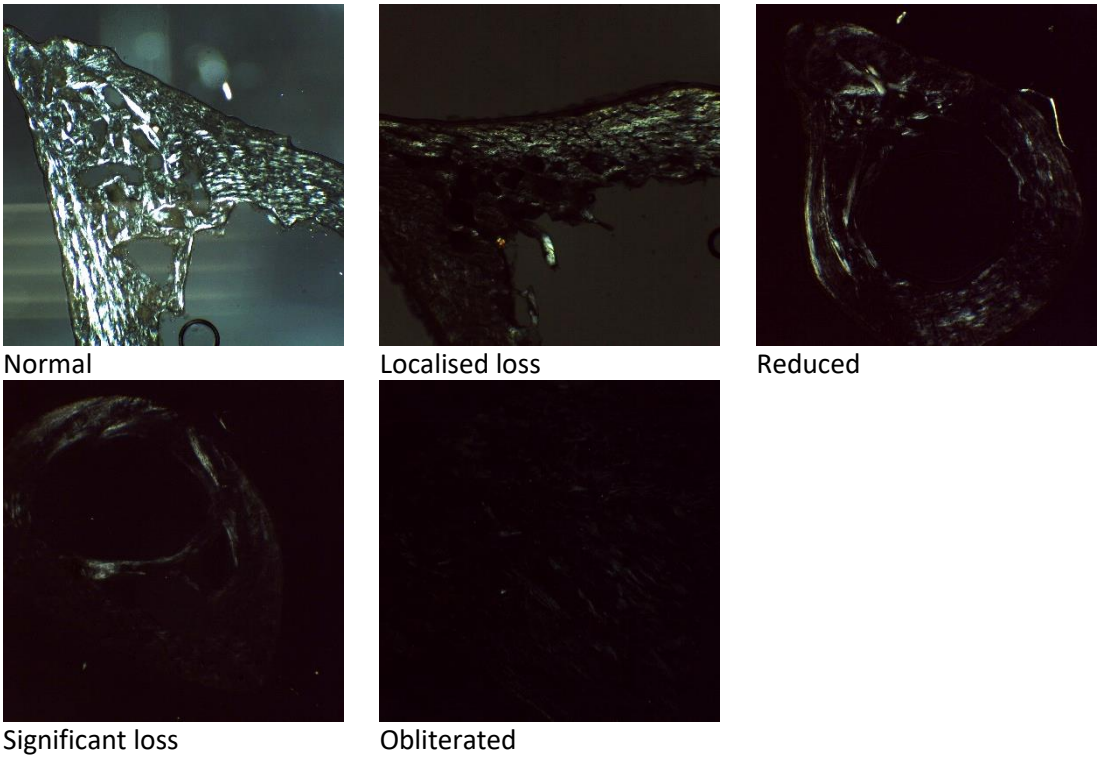
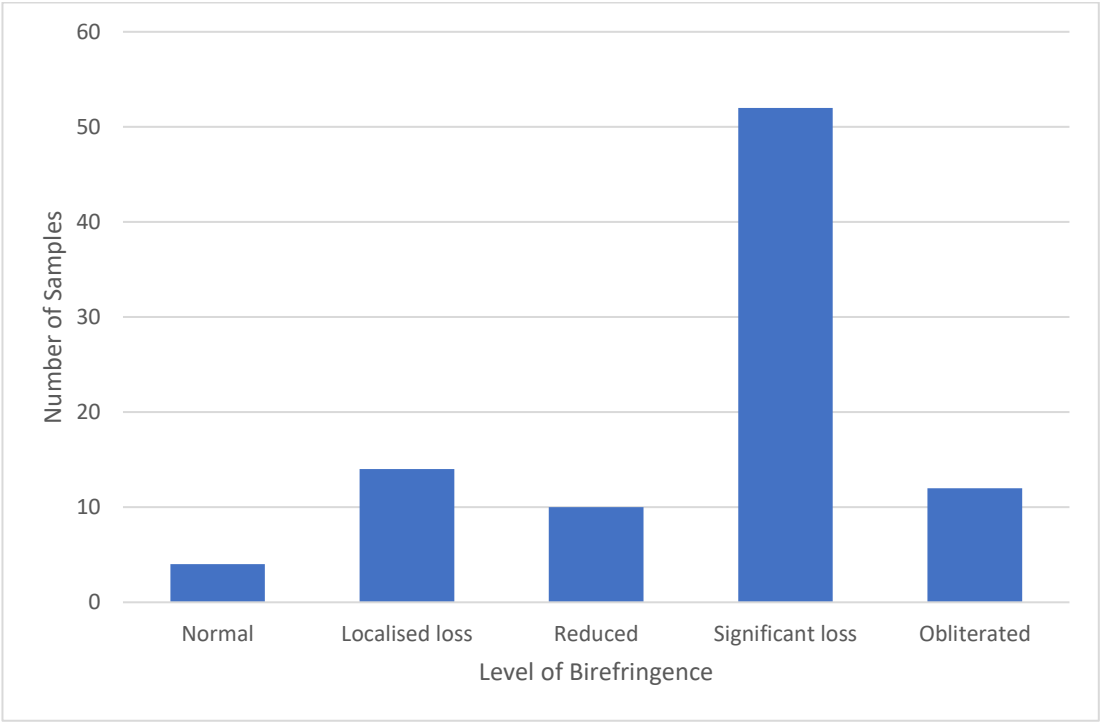


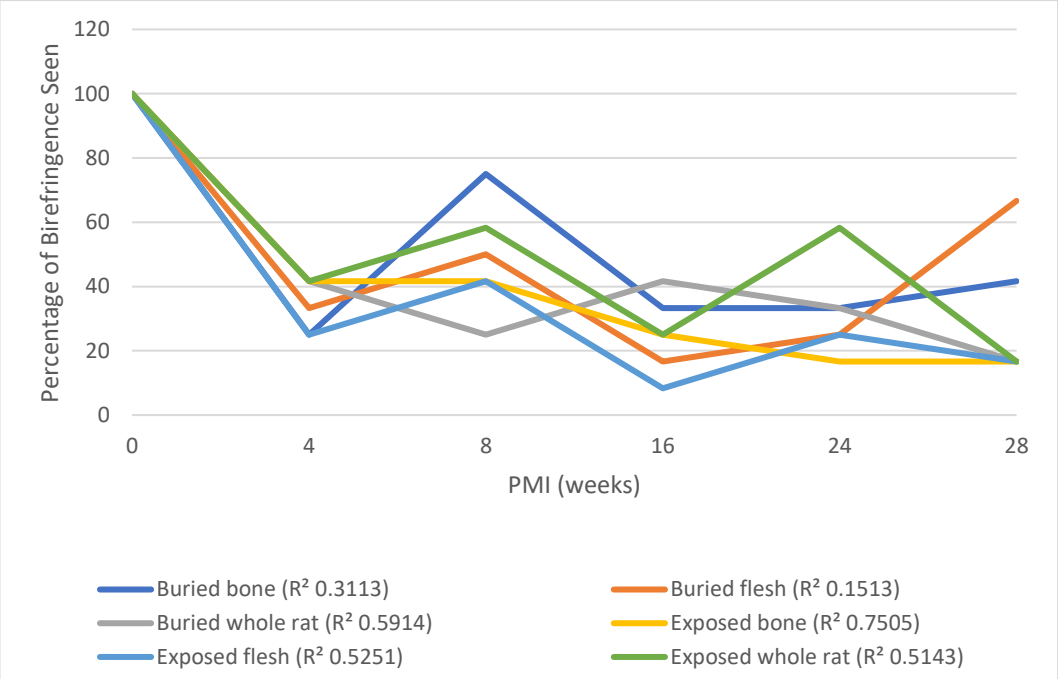
Fig 4.19 Examples of birefringence. It can be seen that each grade gets progressively darker.



Graph 4.10 A histogram to show the changes to the birefringence in all samples. An overview of the BI categories and the number of samples for each one.

Graph 4.10 shows the grades given for birefringence and how many samples fell into each category. 4 samples showed normal birefringence; however, 2 of these were from the fresh day 0 samples and therefore normal was to be expected for them. Only 2 field samples still showed a normal level of birefringence upon analysis; these were from 24 weeks PMI, exposed whole rat, and 28 weeks buried, fleshed limb samples. At the other end of the scale, 12 samples showed a complete loss of birefringence; only 4 of these were from the 28 weeks collection.

Using the amended Birefringence Index, each sample was assigned a percentage where 100% showed normal birefringence and 0% showed all birefringence had been lost. These calculated percentages were used to create graphs to give a visual representation of what was happening over time.



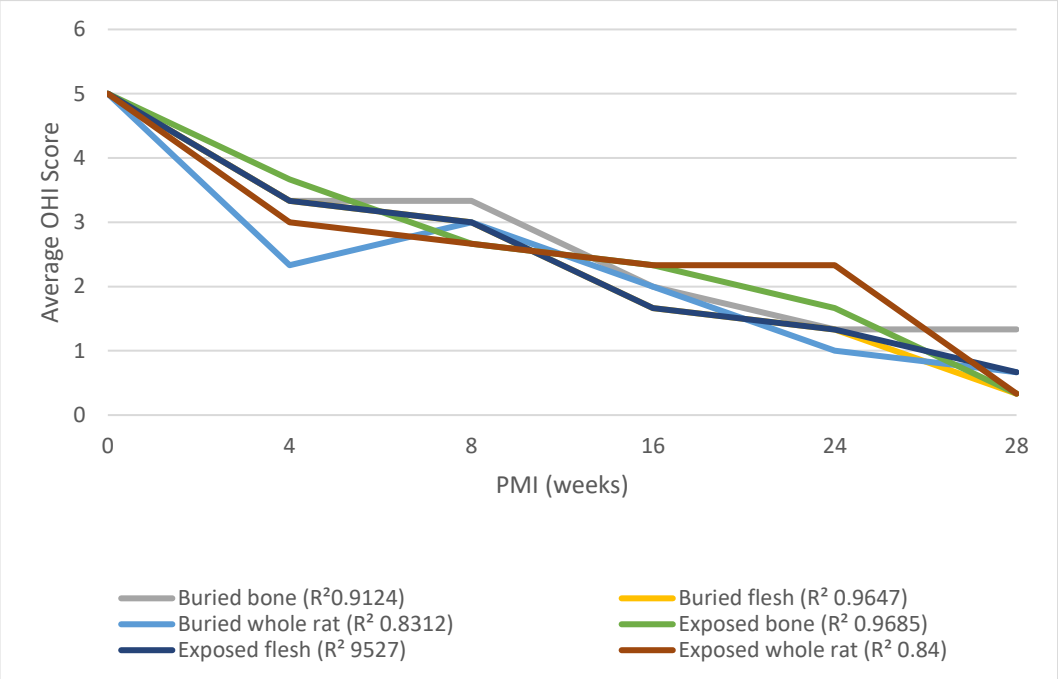
Graph 4.11 A comparison of the birefringence loss observed using an adapted BI Index in all samples over time. Inconsistencies can be seen in the level of birefringence observed.

While many samples did show a loss in birefringence, there was no clear pattern to this (graph 4.11). As can be seen by the R^2 values (ranging from 0.15 – 0.75), birefringence loss over time cannot be predicted from this data set. As some samples were longitudinal samples, further analysis was conducted to exclude these samples, however this still did not yield any significant results. It should also be noted as this was an adapted method of measuring birefringence loss, it was not a validated method. This was also only measured by the researcher and therefore any results given are subjective.

4.7.3 OHI – observable method

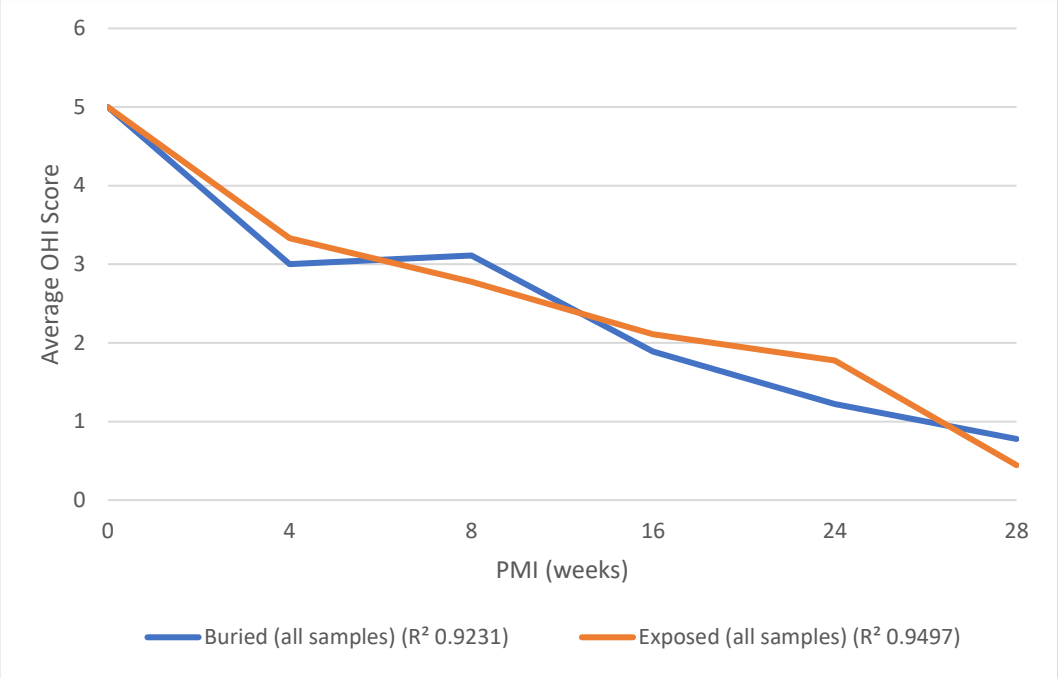
Alongside the analyses described above, the sample were also given an OHI score. This was done by analysing the images obtained on a compound microscope at varying magnifications (40x, 100x, 400x), and using a Keyence microscope for higher magnifications (500x and 1000x); scores were given based on the level of unaltered bone seen. Graph 4.12 shows the scores given for each tissue type and condition at each time interval. These are not whole numbers as the average score was given for each category.

It can be seen that the samples in all conditions degraded over time with the OHI score given decreasing at a fairly steady rate, and the *best fit* lines support a linear regression (R^2 values >0.7).



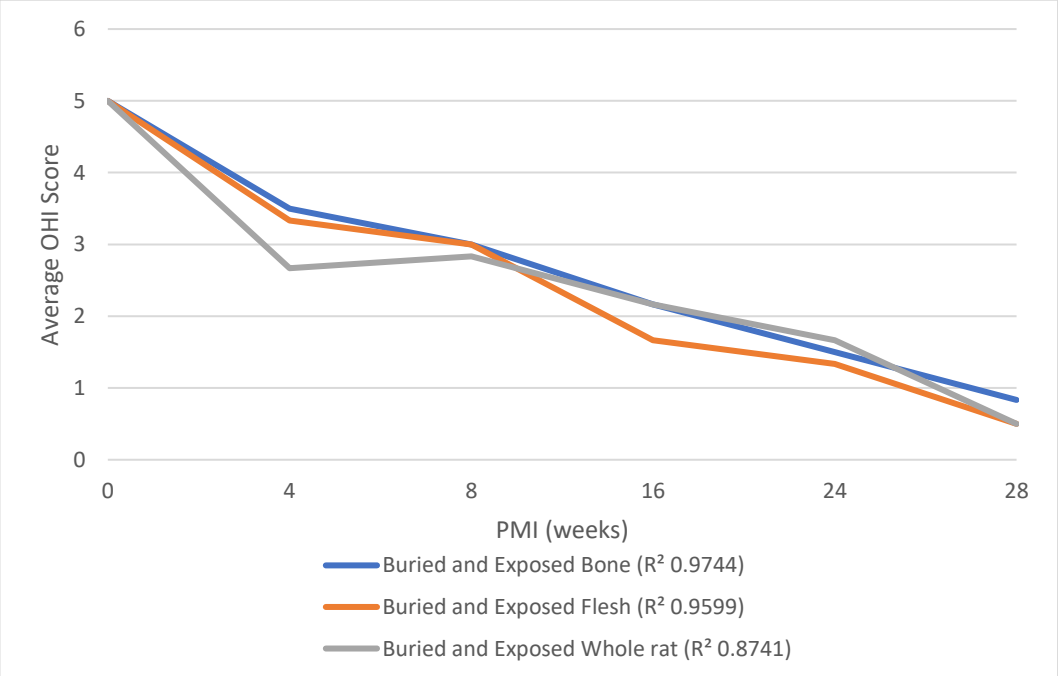
Graph 4.12 A comparison of the changes to the average OHI scores observed in all samples over time. OHI decreased over time for all samples (with few discrepancies seen; these most likely due to averages being used). R^2 values above 0.83 were obtained.

To determine what affect the two deposition environments had on the samples, a separate graph (graph 4.13) was created. As can be seen there was little variance between the buried and exposed samples when all samples were compared together, with the buried samples achieving an R^2 value of 0.92 and the exposed samples achieving R^2 0.95.



Graph 4.13 The average OHI scores observed to all buried samples compared with all exposed samples. Little variation is seen between the buried and exposed samples indicating the burial environment may have little effect on OHI.

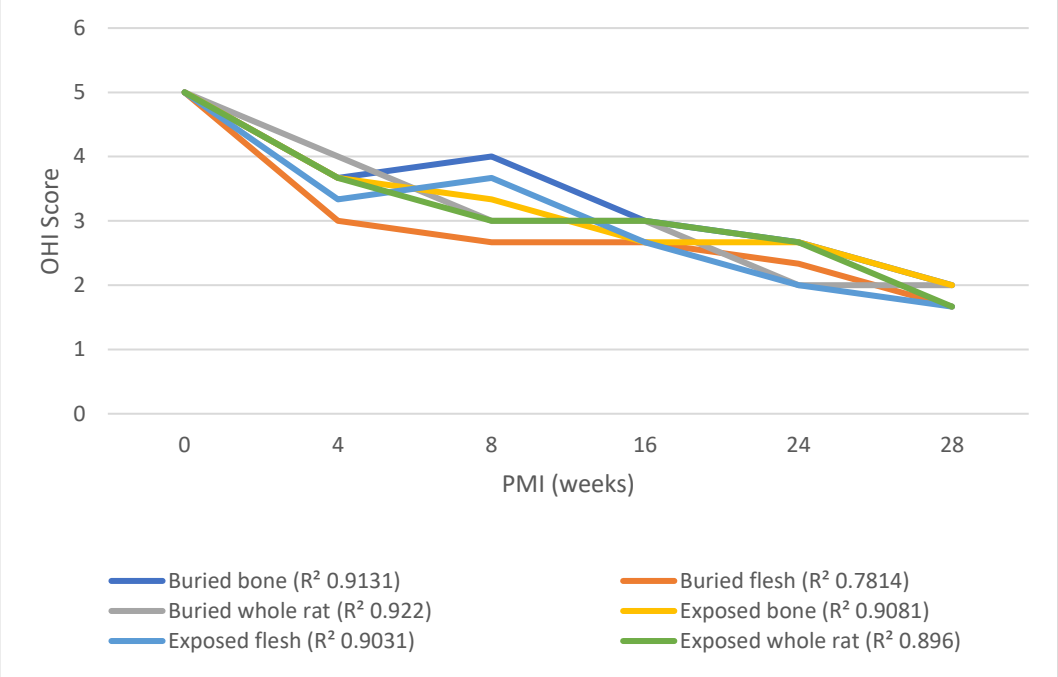
Further to this, the three tissue types were compared (graph 4.14). This was done irrelevant of deposition environment. There appears to be little variation when comparing the defleshed bone and excised, fleshed limb samples with them both following similar decreasing patterns. However, the whole rat samples show a variance, particularly at 4 weeks PMI.



Graph 4.14 The average OHI scores observed between all tissue types. Little variation is seen with the exception of the whole samples at 4 weeks PMI – this is most likely due to the presence of the gut bacteria leading to increased microbial destruction.

4.7.4 OHI – counted method

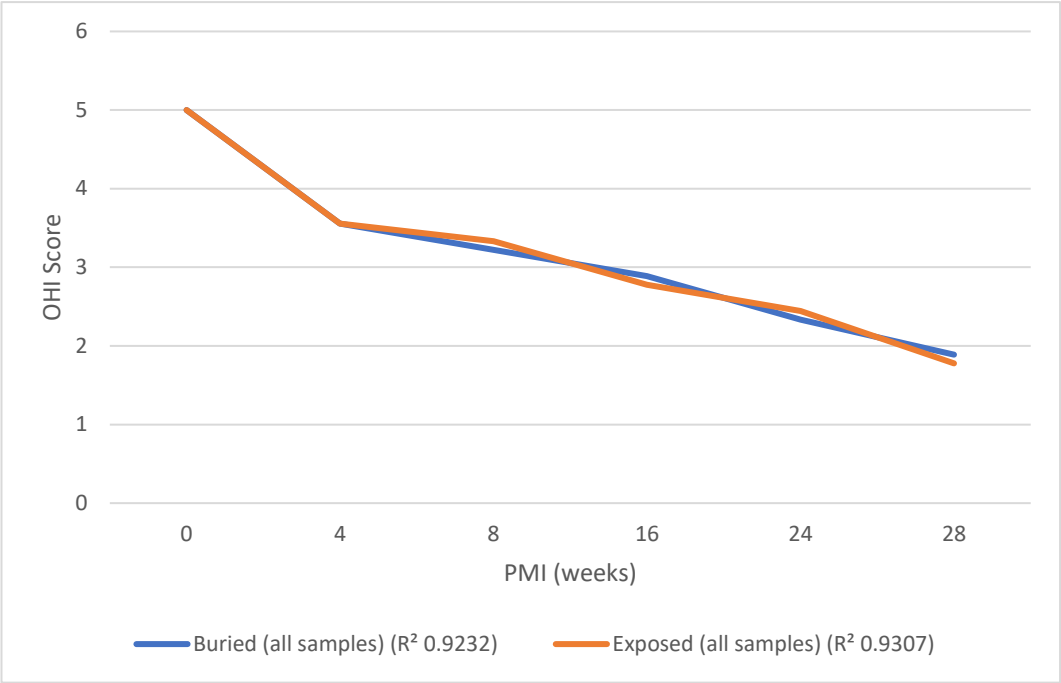
Quantification of loss of histological integrity of the bone was attempted using a counting method. This involved counting the number of osteocyte lacunae seen in each sample; this was then converted into a percentage of intact bone still visible. This percentage was used to assign an OHI score to each sample (graph 4.15).



Graph 4.15 A comparison of the tissue types and deposition environments using the alternative (counted) method of calculating the OHI. It is shown that the OHI score decreased as the PMI increased. R^2 values were not as high as the previous method with one value as low as 0.78 being seen.

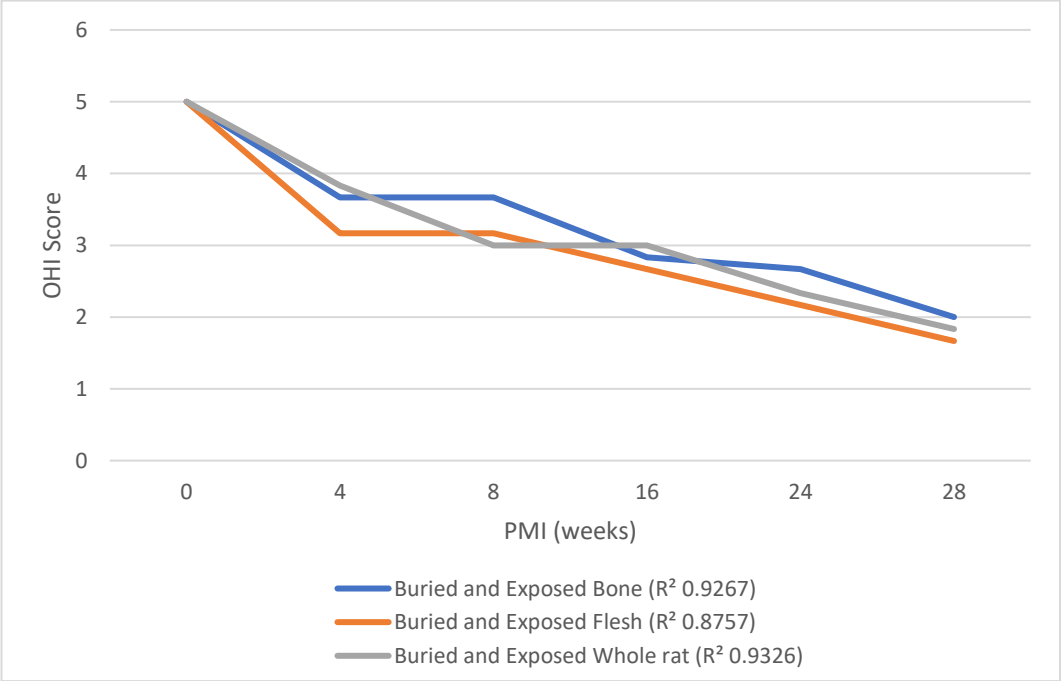
Graph 4.15 shows that the counted method supports a decrease in percentage of intact bone over time. R^2 values of above 0.7 were achieved for all samples.

The deposition environments were compared to determine whether there was a significant difference seen. Graph 4.16 shows that the deposition environment had little effect on the amount of microbial infiltration that occurred.



Graph 4.16 A comparison of the changes to the OHI scores of the two deposition environments over time. There was very little variance between the buried and exposed samples.

Further to this, the tissue types were compared (graph 4.17).

