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# Assessing the extent of bone bioerosion in short timescales – A novel approach for quantifying microstructural loss

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ABSTRACT

The evaluation of bone diagenetic phenomena in archaeological timescales is well established, but little is known about the extent of bone diagenesis in short forensic timescales. In particular, the use of the Oxford Histological Index (OHI) has become synonymous with assessing bioerosion in archaeological bone samples, but it may not be ideal for use on samples with shorter, forensic post-depositional timescales. Here, we present a novel method of quantifying the extent of bioerosion occurring on samples with short post-depositional periods by counting the number of normal and diagenetic osteocyte lacunae observed within the microstructure of the bone, which enables calculation of the percentage of destroyed bone within the sample. Due to the potential for condition of the remains, or depositional environment to affect bone diagenesis, this study investigated multiple conditions; whole rat carcasses, defleshed rat long bones, and excised fleshed rat limbs that were either placed in soil in plastic boxes or exposed on a clean plastic surface, and left to decompose from four to 28 weeks, to allow bone bioerosion to occur. Statistically significant differences in the number of average diagenetic lacunae observed were found between the three conditions, while a statistically significant difference was observed between OHI scores of samples in the two deposition environments. The number of diagenetic osteocyte lacunae increased in all conditions, showing statistically significant increase in the percentage of destroyed bone over time. Diagenetic changes were seen as early as 4-weeks post-deposition. Comparisons between the OHI scores and the lacunae counts showed that, while the use of OHI could not distinguish between samples with discrete microstructural deterioration, counting the normal and diagenetic lacunae could distinguish such diagenetic changes.

## 1. Introduction

Bones are known for their longevity and ability to survive (Turner-Walker, 2019), with discoveries of archaeological skeletal remains being well documented (Reiche et al., 2003; Jans et al., 2004; Nielsen-Marsh et al., 2007; Turner-Walker and Jans, 2008; Booth et al., 2016; Kendall et al., 2018; Kontopoulos et al., 2019). This makes the study of post-mortem bone of interest to many forensic anthropologists as often the skeletonised remains may be the only recoverable evidence to a suspicious death. An understanding of how bone is altered after death over time, and how different intrinsic and extrinsic conditions can influence these alterations, could help in the development of accurate post-mortem interval (PMI) estimation techniques. The histological analysis of such remains have led to the conclusion that, despite many specimens appearing well-preserved (Assis et al., 2015), skeletal tissue can undergo extensive degradation on a microstructural level (Bell et al., 1996; Trueman and Martill, 2002; Jans et al., 2004). A host of processes that can lead to alteration and the eventual destruction of bone fall under the term 'bone diagenesis' (Caruso et al., 2017). Research has identified three main diagenetic pathways; loss of the organic component (collagen hydrolysis); loss of the inorganic component (dissolution); and microbial attack (bioerosion) (Booth et al., 2016; Kontopoulos et al., 2016), with microbial attack being regarded as the main driver of bone diagenesis (Hackett, 1981; Hedges et al., 1995; White and Booth, 2014; Booth, 2016; Kontopoulos et al, 2016, 2019; Brönnimann et al., 2018; Eriksen et al., 2020). Current literature states that these microbes come from the remains themselves (Bell et al., 1996; White and Booth, 2014; Brönnimann et al., 2018), and/or the depositional environment (Reiche et al., 2003; Kendall et al., 2018; Morales et al., 2018).

Carl Wedl first observed the presence of tunnels and bores within the internal microstructure of teeth caused by the post-mortem action of microorganisms (Hackett, 1981) in 1864. Since then, researchers have

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worked to replicate these results in the hope of developing more accurate methods of PMI estimations when dealing with skeletonised remains (Boaks et al., 2014; Cappella et al., 2018). However, advancements have been thwarted by the sensitivity of the diagenetic processes, with differences in the depositional environments (temperature, climate, soil pH) and condition of the remains (cause of death, health of the individual) having an effect on the extent of changes seen (White and Booth, 2014).

Extensive literature is available describing the diagenetic processes that affect bone in archaeological timescales, however there is little research into bone bioerosion occurring in short, or forensic timescales (Bell et al., 1996; Kontopoulos et al., 2016). Until recently, it had been theorised that diagenetic changes to the skeletal tissue only occurred once skeletonisation of the remains had been reached (Bell et al., 1996; Brönnimann et al., 2018), a plausible conclusion considering it is only in the last thirty years or so that taphonomic studies have started to include further analysis of the skeletal tissues (Bell et al., 1996; White and Booth, 2014; Kontopoulos et al., 2016). But studies by Bell et al. (1996) and White and Booth (2014) have shown that this is not the case. Both studies showed that the skeleton begins to change soon after death, with White and Booth (2014) concluding that changes were found before full skeletonisation of the remains, most likely as a result of putrefactive bacteria invading the bone via the vascular network. Both studies observe the extent of bioerosion within very short timescales (three months – one year PMI).[1] This suggests that there may be potential for bioerosion of bone to be used for a method for PMI estimation, one that may be particularly useful if only skeletal remains are found. Research into the effect of bioerosion on bones from short timescales is of importance as it has been shown to be the biggest contributor of bone diagenesis; understanding how bioerosion occurs and what can influence it could enhance our understanding of forensic taphonomy and could help inform forensic practitioners on how to process and analyse skeletal remains.

The Oxford Histological Index (OHI) (Table 1), developed by Hedges et al. (1995) and later modified by Millard (2001), is currently the standard approach for evaluating the amount of bioerosion in a bone sample (White and Booth, 2014). It is used to assess the level of destruction observed within histological bone samples using a light microscope. Scores can be assigned to the specimen from 5 to 0, where 5 indicates unaltered bone that is hard to distinguish from fresh bone, and 0 indicates a sample that has no identifiable features within the microstructure (Hedges et al., 1995; Cappella et al., 2018). This method is flawed for several reasons: is highly subjective; the scores given rely on the observations, opinions and experience of the observer; and assessing inter-observer error between researchers is difficult as different labs can produce different results. In addition, this method was developed using archaeological bone samples likely to show a wide range of observable diagenetic changes; indeed, two of the categories used for scoring specimens are quite wide (score 2 = 15–50% destruction, and score 3 = 50–85% destruction) (Table 1), rendering them potentially inadequate for samples with shorter post-depositional timescales where the level of destruction may not be so obvious or take on the 'expected' form.

