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Evaluating the impact of acetic acid chemical pre-treatment on ‘old’ and cremated bone with the ‘Perio-spot’ technique and ‘Perios-endos’ profiles

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A B S T R A C T

Impacts of diagenesis on archaeological and palaeontological bone complicate the investigation of in-vivo chemical and isotopic characteristics. Such bone is often pre-treated in an attempt to remove diagenetic alteration prior to trace element or isotopic analyses, although very few standardized approaches exist for evaluating pre-treatment effectiveness. In this pilot study, we characterize the diagenetic alteration and assess the impact of acetic acid chemical pre-treatment on the trace element and structural characteristics of four bones from Belgium, including an Early Medieval cremated bone from Broechem and three representative ‘old’ bones of different ages (ca. 40–130 ka) from the Late Pleistocene sedimentary sequence of Schaldna Cave. Each bone was analyzed before and after acetic acid pre-treatment using the ‘Perio-spot’ technique and ‘Perios-endos’ profiles. We measured trace element concentrations with laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) and micro X-ray fluorescence spectroscopy (µXRF). Structural characteristics were investigated with Raman spectroscopy and Fourier transform infrared spectroscopy (FTIR). Our results indicate that chemical pre-treatment had little to no significant impact on the trace element content of the Early Medieval cremated bone, had the most impact on the youngest bone from Schaldna Cave, and had less impact on the trace element content of two older bones from Schaldna Cave. This suggests that the effectiveness of acetic acid chemical pre-treatment is greater for bones undergoing early diagenetic processes, has minimal impact on highly crystalline cremated bone, and may preferentially leach in-vivo signatures from bones undergoing later diagenesis. The weights of leachates removed from each bone also correspond well with their hypothetical diagenetic stages, indicating that researchers could potentially assess the diagenetic state of bones by weighing the leachates produced during acetic acid pre-treatment. Therefore, our new approach may provide a valuable step toward effectively and consistently differentiating among in- and ex-vivo trace element signatures, and, by proxy, those of their isotopes, in archaeological and palaeontological bone.

1. Introduction

Reconstructions of past human and animal behaviour typically rely on the analysis of well-preserved biogenic tissues for diet-related chemical and isotopic compositions. However, the chemical and isotopic characteristics of vertebrate hard parts (i.e., bone, dentin, and enamel) are modified both during life (in-vivo) and after death (ex-vivo; 'taphonomy', Efremov, 1940). As such, archaeological and palaeontological hard parts record evidence of both life and taphonomic histories (e.g., Müller et al., 2003; Bentley, 2006; Trueman et al., 2006; Macfadden et al., 2012; Keenan et al., 2015; Keenan and Engel, 2017; de Winter et al., 2016; McMillan et al., 2017). However, differentiating between in- and ex-vivo chemical and isotopic signals is difficult, complicating life history investigations of archaeological and palaeontological remains.

Vertebrate hard parts each contain various proportions of calcium phosphate biominerals ('bioapatite'), a non-stoichiometric carbonate-bearing calcium apatite; Pasteris et al., 2008; Alexander et al., 2012), organic matter (e.g., proteins and lipids), and water (LeGeros, 1981; Woppenka and Pasteris, 2005; Pasteris et al., 2004, 2008; Keenan and