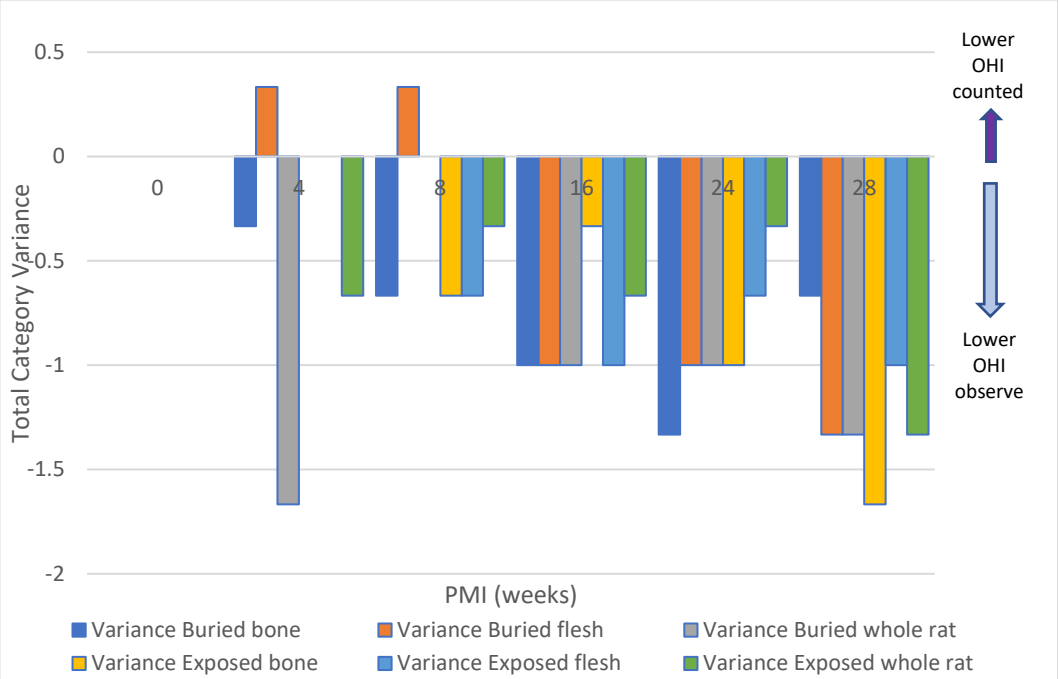


Graph 4.17 A comparison of the changes to the OHI scores of the three tissue types over time. The fleshed, excised samples (all) gave the greatest variance at 4 weeks. This is different to the variance seen using the observational method and could indicate that the observational method was not ideal due to its subjectivity.

4.7.5 Counted vs Observable Method

To work out which method worked best for determining the level of bone diagenesis occurring, the two methods were compared. This was done by creating a graph of variance (graph 4.18),

taking into account the overall OHI scores given for all samples. To create this the counted OHI score was subtracted from the observed OHI score. It can be seen that the observed method gave lower scores to the samples, particularly the later ones.



Graph 4.18 Graph of variance to show differences in OHI scores between observed and counted methods. (Columns above the line indicate a lower score for counted method when compared with observed method). The observable method gave lower scores than the counted method – this could be due to the subjectivity of the observable method, and bias by the researcher (double blind studies would be recommended in future research).

4.8 Confocal LSM

Due to time issues, not all samples were analysed using the Confocal LSM. As this was a new technique in bone diagenesis, samples that were more likely to show some variance were chosen; these were from halfway through the experiment and near the end. Samples from collection weeks 16 and 24 were analysed, as well as fresh samples for testing and control (fig 4.20).

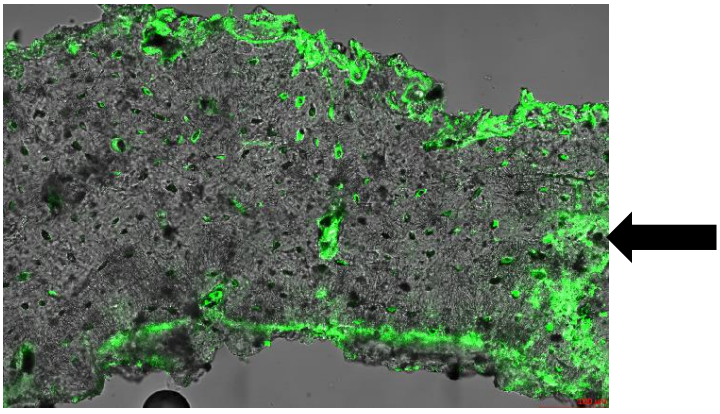


Fig 4.20 Fresh sample showing extensive fluorescence. Arrow indicates fluorescence seen

The fresh sample showed extensive fluorescence at 488 nm. Samples at weeks 16 and 24 showed significant decreases in fluorescence (figs 4.21-4.32).

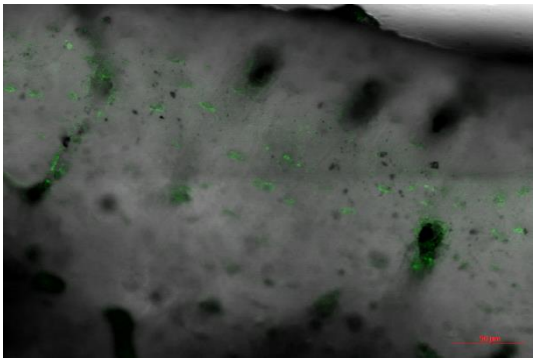


Fig 4.21 Week 16 buried defleshed bone

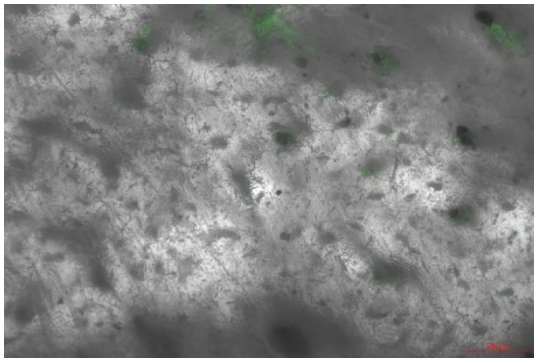


Fig 4.22 Week 24 buried defleshed bone

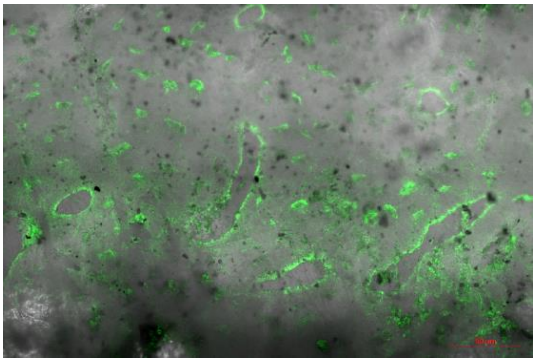


Fig 4.23 Week 16 buried excised, fleshed limb

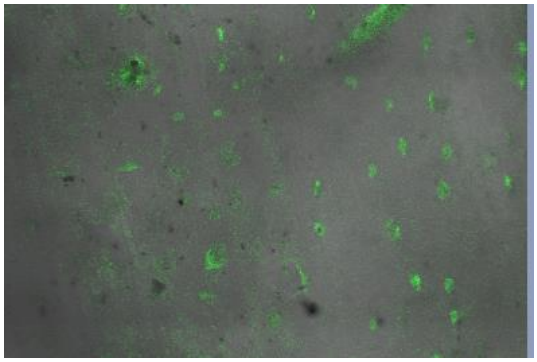


Fig 4.24 Week 24 buried excised, fleshed limb

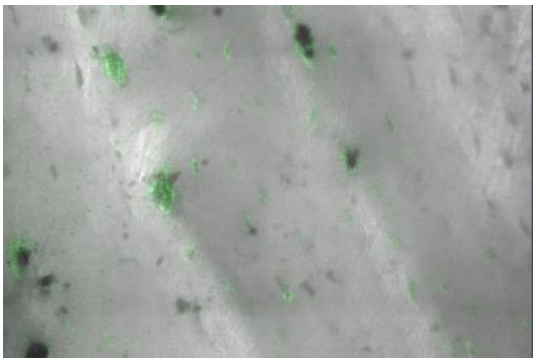


Fig 4.25 Week 16 buried whole rat

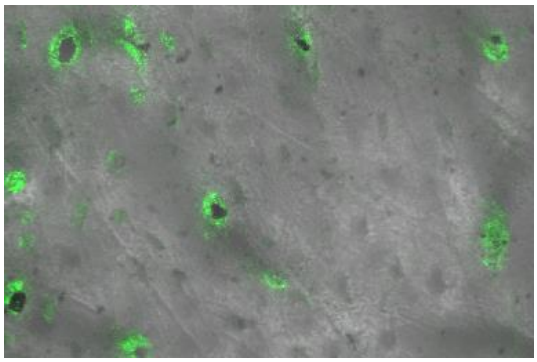


Fig 4.26 Week 24 buried whole rat

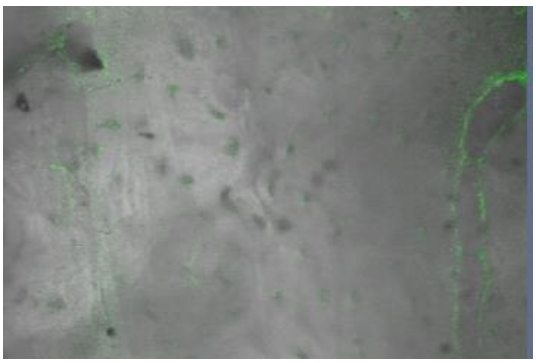


Fig 4.27. Week 16 exposed defleshed bone

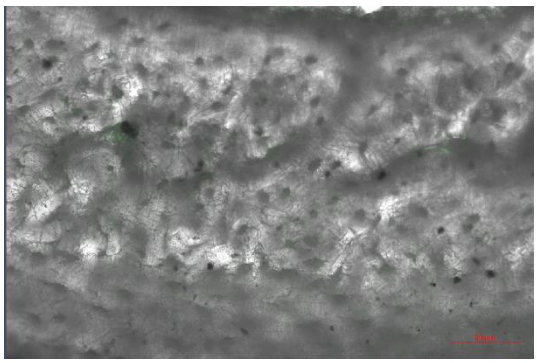


Fig 4.28 Week 24 exposed defleshed bone

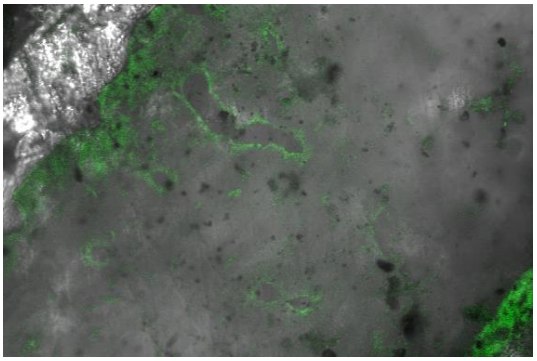


Fig 4.29 Week 16 exposed excised, fleshed limb

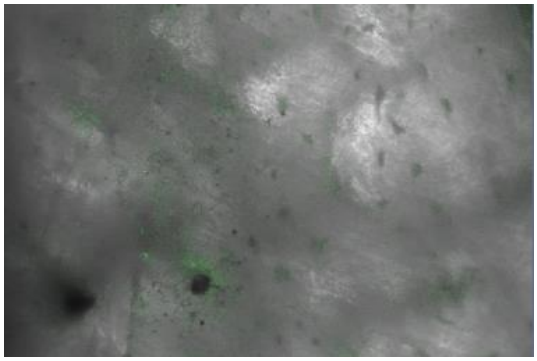


Fig 4.30 Week 24 exposed excised, fleshed limb

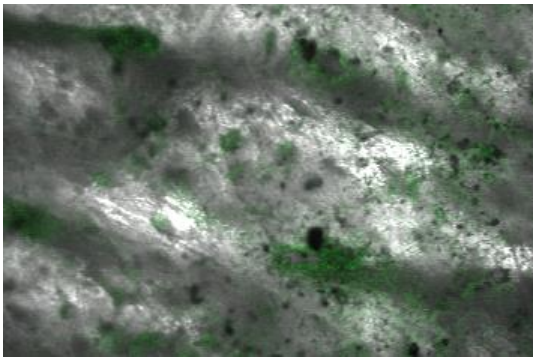


Fig 4.31 Week 16 exposed whole rat

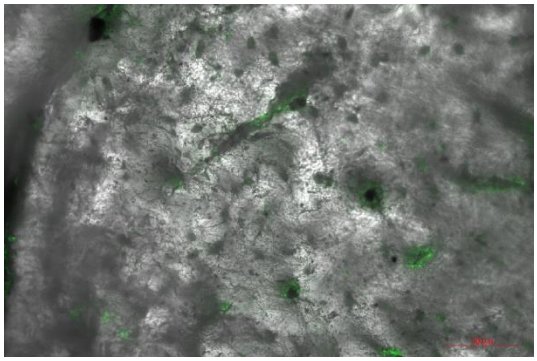
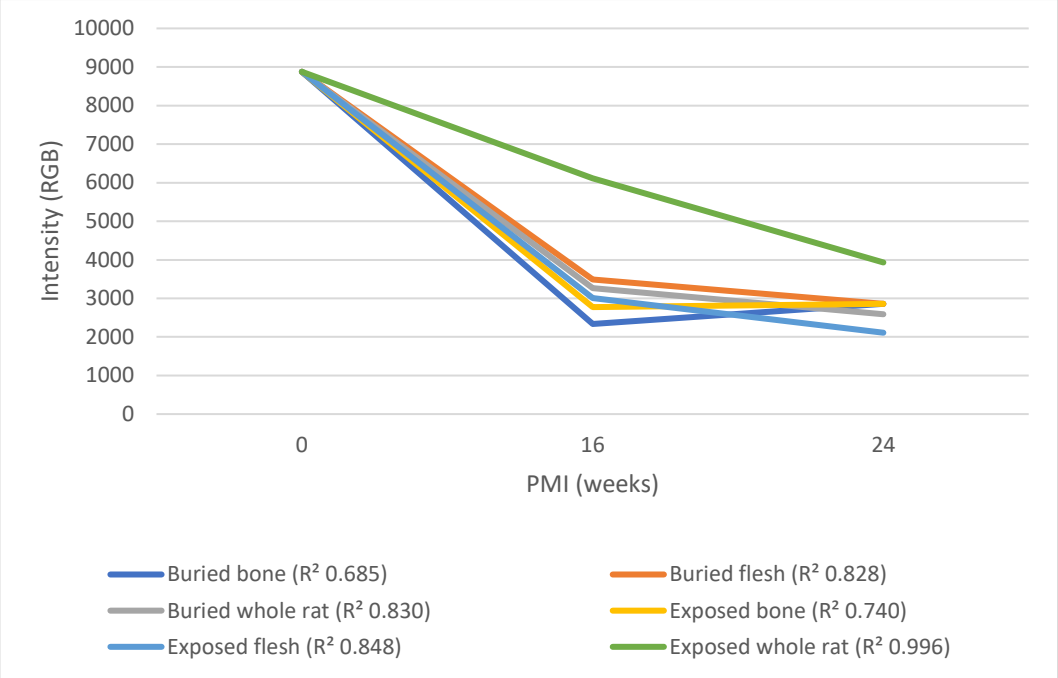


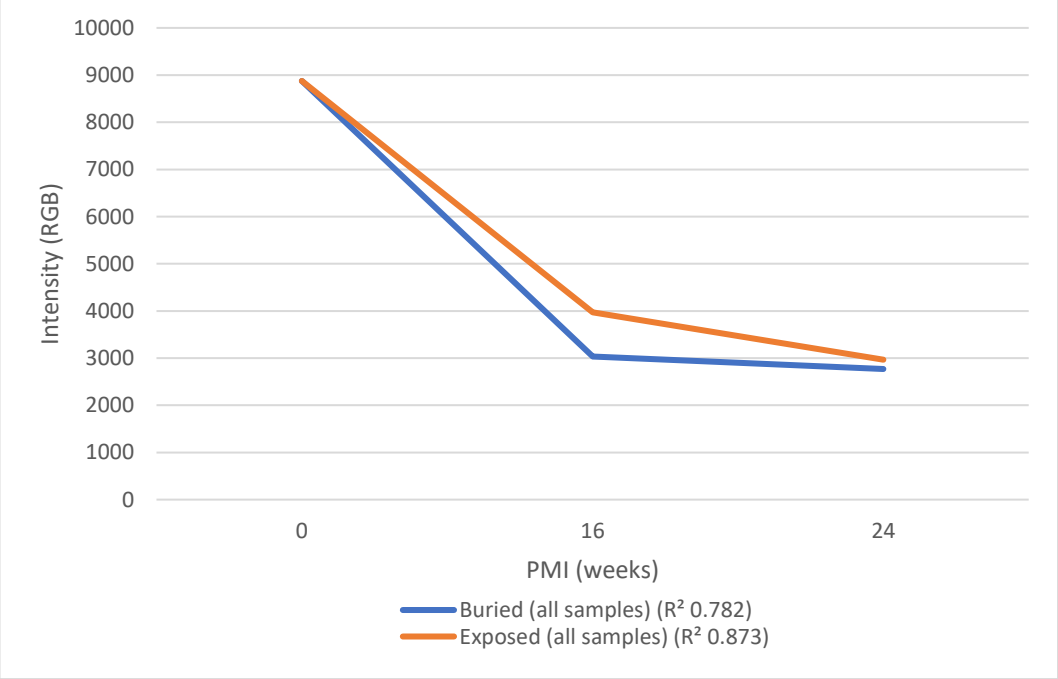
Fig 4.32 Week 24 exposed whole rat

Zeiss software, *Zen black* was used to analyse the intensity of fluorescence. The histogram feature on the software produces an intensity table using the RGB scale of 0-255. The table was set to skip black and white pixels as this was focused mainly on the fluorescence; the averages were calculated from these histogram tables and used to create graphs to show changes occurring over time. Graph 4.19 shows an overall decrease in fluorescence intensity in all samples analysed.

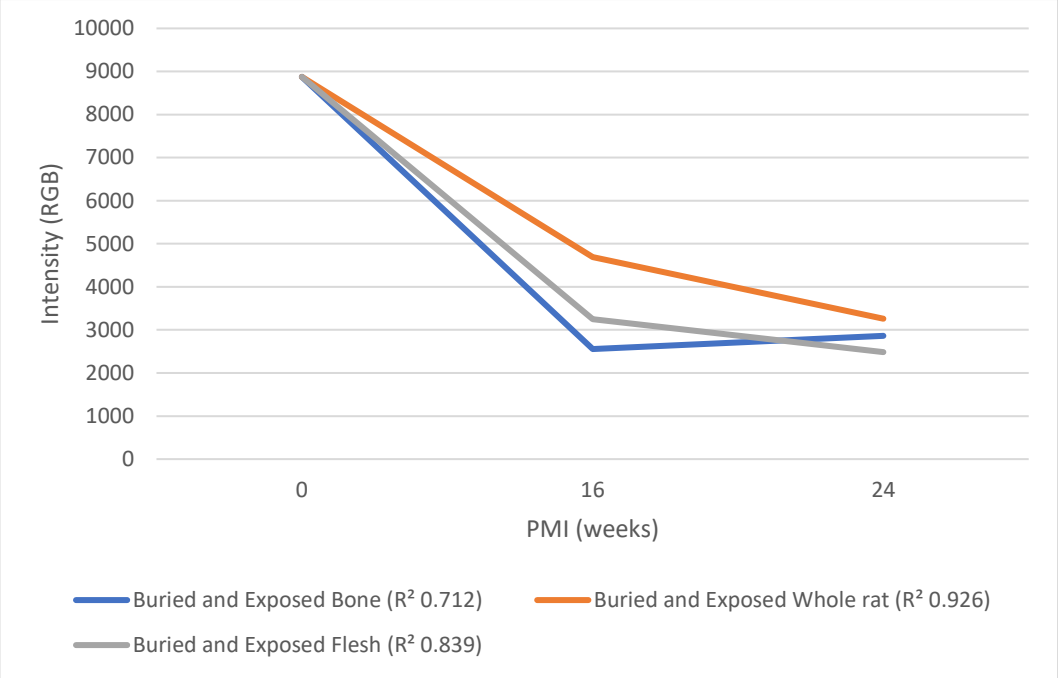


Graph 4.19 The changes to the level of intensity (measured by fluorescence) between all sample conditions. There is little variation between the samples with the exception of the exposed whole rat samples.

Further graphs were produced to compare the effects the deposition environment had on fluorescence, and the different tissue types. Graph 4.20 shows that the deposition environment has little effect on the loss of fluorescence intensity, with the samples showing little variation in the overall loss seen.



Graph 4.20 The changes to the level of intensity (measured by fluorescence) between the two deposition environments. Initially it appears the buried samples lose the most fluorescence, however there is little variance in the later (24 week PMI) samples.



Graph 4.21 The changes to the intensity (measured by fluorescence) between the three tissue types. The whole rat samples showed the lest loss in fluorescence while the defleshed bone and excised, fleshed samples showed some inconsistencies.

The tissue type did appear to have an effect on loss of fluorescence intensity. The whole rat samples showed the least overall loss when compared with the defleshed bones and the excised, fleshed limbs (graph 4.21), with the whole rat samples losing 63% of their fluorescence while the defleshed bone samples and the fleshed, excised limb samples lost 68% and 72% respectively.

There were some samples that showed unusual fluorescence when analysed on the Confocal LSM. These samples appeared to have specific, localised areas of fluorescence (fig 4.33 & 4.34).

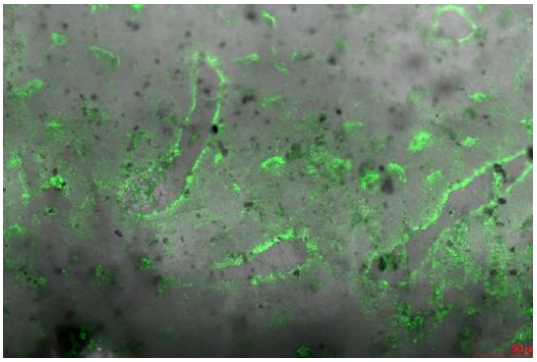


Fig 4.33 Buried excised, fleshed sample, 16 weeks PMI

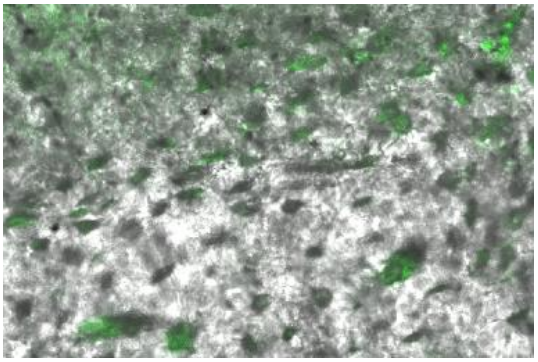


Fig 4.34 Exposed whole rat, 24 weeks PMI

To confirm whether this was due to an issue with the samples, histological samples from Sheffield University were obtained for analysis. These were part of the White & Booth (2014) collection. The localised fluorescence can be seen clearly in Figs 4.35 and 4.36.

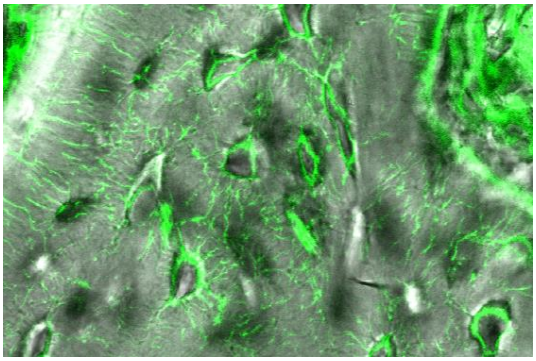


Fig 4.35 Foetal *Sus scrofa*, 6 months exposed PMI

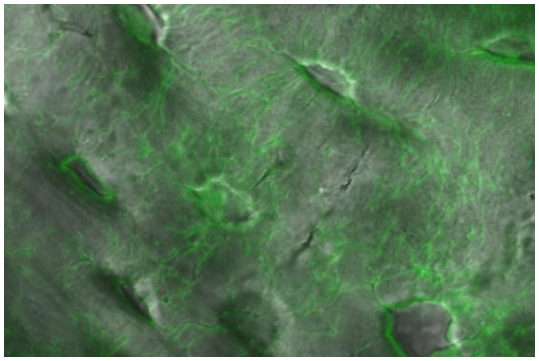


Fig 4.36 Neonate *Sus scrofa,* 6 months PMI

Slides for fig 4.35 and 4.36 borrowed with thanks from Sheffield University Archaeology department (White & Booth 2014 collection)

4.9 Proteomic Analysis

Samples were sent to Dr Noemi Procopio at Northumbria University for proteomic analysis to determine the protein abundance of the bones and analyse whether these changed with increasing PMI, and with the presence of gut bacteria. The samples were selected based on their potential to answer the original research questions; fresh samples for control purposes; buried, defleshed bones and exposed whole rat from week 12; and buried, defleshed bones and exposed whole rat from week 24. Three samples were sent to allow for repeat measurements to be made. The proteins were extracted from the samples using the protocol set out in Procopio & Buckley (2017) and analysed using LC-MS/MS.

The schematic below (fig 4.37) shows the variability of all samples analysed. Each colour corresponds to a different condition:

- Pale blue – fresh (control)
- Pink – buried defleshed bone (12 weeks)
- Purple – exposed whole rat (12 weeks)
- Blue – buried defleshed bone (24 weeks)
- Orange – exposed whole rat (24 weeks)

Fig 4.37 shows a PCA map of all the samples analysed. Principle Component Analysis was done to analyse the variation, if any, between the samples. The grey labels are the names of the proteins found while the coloured dots represent the samples. The distance between the dots represents the variance in their protein content, and specifically, the abundance of the individual proteins. The Principle Component 1, found on the *x* axis, accounts for more difference in the protein content than differences in Principle Component 2; the greatest variability occurs between the light blue dots (fresh) and the blue dots (buried defleshed bone, 24 weeks PMI).

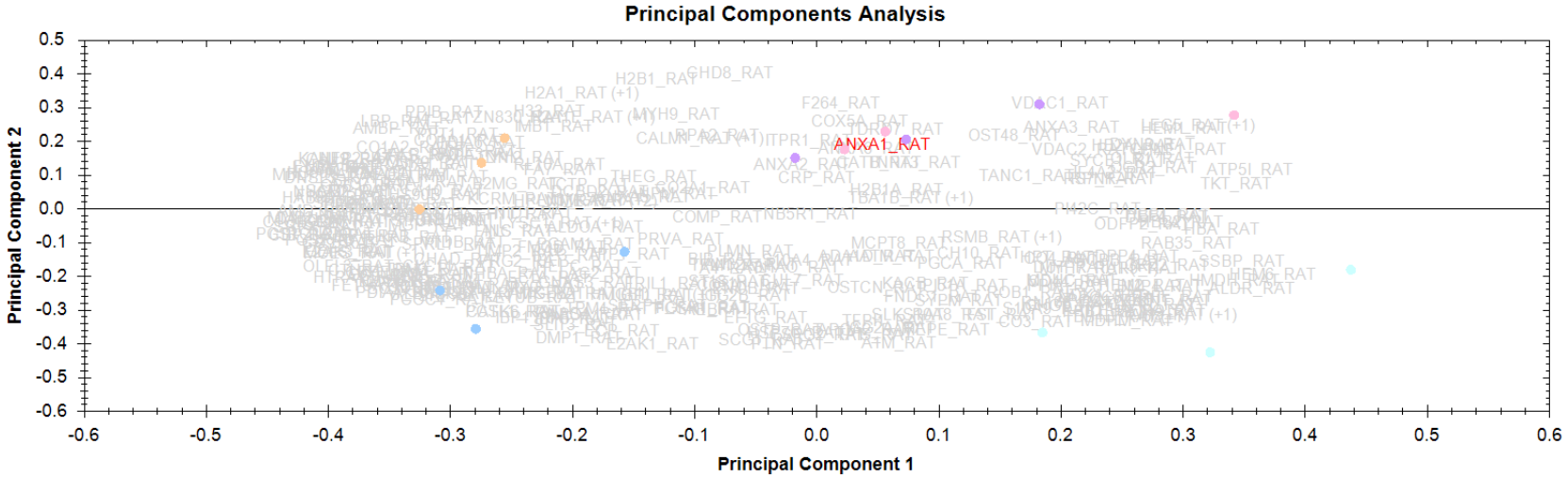


Fig 4.37 PCA showing all samples analysed. Pale blue dots – fresh (control), pink dots – buried defleshed bone at 12 weeks PMI, purple dots – exposed whole rat at 12 weeks PMI, blue dots – buried defleshed bone at 24 weeks PMI, orange dots – exposed whole rat at 24 weeks PMI

The fresh samples show a difference in their protein content compared with the week 12 and week 24 PMI samples, with the biggest variance being seen between the fresh and 24 weeks PMI.