**Table 1**
The Oxford Histological Index
Oxford Histological Index. Source: Millard (2001), modified from Hedges et al. (1995).

| 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 patterns 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 |

**Table 2**
Type and number of samples per deposition/condition.

| Deposition | Condition | Number of Samples |
|---|---|---|
| Buried | Bone | 15 long bones |
| | Fleshed, excised limb | 15 limbs |
| | Whole | 5 whole rats |
| Exposed | Bone | 15 long bones |
| | Fleshed, excised limb | 15 limbs |
| | Whole | 5 whole rats |

This issue was encountered by White and Booth (2014) when studying bone bioerosion six and 12 months after (the) deposition. They found that the diagenetic changes they were observing were not consistent and therefore were too subtle for the OHI. They concluded they were potentially observing 'early' forms of diagenetic change, not seen before in archaeological samples. To overcome this, they used an adaptive 'Tunnelling Score' (White and Booth, 2014) which, while working in a similar manner to the OHI, allowed them to observe more subtle changes within the microstructure of the specimens. This method used a similar subjective scoring system to the OHI, but was not designed to become an accepted method of measuring bioerosion (White and Booth, 2014). These subtle changes were enlarged, misshapen osteocyte lacunae, observed within the microstructure of the bone samples. They (White and Booth, 2014) called these discrete changes 'diagenetic lacunae', and later research conducted by Kontopoulos et al. (2016) proposed that these changes were a very early form of bioerosion. Osteocytes are bone cells that reside within lacunae; small cavities within the bone (Schultz, 2006). They are connected to each other by small canals (canaliculi). It is estimated that the cortical bone within the human femur has in the region of 10 million osteocytes per cubic cm (Papageorgopoulou et al., as cited in Kendall et al., 2018).

Presented here is a novel approach for quantifying bioerosion in histological bone samples with short post-depositional intervals. This Osteocyte Lacunae Counting (OLC) method, influenced by the findings of White and Booth (2014) and Kontopoulos et al. (2016), requires counting the number of normal and abnormal (diagenetic) osteocyte lacunae that can be observed within the microstructure of bone. This allows the researcher to calculate the percentage of intact and/or damaged lacunae and determine how they are changing over time. This method was chosen as the authors wanted to investigate the theory that diagenetic lacunae were produced by early bioerosion; as this appears to occur very early in the post-mortem period, it was felt this could enhance current PMI estimation techniques. To assess the potential for evaluating and quantifying diagenesis in short timescales, we present here (a) a novel method for evaluating and quantifying the extent of bioerosion in bone samples with a short post-depositional period, in an attempt to overcome the limitations of the current methods, which were based on archaeological specimens and (b) an assessment of multiple variables to determine whether the novel method can help in understanding the microbial drivers that affect the diagenetic process. The working hypothesis for this research was that there would be a statistically significant increase in the number of diagenetic osteocyte lacunae observed within the bone samples over time. The *null* hypothesis was that no statistically significant increases in diagenetic osteocyte lacunae would be observed over time.

[1] It has to be noted that the Bell et al. (1996) sample came from predator scat and therefore may not be a true indication of diagenetic effects (Bell et al., 1996; Jans, 2008).

## 2. Materials and methods

### 2.1. Field experiment

The experimental set up was the same as that described in Procopio et al. (2021) and is briefly illustrated in Fig. 1. The femur and humerus bones of 26 medium-sized (weight range = 151–250g) domestic rats (*Rattus rattus*), purchased dead and frozen from a reptile food supplier (Northampton Reptile Centre) operating in compliance with the Animal Welfare Act 2006, were used for this study. The authors note the preferred use of *Sus scrofa* for taphonomy research however, as space was limited at the taphonomy site, smaller specimens were required for this research. Domestic rats were chosen as subjects for this investigation as they allowed for multiple repeated measurements to be taken with minimal inter-individual variability. Research has shown there is the possibility for different skeletal elements to show varying degrees of bone diagenesis, even within the same set of remains (Hanson and Buikstra, 1987; Bell et al., 1996; Jans et al., 2004), so the long bones were chosen because of their high cortical bone content and robusticity (Booth, 2016; Booth and Madgwick, 2016). The rats were flash frozen within 2–3 h after death, delivered frozen via 24-h delivery and immediately transferred to a freezer at −20 °C for storage. To prepare the field samples:

1. Prior to dissection, the rats were defrosted overnight at 4 °C. The authors were aware of the effects that freeze-thawing can have on the intrinsic bacterial communities (Hyde et al., 2017) and on soft tissues (Micozzi, 1997), however several studies have shown that bacteria are able to survive during freezing procedures (Speck and Ray, 1977; Dieser et al., 2013; Procopio et al., 2019). Since only the skeletal tissues were analysed in this study, and all animals were subjected to the same duration of freezing and thawing, the authors believe that the results should be compatible and comparable with each other. Furthermore, the soft tissue decomposition of the exposed whole samples was observed throughout and no deviations from the normal rate or pattern of putrefaction were noted.
2. The samples were split into three groups consisting of 10 whole carcasses; 30 excised, fleshed limbs (fore and hind limbs); and 30 defleshed long bones (femora and humeri). The whole remains group were deposited with no preparations, while the fore and hind limbs were removed for the excised fleshed limbs group using a small #10 scalpel, and the femora and humeri were extracted for the defleshed bones group, also using a #10 scalpel. The dissection and preparation process took place over two days and the samples were kept refrigerated overnight at 4 °C in between the two days. Two further long bones (a femur and humerus) were taken from a fresh, non-decomposed rat to use as control (day 0) samples.
3. The experimental samples were divided into two equal groups for each of two depositional environments (Table 2): exposed on the surface of a clean, plastic box or kept in soil in a plastic box. The exposed samples were placed into three large 42L plastic boxes, one box for each type of sample type (e.g., one box for the whole rats, one for the excised limbs and one for the defleshed bones). The boxes had holes drilled in the base to allow drainage of rainwater and were placed within large, locked wire cages to protect them from scavengers or interference (Fig. 2A–C). The soil samples were placed into smaller soil-filled 3L boxes; to prevent disturbing the samples, different boxes were used for each collection date. The samples were placed on top of ~1.35L of *Godwins topsoil* and further covered with 1.35L of the same topsoil. The boxes were closed and weighted down to protect the contents from scavengers (Fig. 2D). The soil condition was chosen to include soil bacteria, while the exposed condition allowed bacteria from environmental factors (such as insects) to be included.
4. The samples were deposited at "HuddersFIELD", the University of Huddersfield's animal taphonomic facility, from mid-November 2018 to May 2019. The facility was situated on grassy farmland in Halifax, West Yorkshire (UK). Local temperature and rainfall information was collected using a local weather station, World Weather Online, and can be found at (https://www.worldweatheronline.com/halifax-weatheraverages/west-yorkshire/gb.aspx). Average monthly temperature and rainfall readings/values were recorded from November 2018 to May 2019, the duration of the field experiments (Supplementary Fig. S1).
5. Sample collections were performed at 4, 8, 16, 24, and 28 weeks; at each collection timepoint two whole carcasses (one soil, one exposed), six excised, fleshed limbs (three soil, three exposed), and six defleshed long bones (three soil, three exposed) were collected. Upon collection, the bones were cleaned of any soil or debris using distilled water. The whole remains and the excised fleshed limbs were defleshed where necessary using a #10 scalpel. Three long bones were extracted from each whole rat for analysis. All complete long bones were cleaned using distilled water and stored in the freezer until their further preparation for the histological analysis.