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Engel, 2017). In bone, bioapatite exists as small crystallites (~2–3 unit cells thick; Pasteris et al., 2008) within an organic matter framework composed primarily of collagen (protein) fibrils (Alexander et al., 2012). In-vivo chemical modifications of the bioapatite crystal lattice typically facilitate ion exchange and regulate acid-base chemistry (Bergstrom and Wallace, 1954; Green and Kleeman, 1991; Rollin-Martinet et al., 2013; Keenan and Engel, 2017). Once a bone is removed from biological control, both microbial (e.g., Pfretzschner, 2004) and abiotic (Leikina et al., 2002; Keenan et al., 2015) processes degrade organic material, increasing the bone’s porosity (Nielsen-Marsh and Hedges, 2000) while simultaneously decreasing the stability of associated bioapatite crystallites (Hedges, 2002; Keenan and Engel, 2017). As porosity increases, surrounding sedimentary pore fluids transport exogenous (ex-vivo) trace elements into the bone (e.g., Kohn,
Fig. 2. Box plots of trace element concentrations in ‘Perio-spots’ collected by LA-ICP-MS for bones from Scladina Cave. Boxes are 1st and 3rd quartiles, separated by the median, and filled green for treated samples and red for untreated samples. Whiskers are min-max with no outliers removed. From youngest to oldest, samples include OSGRO-1 (Complex 1B; left in each column), OSGRO-24 (Unit 3-SUP; middle in each column), and OSGRO-14 (Unit 6A; right in each column), all from Scladina Cave. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2008) that concurrently exchange with endogenous (in-vivo) chemical elements. The timespan when organic matter is degrading and associated bioapatite crystallites are highly unstable is commonly referred to as ‘early diagenesis’.

Exogenous trace elements, including both their radionuclides (e.g., of Sr and Pb) and stable isotopes (e.g., of Fe and Cu), have three possible fates once they have entered a bone during early diagenesis: 1) they adsorb onto the outer surfaces of bioapatite crystallites or onto the remaining organic material, 2) they precipitate into pore spaces as components of diagenetic and exogenous mineral species (‘permineralisation’; e.g., Daniel and Chin, 2010; Pfretzschner and Tüeken, 2011), or 3) they are incorporated into the destabilized bioapatite crystallites during early diagenetic recrystallization (Trueman, 1999; Nielsen-Marsh and Hedges, 2000). The ionic and structural flexibility of the bioapatite crystal lattice, as well as site- and context-specific hydrological, mineralogical, geochemical, and depositional characteristics, result in overprinting of in-vivo lattice-bound chemical and isotopic signatures with diagenetic signals related to the bone’s ex-vivo history (e.g., Berna et al., 2004; Trueman et al., 2004; Trueman et al., 2006; Keenan and Engel, 2017; McMillan et al., 2017). Such early diagenetic recrystallization results in relatively rapid (>1–50 ky; e.g., Trueman et al., 2006; Keenan and Engel, 2017) elemental and isotopic exchange between a bone and its surrounding environment and also modifies the bone’s structural characteristics (e.g., Trueman et al., 2008; McMillan et al., 2017).

In calcined bone burned at temperatures above 650°C, all organic matter is destroyed during the process of cremation and only the bio.

mineral component remains (e.g. Stiner et al., 1995). The proportion of carbonates also decreases, and the structure of bioapatite concurrently becomes more crystalline (Lebon et al., 2010, 2014; Snoeck et al., 2014). Therefore, calcined bone can be reliably used for radiocarbon dating (e.g. Lanting et al., 2001) and Sr isotope analyses (Snoeck et al., 2015). Laboratory controlled contamination experiments showed that, in calcined bone, exogenous strontium is mostly adsorbed onto the bone surface and can be efficiently removed with acetic acid pre-treatment (Snoeck et al., 2015). Still, much remains unknown about the behaviour of trace elements in highly crystalline calcined bone after burial.