The PMI analysis was further investigated. This gave the following:

- Purple – fresh (control)
- Pink – week 12
- Blue – week 24

These PCA maps (Fig 4.38 and 4.39) show the variance when comparing the PMI. Fig 4.38 shows the fresh samples and the grouped (defleshed bone and whole rat) samples for weeks 12 and 24. It can be seen that there is less variance between the fresh and week 12 samples compared to the fresh and week 24 samples. Fig 4.39 shows the grouped samples for weeks 12 and 24, minus the fresh samples. The variance between the two PMI's can be seen through the separation on the Principle Component 1 axis. The week 24 samples showed an increase in protein abundance therefore, the PCA map was further studied to determine which proteins were causing this shift.

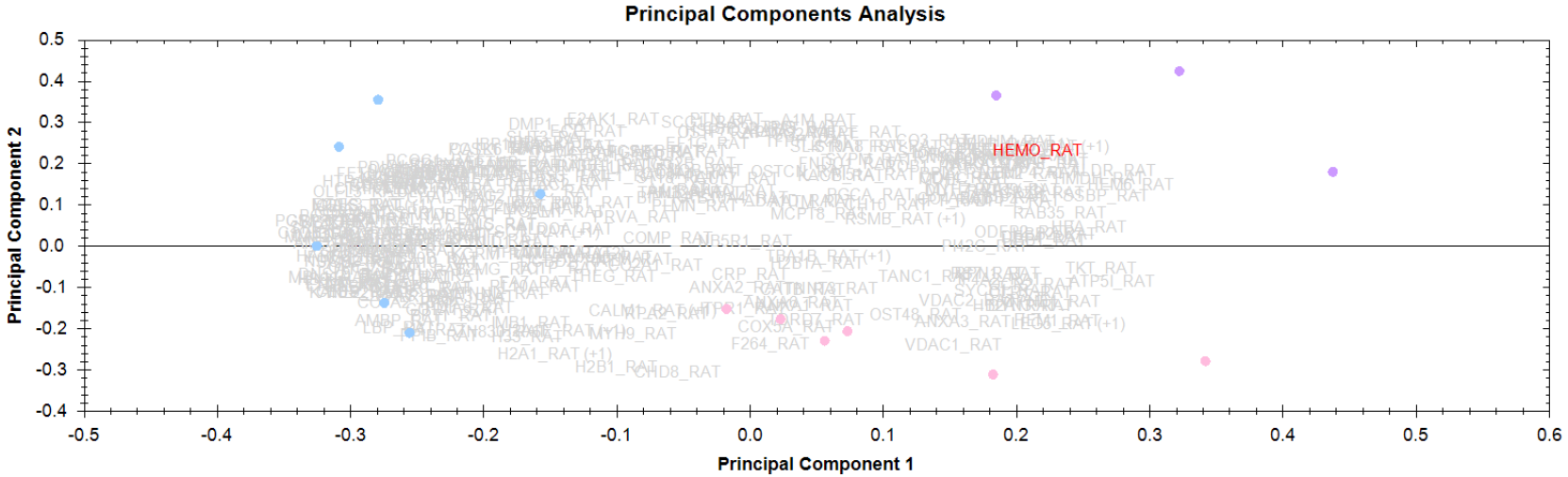


Fig 4 38. PCA of PMI including fresh. Purple dots – fresh (control), pink dots – all samples at 12 weeks PMI, blue dots – all samples at 24 weeks PMI

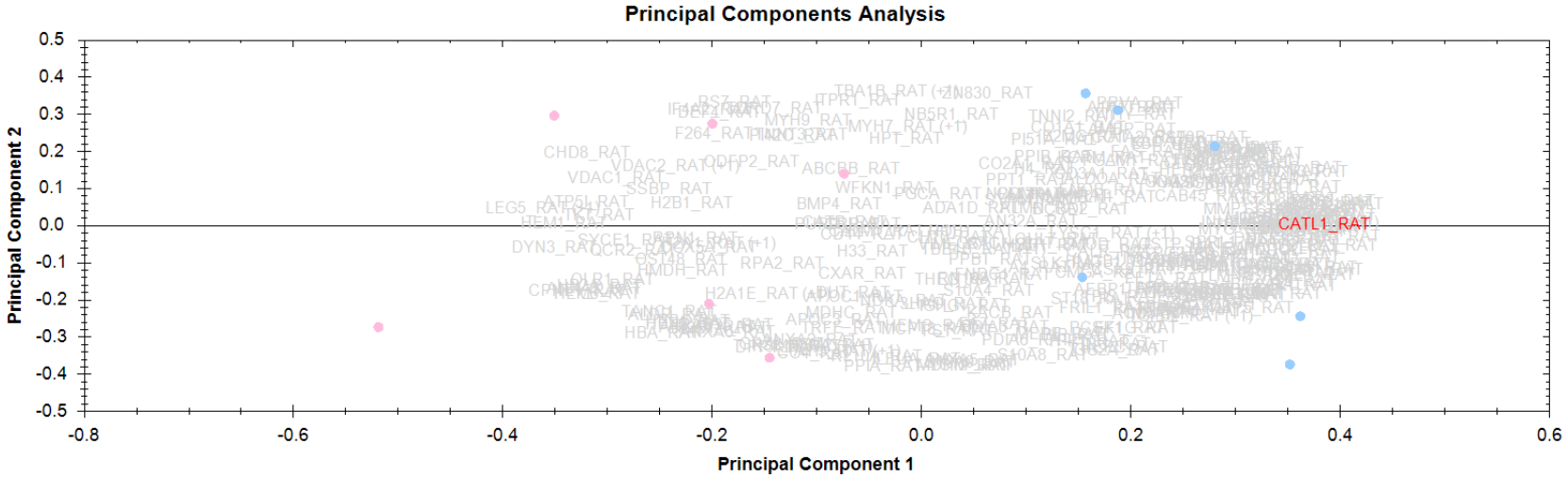


Fig 4.39 PCA of PMI only. Pink dots – all samples at 12 weeks PMI, blue dots – all samples at 24 weeks PMI

Fig 4.40 shows vector maps which show the proteins that had the biggest influence. The colours correspond to:

- Red – fresh (control)
- Green – 12 weeks
- Yellow – 24 weeks

The bar charts below the vector maps show the 15 most significant proteins, with the size of the bar indicating how much they contributed to the separation of the samples on the vector map. Those over the red line collectively contribute over 50% to the two dimensions (Dim 1 for left chart, Dim 2 for right chart). The proteins (up to MBL1 for Dim1 and PGS1 for Dim2) explain at least 50% of the contribution to the first and second dimensions respectively.

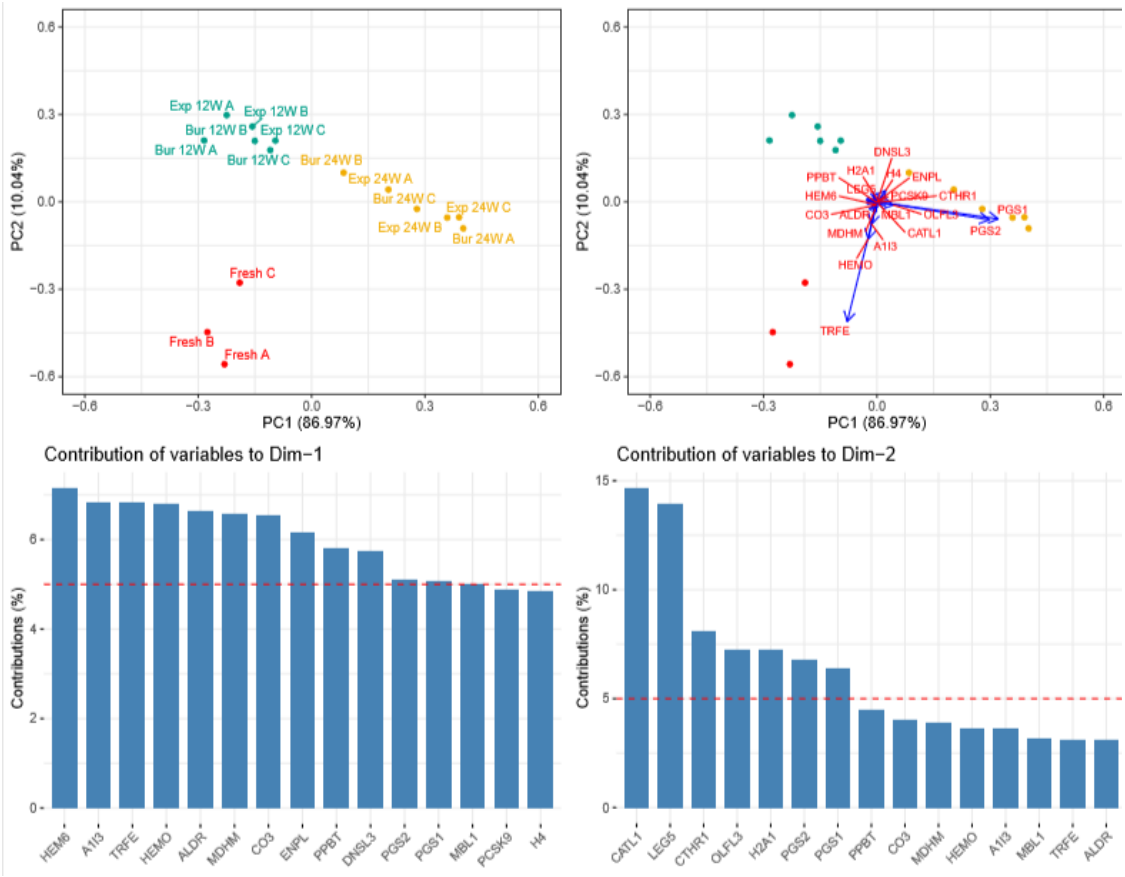


Fig 4.40 Analysis of specific proteins causing the shift between PMI. Red dots – fresh (control), green dots – all samples at 12 weeks PMI, yellow dots – all samples at 24 weeks PMI. The histograms indicate the proteins having the biggest effect on the samples.

The left-hand side shows that the proteins HEMO and TRFE (blood proteins) are pushing the fresh samples down on the Principle Component 2 axis. The bar chart to the left shows that these two proteins are making a contribution of about 6.5% in pushing the fresh samples down in the first dimension.

The right-hand side shows the proteins PGS1 and PGS2 pushing the week 24 samples to the right on the vector map, separating them from the other samples. The bar chart shows they are important contributors to the second dimension on the PCA map.

PGS1 and PGS2 correspond with Biglycan and Decorin, bone proteins (Procopio & Buckley, 2016). These showed higher in abundance in the week 24 samples compared with the fresh and week 12 samples. This was not as expected; it had been anticipated that the proteins would be lower in abundance as time increased. One hypothesis is that the degradation of the bones may have led to easier extraction of the proteins.

As with the other analyses, the deposition environments were compared to determine whether being buried or left exposed had an effect on protein content (fig 4.41). The colours correspond to:

- Purple – fresh (control)
- Blue – exposed samples
- Pink – buried samples

The collection weeks were combined for this analysis in order to take only the deposition environment into account. As can be seen below, there was not a big difference between the buried and exposed samples. This can be determined as the samples were not well separated along the *x* axis. As the buried samples were defleshed bones only and the exposed samples were from whole rats; the PCA below can also be used to analyse any differences between the two tissue types. It can be concluded that the tissue types made no difference on protein content.

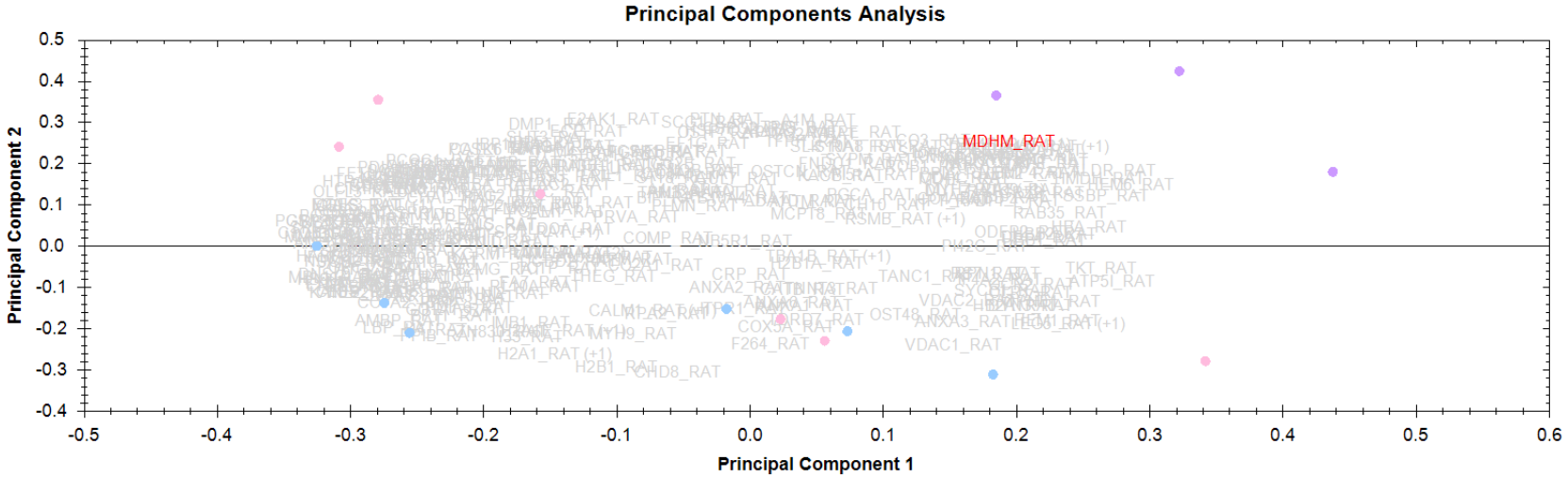


Fig 4.41 PCA of the deposition environments. Purple dots – fresh (control), blue dots – all exposed samples, pink dots – all buried samples

4.10 Statistical Analysis

Statistical analysis was conducted where possible; due to the small number of repeats and often limited variation of data values this wasn't always possible. T-tests were performed for the OHI scores using the visual method, however due to the low samples size, it was not possible to get anything significant. Therefore the counted method was used to determine statistical significance.

Due to the irregular patterns of fluorescence using UV light, a t-test was performed pairing all 4 weeks PMI samples with all 28 weeks PMI samples. This resulted in a P value of 0.507, as this was >0.05 (Rumsey, 2011) it indicated that there was no statistical significance to the results seen[6].

Table 4.8 T-test for weeks 4 and 28 PMI (UV fluorescence)

	Week 4	*Week 28*
Mean	131.9606	136.1111
Variance	232.664	222.51
Observations	12	12
Pooled Variance	227.587	
Hypothesized Mean Difference	0	
df	22	
t Stat	-0.67391	
P(T<=t) one-tail	0.253694	
t Critical one-tail	1.717144	
P(T<=t) two-tail	0.507388	
t Critical two-tail	2.073873	

The t-test analysis was conducted for the osteocyte counting method to determine whether the results being seen were of statistical significance. When comparing the 4 weeks PMI samples with 28 weeks PMI, a P value of 2.34×10^{-14} was calculated. This shows that there is less than 5% chance of the results seen being due to random variations, making them statistically significant. T-tests were also calculated comparing defleshed bone to the whole rat samples, and the buried environment to the exposed environment. These gave P values of 0.428 and 0.778 respectively, showing that these variations were not statistically significant and could be due to other factors[6].

Table 4.9 T-test for week 4 and week 28 PMI (counted lacunae)

[6] If a P value of less than 0.05 was calculated the results were considered statistically significant and the null hypothesis was rejected.

	Week 4	*Week 28*
Mean	12.37888889	4.093889
Variance	5.891469281	1.899237
Observations	18	18
Pooled Variance	3.895353105	
Hypothesized Mean Difference	0	
df	34	
t Stat	12.59332327	
P(T<=t) one-tail	1.16924E-14	
t Critical one-tail	1.690924255	
P(T<=t) two-tail	2.34E-14	
t Critical two-tail	2.032244509	

Table 4.10 T-test for bone and whole rat samples (counted lacunae)

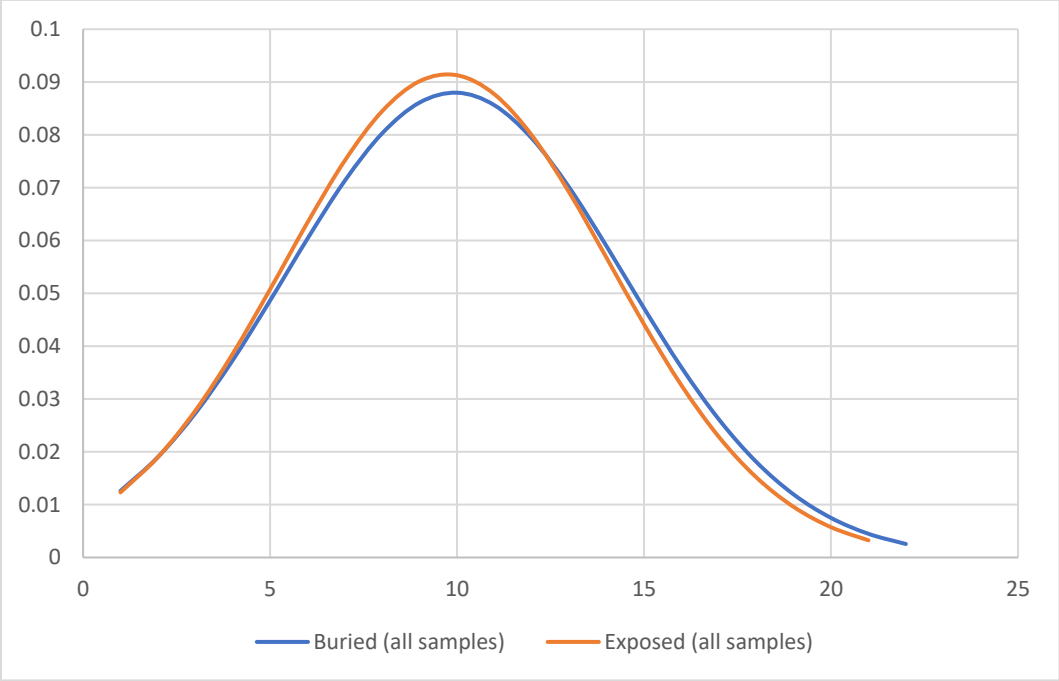
	Bone	*Whole*
Mean	9.494444444	8.8
Variance	11.68100255	10.98544
Observations	30	30
Pooled Variance	11.33322158	
Hypothesized Mean Difference	0	
df	58	
t Stat	0.798925802	
P(T<=t) one-tail	0.213796499	
t Critical one-tail	1.671552762	
P(T<=t) two-tail	0.427592999	
t Critical two-tail	2.001717484	

Table 4.11 T-test for buried and exposed samples (counted lacunae)

	Buried	*Exposed*
Mean	8.937037	8.733333
Variance	12.08244	11.19242
Observations	45	45
Pooled Variance	11.63743	
Hypothesized Mean Difference	0	
df	88	
t Stat	0.283245	
P(T<=t) one-tail	0.388827	
t Critical one-tail	1.662354	
P(T<=t) two-tail	0.777655	
t Critical two-tail	1.98729	

A normal distribution graph was created for the counted lacunae method. It can be seen that the data points are evenly distributed across the mean, with the averages being 8.937 for all buried samples and 8.733 for the exposed samples. The curves for both buried and exposed

samples are shown to be very similar across both sides of the mean, indicating that microbial destruction was very similar, regardless of the deposition environment.



Graph 4.22 Normal Distribution of the OHI scores using the counted lacunae method

T-tests were performed on the Confocal LSM data to determine whether there was any statistical significance to them. This showed there was statistical significance when comparing the day 0 samples with the 24 weeks PMI samples, with a P value of 2.06×10^{-6}. The same was found when comparing the defleshed bone and whole rat samples, with a P value of 0.007. However, when the two deposition environments were compared no statistical significance was found (P value = 0.137).

Table 4.12 T-test day 0 vs 24 weeks PMI

	Day 0	*Week 24*
Mean	8875.854	2867.787
Variance	0	356949.4
Observations	6	6
Hypothesized Mean Difference	0	
df	5	
t Stat	24.63242	
P(T<=t) one-tail	1.03E-06	
t Critical one-tail	2.015048	
P(T<=t) two-tail	2.06E-06	
t Critical two-tail	2.570582	

Table 4.13 T-test buried vs exposed

	Buried	*Exposed*
Mean	2900.288	3466.998

Variance	626098.1	4418487
Observations	36	36
Hypothesized Mean Difference	0	
df	45	
t Stat	-1.51391	
P(T<=t) one-tail	0.068521	
t Critical one-tail	1.679427	
P(T<=t) two-tail	0.137042	
t Critical two-tail	2.014103	

Table 4.14 T-test bone vs whole

	Bone	*Whole*
Mean	2597.488	3599.148
Variance	309828.7	973602.5
Observations	12	12
Hypothesized Mean Difference	0	
df	17	
t Stat	-3.06284	
P(T<=t) one-tail	0.003522	
t Critical one-tail	1.739607	
P(T<=t) two-tail	0.007043	
t Critical two-tail	2.109816	

Statistical analysis was conducted on the proteomics. The abundance of proteins PGS1 and PGS2 was very similar between the fresh samples and the samples from week 12, but between the fresh samples and the 24 week samples, there was a statistically significant difference ($p < 0.05$) in the abundance of PGS1 and PGS2. This indicates that the results seen were not due to random variations. The levels of these proteins were higher in the samples with the longer deposition interval.

Chapter Five:

Discussion

5.1 Introduction

The main purpose of this research was to attempt to answer the long-standing question of where the bacteria responsible for bone diagenesis originates (Hackett, 1981; White & Booth, 2014). As discussed in previous chapters there are two main themes for this;

1. That the intrinsic gut bacteria are the driving force behind bone diagenesis; having been kept in place by the immune system throughout life, the gut bacteria are able to move freely throughout the body after death, leading to infiltration of the skeletal material (White & Booth, 2014),
2. That bacteria in the environment are responsible for diagenesis; particular focus is usually placed on the bacterial colonies in the burial soil (Hackett, 1981).

This chapter will analyse and critically discuss the results obtained throughout this project. The research questions presented in Chapter One will also be revisited.

5.2 Soft Tissue Decomposition and TBS

The decomposition of the soft tissue was not the purpose of this research, however as this project used whole and fleshed samples, it was decided to document the process to determine whether the burial and/or tissue conditions had any effect on the rate of decomposition seen.

The samples were deposited on the 14th November 2018 and left for a total of 196 days. The first set of samples were collected four weeks later; it was anticipated that due to the cold weather, the decomposition process would be slow. However, the exposed, whole samples were full of entomological activity and some skeletonisation had already started to occur. The fleshed, excised legs took longer to attract insect activity and for skeletonisation to begin; this did not occur until 12 weeks PMI. A comparison of the limbs from the whole samples and the excised samples shows the difference in rate of decomposition. It can be seen on Graph 4.4 that the limbs from the whole samples showed an increased rate of decomposition, with a TBS of 6 being achieved by 4 weeks PMI compared with the excised limbs TBS of 2. Unlike the whole samples which quickly attracted insects, which in turn accelerated the decomposition process, the fleshed, excised legs did not attract insects until sometime between the week 8 and week 12 collections. This delay was most likely due to the lack of odour; the scent of decomposition caused by the intrinsic gut bacteria in the whole samples attracted the insects to the samples. The ensuing feeding frenzy led to the extensive skeletonisation seen at the 8 week collections.

Graph 4.3 gives a clear indication of the effect of insect activity on the whole samples, which showed a sudden increase in TBS score from 3 to 17 in just four weeks. By 8 weeks PMI, the TBS had only increased to 22, which suggests that the lack of insect activity between 4 and 8 weeks PMI caused the rate of decomposition to slow.

The buried samples showed a substantial difference in decomposition compared with the exposed samples as can be seen on Graph 4.3. Unlike the exposed samples, the rate of decomposition was much slower with the whole samples achieving a TBS score of 6 at 4 weeks PMI. As these samples were in lidded boxes, it is safe to assume there was a significant lack of entomological activity. This lack of insect activity, as well as the protection the soil will have provided against temperature changes had an effect on the decomposition seen. The limbs from the buried samples, both whole and excised, show little difference in rate of decomposition; the whole samples had a slight increased TBS at times but this was not to any significant extent. Both sets did succumb to adipocere; this had occurred by 16 weeks PMI. It was more extensive in the whole samples due to the increased amount of soft tissue. As adipocere often occurs in moist, anaerobic conditions (Langley & Tersigni-Tarrant, 2017), this was to be expected due to the enclosed conditions the samples were left in – drainage holes were drilled into the bottom of the boxes, but these did not adequately allow all the water collected over the winter months to drain.

5.3 Weathering and Surface Modifications

Significant changes to the bone surfaces were not seen. It had been anticipated that, due to the weather changes, some change to the surface would occur, however this was not the case. Behrensmeyer (1978) does state it can take up to six months for bones to achieve a weathering score of 1. This experiment ran for a total of 28 weeks, and most bones were given a score of 1 by that time, so while this was not as expected, it was in line with the time range given.

The exposed samples were showing surface degradation by 4 weeks PMI with pitting and some flaking occurring. This did not change significantly throughout the course of the project; microcracks were sometimes seen but nothing to substantiate a weathering score of above 1. Towards the end of the experiment, many of the exposed bones were beginning to show a green discolouration; this was quite moist and was possibly due to moss growth on the samples. The samples had been placed into large plastic boxes, in part to protect them from scavenging, however these may have also kept the environment damp/wet, as they did not drain very well. This could have allowed the moss to grow. It also has to be noted that the plastic box may have protected the samples from any effects the wind could have potentially had on weathering.