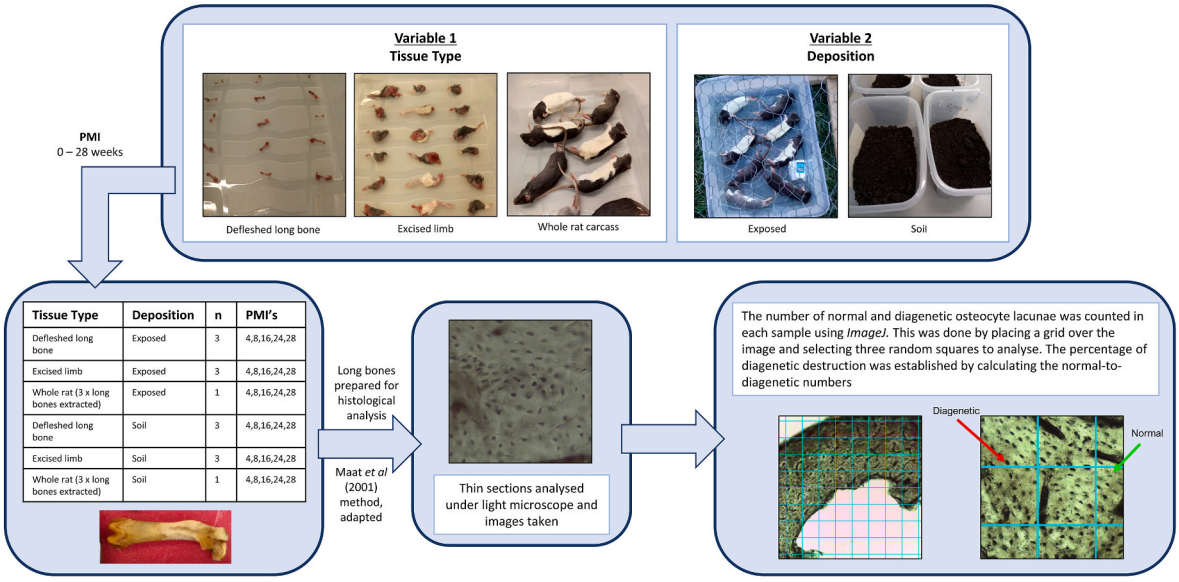


**Fig. 1.** Illustration to show a brief summary of the methodology used for this study.


**Fig. 2.** Samples placed *in situ* at the "HuddersFIELD" taphonomic facility. **A)** Exposed whole rats in the plastic box. **B)** Exposed excised limbs in the plastic box. **C)** Exposed rat bones in the plastic box. **D)** Soil samples (whole carcasses, excised limbs and defleshed bones) in the plastic boxes. Back row – whole remains; middle row – excised, fleshed limbs; front row – defleshed long bones.

### 2.2. Preparation of collected samples

To prepare the collected samples:

1. While the collected long bones were still frozen, the proximal and distal ends of the bones were cut using a junior hacksaw (Wilkinson) and mitre box (Modelcraft) and discarded.
2. The mid-section was further prepared for the histological analysis. The diaphyses were prepared using an adapted version of the Maat et al. (2001) manual preparation method for dry specimens, in order to obtain transverse histological samples. The adaptation of the Maat et al. (2001) method included the use of distilled water and higher grit sandpaper (240-grit and 1200-grit, both Titan hand-sanding) to give a smoother finish.
3. Samples were mounted onto glass microscope slides (Academy by CamLab) and distilled water was used as a mounting medium with a glass coverslip (Academy by CamLab). All samples were analysed using a Leica ICC50HD light microscope using 10x and 40x lenses (final magnification of 100x and 400x, respectively) and images taken. Samples were unstained and no filters were applied to the images.

### 2.3. Histological analysis

The OHI (Hedges et al., 1995) was used to assess the extent of bio-erosion within the samples. This new method was used alongside the OHI. For this the number of normal and diagenetic osteocyte lacunae were counted as described below. Osteocyte lacunae are elliptical in shape and have a diameter of less than 15 μm (Wang et al., 2022). This study focused on changes to the width of the lacunae as they appeared to swell when diagenetically changed. The width of lacunae from fresh samples measured 5–10 μm in width; lacunae was considered diagenetic if the width measured >10 μm and was of similar measurement to the length (Fig. 3). The method consists of:

1. Using *ImageJ*, a grid of 30,000-pixel per square was overlaid onto the 100x magnification images (Fig. 4A–B).
2. Six squares were chosen randomly per image (three images per sample to give 18 squares quantified per sample) and the normal and diagenetic osteocyte lacunae within were counted. Lacunae was considered to be diagenetic if they appeared enlarged (width >10 μm), misshapen (rounder rather than elliptical) and darker (Fig. 3), such as those described by Kontopoulos et al. (2016) and White and Booth (2014), than those observed within the control samples.
3. The mean number of diagenetic lacunae, and a mean of the total lacunae was calculated using the results from the grid counts. These mean values were used to calculate the percentage of destroyed bone within each sample.