To remove diagenetic alteration prior to trace element or isotopic analyses, bones may be chemically or mechanically pre-treated (e.g. Garvie-Lok et al., 2004; Trueman et al., 2006; Snoeck et al., 2015; Snoeck and Pellegrini, 2015). Cremated bones and bones of different ages inherently have different physical and chemical properties that affect their responses to both taphonomic alteration and chemical pre-treatment. Any procedure selected to evaluate the effectiveness of pre-treatment thus depends on the material and chemical or isotopic system of interest. These variables challenge the development of a standardized approach for evaluating the effectiveness of pre-treatment procedures. A number of proxies already exist for specific analytes and associated geochemical systems; however, few, if any, frameworks are currently available for evaluating the effect of diagenesis and pre-treatment procedures on trace elements and their isotopes, and those that exist are limited to a single element and/or alteration type (e.g., Snoeck et al., 2015; Greene et al., 2018).
We characterized the effects of acetate acid chemical pre-treatment for a suite of cations (Mn, Fe, Cu, Sr, La, Ce, Pb, and U) on three bones of different Pleistocene ages and one cremated Early Medieval human bone, all from Belgium. We selected these elements based on their capacity to substitute Ca in bioapatite both in- and ex-vivo (due to bioavailability, ionic radius, and valence) and/or are documented geochemical proxies for life and death histories (e.g., Müller et al., 2003; Bentley, 2006; Trueman et al., 2006; Jouven et al., 2012, 2013; McMillan et al., 2017). Although we do not investigate the full rare earth element (REE) series in this work, we use La and Ce concentrations as proxies for the light REE, which are more readily incorporated in the bioapatite crystal lattice than the heavier REE due to their more similar ionic radii to Ca. We also use variations in the ex-vivo trace element content of bone as tentative proxies for the alteration of biogenic radiogenic and stable isotopic signatures, the covariance of which is, for example, demonstrated for strontium by Budd et al. (2000). In addition, we use the structural characteristics of each bone to investigate the effects of recrystallization and differentiate among lattice-bound and adsorbed trace element characteristics. The main objective of this study is to compare different techniques for identifying diagenetic alteration of the trace element content of bone by developing an analytical framework that allows researchers to simultaneously 1) characterize the impacts of diagenesis within and among bones at high spatial resolution, and 2) evaluate the effectiveness of chemical pre-treatment for bones of different ages and with various states of alteration.

2. Materials: sample types, collection, and context

Bone samples were collected from two settings: 1) a Pleistocene sedimentary sequence in a cave and 2) an Early Medieval archaeological site where the deceased were cremated. These two juxtaposing contexts present some of the greatest differences in the post-mortem alteration of bone. ‘Old’ bone is commonly highly diagenetically altered and can exhibit varying degrees of recrystallization (Trueman et al., 2006; Trueman et al., 2008; McMillan et al., 2017), and the chemical and structural properties of cremated bone are inherently difficult to alter after burning due to the recrystallizing effects of heat treatment (Lebon et al., 2010, 2014; Snoeck et al., 2014). The highly crystalline
anthropogenically altered cremated bone can thus be considered an end member for the lowest potential exogenous trace element concentrations and the highest degree of structural order in archaeological bone, to which we can directly compare the much older bones from Schladina Cave. We chose to analyse bone because of its greater susceptibility to diagenetic alteration when compared to enamel (e.g., Budd et al., 2000), and because it is more common in the archaeological record than dentine.

Samples of ‘old’ (Late Pleistocene) bone were obtained from well-documented sedimentary profiles within Schladina Cave, Belgium. Bone samples from Schladina were chosen based on their sedimentary context and ‘diagenetic facies’ or ‘diagenetic period’ (McMillan et al., 2017). ‘Diagenetic periods’ are groups of sedimentary facies first identified at Schladina Cave that contain bones with similar states of diagenetic alteration. Schladina samples were all cortical bone from indeterminate megafauna. We chose one representative sample from within each ‘diagenetic period’ at Schladina Cave that was shown by McMillan et al. (2017) to have not been intensively reworked by erosional processes and also provided enough material for analysis. Sample OSGRO-1, the youngest bone, was collected from Sedimentary Complex 1B, which is radiocarbon dated to >45–40 ka and was deposited during MIS 4 or 3 (ca. 71–29 ka). Sample OSGRO-24 was collected from Unit 3SUP, which dates to beyond the range of radiocarbon dating and has been situated in MIS 5 or 4 (ca. 130–57 ka). Sample OSGRO-14 from Unit 6A, the oldest analysed bone from Schladina, is also beyond the range of radiocarbon dating and has been placed confidently in MIS 5 (ca. 130–71 ka) (Pirson et al., 2008; McMillan et al., 2017). The human calcined cranial bone fragment was collected from the Early Medieval Cemetery of Broechem, Belgium, where both inhumation and cremation co-occur.