The buried bones, while not likely to succumb to any weathering changes, were scored based on any changes that occurred due to the soil interactions with the surface of the bone. In this case, it was anticipated that no changes would occur with the exception of some erosion due to soil. However, it was a buried defleshed bone sample that gave the highest score (of 2) at 24 weeks PMI. This score was given due to extensive pitting and a crack that ran through the sample. This appears to have been an anomaly as it was the only sample to be given a score of 2 and the corresponding samples at 28 weeks PMI did not show the same level of degradation.

The whole rat samples and excised samples did succumb to adipocere and this can be seen in images in section 4.5. In some samples, the adipocere appeared to coat the bone; while this was not removed to analyse the surface underneath, it is likely that it protected the bones from any surface degradation (Munro & Munro, 2008).

While it has to be noted that in forensic cases, factors such as scavenging would need to be taken into account, this research does show that weathering and surface modifications by themselves/alone cannot be used to accurately determine PMI. This may be different in scenarios with longer PMI's, however further research would be needed. In this case, it is also clear that weathering does not change as a result of the deposition environment or tissue type (again the lack of scavenging here needs to be considered); and while the Behrensmeyer (1978) weathering score is for exposed remains, this shows it can be used to some extent on buried remains due to the effects the soil can have on the bone surface.

5.4 UV Fluorescence

The autofluorescence of the samples were analysed to determine whether any pattern could be distinguished to give an indication of PMI. It is thought that the collagen in bone allows for fluorescence to be seen when the bone is subjected to light within the UV range; this experiment used light at a wavelength of 375 nm. The theory states that as the collagen is destroyed through bone diagenesis, the level and intensity of fluorescence will decrease (Hoke *et al*, 2013). This proved to be inconsistent and no correlation could be found between intensity of autofluorescence and PMI. The 'sandwich' effect; a phenomenon whereby the intensity of fluorescence decreases over time from the outer and inner rims of the cross section (Saukko & Knight, 2015), was not seen. The use of image software, *ImageJ*, also did not give any significant results when used to analyse the fluorescence intensity. As the graphs in section 4.6 show there was a very poor correlation and R^2 values of <0.3 were calculated. There are several possible reasons for this; for example, while the samples were biologically similar, differences in their size, age at death, health, could have caused variations in collagen levels at the start of the

experiment. Inconsistent collagen levels on day 0 are bound to skew results as collagen loss cannot be consistently tracked. A better method would have been to analyse each individual sample on day 0 so it could be compared with the end loss. However, as this experiment analysed cross sections of the bone, that would have been an impractical approach. Other factors such as the type of bone analysed also need to be considered; this experiment used the long bones, however a selection of femur, humerus, and tibia were used, therefore the initial collagen levels in each type of bone have to be considered. It would have been more practical to use the same type of bone throughout this study, but due to limited available long bones and the need for repeated samples, different types were used. The question also has to be asked; what is fluorescing in the later samples? Can it be accurately stated that it is the collagen fluorescing? It has already been determined that bacteria are present on the samples as these drive the bone diagenesis, therefore could it be bacteria that are causing the inconsistent results? To answer this, one would need to isolate the bacteria on the samples and analyse them to determine whether they would fluoresce at the wavelength used. Unfortunately, bacterial analysis was not an option for this project, but it would be wise to consider this for future research.

Despite this inconsistency, the samples were compared to determine whether deposition environment or tissue type had an effect on autofluorescence. As expected, the inconsistent results affect what can be seen in the comparisons, but it is possible to determine that both the exposed and buried samples followed a very similar pattern. The same can be concluded when comparing the fleshed and whole samples: - Graph 4.7 shows a similar pattern between the two tissue types, while the buried bone shows much lower levels of autofluorescence. Unfortunately, the inconsistencies mean it is impossible to determine whether this difference is due to the bone being defleshed and therefore exposed to the elements sooner, or due to variations in the subjects.

5.5 Histology

5.5.1 Microscopical Foci of Destruction

Two types of microscope were used for histological analysis; compound and digital, to determine whether any diagenetic alterations were occurring over time. Overall, it was found that while the compound microscope could be used to obtain clear images of the samples, the digital microscope was not as good – this was most likely due to the preparation technique that was used. Digital Calipers were used to measure the thickness of the samples, however there was still room for error in the measurements – a thickness of 0.1 mm was aimed for, but due to

the sanding techniques, there will have been slight variations between the samples. This may have caused the focus issues that were encountered on the digital microscope.

The first samples were collected at 4 weeks PMI, and signs of microbial infiltration could be seen at that point. This indicates that the first signs of diagenetic alterations occur before this time. These changes were observed in samples from all conditions, including the samples that required defleshing upon collection, indicating that diagenesis occurs before the skeletal stage of decomposition. The buried whole rats showed the most extensive signs of MFD with two of the three samples scoring a 2 on the OHI. While a score of less than 5 had been anticipated, such a low score was surprising. Potential reasons for this are given as the presence of the gut bacteria, the soft tissue of the buried samples were much slower to decompose suggesting the gut bacteria may have had longer to infiltrate the bones compared with the exposed whole rat samples, which gave an OHI score of 3.

Over the course of this experiment, the MFD as categorised by Hackett (1981) were seen. As with previous bone diagenesis research, these MFD increased as the PMI increased, however there were other diagenetic alterations seen that had not been categorised by Hackett (1981). These took the forms of dark banding, or shadows, on the cross sections of the samples and enlarged, misshapen osteocyte lacunae. These appeared similar to the early diagenetic changes noted by White & Booth (2014), where they were given the terms 'amalgamations' and 'diagenetic lacunae'. The same terms were used here to describe the changes being seen. These amalgamations and diagenetic lacunae were seen extensively in most samples analysed over the course of the experiment; however there is very little in the literature about this. As most bone diagenesis research is conducted on skeletal remains, it can be concluded that these are most likely early forms of MFD often seen during and soon after the soft tissue decomposition. While these were still observable in most samples at 28 weeks PMI, it could be theorised that over a longer time scale, the amalgamations and diagenetic lacunae are obliterated by the other forms of MFD and therefore are rarely seen in older skeletal remains, for example archaeological remains. There were three samples from week 28 that showed zero diagenetic lacunae present, this was due to the osteocyte lacunae being obliterated by non-Wedl MFD; they were consequently given an OHI of 0 and showed no birefringence. These samples would support the theory that amalgamations and diagenetic lacunae can be obliterated over time by other forms of MFD.

As this experiment used domestic rats as subjects, it had been anticipated that the samples would succumb to Wedl tunnelling. Jans *et al* (2004) and Brönnimann *et al* (2018) both found

that animal bones were less likely to succumb to microbial tunnelling and when they did, they were more prevalent to Wedl tunnelling than non-Wedl tunnelling when compared with human bones. However, this was not the case here. Very few samples showed signs of fungal infiltration; of the 90 field samples that were analysed over the 28 weeks, only 30 showed the presence of Wedl tunnels, while 85 showed some form of non-Wedl tunnelling. According to Hackett (1981), Wedl tunnelling is caused by the infiltration of fungal spores while non-Wedl tunnelling occurs as a result of bacterial infiltration. Wedl tunnelling has shown to be more prevalent in water burials than soil, although if the soil is damp enough, they have been observed (Brönnimann *et al*, 2018). The Wedl tunnels that were seen were mostly type 2, the enlargement of the canaliculi; given the structure of the canaliculi it may be that they are more prone to microbial infiltration and this is why some samples were attacked in this way. No reason has been given in the literature as to why animal bones succumb to Wedl tunnels more easily than human bones, but it is thought that it could possibly be due to the difference in microstructure, such as the absence of osteon systems which plays a part (Jans *et al*, 2004); however the results here did not support this theory.

5.5.2 Birefringence

The birefringence of the samples were observed throughout the experiment. This was carried out using a polarised compound microscope and the samples were scored based on the level of birefringence seen compared to a fresh (control) sample. The birefringence index was used to rate these samples; however it was found that the three-category scale was not enough for the levels of birefringence being seen throughout the course of the 28 weeks. Due to this, two more categories were added to the index. These took into account samples that showed localised losses in birefringence, which were not considered to be enough to give a rating of 'reduced'; and those samples that showed significant loss of birefringence, typically these samples showed localised areas that were still birefringent. As this was an adaptation to the BI by the researcher, there was a limitation in that the method of analysis was not validated. This was also a measurement only conducted by the researcher and therefore the results are subjective; another researcher may not show the same results when observing the same samples. This appears to be an issue with this type of measurement and better methods of analysis, such as double-blind analysis, should be considered in any future work.

Only two samples showed normal birefringence during the experiment, these were one from the week 24 collections, and one from week 28, while twelve showed obliterated birefringence as defined in section 4.7.2. Four of the obliterated samples were from the week 28 collections, with three of them also showing no observable lacunae. The week 4 collections had two

obliterated samples; one of these was a longitudinal section while the other was transverse. While transverse sections were preferable for this type of analysis, due to the preparation method used, they were not always possible to obtain. The longitudinal sections were not as clear to analyse under the microscope as the transverse, and were often slightly thicker, this could explain why this sample was showing no birefringence after only 4 weeks PMI.

As with the UV fluorescence analysis, the birefringence showed no consistency over time. While most samples (88 field samples) showed some loss in birefringence, there was no linear pattern to this and as the R^2 values show (0.15-0.75), no correlation could be found to indicate that birefringence decreased as time increased. As Graph 4.11 shows, the level of birefringence was inconsistent throughout. This could be explained by the preparation method used. The preparation method was imperfect, the samples had to be hand sanded to the required thickness. This had to be done with care so not to destroy the microstructure of the samples while also allowing for an optimal thickness to be obtained to allow the transmitted light of the microscope to pass through the sample. This method meant the samples were not identical in thickness, and while the variances may only be slight, they will have been enough to hinder the birefringence of the samples. A better preparation method would have been preferred and must be considered in any future research.

5.5.3 Oxford Histological Index

The Oxford Histological Index as devised by Hedges & Millard (1995) was used to rate the samples on their level of intact structural integrity. As the OHI relies on the judgement of the person analysing the samples, which may not necessarily be without bias, an attempt was made to quantify the level of microbial destruction occurring. The two methods were run side by side; one was based on the judgement of the researcher, while the other used *ImageJ* to count the number of osteocyte lacunae visible in the samples. The graphs in sections 4.7.3 and 4.7.4 show that while both methods indicate a loss in structural integrity over time, the observer did score the samples lower than they were as the PMI increased (see Graph 4.18 for the variance between the two methods). Both methods gave good linear regression lines and the R^2 values (>0.7) indicated a very large correlation between decreasing OHI scores with increasing time. The lowest R^2 value (0.78) calculated was for the buried excised, fleshed samples using the counted method. Inconsistencies such as this could be due to the types of bones used; this experiment used humerus, femur and tibia for analysis; the size and age of the subject could also have an effect. Despite these potential issues, overall it was shown that as the PMI increased, so did the amount of microbial infiltrations. A t-test was carried out on the counted method to determine whether the results being obtained were of significance. This gave a P

value of $2.34x10^{-14}$; as this is lower than the accepted 0.05 value (Rumsey, 2011) it shows that the results being obtained were statistically significant and it can be concluded that OHI score decreases as the PMI increases.

To determine whether the deposition environment had an effect on the level of bone diagenesis occurring, the buried samples were compared with the exposed samples. Using Graphs 4.13 and 4.16 it can be seen that the deposition environment had little effect on the samples. Using the observable method, the exposed samples show a slightly more consistent loss when compared with the buried samples, however this could be due to observer error rather than the samples themselves. When comparing this with the counted method, it is clear that the two environments show an almost identical rate of bone diagenesis. This would indicate that the environment is of little consequence to microbial infiltrations. However, this may not be true in a forensic case; in this experiment the buried samples were enclosed in lidded boxes limiting the exposure to insect activity and environmental fluctuations, such as temperature changes and rainfall. These factors may have affected the amount of microbial tunnelling seen in these samples. The exposed samples were also placed into plastic boxes, while this was deliberate to limit the samples coming into contact with the soil on the ground, and to stop scavenging animals, this may also have affected microbial tunnelling. Therefore, it can only be concluded that the deposition environment had no effect on bone diagenesis in this experiment only.

As this experiment used three different tissue types, they were compared to determine what, if any, differences were occurring between them. Here the two different methods used to observe microbial tunnelling showed a few differences. The observable method showed a significant decrease in OHI score between 0 and 4 weeks PMI for the whole rat samples; initially this was believed to be due to the bloat stage of decomposition, this would confirm the theory that intrinsic gut bacteria can affect bone diagenesis, particularly during the soft tissue decomposition stage. However, upon analysing Graph 4.17 for the counted method, it can be seen that the whole rats do not show significantly different results to the excised, fleshed limb samples and the defleshed bone samples during the first 4 weeks PMI. Here, however it can be seen that the OHI shows a consistent decrease in the whole samples for the first 8 weeks post-mortem, with the OHI decreasing from 5 at day 0 to 3 by week 8. This could show that the intrinsic gut bacteria affect the skeletal tissue for the first 8 weeks; this would coincide with the insect activity for the exposed samples, that had left the samples by the week 8 collections having left very little tissue remaining. The defleshed bone samples gave the least OHI decrease overall. This could be due to the removal of the soft tissue and lack of gut bacteria present and would confirm the theory that the intrinsic gut bacteria drive bone diagenesis.

5.6 Confocal LSM

The use of the Confocal LSM was a novel approach to this experiment as, despite its potential, it has been little utilised in the area of bone diagenesis. The ability to use the reflection mode to obtain images of the microstructure of the bone, as well as the fluorescence analysis means this has the potential to be very useful in bone diagenesis research.

As this was a novel technique at the University of Huddersfield, it took a lot of time to establish the ideal settings to allow for clear fluorescence analysis to take place – this meant a lot of time was lost that had not been anticipated. As a result, only two sets of samples were analysed alongside fresh samples for control. These samples were from collection weeks 16 and 24. Despite the small sample numbers, differences can be seen.

Software from Zeiss make it possible to convert the intensity of the samples into easy to understand tables; these were then used to calculate averages which could be used to create graphs to show what was occurring to the samples over time. This was particularly useful when comparing images of the samples. From the images obtained, it initially looked like the buried fleshed, excised limbs and the buried whole rats were maintaining their levels of fluorescence – something that was not expected, particularly in the whole samples due to the presence of the gut bacteria; however upon further analysis using the intensity tables this was found to not be the case. As Graph 4.19 shows, there was an overall decrease between the control samples and week 24 collection samples indicating that the intensity of fluorescence was lost over time. There was some inconsistency in the week 16 collection samples, however this could be due to the samples not being identical in thickness; as previously discussed the preparation method was not ideal and did limit some of the results seen. When the t-test was performed using the day 0 samples and week 24 collections, a P value of 2.06×10^{-6} was given, this would indicate that the loss of fluorescence was statistically significant, however more research needs to be considered using this technique.

When comparing the deposition environments, there was little difference seen. The exposed samples showed a slightly higher intensity in fluorescence at 16 weeks PMI, however by 24 weeks PMI there was little variation between the levels of fluorescence intensity seen. The t-test was performed to calculate a P value (0.137) which showed the results were not statistically significant.

As with the other analyses, the tissue types were compared. Graph 4.21 shows there is a vast difference between the whole rat samples and both the defleshed bones and the excised, fleshed leg samples. The whole rat samples showed less loss in fluorescence intensity when

compared to the other tissue types. As with the images, this was unexpected as previous research (White & Booth, 2014) has shown that the presence of gut bacteria drive bone diagenesis. A t-test gave a P value of 0.007 when comparing the defleshed bone samples with the whole rat samples; this would indicate that the results being seen here are statistically significant and not due to random variation within the samples. This would indicate that the presence of the gut bacteria does not have a significant effect on bone diagenesis as previously thought. Other potential causes for fluorescence, such as the presence of decomposition bacteria, were considered but no literature could be found to state that bacteria would fluoresce at the wavelengths used.

Despite what these results show, they do have to be taken with caution. There is still some debate over why bones have the ability to autofluoresce; the most popular theme being that the collagen fibres within the microstructure of the bone are the cause although no one knows why this is (Capasso *et al*, 2017). Research undertaken by Capasso *et al* (2017) found links between collagen content and fluorescence intensity, however, as with this experiment, they also found localised fluorescence within their samples. Further investigation showed that this fluorescence appeared to be due to the 'Rouget-Neumann sheath'. This substance is found in spaces between the osteocytes and the walls of the canaliculi (Capasso *et al*, 2017). As can be seen in Figs 4.35 and 4.36, the fluorescence is specific and does appear to be where this Rouget-Neumann sheath would be expected. As this fluorescence could affect the results using Confocal LSM and the intensity table from Zeiss software, it may not be possible to get a true indication of fluorescence loss due to collagen destruction without knowing the full extent of this localised fluorescence. Further research is needed to explore this.

5.7 Proteomic Analysis

The samples sent to Northumbria University were selected based on their potential to answer the original research questions; what are the origins of the bacteria that drive bone diagenesis? For this three each of the buried defleshed bones and the exposed whole rat samples from collection weeks 12 and 24 were sent along with fresh bones (three bone samples). The purpose of analysing the protein content of the defleshed bone samples was to determine what, if any, effects the burial soil had on the protein content, while the analysis of the exposed whole rats was done to determine whether the presence of the gut microbiome during the decomposition stage had led to any shifts in protein abundance.

Progenesis software was used to analyse the data obtained from the LC-MS/MS. Using this it was possible to analyse all the data together; and to separate them into groups to allow for comparisons.

At first glance, it could be seen that there was a good variability between all samples; the fresh samples stood apart from the field samples as expected, and the level of variance increased at the deposition time increased.

Comparing the fresh samples with the samples collected in week 12 and week 24, it could be seen that the week 24 samples were showing the greatest variance from the fresh samples. Originally it had been expected that this would be due to proteins breaking down as part of bone diagenesis and this would lead to fewer proteins being extracted for the LC-MS/MS analysis. However, what was seen was a greater abundance of proteins in the week 24 samples. It is hypothesised that this was due to easier extraction of the proteins in the later samples due to the breakdown of either the proteins themselves, or of the bone material through the decomposition process. As the PMI analysis used samples from more than one subject, it is believed that the results being seen are not due to biological variability between the animals used, and are due to the effects of the deposition time.

Further analysis was conducted to determine which proteins were causing the variations seen. The proteins causing the most significant variances were plasma proteins, particularly in the fresh samples, and bone specific proteins, mainly in the later PMI samples. It was found that the collagenous proteins were not causing any statistically significant changes as they did not appear to be causing any of the variances seen on the PCA maps; this would agree with the results seen for the UV analysis and the birefringence, which also related to collagen content. Due to this, the non-collagenous proteins were focused on; these were shown to be more sensitive to damage and significant increases were found. Further analysis showed the two most influential proteins contributing to the differences being observed between the fresh and 24 weeks PMI samples to be PGS1 and PGS2. These were bone proteins; biglycan and decorin and are found within the bone matrix. ANOVA showed the P value to be statistically significant ($P < 0.05$) indicating that the results seen were not due to random variations.

Research previously conducted by Procopio *et al* (2018) also found that biglycan increased with increasing PMI; the analysis presented here did not reach six months to compare with the research by Procopio *et al* (2018), but in the case of Procopio *et al* (2018) it was found that these levels peaked between four- and six-months PMI. These results could indicate a potential new

biomarker for PMI estimation, however as this experiment and those conducted by Procopio *et al* (2018) and Prieto-Bonete *et al* (2019) are the only ones of this kind, more research is needed.

As well as the increasing PMI, the deposition environment conditions were compared to determine whether they had an effect on the proteome of the samples. It was found that there was no difference between the buried samples and the exposed samples; the samples were not well separated on the Principle Component 1 axis when shown on the PCA chart. This corresponds with what has been seen in the other analyses presented in this experiment; whether the remains are buried or left exposed, there is little to no difference in the rate of bone diagenesis.

As the buried samples were all defleshed bone samples and the exposed samples were all whole rat samples, the deposition results can also be used to compare the tissue types. Again, it can be determined that the presence of the gut microbiome made little difference to the protein abundance ($p > 0.05$). The presence of the bacterial colonies within the soil also had little effect.

5.8 Implications and Limitations

5.8.1 Implications

The results seen in this study could have implications in the field of bone diagenesis as it shows that what has previously been believed may not actually be true in all scenarios. The White & Booth (2014) study showed that the presence of gut bacteria drove bone diagenesis while what has been shown here is that this may not always be the case. Here we can see that while the presence of the gut bacteria does lead to an initial increase in bone diagenesis, this slows once the soft tissue decomposition has ceased; the later samples did not show a big variation when comparing the defleshed bones with the whole rat samples. It has also shown that while the deposition environment may greatly affect the rate at which the soft tissues decompose, it doesn't have as big an influence on the rate at which bone diagenesis occurs.

This research could have implications in both forensic anthropology and archaeology. Research in bone diagenesis could help to establish the rate at which the structural integrity of skeletal tissue is lost; something that is important to both these fields of study.

Archaeological studies are more concerned with how people lived, and as the skeleton is often all that remains, studies using bone samples are often undertaken. The problem is that most techniques are destructive; as they are dealing with human remains, there is reluctance to doing many of these tests if the results are not likely to be productive. Knowing the point at which certain tests are likely to be unreliable could be very useful here.

Forensic anthropology, however, has the question of PMI to answer. Many have suggested that more research in bone diagenesis could allow this question to be answered one day. However, it has to be noted that despite much research into this, little progress has been made. This could be due to most research being conducted on remains of archaeological interest; it is only in recent years that forensic interest has peaked in bone diagenesis.

5.8.2 Limitations

This experiment used domestic rats as human analogues due to UK legislation that currently restricts the use of donated human remains for research in forensic taphonomy. While rats were suitable for this experiment, future research ideally needs to be undertaken with human subjects if significant results are to be gained. Rats were used here for ease, but it needs to be remembered that there are vast differences between the human skeleton and that of an animal. Variations in lifestyle, such as diet and potential disease, can make a difference to the subject; a rat will have a vastly different microbiome to that of a human due to their restricted diet, which needs to be considered when looking at the results of this experiment (Nagpal *et al*, 2018). Did the diet of the subject play a part in the lack of gut microbiome-driven bone diagenesis seen here? The difference in bone microstructure also needs to be addressed; it is known that many animal bones generally lack the osteon systems found in human bones. While this research appears to show that this does not play a significant part in bone diagenesis, it may be that there are variations yet to be discovered. There is a lack of comparison research for human and animal bone, within forensic timescales, to know what limitations the use of animal subjects has on forensic anthropology.

The timescale for this experiment also presented a problem. As for a Masters by Research, time was limited and therefore the field experiments only ran for a total of 28 weeks. Other forensic taphonomy studies have run for much longer, allowing for more changes to be seen. While there have been changes here, inconsistencies in some analytical techniques have hampered results. Had the time allowed, a much longer experiment with less frequent data collections may have been preferable. These samples were also left in the field from November – May, meaning the summer months were not covered. While the winter of 2018/19 was mild, the diagenetic changes may have been much different had the experiment run for longer, covering the more variable UK weather.

The preparation method presented a particular problem with this research. It would have been preferable to embed the samples and use a microtome to slice them, however a lack of resources meant that this was not an option. Instead the manual bone preparation method

adapted by Maat *et al* (2001) was used. This required wet sanding the samples to a preferred thickness, in this case 0.1 mm, before placing them onto a glass slide. While Digital Calipers were used to measure the thickness of the samples, this was not ideal, slight variations between samples were encountered. This may have hindered some of the results seen here; unfortunately without repeating the conditions of the experiment and using the preferred embedding method, it is impossible to estimate the exact influence the preparation method may have had.

5.9 Further Work and Adaptations

This project was not without its difficulties. As this was a Masters by Research the project could only run for one year; with planning and time for analysis this was cut down to 28 weeks. While degradation to the samples was seen, it would have been beneficial to have a longer deposition period. In hindsight it has been noted that more samples should have been used to allow for more repeats and therefore obtain better statistics. The samples in this research were only observed by one person, this was not ideal as the changes observed are subjective. A method such as double-blind observations would be recommended in any further research.

There were also issues encountered with the materials used; the plastic boxes used for the buried samples did not have sufficient drainage, as a result water did gather in the bottom of the boxes. The larger boxes used for the exposed samples also required better drainage, water did gather in the base due to the uneven surface leading to some of the samples turning green with suspected moss growth. It is also possible that the high sides of the large plastic boxes may have protected the samples from the effects of weathering. In hindsight it may have been better to place the samples directly on the ground with the cage above to protect from scavengers. While it was not the focus of the project, weekly monitoring in the form of photographs of the soft tissue decomposition could have been beneficial for the TBS section. The biggest hurdle for this project was the histology preparation technique. Due to limited resources a manual technique was used which was abrasive and may have affected some of the results seen. Any future research should be done with better techniques, such as fixing and embedding the samples and using a microtome to obtain thinner, more consistent samples.

It is also noted that soil analysis should have been conducted throughout the project. Microbial testing of the soil (and analysis of the gut microbiome) was discussed and rejected due to financial and time constraints. However, simple tests such as pH and hydrology would have been possible. This is something that would be done in any future research.

5.10 Summary

This experiment has given some interesting and unexpected results. It was initially thought that the whole rat samples would show the greatest amount of diagenetic alteration, however this has not been the case. While the soft tissue decomposition did follow the expected path of decomposition, the skeletal tissue did not. The bloat stage did appear to have an increased effect on bone diagenesis when assessing the samples using the OHI, however the other tissue types appeared to catch up by the end of the experiment. There also appeared to be little difference between the two deposition environments, with the exception of the soft tissue decomposition. While the results presented here appear to deviate from other research in this area, the samples used have to be considered. Other researchers have used domestic pigs as their human analogues, while it was decided to use domestic rats here due to the number of subjects required; the subjects used may be the cause of the discrepancies being seen.

5.10.1 Future Research

This project has given rise to some interesting results for forensic taphonomy. While some of the results seen were not as expected, there has been some statistically significant knowledge gained. It was found that some analytical techniques, such as weathering and UV analysis, are not ideal when working with smaller timescales/PMI, however longer field studies could determine if these could be viable methods of longer PMI estimation.