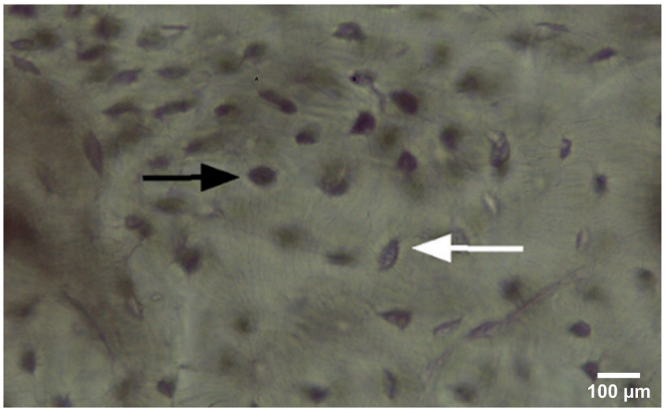


**Fig. 3.** Histology sample, 16 weeks after exposed deposition (400x magnification). Black arrow – diagenetic osteocyte lacunae, identified as it is larger and misshapen. White arrow – normal osteocyte lacunae.

This method was based on the evidence that microbes destroy the histological features of the bone (White and Booth, 2014), leading to a decrease in the number of normal observable osteocyte lacunae. The results of this novel OLC method were compared with the results using the OHI.

### 2.4. Statistical analysis

In order to determine if there was any statistical significance in the

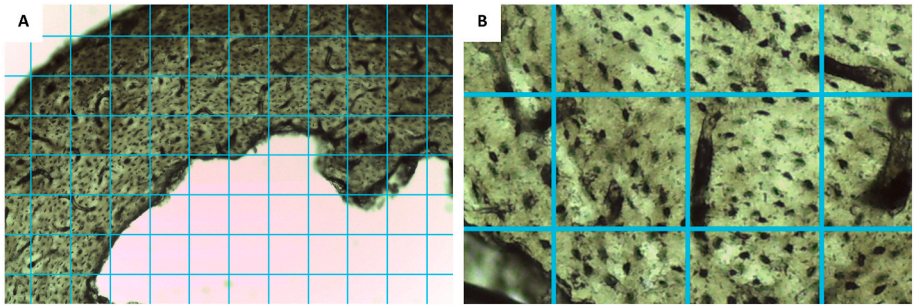


**Fig. 4.** Histology image from an exposed, whole carcass at 4 weeks PMI (100x magnification). Image opened using *ImageJ,* overlaid grid is set to 30,000 pixel$^2$. **A)** Original image. **B)** Zoomed in image as used to count the osteocyte lacunae.

extent of bioerosion occurring in the samples over short timescales, the average (mean) diagenetic lacunae count was compared across environments, conditions and PMI. The Shapiro-Wilks test for normality was used to determine which statistical test to perform. The distribution was not a normalised dataset, therefore the non-parametric Kruskal-Wallis test for independent samples was used with pairwise comparisons. Unadjusted *p* values were used for analysis, however adjusted values are given throughout in brackets (). SPSS was the statistical software package used to perform statistical evaluations.

In addition, the authors also assessed whether there were any measurable differences in the bioerosion of the whole carcasses (subject to autolysis and to the action of gut bacteria) in comparison with the excised limbs (subject to autolysis only) and the defleshed bone (subject to environmental effects) from both depositions. The two depositions were compared to determine whether there were statistically significant differences in bioerosion of the soil samples (subject to soil bacteria) compared to the exposed ones (subject to environmental factors).

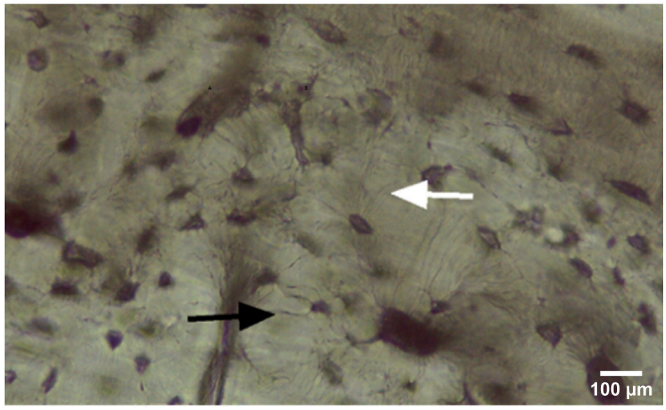


**Fig. 6.** Histology sample, 16 weeks after exposed deposition (400x magnification). Black arrow – enlarged canaliculi (Wedl type 2). White arrow – normal canaliculi.

## 3. Results

Diagenetic alterations (diagenetic osteocyte lacunae, as described by White and Booth (2014)) were observed as early as four weeks post-deposition across all conditions and both depositions, and were seen throughout the study in increasing numbers (Fig. 5A–B). Wedl type 2 tunnelling (Brönnimann et al., 2018) was observed in over a third of the samples (n = 80), with the canaliculi increasing in size (Fig. 6). Fifteen samples per depositional environment showed Wedl type 2 tunnelling. The defleshed bone and whole carcasses exhibited more Wedl type 2 tunnels (11 out of 30 samples and 15 out of 30 samples, respectively) compared with the excised, fleshed limbs (4 out of 30 samples).

The structural integrity of the samples degraded over time, with samples becoming visibly more damaged (Fig. 7, Supplementary Fig. S2). The average number of normal osteocyte lacunae decreased, as the average number of diagenetic osteocyte lacunae increased, leading to an increase in the calculated percentage of destroyed bone over increasing PMIs (Fig. 8A–B, Supplementary Fig. S3). The number of diagenetic osteocyte lacunae appeared to suddenly increase from 16 weeks PMI (Fig. 8A–B), indicating something may have occurred at this timescale to influence bioerosion. It was at this point the remains were exhibiting almost complete skeletonisation in the exposed samples, which may have allowed easier access for microbes to burrow into the bone. This sudden change in bone destruction was not observed using the OHI method however, with steady changes observed throughout (Fig. 8C). When comparing the three conditions, the whole carcasses appeared to have an increased number of diagenetic lacunae (mean = 11.38 at 28 weeks PMI), and therefore an increased percentage of destroyed bone (mean = 70.26% at 28 weeks PMI), occurring over time (Fig. 9A–B), however this was not so obvious when assessing the samples

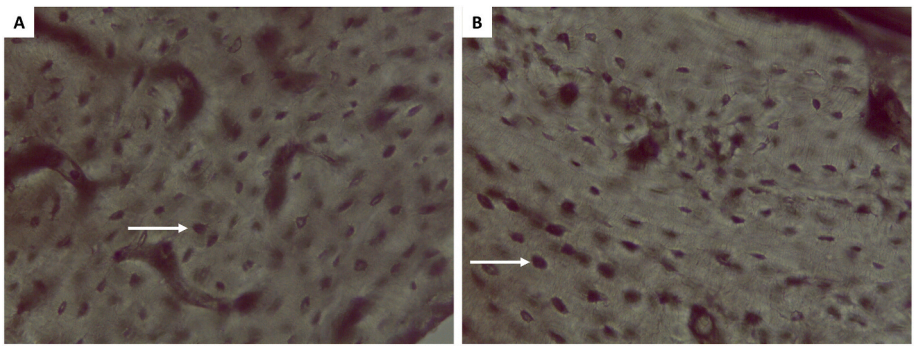


**Fig. 5.** Histology samples, 400x magnification. **A)** Exposed, whole bone sample, 4 weeks PMI, **B)** Exposed, whole sample, 16 weeks PMI. Arrows indicate diagenetic lacunae. More diagenetic lacunae can be observed in the later sample.

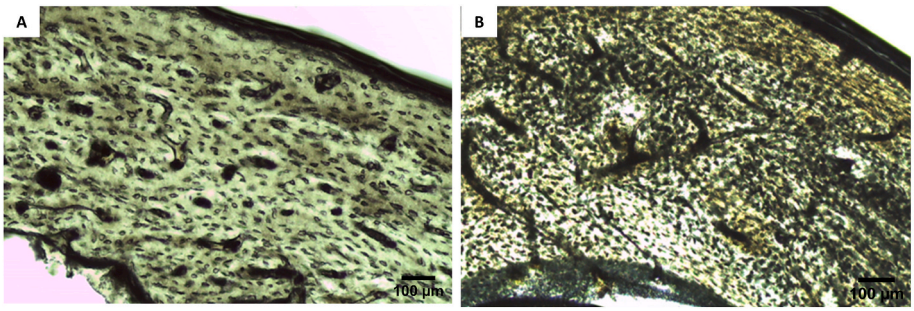


**Fig. 7. A)** A fresh histological sample of rat cortical bone showing normal osteocyte lacunae and **B)** a soil histological sample at 28 weeks deposition. Microbial infiltration seen as dark spots and shadows as indicated by the black arrow (100x magnification).

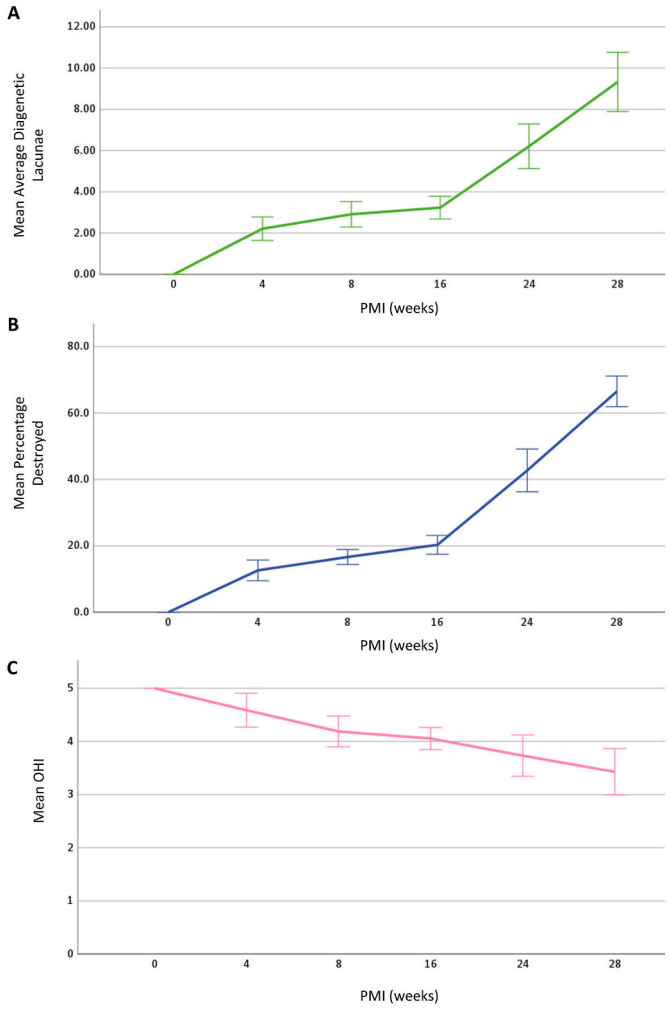


**Fig. 8.** Simple line graphs to show increasing histological damage occurring over increasing PMIs. **A)** Average number of diagenetic osteocyte lacunae in the samples. **B)** Percentage of destroyed osteocyte lacunae. **C)** Scores given using the Oxford Histological Index. Error bars represent 95% confident intervals.

using the OHI (Fig. 9C). Using the OLC, a difference between the deposition types was seen. The soil samples showed more diagenetic lacunae by the end of the study (mean = 69.88% at 28 weeks PMI) than the exposed samples, however it was not until >24 weeks PMI that this occurred (Fig. 9D–E). Prior to this timescale, the samples kept in soil showed less diagenetic damage than their exposed counterparts. The OHI however, did not show this sudden change >24 weeks PMI as the samples placed in soil were almost consistently less affected by bioerosion than the exposed samples (Fig. 9F); this difference between the two deposition types became more apparent >24 weeks PMI.

Statistical analysis was conducted (Table 3). The OHI scores were compared to the OLC scores to determine if there was a statistically significant difference between them. The extent of bioerosion occurring to the samples over time was shown to be statistically significantly changing with $p$ values of <0.001 calculated for all dependant variables (OHI; number of diagenetic osteocyte lacunae; percentage of destroyed bone) using non-parametric tests (Fig. 10A–C). Correlation analysis was run using Spearman's rho correlation test to determine the relationship between increasing PMI and the three dependant variables (OHI; number of diagenetic osteocyte lacunae; percentage of destroyed bone). This showed a strong positive and significant correlation between PMI and the number of diagenetic osteocyte lacunae ($r$ = 0.822, n = 82, $p$=<0.001); a strong positive and significant correlation between PMI and percentage of diagenetic lacunae ($r$ = 0.880, n = 82, $p$=<0.001); and a significant but moderate negative correlation between PMI and OHI ($r$ = −0.583, n = 82, $p$=<0.001) (Table 3). Separating the three conditions (defleshed bone, excised, fleshed limb, whole carcass) continued to show significant changes occurring over time for the number of diagenetic lacunae, and the percentage of destroyed lacunae ($p$=<0.001) (Table 3). Significant changes were found for defleshed bone and excised, fleshed limb over time using the OHI, however these were not as significant as the OLC method ($p$=<0.02), and no significance could be found over time using the OHI for the whole carcass samples (Table 3). Observing the two deposition environments, all three dependant variables showed significant results over time with $p$ values of <0.001 given for the exposed samples using all three measurements (Table 3). For the soil samples, $p$ values of <0.001 were obtained using the OLC method, while the OHI gave a $p$ value of 0.023 for changes occurring over time (Table 3). Significant and strong positive correlations were found for increasing PMI and changes to the number of average diagenetic lacunae and percentage of destroyed lacunae and a significant, negative correlation was present for OHI changes over time for all three conditions (Table 3).