Histological analysis and proteomic analysis did yield significant results in bone diagenesis. Histologically, the samples all showed increasing levels of MFD as deposition time increased. The method of quantifying MFD within samples using osteocyte lacunae counting showed promise, with statistically reliable results gained ($P = 2.34x10^{-14}$). More work would be needed to develop a less time-consuming method than was used here but for a novel technique, these results show potential; R^2 values of >0.7[7] ($R^2 = 0.922$, $R^2 = 0.9131$, $R^2 = 0.9081$, $R^2 = 0.9031$, $R^2 = 0.896$, R2 = 0.7814) were obtained and a statistically significant P value ($2.34x10^{-14}$) for the deposition time was calculated. The use of the Confocal LSM led to the finding that the PMI had an effect on the samples; more research would need to be conducted using this technique as it is a new approach to bone diagenesis. The proteomic analysis also showed a relationship between increasing deposition time and protein abundance; with the greatest variances being seen at 24 weeks deposition ($P = < 0.05$ compared to fresh samples). The proteins causing this shift were isolated and corresponded with the results of previous research. It is hoped that with more work, a potential new biomarker for PMI estimation may have been found.

[7] R^2 values above 0.7 were considered to show a very large correlation (McCormick *et al,* 2015)

Conclusions

This research has produced some interesting and significant results. Relating to the hypotheses stated in Chapter One, the following was observed:

- The hypotheses for the weathering of the samples were rejected as the time scale was not long enough to see any significant changes to the bone surfaces.
- The hypotheses relating to the presence of the gut microbiome were accepted for the initial eight weeks PMI. The gut microbiome did appear to accelerate the destruction of the internal structure of the bone and the presence of MFD, however this slowed in the later stages of decomposition, resulting in a lack of comparable changes between the later samples.
- The hypothesis relating to the collagen content was rejected as the results seen were inconsistent.
- The hypotheses relating to the integrity of the internal structure of the bone and the presence and extent of MFD seen were accepted. As the PMI increased, so did the level of destruction seen, this was measurable and did have statistically significant results ($P = 2.34x10^{-14}$).
- The hypotheses relating to the tissue types; defleshed bone (soil bacteria); excised, fleshed limbs (autolysis); whole rats (gut microbiome), were rejected. Once the initial bloat stage had ended for the whole samples, there were no statistically significant differences between the levels of microscopical destruction seen.

Statistically significant results obtained were:

- Very large R^2 values of >0.7 (McCormick *at el*, 2015) were found for the OHI using both methods of analysis, showing the link between increasing PMI and increasing levels of MFD
- The osteocyte lacunae counting method gave a P value for PMI week 4 vs week 28 was $2.34x10^{-14}$, indicating a strong link between increasing PMI and increasing levels of MFD
- Confocal LSM showed potential for the analysis of bone diagenesis when comparing the fluorescence intensity of fresh samples with 24 weeks deposition samples ($P < 0.05$).
- Confocal LSM analysis showed a statistically significant difference in fluorescence intensity when comparing the whole rat samples with the defleshed bone samples ($P = 0.007$).

- Proteomic analysis showed that the abundance of specific bone proteins (PGS1 and PGS2) were statistically significant between fresh samples and 24 weeks PMI samples ($P = <0.05$).

This research has given some significant results to the field of forensic anthropology and the study of bone diagenesis. It has shown that the presence of microbes, such as those found in the gut microbiome and those found in soil, do play a part in bone diagenesis. The intrinsic gut bacteria appear to accelerate bone diagenesis but only for a short period of time. This research is of importance for the field forensic science as it could help resolve the inaccuracy of PMI estimation, however more research with longer time scales is needed.

Appendix One:

Total Body Score (Humans)

TBS for head and neck

Descriptions	Score
Fresh, no discoloration	1
Pink-white appearance with skin slippage and some hair loss	2
Gray to green discoloration; some flesh is still relatively fresh	3
Discoloration and/or brownish shades, particularly at edges; drying of nose, ears, and lips	4
Purging of decomposition fluids out of eyes, ears, nose, and mouth; some bloating of neck and face may be present	5
Brown to black discoloration of flesh	6
Caving in of the flesh and tissues of eyes and throat	7
Moist decomposition with bone exposure less than one half that of the area being scored	8
Mummification with bone exposure less than one half that of the area being scored	9
Bone exposure of more than half of the area being scored with greasy substances and decomposed tissue	10
Bone exposure of more than half of the area being scored with desiccated or mummified tissue	11
Bones largely dry but retain some grease	12
Dry bone	13

TBS for abdomen

Descriptions	Score
Fresh, no discoloration	1
Pink-white appearance with skin slippage and marbling present	2
Gray to green discoloration; some flesh is still relatively fresh	3
Bloating with green discoloration and purging of decomposition fluids	4
Post-bloating following release of the abdominal gases, with discoloration changing from green to black	5
Decomposition of tissue producing sagging of flesh; caving in of the abdominal cavit	6
Moist decomposition with bone exposure less than one half that of the area being scored	7
Mummification with bone exposure less than one half that of the area being scored	8
Bones with decomposed tissue, sometimes with body fluids and grease still present	9
Bones with desiccated or mummified tissue covering less than one half of the area being scored	10
Bones largely dry but retain some grease	11
Dry bone	12

TBS for limbs

Descriptions	Score
Fresh, no discoloration	1
Pink-white appearance with skin slippage on hands and/or feet	2
Gray to green discoloration; marbling; some flesh is still relatively fresh	3
Discoloration and/or brownish shades, particularly at edges; drying of fingers, toes, and other extremities	4
Brown to black discoloration; skin having a leathery appearance	5
Moist decomposition with bone exposure less than one half that of the area being scored	6
Mummification with bone exposure less than one half that of the area being scored	7
Bones exposure over one half of the area being scored; some decomposed tissue and body fluids are remaining	8
Bones largely dry but retain some grease	9
Dry bone	10

Total Body Score as adapted by Megyesi et al (2005). Source: Langley & Tersigni-Tarrant (2017) pp. 293-294

Appendix Two:

Total Body Score (Rabbits)

TBS for head and neck

Stage	Points	Description (head and neck)
Fresh	1	Fresh, no discoloration
Early decomposition	2	No skin discoloration, maggots visible
	3	Some flesh relatively fresh, fur loss
	4	Discoloration, brownish, drying of nose and ears, and heavy maggot activity
	5	Purging of decompositional fluids, wet flesh
	6	Skin brown to black
Advanced decomposition	7	Caving in of flesh and tissues of eyes and throat
	8	Wet decomposition, bone exposure <50% scored area
	9	Dessication, bone exposure <50% scored area
Skeletonization	10	Bone exposure >50% scored area, wet tissue
	11	Bone exposure >50% scored area, dessicated tissue, incisor loss, and disarticulation

TBS for abdomen

Stage	Points	Description (abdomen, including pectoral and pelvic girdle)
Fresh	1	Fresh, no discoloration
Early decomposition	2	Skin appears fresh, fly eggs, few maggots
	3	Flesh appears red-brown, small amount fur loss (<30%)
	4	Bloating, purging of decompositional fluids, heavy maggot activity
	5	Bloat lost, severe fur loss (>70%), heavy maggot activity
Advanced decomposition	6	Wet decay, abdominal collapse where internal structure lost, flesh grey green
	7	Wet decay, bone exposure <50% scored area
	8	Surface mummification, bone exposure <50% scored area
Skeletonization	9	Black skin, bones greasy, body fluids occasionally present
	10	Bones with dessicated black skin over <50% scored area
	11	Bones largely dry and white, mummified skin
	12	Bones beginning to weather

TBS for limbs

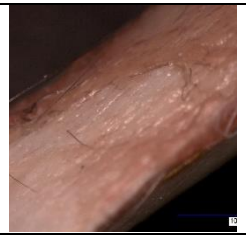
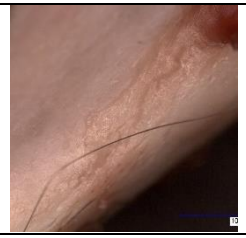
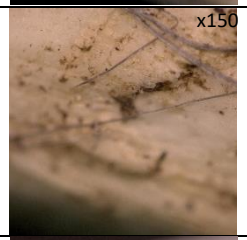
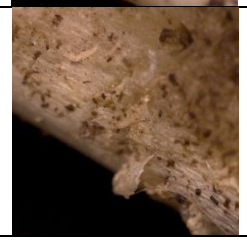
Stage	Points	Description (limbs)
Fresh	1	Fresh, no discoloration
Early decomposition	2	Flesh appears fresh, some maggots
	3	Some flesh still fresh, fur loss
	4	Discoloration of skin to brown, drying of extremities
	5	Black skin, leathery appearance
Advanced decomposition	6	Wet decomposition, bone exposure <50% scored area
	7	Wet decomposition, some disarticulation
Skeletonization	8	Bone exposure >50% scored area, dry papery skin
	9	Bones largely dry and disarticulating
	10	Bones dry and white

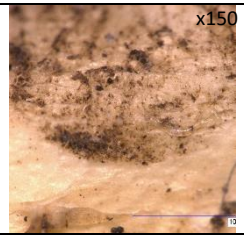
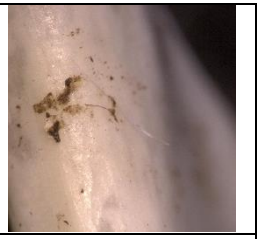
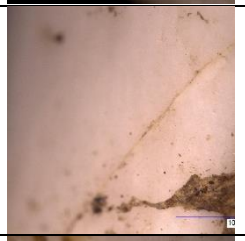
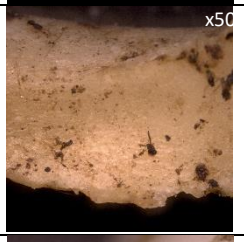
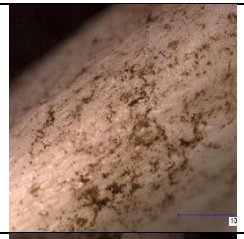
Total body score adapted for rabbits. Source: Adlam & Simmons (2007)

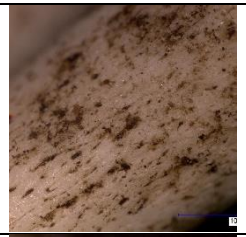
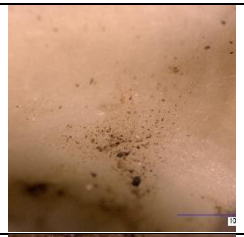
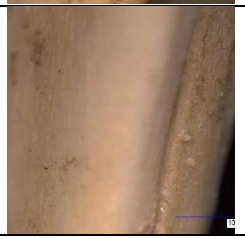
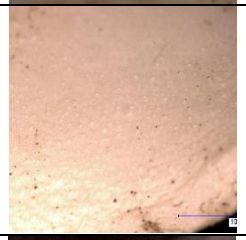
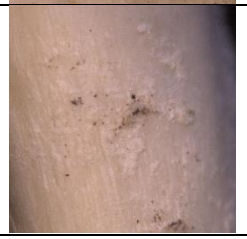
Appendix Three:

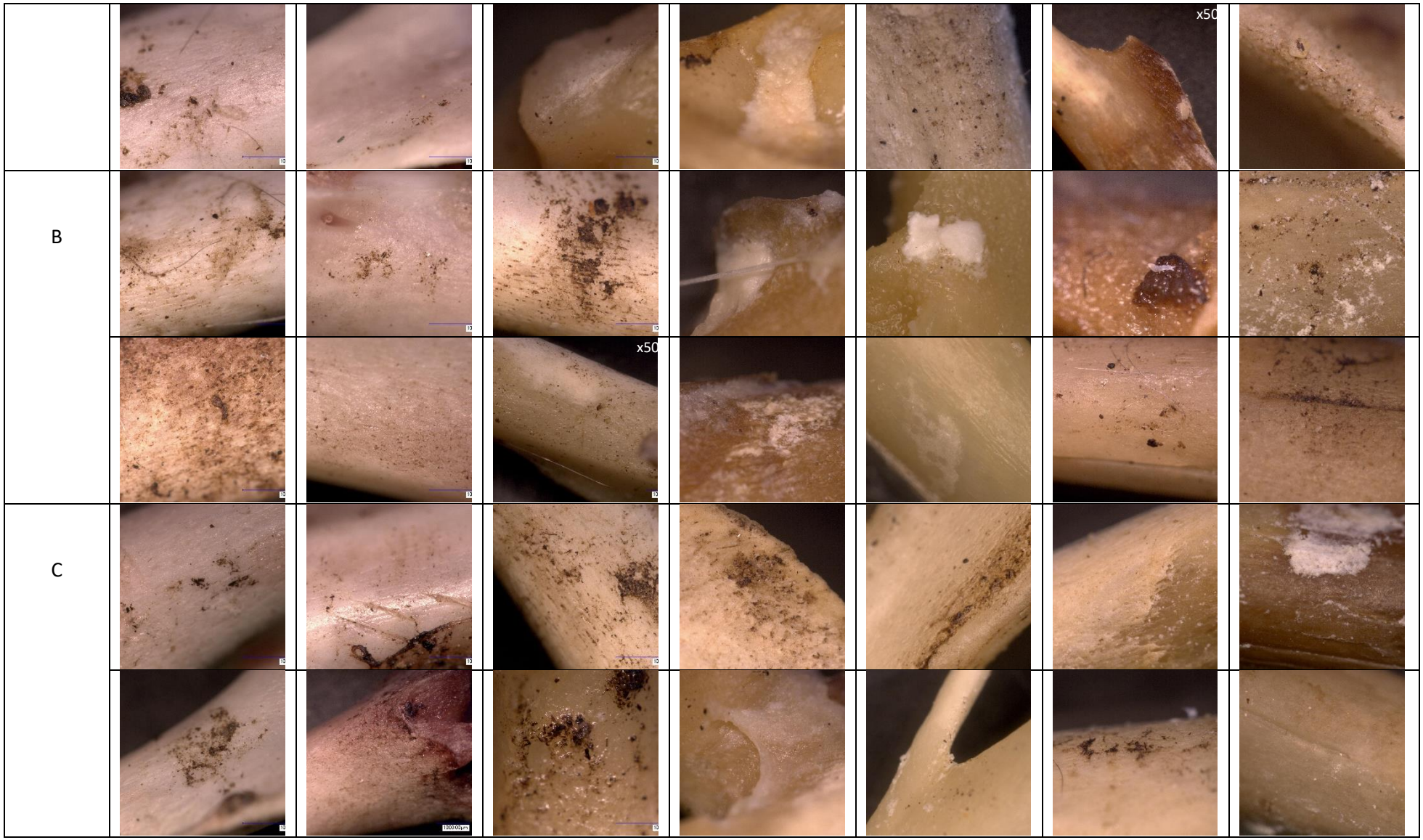
Weathering and Surface Modifications

All images are x100 magnification unless indicated.

Fresh							
	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks	28 weeks
Buried bone A							
							
B							

							
C	N/A						N/A
	N/A						N/A
Buried flesh A							
							

B							
							
C							
							
Buried whole A							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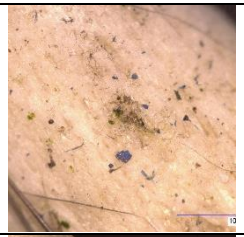
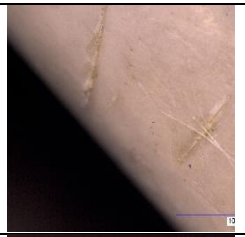
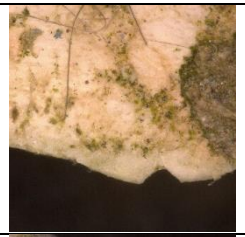
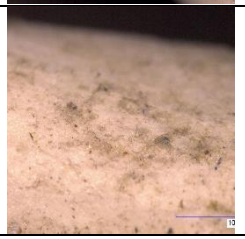
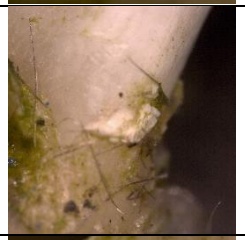
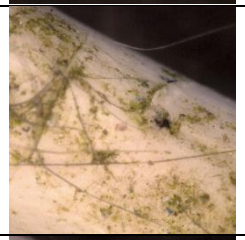
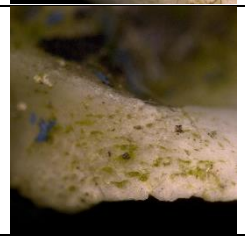
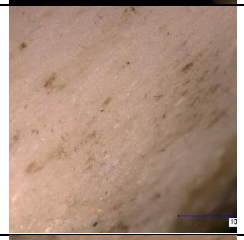
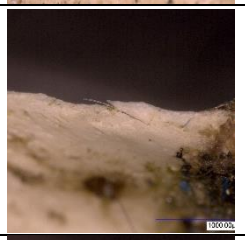
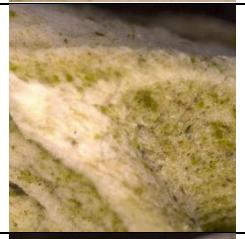
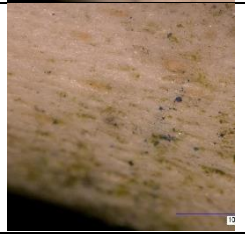
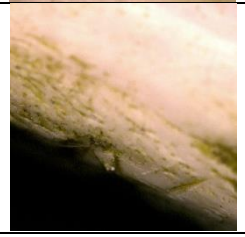
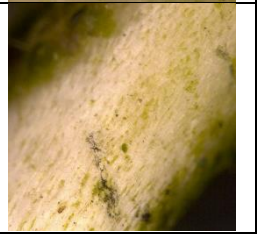
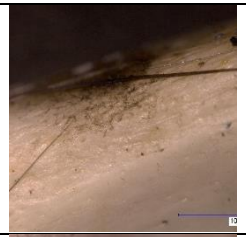
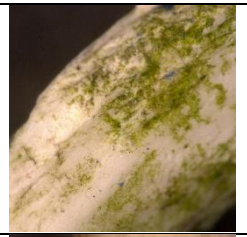
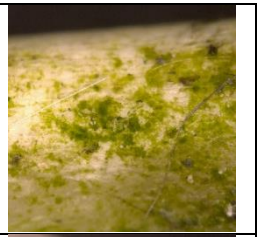
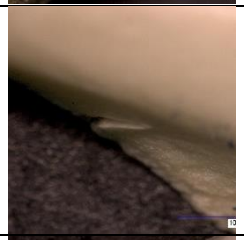
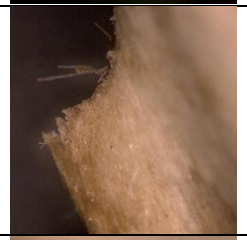
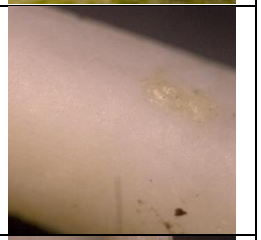
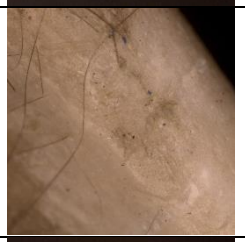
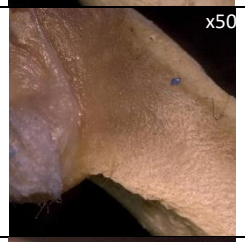
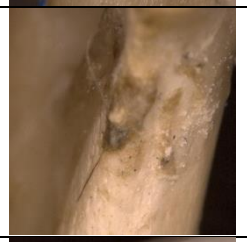
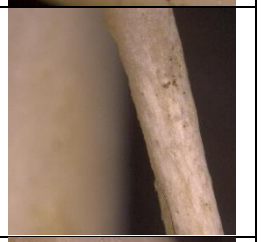
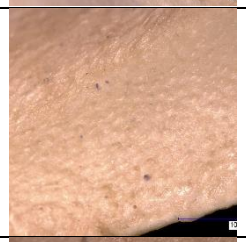
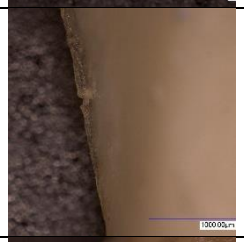
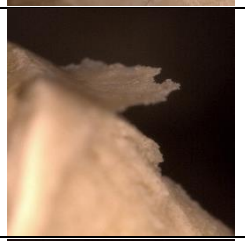
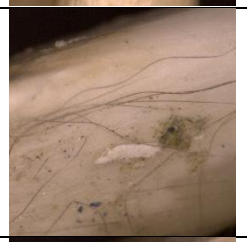
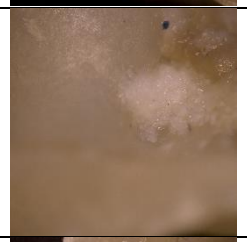
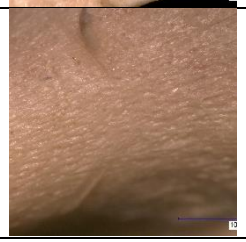
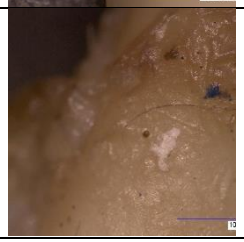
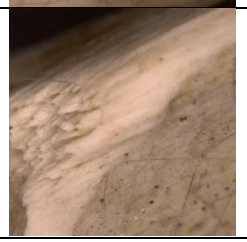
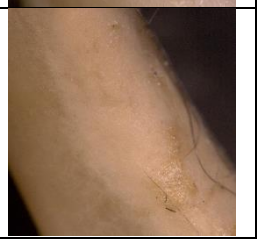
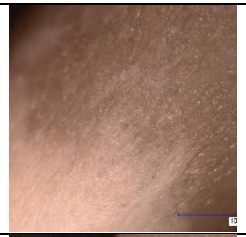
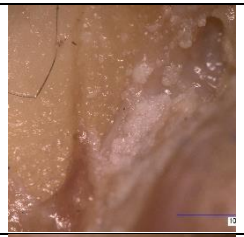
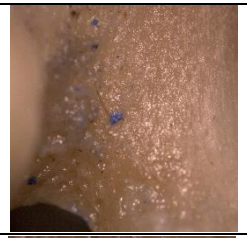
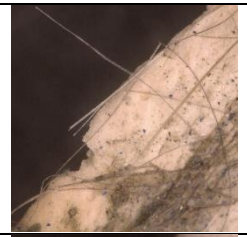
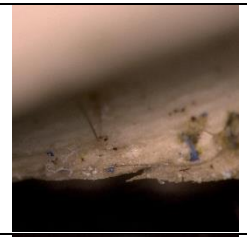
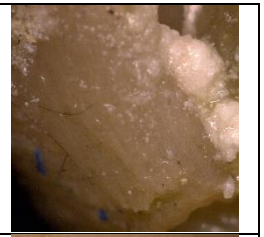
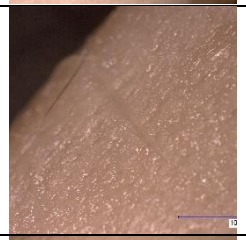
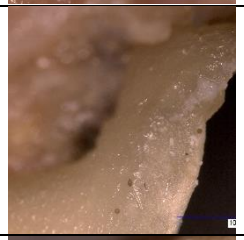
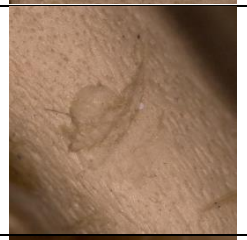
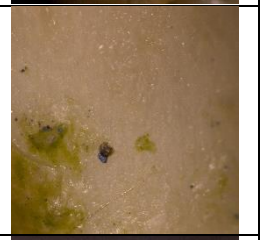
Exposed bone A							
							
B							
							
C							

							
Exposed flesh A							
							
B							
							

C							
							
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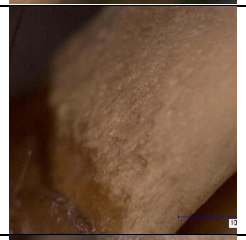
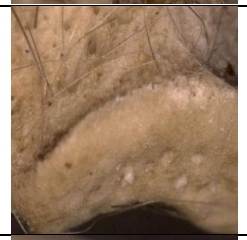
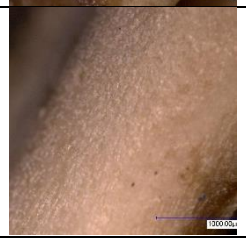
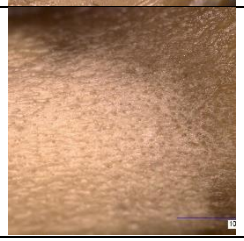
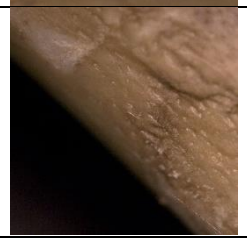
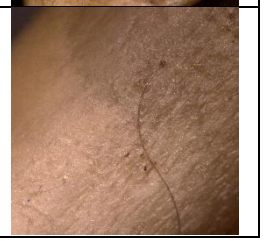
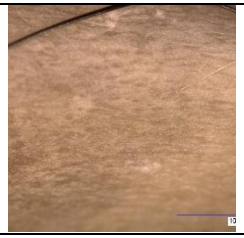
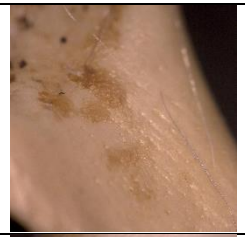
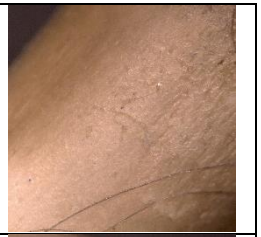
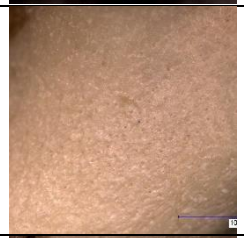
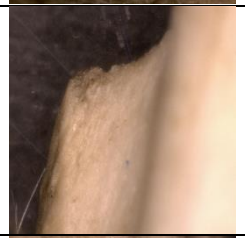
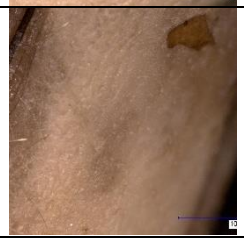
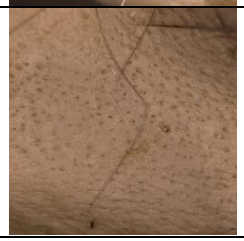
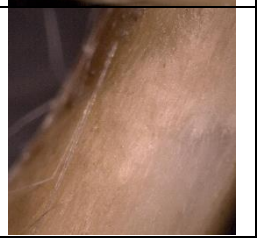
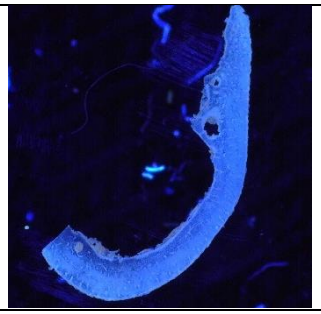
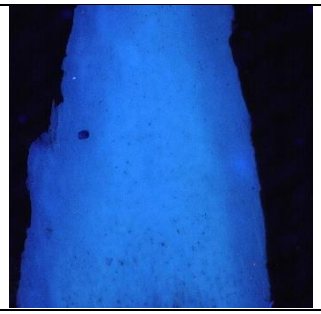
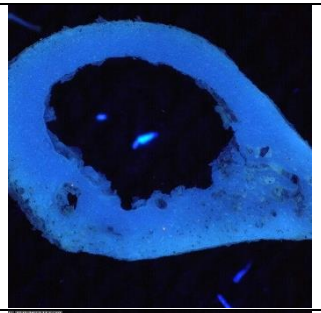
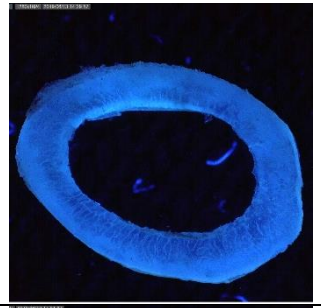
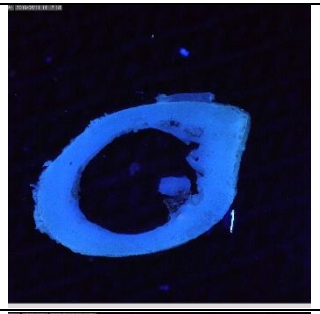
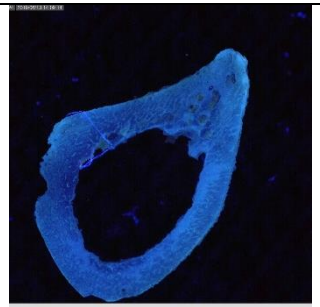
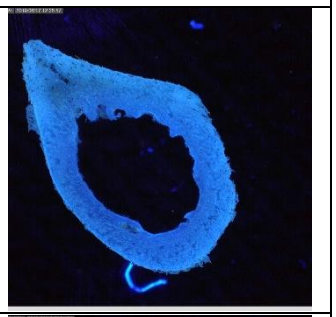
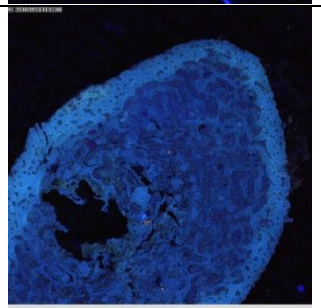
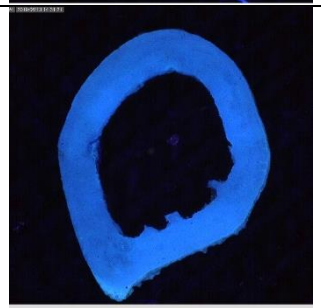
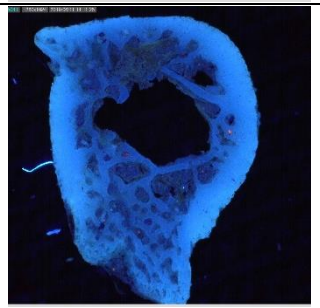
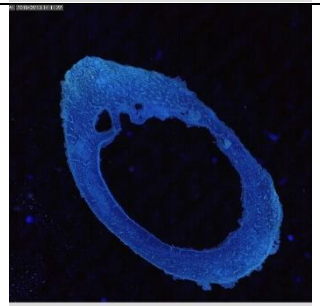
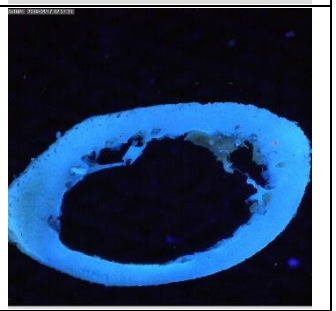
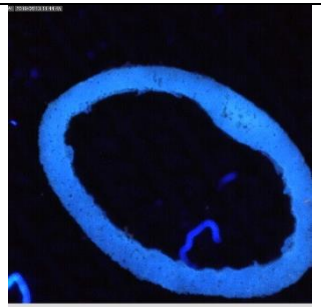
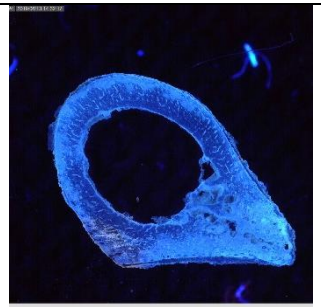
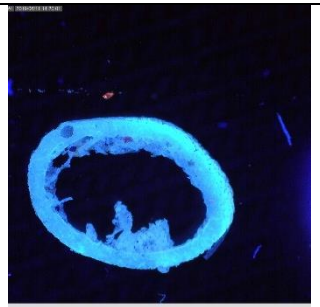
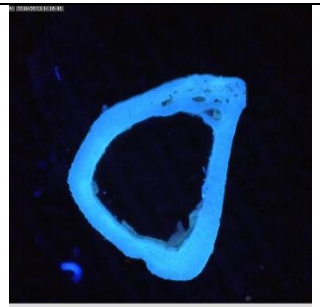
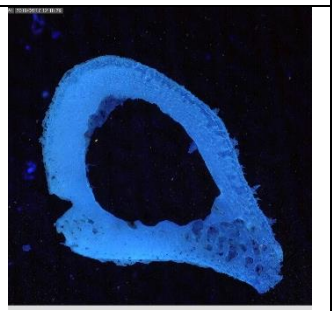
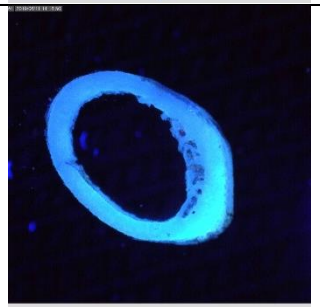
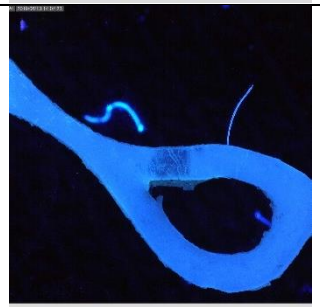
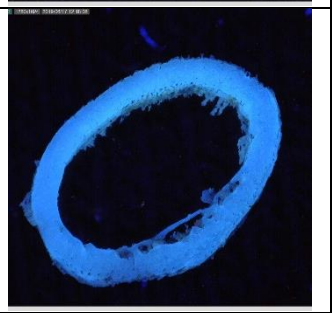
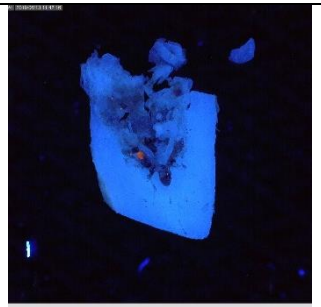
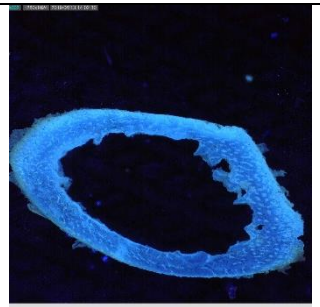
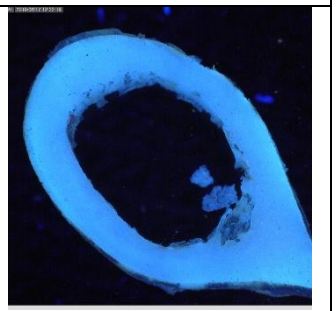
							
B							

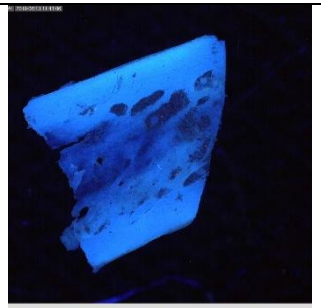
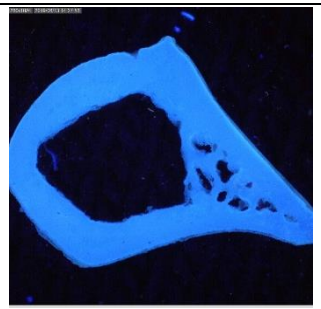
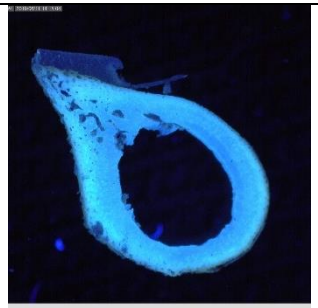
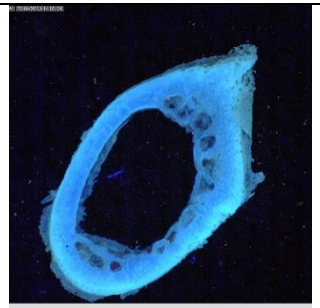
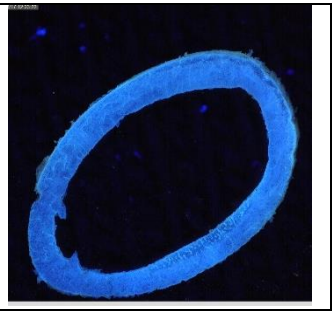
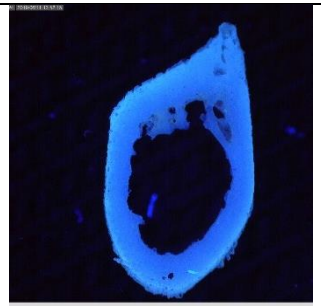
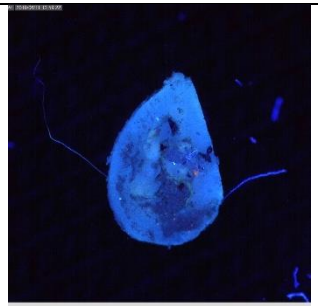
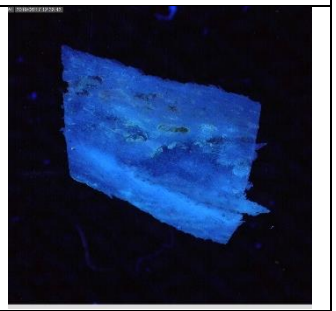
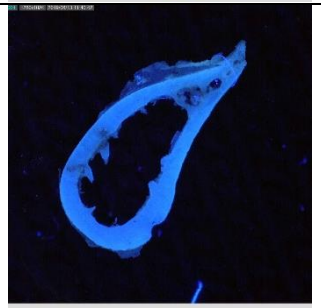
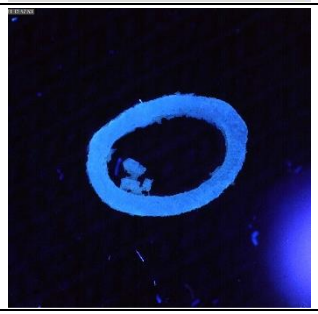
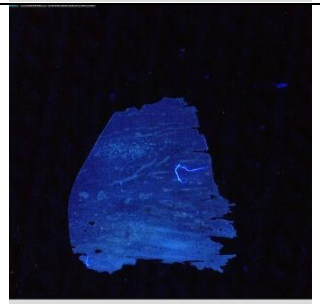
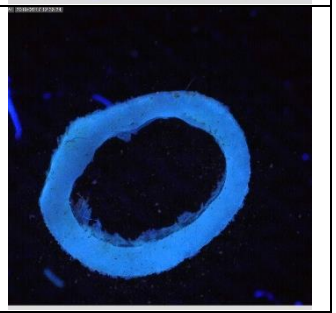
							
C							
							

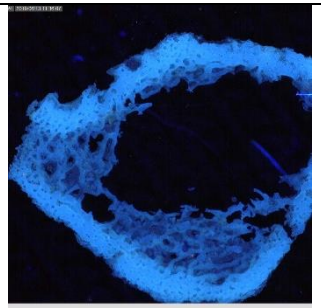
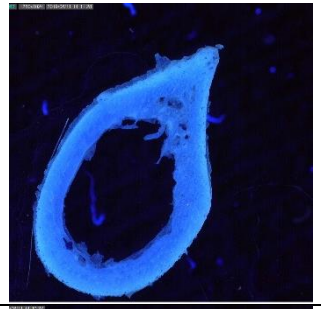
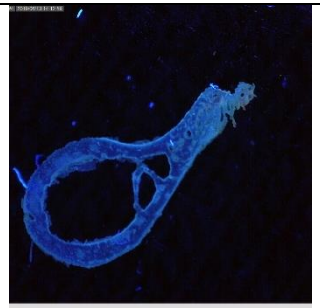
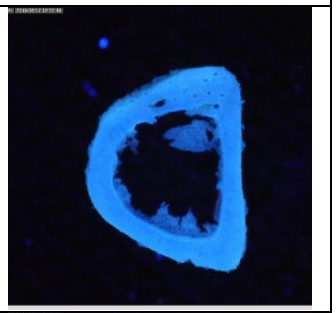
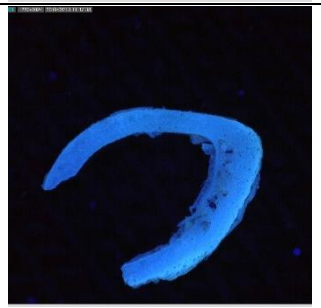
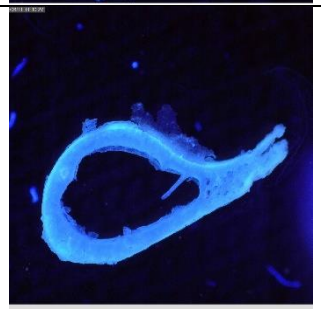
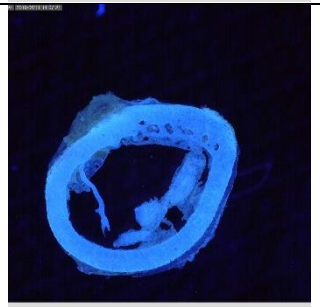
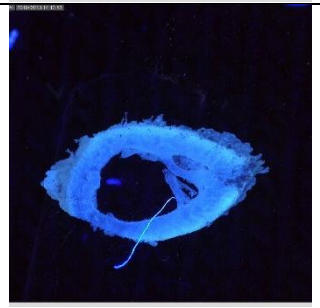
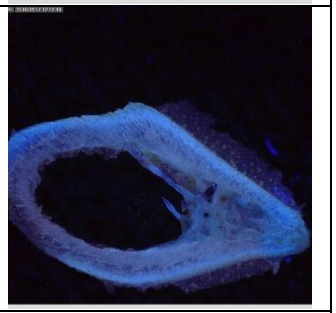
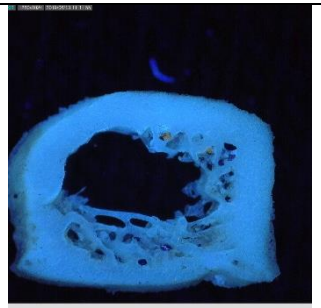
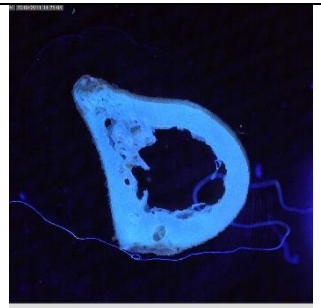
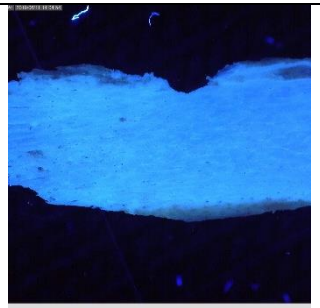
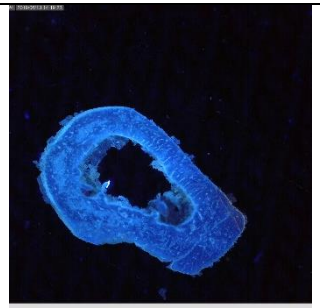
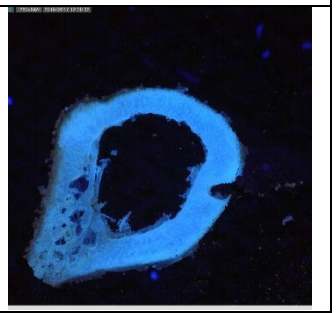
Appendix Four:

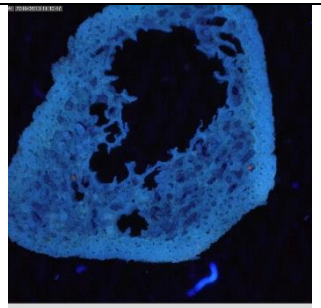
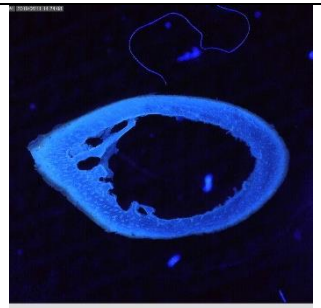
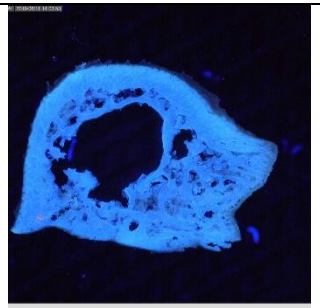
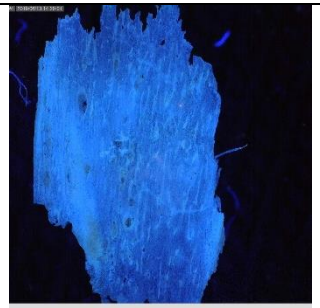
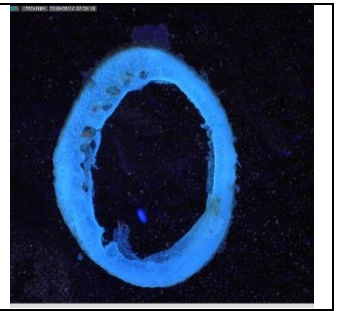
UV fluorescence

Fresh					
Buried Bone	4 weeks	8 weeks	16 weeks	24 weeks	28 weeks
A					
B					

Buried Flesh	4 weeks	8 weeks	16 weeks	24 weeks	28 weeks
A					
B					
Buried Whole	4 weeks	8 weeks	16 weeks	24 weeks	28 weeks
A					

B					
Exposed Bone	4 weeks	8 weeks	16 weeks	24 weeks	28 weeks
A					
B					

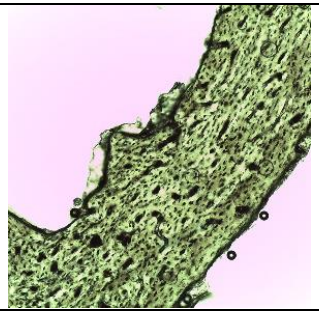
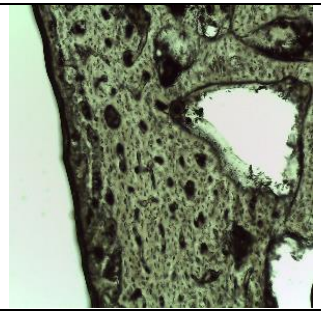
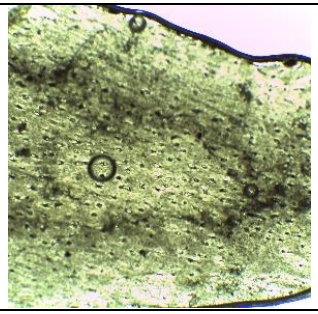
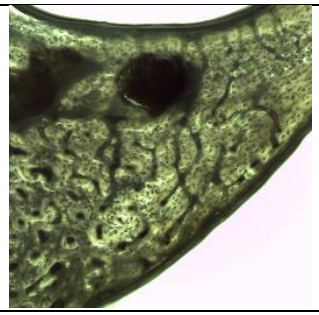
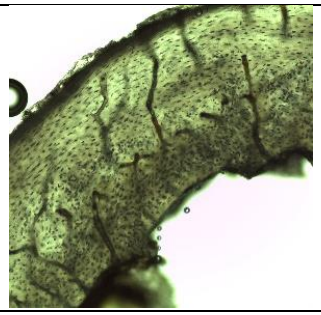
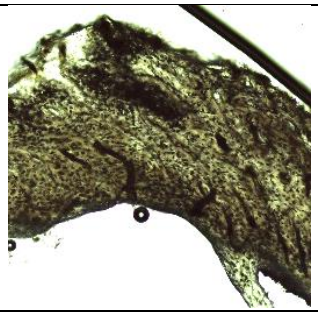
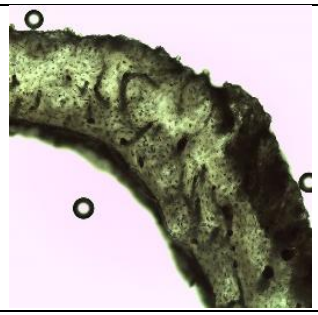
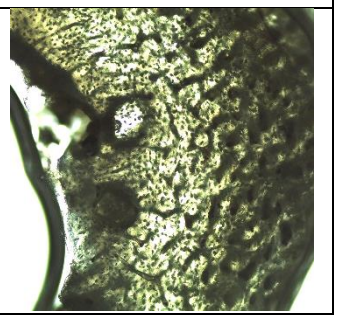
Exposed Flesh	4 weeks	8 weeks	16 weeks	24 weeks	28 weeks
A					
B					
Exposed Whole	4 weeks	8 weeks	16 weeks	24 weeks	28 weeks
A					

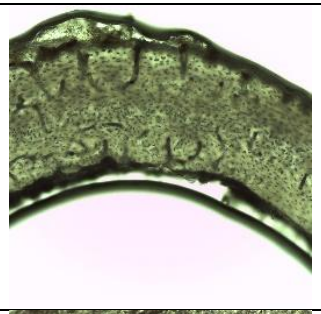
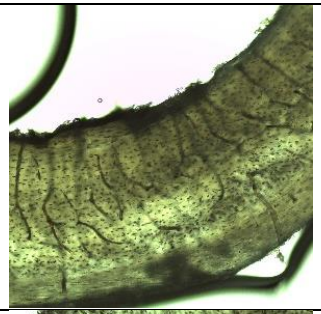
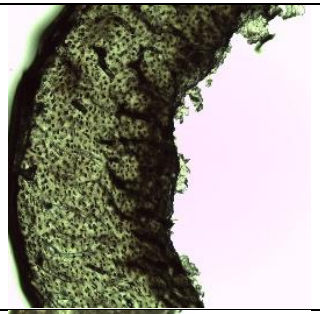
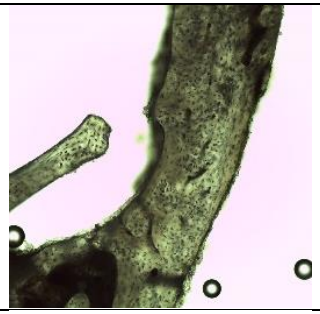
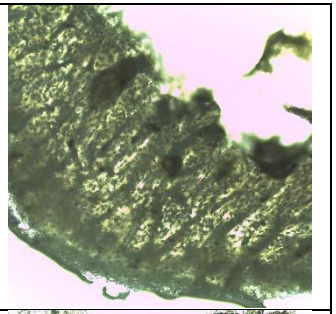
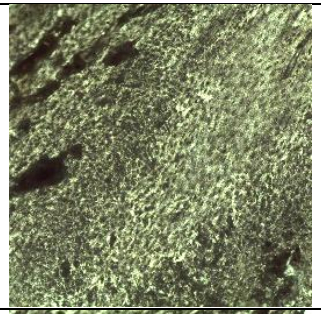
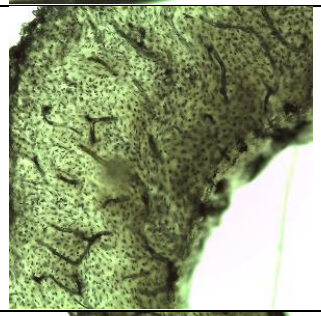
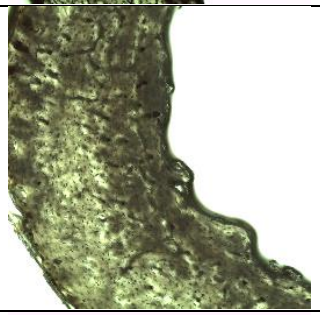
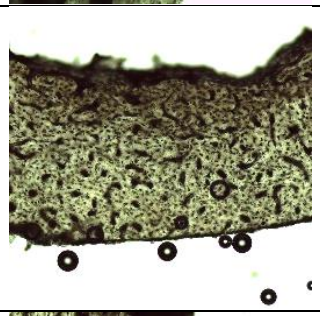
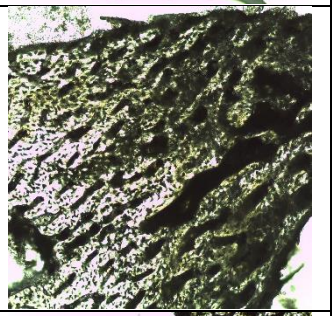
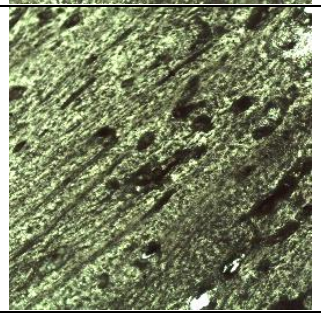
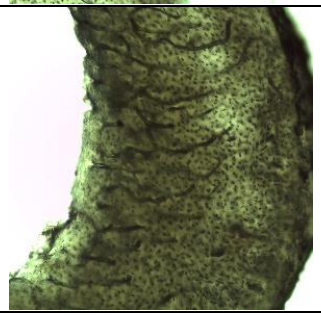
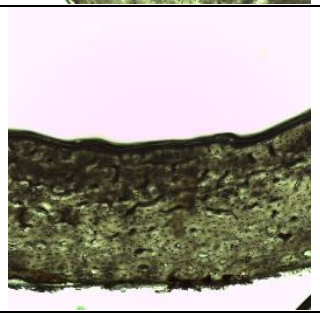
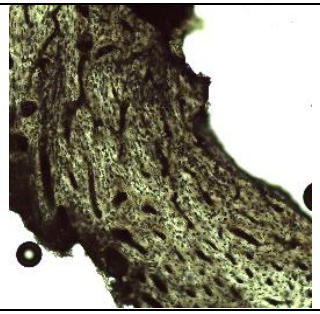
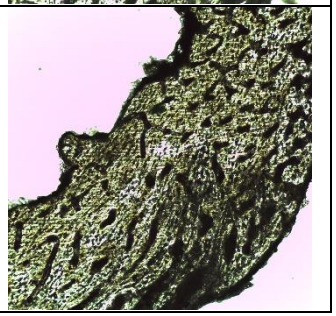
B					