Upon comparing samples from the two deposition environments, significance ($p$=0.021) was found between the two when using the OHI for assessment, with the soil samples showing better preservation compared with the exposed samples (Fig. 11A). Using the average number of diagenetic lacunae, significant differences were found between the three conditions; defleshed bone, excised fleshed limb, whole carcass ($p$ = 0.019), with pairwise comparisons showing significant differences between the whole carcass samples and defleshed bone, with a $p$ value of 0.037 (adjusted $p$ = 0.110), and between the whole carcasses and the excised, fleshed limbs, with a $p$ value of 0.007 (adjusted $p$ = 0.022) (Fig. 11B). Spearman's rho showed strong, significant correlations between PMI and the three dependant variables (number of average diagenetic lacunae; percentage of destroyed lacunae; OHI) for both depositions (Table 3).

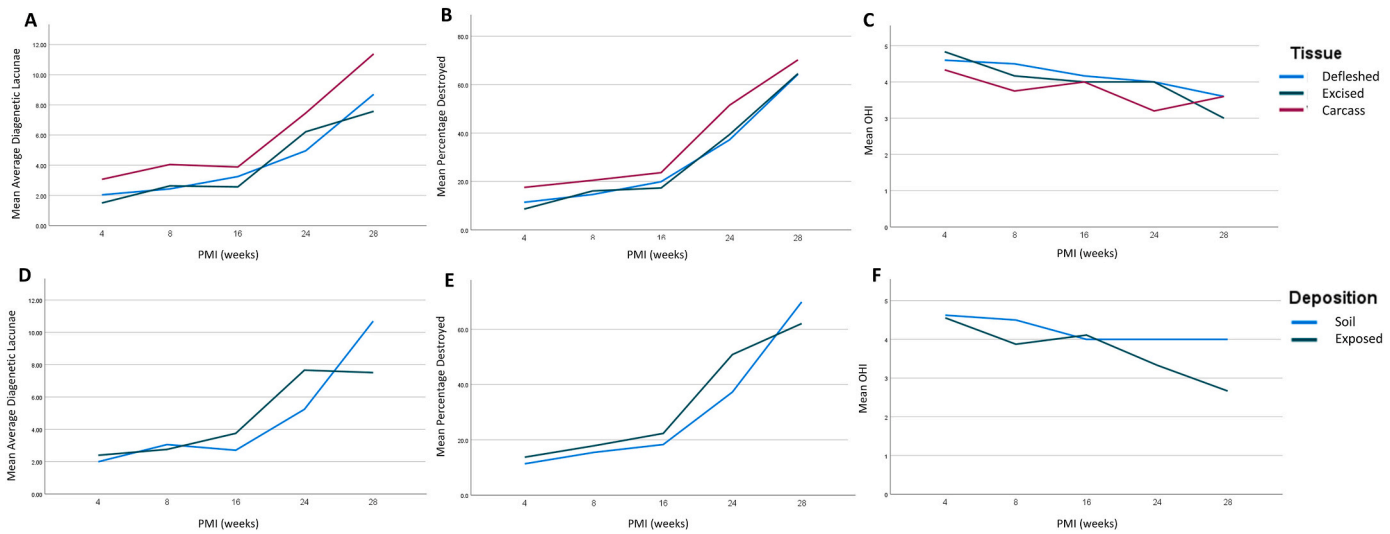


**Fig. 9.** Line graphs to show the number of diagenetic lacunae, percentage of destroyed bone, and OHI scores of the samples over time. Top row (**A-C**) shows the differences between the three conditions; defleshed bone, excised, fleshed limbs, whole carcasses. Bottom row (**D-F**) shows the two depositions used; kept in garden soil, exposed on a clean, plastic surface.

## 4. Discussion

We have attempted to quantify the extent of bioerosion occurring in the bone samples within short, forensic post-depositional timescales, using a novel OLC method, based on White and Booth (2014) and Kontopoulos et al. (2016)'s premise, that microbial attack results in the deterioration of the osteocyte lacunae within the internal microstructure of bone tissue. Our novel method involves counting the number of normal and diagenetic lacunae to determine how these numbers change over time, and how this affects the percentage of intact and destroyed bone. The standard method of analysing bioerosion uses the OHI, a scale of 0–5, to assess the percentage of intact bone observed within the sample (Hedges et al., 1995). As this scale was developed using archaeological bone, it was felt that it might not be suitable for samples within short, forensic timescales as it would not take into account subtle changes within the microstructure. This issue was also encountered by White and Booth (2014), who determined the changes they were seeing in their samples were too discrete and inconsistent for the OHI to accurately distinguish them. To determine whether this was the case in the study presented here, the OHI was used to assess the samples alongside the OLC. It can be seen in Supplementary Fig. S3 that many samples (49/80) were given a score of 4 using the OHI scale, but with the use of the OLC it was shown that perceptible diagenetic changes had occurred in each timescale. Here, it was found that the deterioration of lacunae increased significantly as the PMI increased ($p < 0.001$).

Previous bone diagenesis studies have not considered the potential for microbial infiltrations occurring prior to skeletonisation of the remains until recently (Bell et al., 1996; White and Booth, 2014). Due to this, there is very little literature available about the extent of diagenesis occurring within short timescales (Bell et al., 1996; Kontopoulos et al., 2016). Bell et al. (1996) observed changes within three-months post-mortem on a human tibial fragment, however it was noted that this sample had come from predator scat and therefore the observed changes may not be representative of other environments. They also observed diagenetic changes on a human rib bone, recovered from a waterlogged muskeg bog, at 15-months post-mortem. White and Booth (2014) showed diagenetic alterations at 6-months PMI, although they were not the expected non-Wedl tunnels that are typical of microbial attack, but abnormal, enlarged osteocyte lacunae, which they referred to as 'diagenetic osteocyte lacunae' (Bell et al., 1996; Kontopoulos et al., 2016). Further work by Kontopoulos et al. (2016) hypothesised that these were a very early form of microscopic foci of destruction (MFD), a precursor to non-Wedl MFD that can appear in short timescales in comparison with studies conducted on longer timescales. As they do not appear to have been quantified before, it is the presence and frequency of these diagenetic lacunae that are assessed here. More recently, a study by Eriksen et al. (2020) analysed bioerosion on bone samples within short timescales (4, 14, 28, and 52 weeks) in aquatic and terrestrial environments, but observed no changes before one-year post-deposition. However, this study used partial animal remains that were cooked prior to deposition, which undoubtedly affected the bone structure. We have demonstrated that diagenetic changes, such as the presence of enlarged osteocyte lacunae, can be observed as early as four-weeks post-deposition, and before skeletonisation of the whole carcasses. Nothing could be found within current literature to show diagenetic changes this early in the post-mortem period therefore, this is the earliest that diagenetic bone changes have been observed using histological techniques. The number of diagenetic lacunae increased constantly throughout the study, with this degradation of the osteocyte lacunae suddenly increasing from 16 weeks PMI. These changes were seen alongside the presence of Wedl type 2 tunnelling (enlarged canaliculi), also starting from four-weeks post-deposition. A steady decline of the percentage of intact bone was observed from four-weeks post-deposition through to 28-weeks. This supports Kontopoulos et al. (2016)'s theory, that an early form of non-Wedl MFD can be observed soon after deposition, however more taphonomic research is needed to further support this observation and to determine how they progress over time. There was an increase in the rate of osteocyte lacunae showing diagenetic traits from 16 weeks PMI. The fact that this increase was observed >16 weeks, around the time the exposed samples became fully skeletonised, may indicate that the exposure of the bone to external factors plays a pivotal role in bone diagenesis as it allows easier access for microbes.

In addition, we aimed to clarify whether there was a preference for microbial attack in certain environmental conditions or in the presence of specific microbes/environmental factors. We found similar levels of destruction between the three conditions when using the OHI as an assessment tool, however when using the OLC methods, the whole carcasses were found to have statistically significantly more diagenetic lacunae compared with the two other conditions. This suggests that the presence of gut bacteria during the putrefactive stage does affect bone diagenesis, which corroborates White and Booth (2014)'s results, which showed increased levels of microbial infiltrations in their juvenile samples compared to their neonate samples. Using the OLC method, the soil samples showed an increased level of lacunae destruction than their

**Table 3**
Statistical significance of differences recorded in the extent of bioerosion observed in the samples over time, and when the samples were grouped according to their depositional environment, and condition. Statistically significant results are shown (*$p < 0.001$ '***', $p < 0.01$ '**', $p < 0.05$ '*'; $p > 0.05$ 'ns'*).

| Grouping Variables | Variable | Kruskal-Wallis Significance *p* | Spearman's rho Correlation r (*p*) |
|---|---|---|---|
| Bioerosion vs Time (including control samples) | OHI | *** | −0.583 (***) |
| | Percentage Destroyed Bone | *** | 0.880 (***) |
| | Diagenetic Lacunae Count | *** | 0.822 (***) |
| Bioerosion vs Time (excluding control samples) | OHI | *** | −0.554 (***) |
| | Percentage Destroyed Bone | *** | 0.870 (***) |
| | Diagenetic Lacunae Count | *** | 0.808 (***) |
| Bioerosion vs Time (defleshed bone samples) | OHI | * | −0.686 (***) |
| | Percentage Destroyed Bone | *** | 0.916 (***) |
| | Diagenetic Lacunae Count | *** | 0.867 (***) |
| Bioerosion vs Time (fleshed limb samples) | OHI | ** | −0.711 (***) |
| | Percentage Destroyed Bone | *** | 0.914 (***) |
| | Diagenetic Lacunae Count | *** | 0.870 (***) |
| Bioerosion vs Time (whole carcass samples) | OHI | *ns* | −0.523 (**) |
| | Percentage Destroyed Bone | *** | 0.908 (***) |
| | Diagenetic Lacunae Count | *** | 0.853 (***) |
| Bioerosion vs Time (exposed samples) | OHI | *** | −0.768 (***) |
| | Percentage Destroyed Bone | *** | 0.875 (***) |
| | Diagenetic Lacunae Count | *** | 0.840 (***) |
| Bioerosion vs Time (soil samples) | OHI | * | −0.533 (***) |
| | Percentage Destroyed Bone | *** | 0.903 (***) |
| | Diagenetic Lacunae Count | *** | 0.846 (***) |
| Deposition: Soil vs Exposed | OHI | * | n/a |
| | Percentage Destroyed Bone | *ns* | n/a |
| | Diagenetic Lacunae Count | *ns* | n/a |
| Condition Type: Defleshed Bone vs Fleshed Limb vs Whole Carcass | OHI | *ns* | n/a |
| | Percentage Destroyed Bone | *ns* | n/a |
| | Diagenetic Lacunae Count | * | n/a |
| Condition Type: Defleshed bone vs Fleshed Limb | OHI | *ns* | n/a |
| | Percentage Destroyed Bone | *ns* | n/a |
| | Diagenetic Lacunae Count | *ns* | n/a |
| Condition Type: Defleshed Bone vs Whole Carcass | OHI | *ns* | n/a |
| | Percentage Destroyed Bone | *ns* | n/a |
| | Diagenetic Lacunae Count | * | n/a |
| Condition Type: Fleshed Limb vs Whole Carcass | OHI | *ns* | n/a |
| | Percentage Destroyed Bone | *ns* | n/a |
| | Diagenetic Lacunae Count | * | n/a |