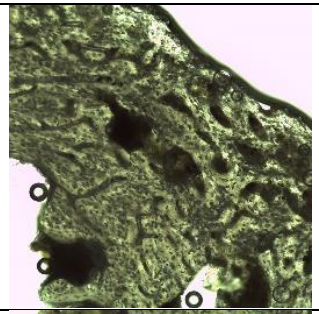
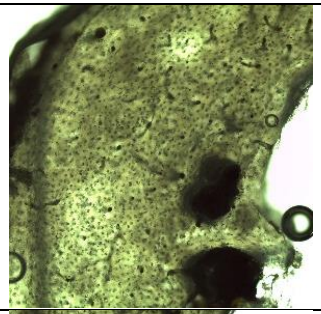
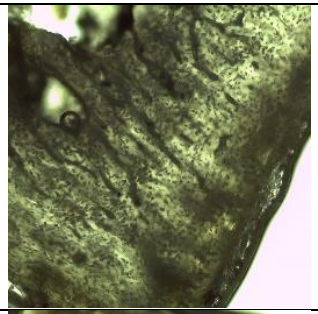
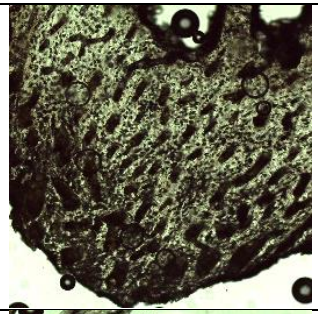
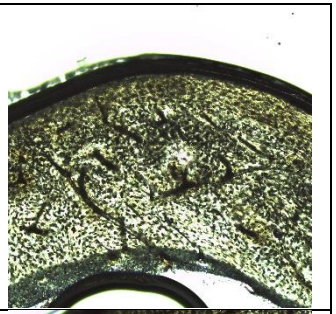
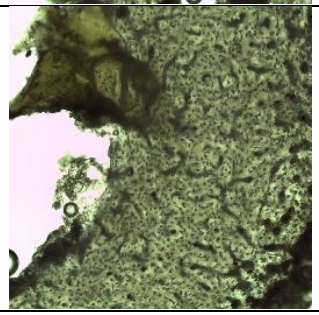
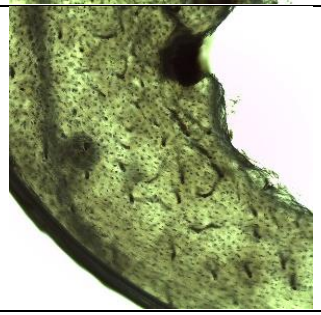
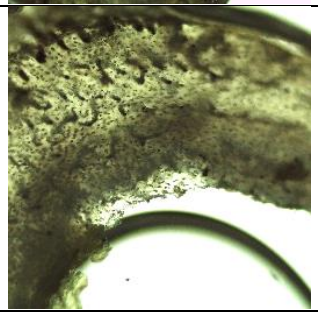
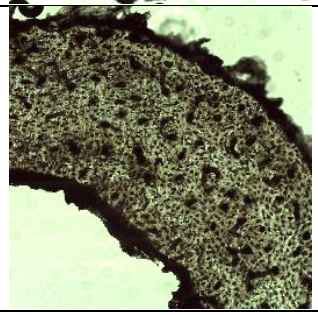
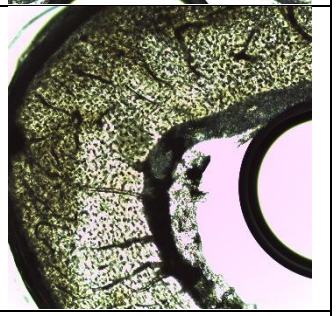
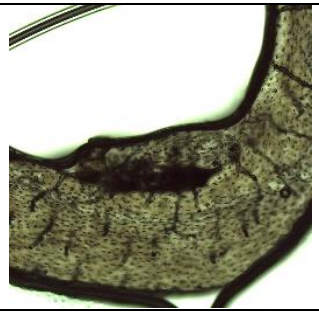
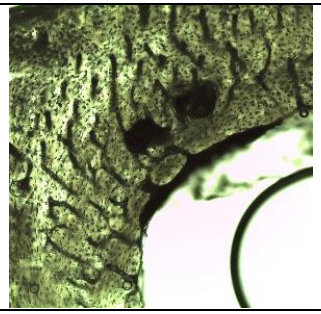
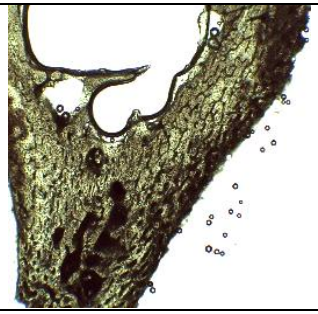
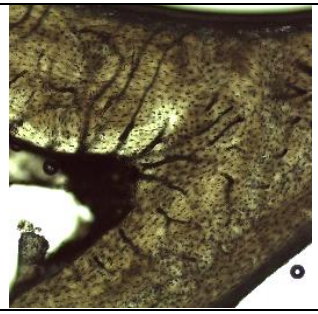
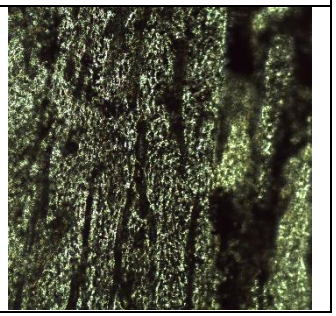
Appendix Five:

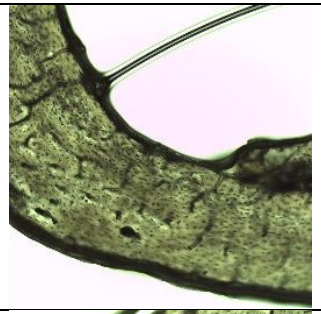
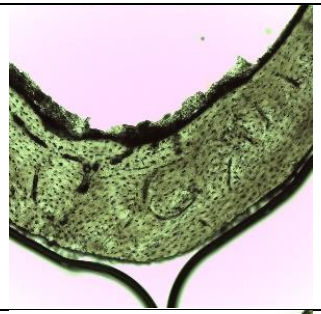
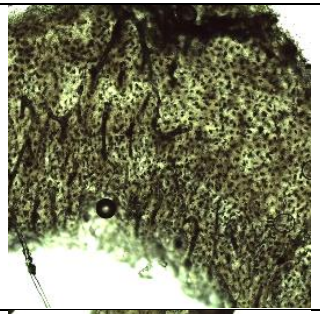
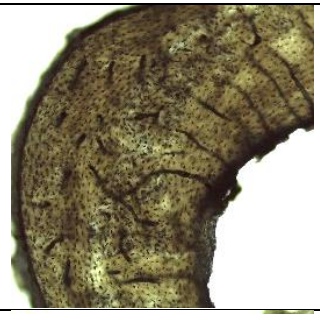
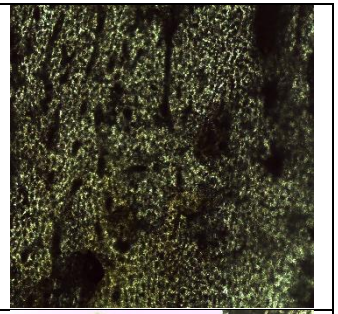
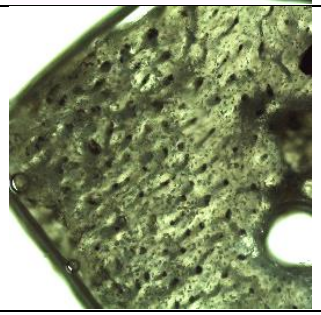
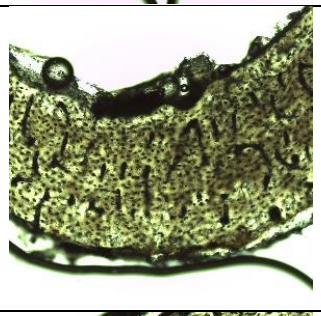
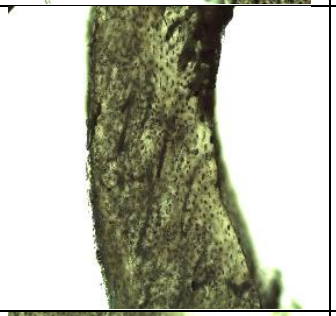
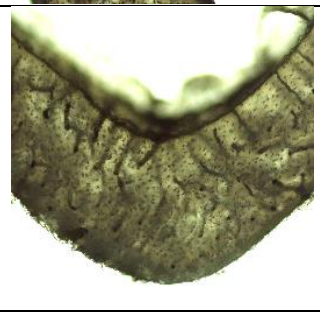
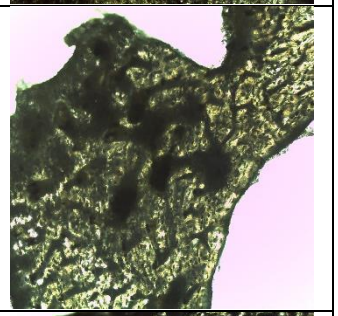
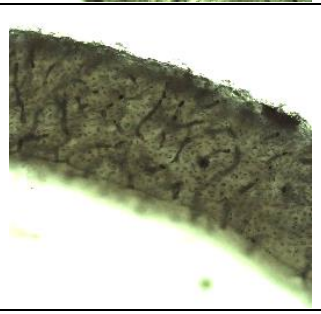
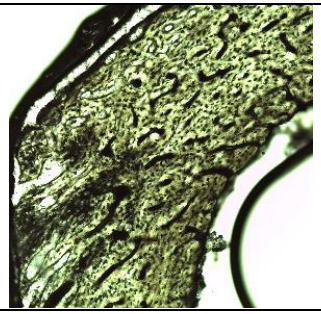
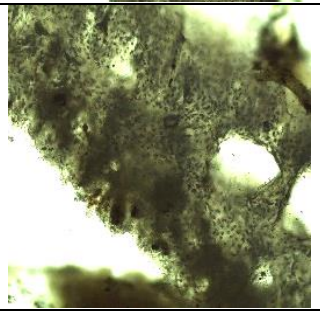
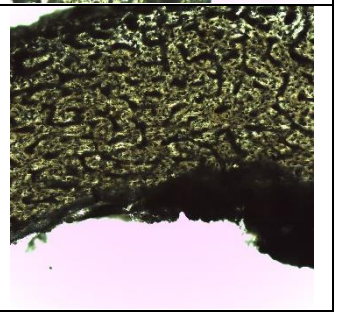
Compound Microscope

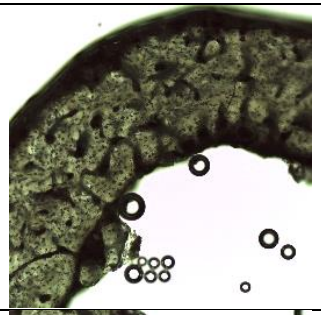
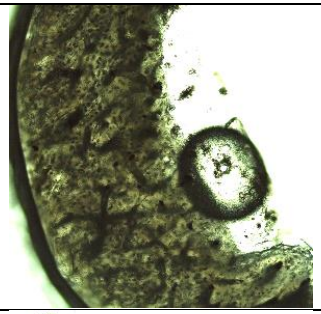
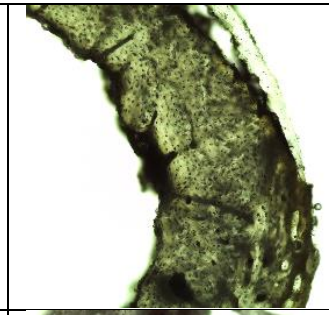
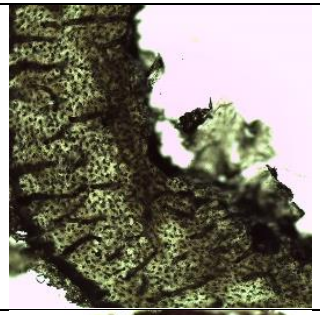
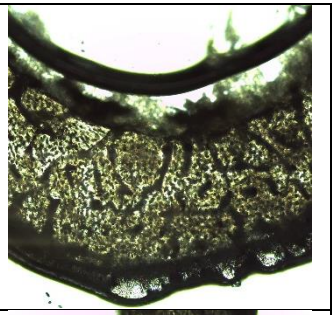
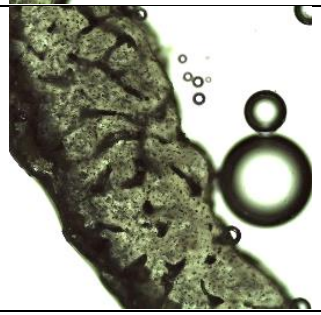
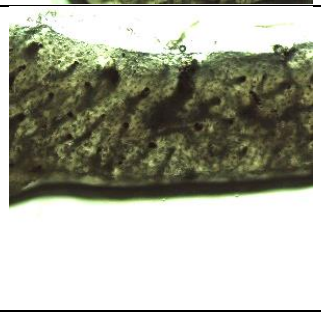
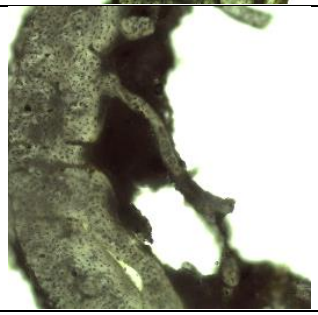
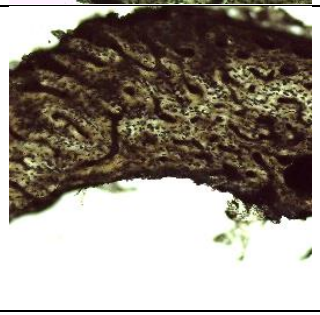
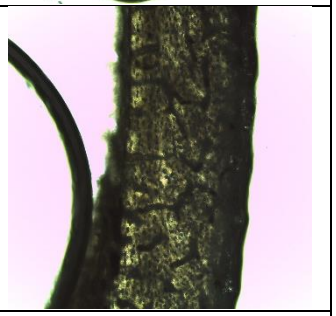
All images are x100 magnification

Fresh					
Buried Bone	4 weeks	8 weeks	16 weeks	24 weeks	28 weeks
A					

					
B					
					

C					
					
Buried Flesh	4 weeks	8 weeks	16 weeks	24 weeks	28 weeks
A					

					
B					
					

C					
					
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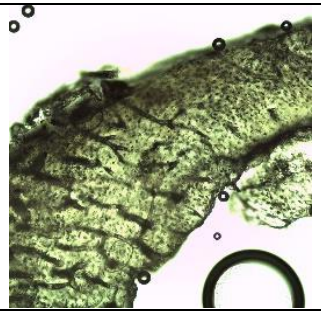
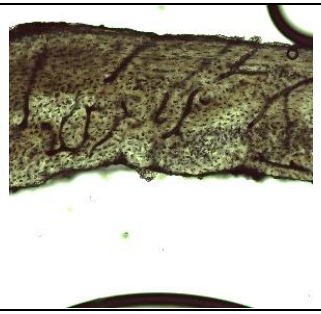
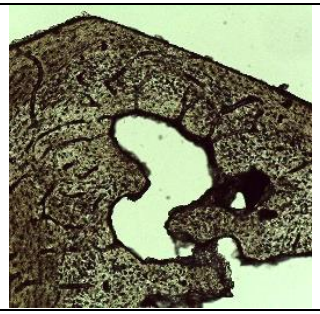
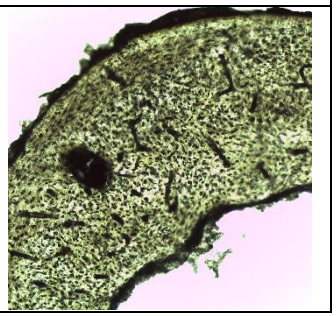
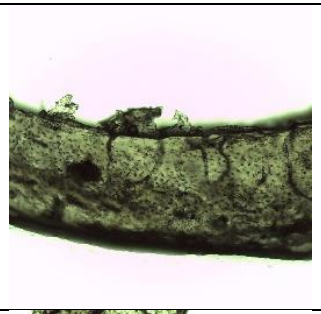
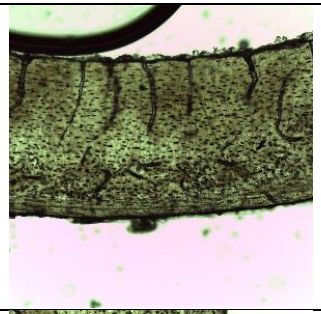
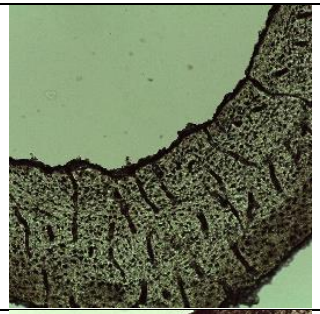
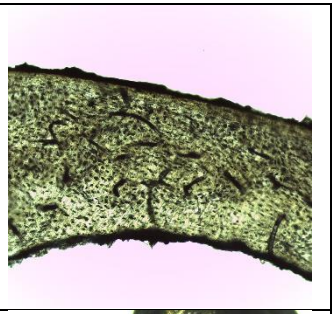
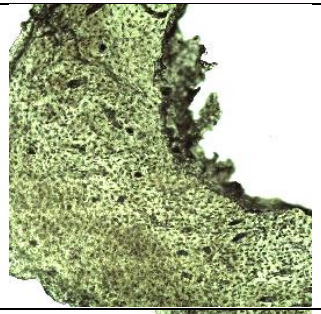
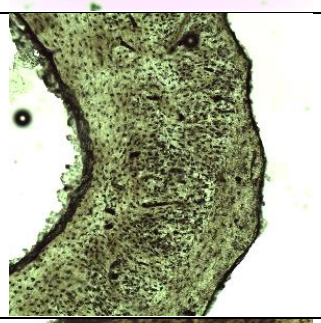
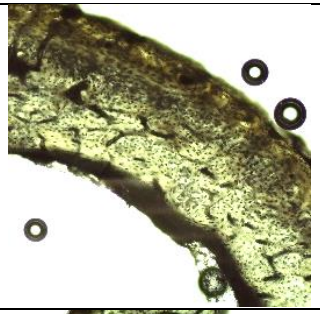
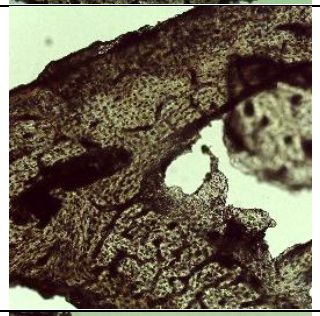
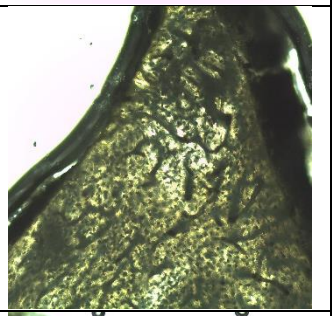
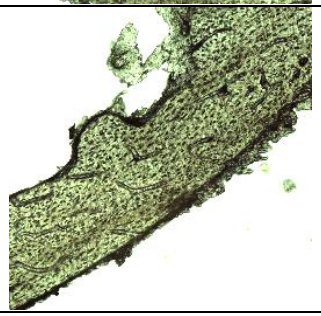
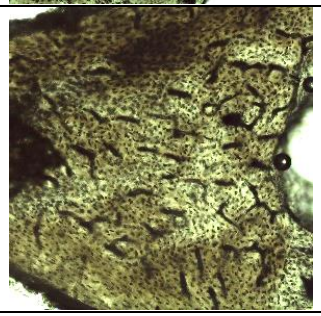
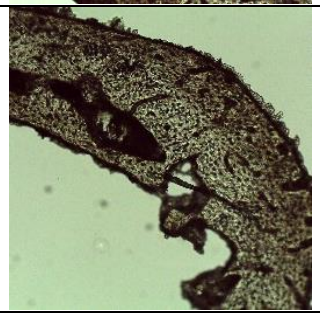
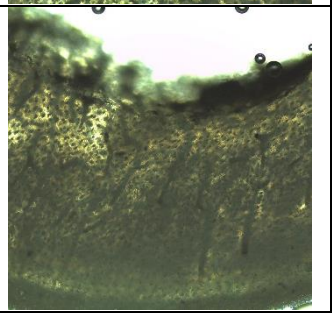
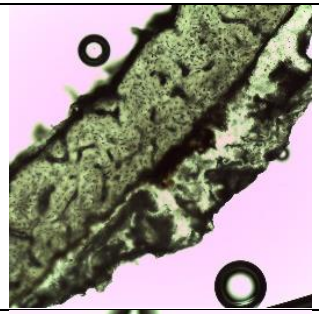
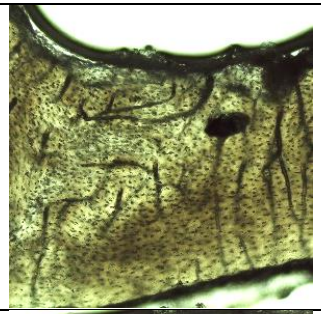
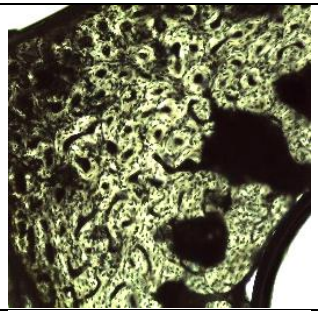
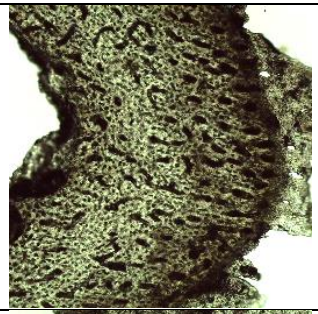
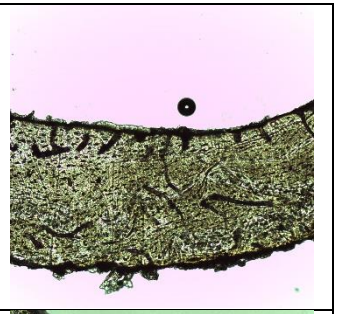
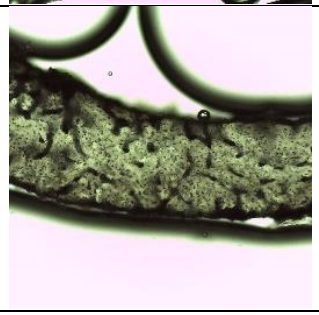
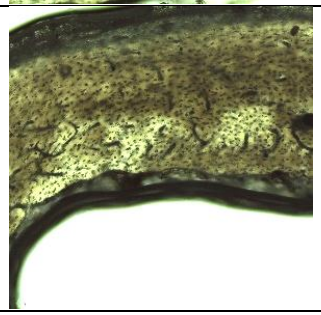
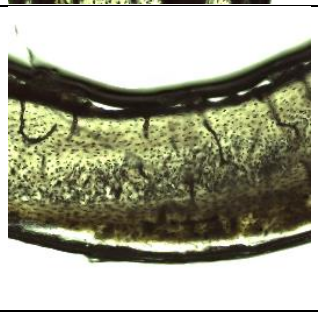
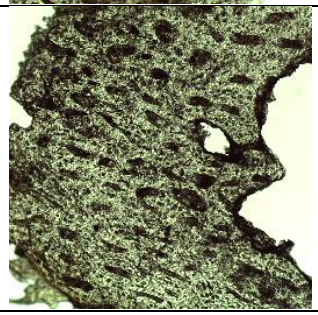
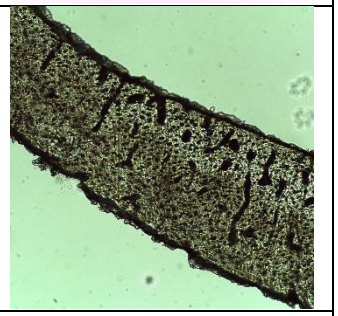
A					