exposed counterparts, although this only occurred >24 weeks PMI; prior to this timescale, the samples placed in soil showed less diagenetic change than the exposed samples. It is unclear why this occurred; the exposed samples were skeletonised quicker due to their deposition environment and no sudden increase in skeletonisation of the soil samples was observed around this timescale to explain this sudden change. The increased diagenetic change in the soil samples by 28 weeks were not statistically significant, however this result does agree with research conducted by Bonicelli et al. (2022), who found that the presence of soil bacteria has a big effect on protein degradation in bones. In contrast, the OHI method showed that the soil samples were statistically significantly better preserved than the exposed samples, most likely as a result of the defleshed bone samples being better preserved in the soil deposition than exposed deposition ($p < 0.05$). These discrepancies could be due to the subjective nature of the OHI, and/or that the OLC method only takes into account diagenetically altered lacunae and not the rest of the bone matrix. But, considering the placed-in-soil samples in this research were placed into plastic boxes rather than in the ground (limiting the number and variety of bacteria present), it cannot be ruled out that the results were influenced by the experimental method. To determine this, it would be beneficial to repeat this study incorporating different depositions (with a focus on open/natural burials) and larger specimens. It would also be recommended to consider different bone types. This research focused on the long bones as these are generally used in histological research due to their high cortical bone content, but it has been proposed that different bone types may be affected by diagenesis at different rates (Gallello et al., 2015; López-Costas et al., 2016) The repeatability of this novel method also needs to be assessed; as this research was conducted within a small research group no one was available to undertake inter-observer error analysis. A selection of samples was reassessed for intra-observer error however, although only very slight differences in scores were noticed between the new and original results, these did not produce a high level of agreement using the Cohen's Kappa statistical test, most likely due to the small number of samples analysed (<25%) and due to working from multiple images.

## 5. Conclusion

This research has added to the current knowledge in forensic anthropology and to the understanding of how bones is altered in the early taphonomic period. The results reported here show clear evidence for microbial attack in the early post-mortem period (by 4 weeks PMI). However, these changes did not appear as tunnels as reported in the archaeological literature (Hackett, 1981; Trueman and Martill, 2002; Jans et al., 2004), but were recorded as altered osteocyte lacunae. The microbial attack found here corroborates current findings from White and Booth (2014) and Kontopoulos et al. (2016), who conducted research into bone diagenesis within relatively short timescales and found the presence of enlarged, diagenetic lacunae, but did not attempt to quantify their presence.

Results obtained here have shown that the depositional environment may affect the extent of bioerosion seen, however conflicting results mean more research is required to clarify the extent of its influence. The influence of different depositions, such as shallow burials or wrapped remains, also on bone diagenesis also need to be considered for future research. Whole carcass samples were found to have a statistically significantly higher level of bioerosion than defleshed bone, or excised, fleshed limbs, which corresponds with results found by other

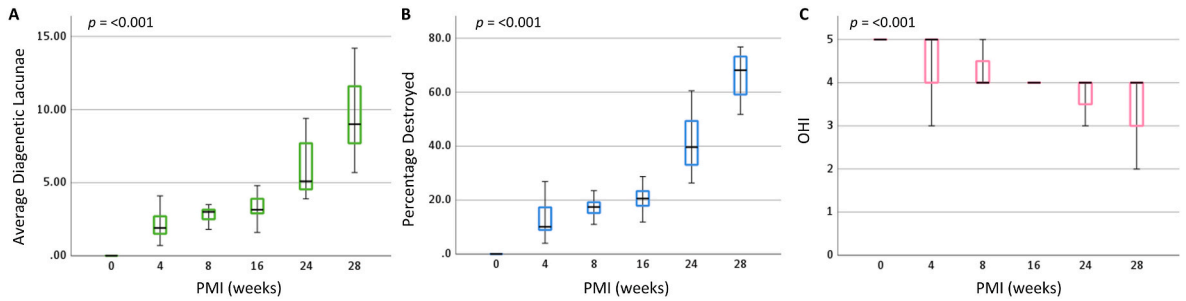


**Fig. 10.** Box and whisker plots showing distributions over increasing PMIs of **A)** average number of diagenetic lacunae, **B)** percentage of destroyed lacunae, and **C)** OHI. Box represents the 25% and 75% quartiles, median is shown, whiskers represent minimum and maximum values (excluding outliers). Kruskal-Wallis *p* values are given.

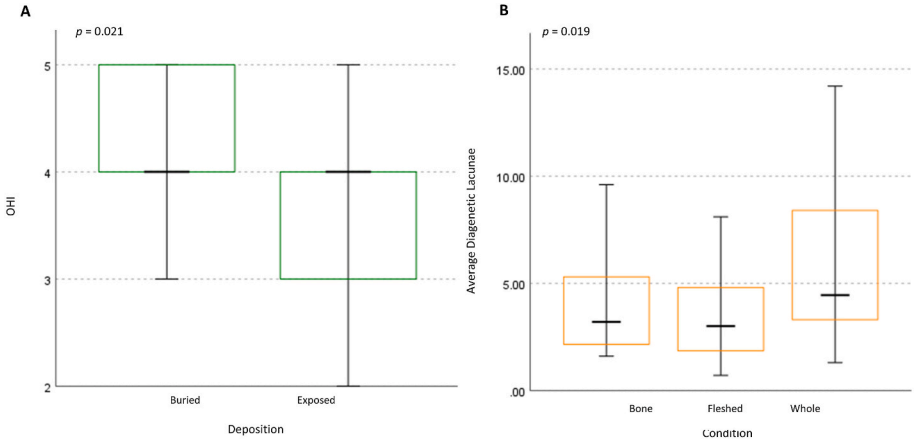


**Fig. 11.** Box and whisker plots showing distributions of **A)** OHI scores when comparing the two deposition environments and **B)** diagenetic lacunae when comparing the three conditions. Box represents the 25% and 75% quartiles, median is shown, whiskers represent minimum and maximum values (excluding outliers). Kruskal-Wallis *p* values are given.

researchers and indicates that intrinsic gut bacteria play a bigger role in bone bioerosion than extrinsic bacteria.

It has been demonstrated here that the extent of bioerosion within skeletal remains from short timescales can be quantified using a method of counting the normal and diagenetic lacunae within the microstructure of the samples. Unlike the OHI, which is adapted for archaeological samples, counting the lacunae changes in short timescales can take into account deterioration to the samples that are too subtle for the OHI to detect. More research is required, particularly further research that includes intra- and inter-observer errors to establish the repeatability of this method, but it is anticipated that this new method of quantifying bone diagenesis may be applicable to samples from other conditions and depositions. One limitation could be its application to archaeological samples; this method relies on the lacunae being observed for counting, which may not be possible on samples with large areas of destruction, however archaeological samples were not the focus here.

With further research, this novel method could be useful forensically; as it has been shown that the number of diagenetic lacunae increase as the PMI increases, this method could contribute to PMI estimation techniques, particularly in cases where only skeletal remains are recovered or where the current methods of determining early PMI are no longer applicable.

## Author contributions

Caley Mein: Conceptualisation, Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualisation. Anna Williams: Conceptualisation, Methodology, Resources, Writing – Original Draft, Writing – Review & Editing, Supervision, Project Administration.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

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