					
B					
					

C					
					
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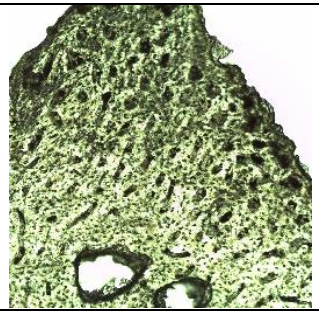
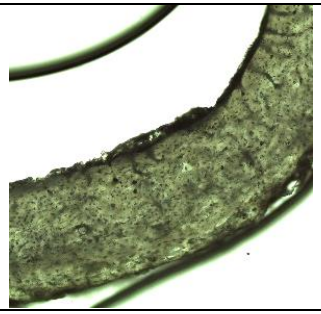
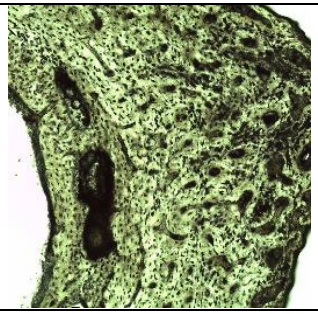
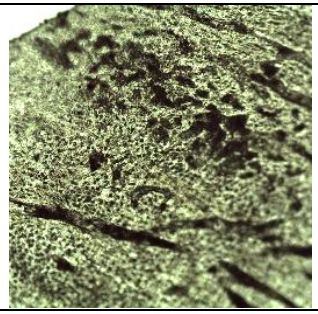
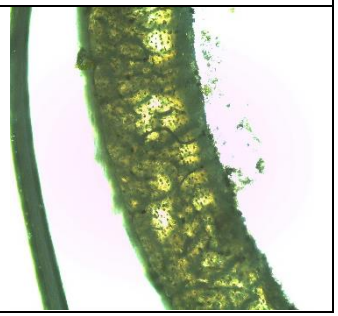
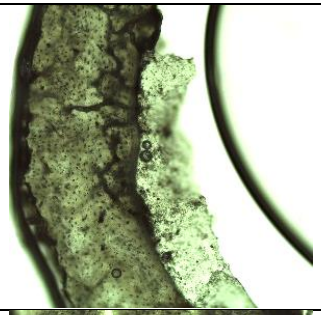
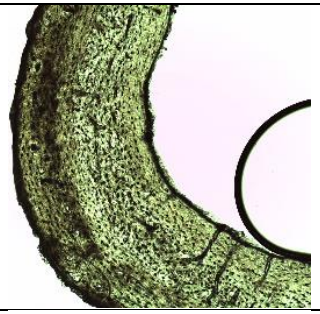
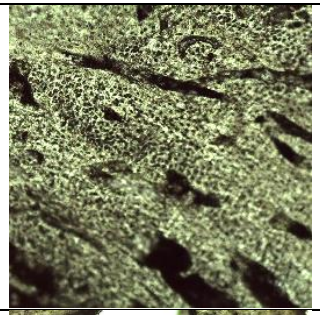
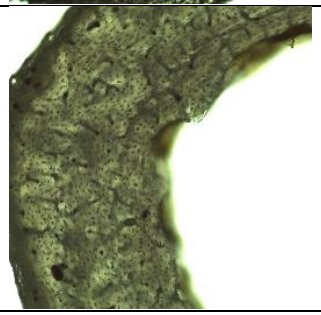
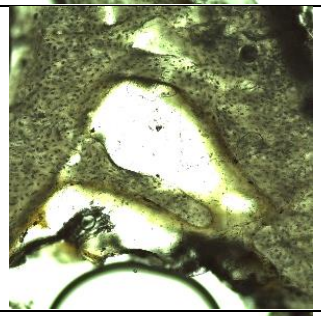
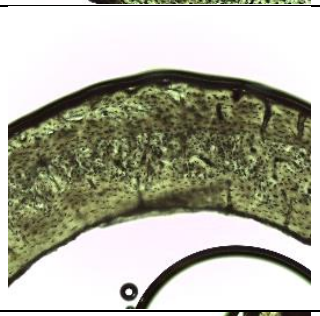
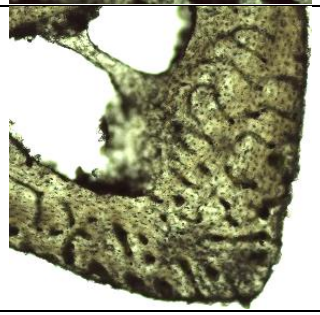
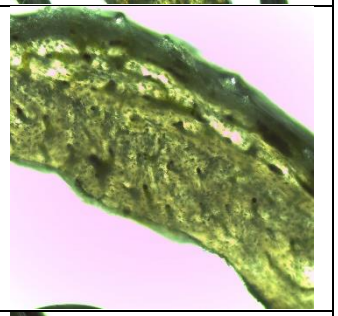
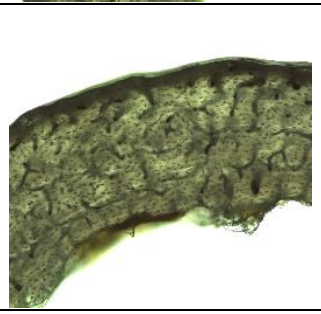
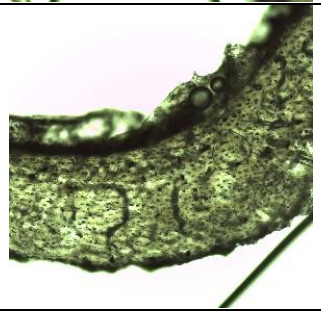
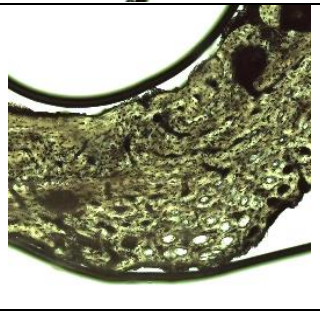
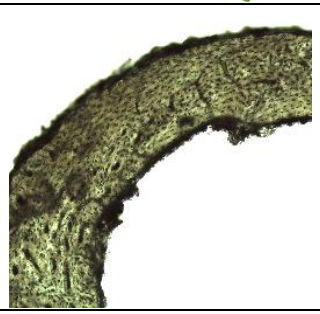
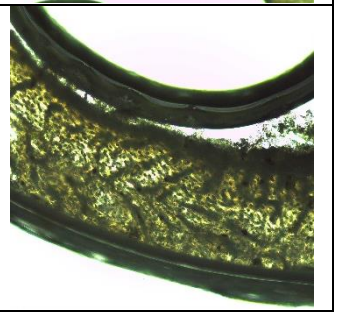
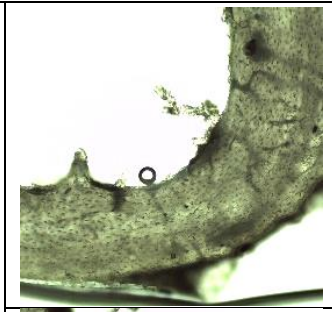
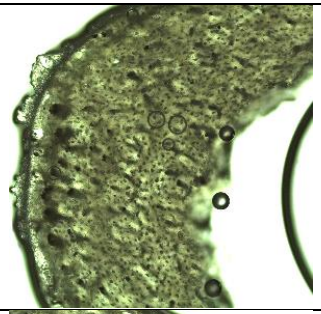
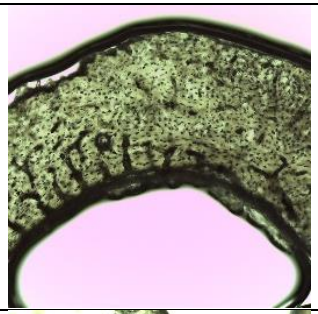
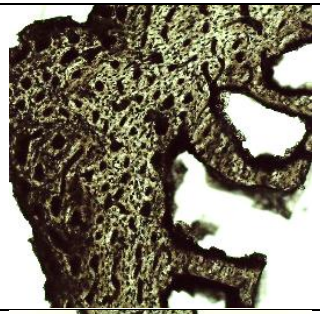
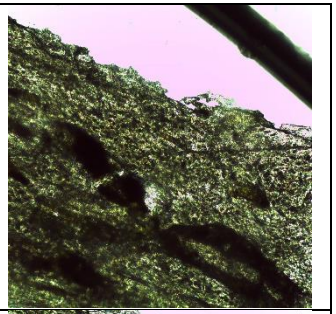
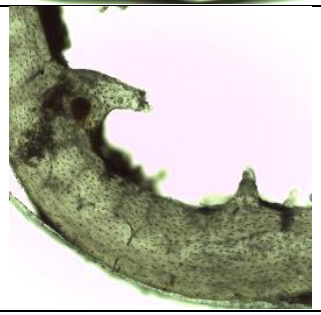
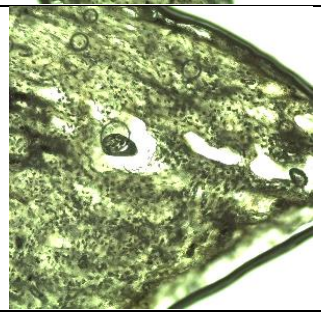
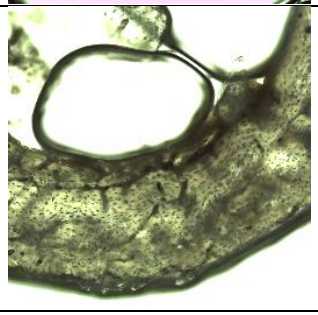
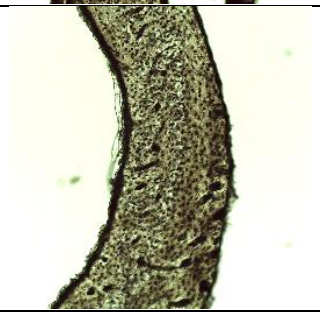
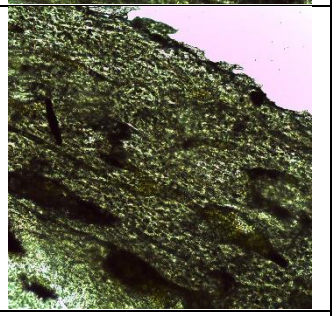
A					

					
B					
					

C					
					
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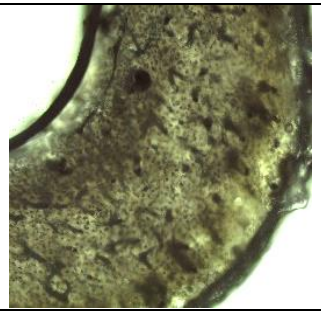
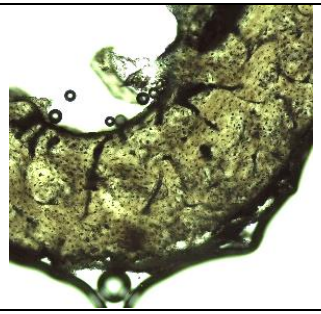
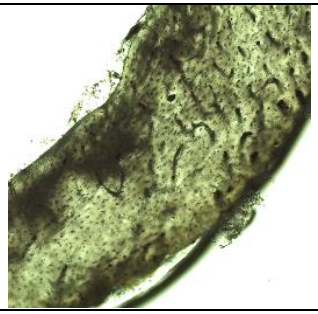
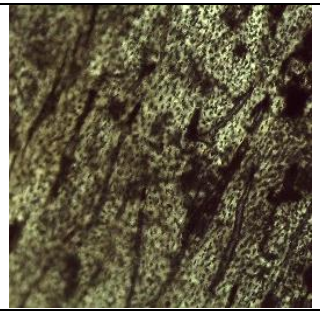
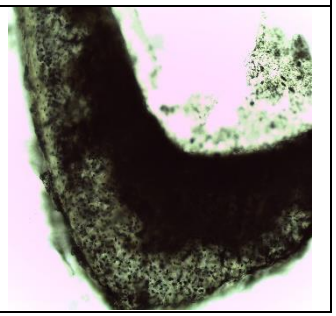
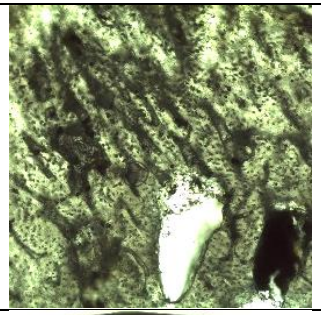
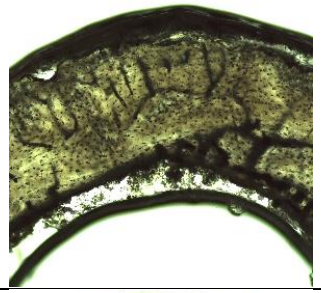
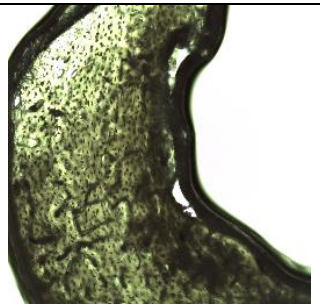
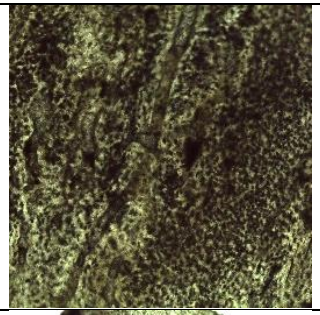
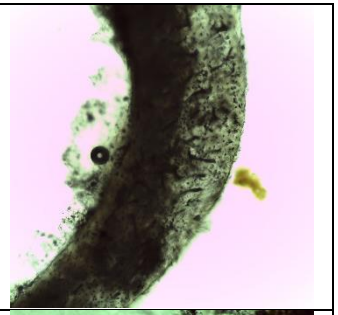
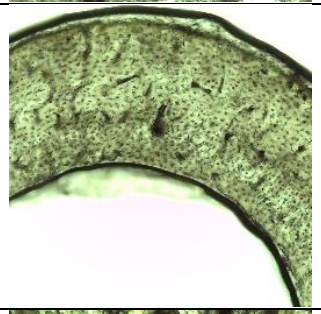
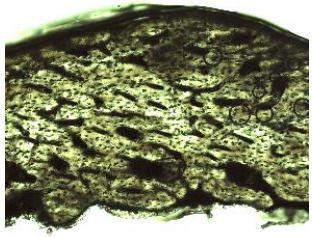
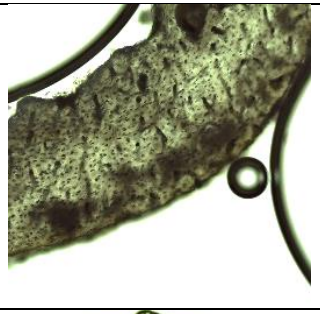
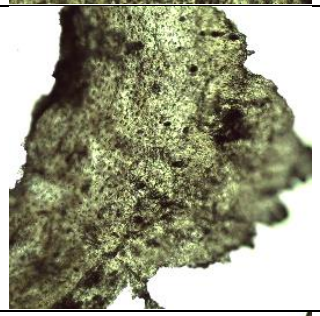
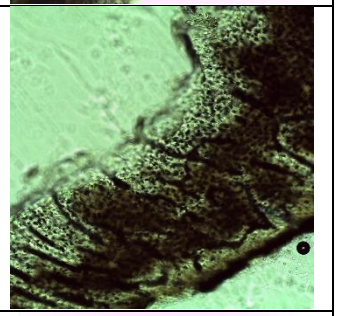
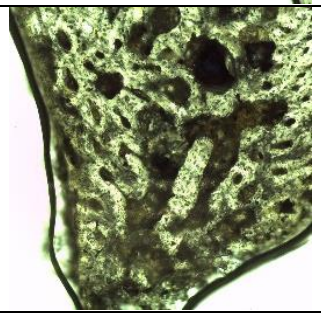
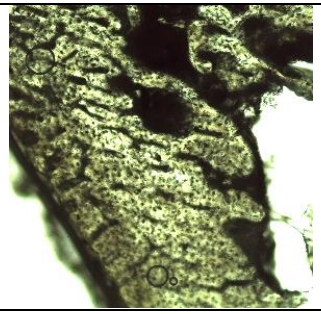
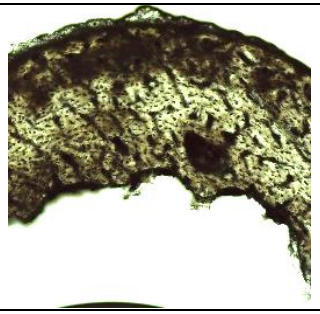
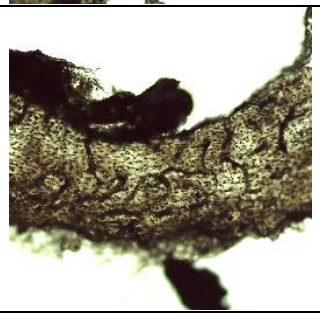
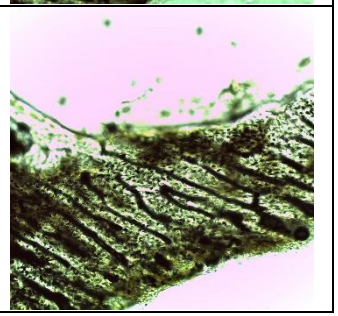
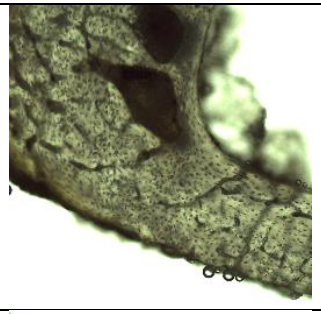
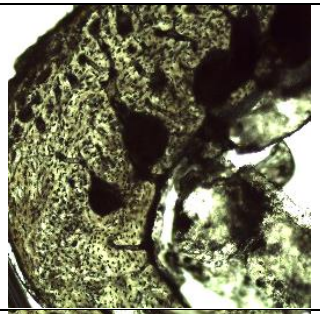
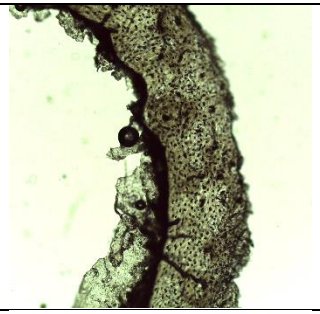
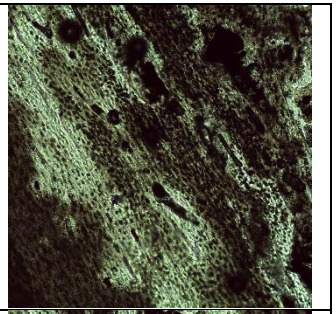
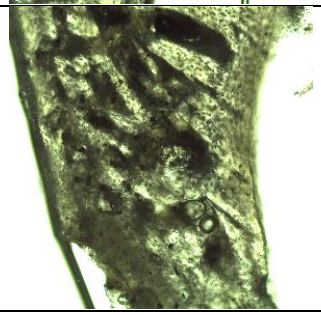
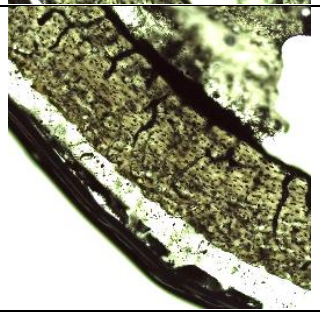
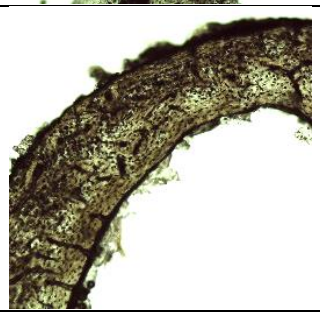
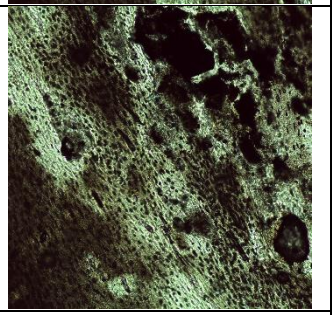
A					

					
B					
					

C					
					
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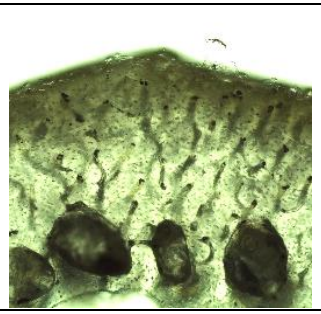
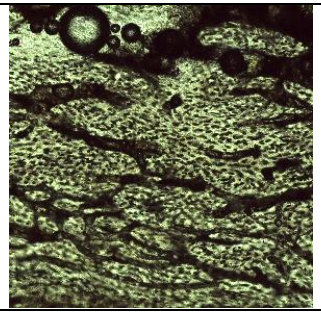
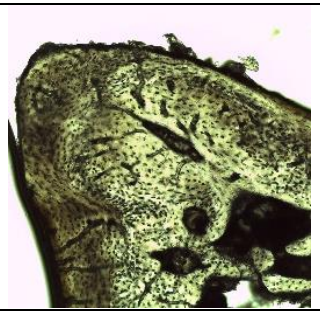
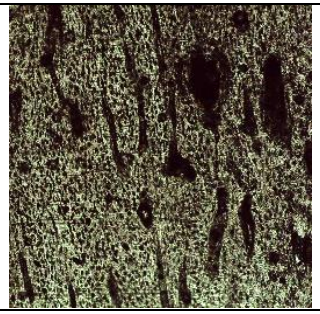
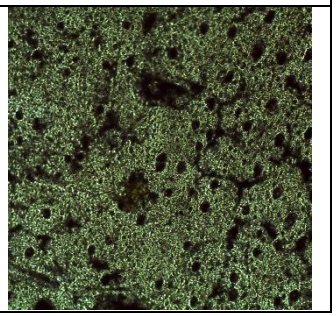
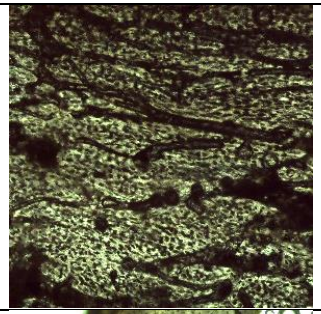
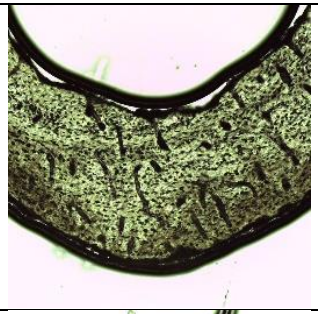
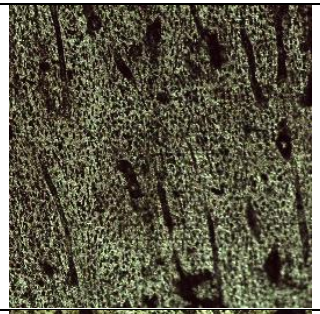
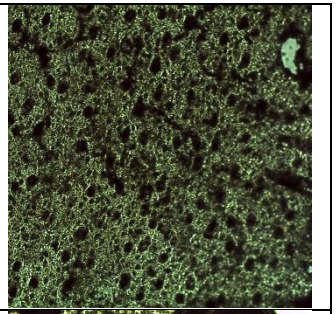
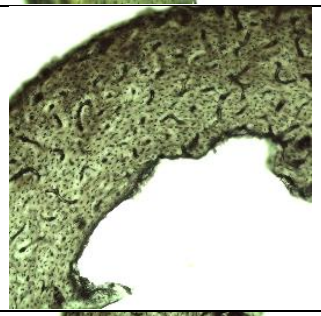
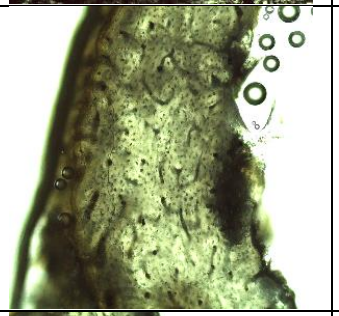
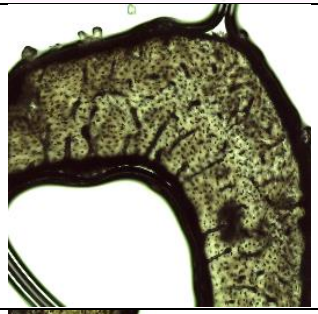
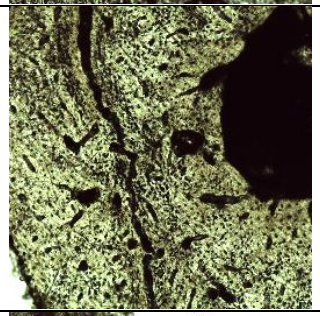
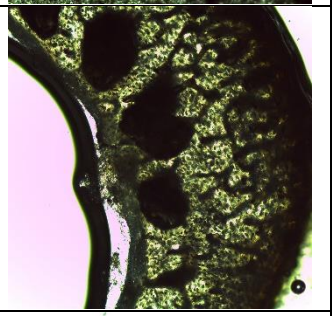
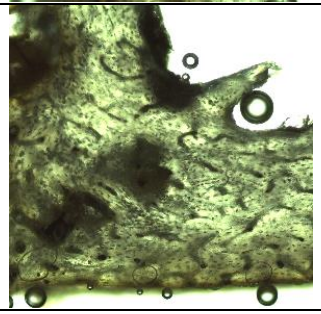
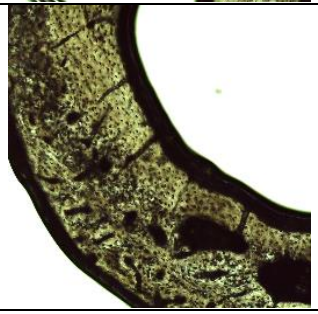
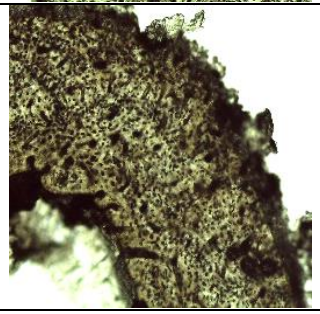
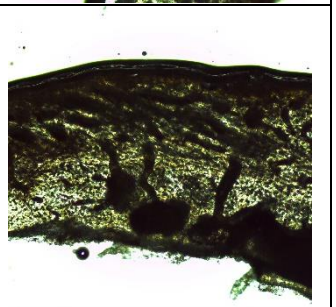
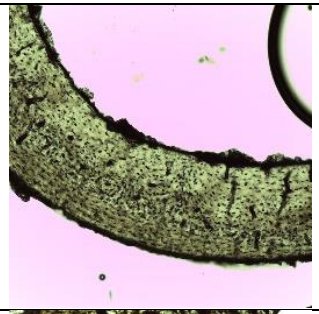
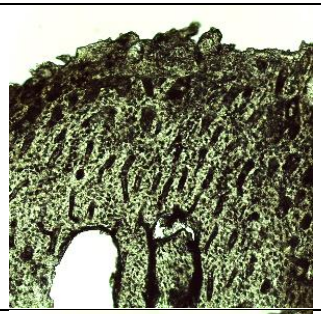
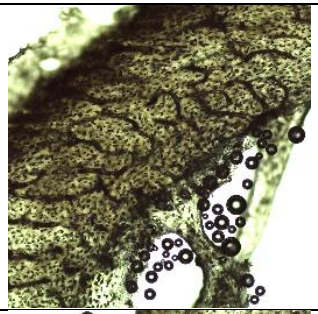
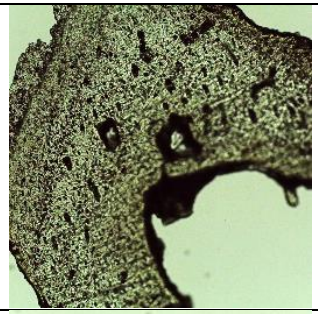
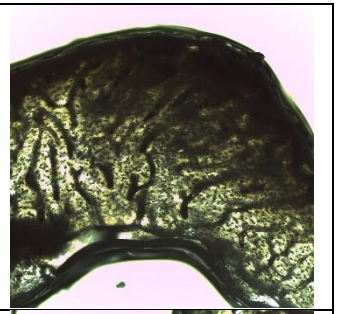
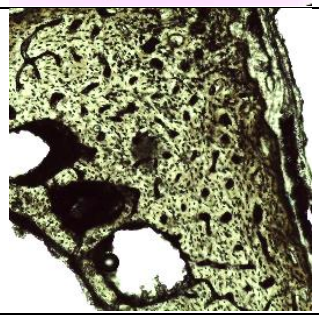
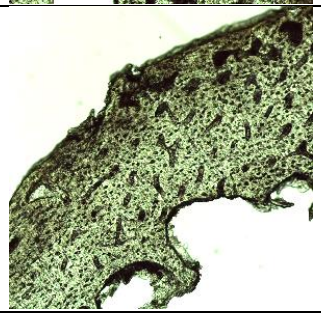
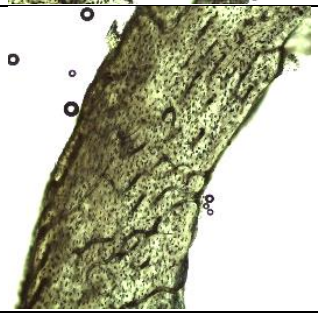
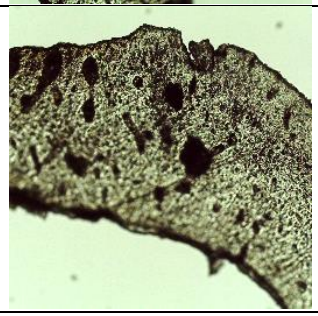
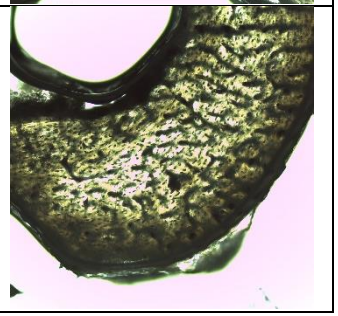
A					

					
B					
					

C					
					

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