3. Methods

3.1. Sample preparation and acetic acid pre-treatment

Prior to trace element and structural analyses, bone samples were sectioned along their transverse planes with a Buehler Isomet 4000 Linear Precision saw (~2700 RPM blade speed). Half of each bone sample was submerged in 18.2MQ cm water and sonicated for 20 min followed by an additional 20 min of sonication in 1 M acetic acid, which was repeated for two cycles (until the solution remained clear after sonication). Acetic acid was selected for pre-treatment, as it is often used during sample preparation prior to radiocarbon dating, C, O, and Sr isotope analyses, as well as trace element analyses of bone (e.g. Sillen, 1986, 1989; Sealy et al., 1991; Price et al., 1992, 1994; Sillen and Sealy, 1995; Nielsen-Marsh and Hedges, 2000; Lanting et al., 2001; Garvie-Lok et al., 2004; Trueman et al., 2006; Snoeck et al., 2015; Pellegrini and Snoeck, 2016). To quantify the amount of material lost during each step, each bone sample was dry-weighed three times before the chemical pre-treatment, and the leachate and supernatant from each step were dried down overnight at 95 °C and also weighed three times. Both the treated and untreated halves were then embedded in Buehler (Lake Buff, Illinois) Epoxi-Cure resin.

3.2. The ‘Perio-spot’ technique and ‘Perios-ends’ profiles

The ‘Perio-spot’ technique (McMillan et al., 2017) involves conducting in-situ geochemical and structural analyses within close proximity to one another, typically at two or more locations directly adjacent to a bone’s periosteal surface (‘Perio-spots’; Fig. 1), the part of a bone that has the most interaction with sedimentary pore waters ex-vivo. We avoid permineralized and otherwise visibly altered components of each bone using the high spatial resolution of our techniques. For example, we only sampled the intact circumferential lamellae of OSGRO-1 and avoided the internal, more porous, components of the bone that appear to have undergone much carbonate permineralisation. We also avoided the fractures in-filled with dark exogenous minerals when analysing OSGRO-14 (Fig. 1). The extent of the ‘diagenetic front’ is recorded in concentration gradients of ex-vivo trace elements that extend inward from the periosteal surface (e.g., Millard and Hedges, 1996; Henderson et al., 1983; Kohn, 2008; Herrwitz et al., 2011; Kohn and Moses, 2013; Herrwitz et al., 2013; Greene et al., 2018). ‘Perios-ends’ profiles are investigated by extending spot analyses toward the bone interior along a line oriented perpendicular to the periosteal surface (Fig. 1). These profiles include one ‘Perio-spot’ at the periosteal surface (Fig. 1) and allow the direct investigation of concentration profiles of exogenous trace elements incorporated into a bone ex-vivo. Low-concentration diageneric indicators, such as the lanthanides, are typically not present in measurable quantities by ~250 µm away from the periosteal surface of Late Pleistocene bone. Diagenetic indicators at higher concentrations (e.g., Mn, Fe) can extend much farther (>500 µm) into the bone. Both ‘Perio-spots’ and ‘Perios-ends’ profiles allow simultaneous investigation of inter- and intra-bone diagenetic alteration from the most affected region of a bone and with high spatial resolution. ‘Perio-spot’ analyses and ‘Perios-ends’ profiles were conducted on the same ‘mirrored’ locations on both pre-
treated and untreated sides of each sectioned bone sample. A minimum of one ‘Perio-ends’ profile and two ‘Perio-spots’ were investigated on each half of each bone sample, and additional ‘Perio-spot’ and ‘Perio-ends’ analyses were carried out on the untreated halves where possible to investigate intra-bone heterogeneity of diagenetic alteration (Table 1).

### 3.3. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)

Trace element analyses were carried out with LA-ICP-MS after McMillan et al. (2017) at the Pacific Centre for Isotopic and Geochemical Research (PCIGR) at the University of British Columbia (Vancouver, Canada) with a RESolution M-50-LR ArF excimer laser system (193 nm, 20 ns pulse width) coupled to an Agilent 7700x Quadrupole ICP-MS. Bones, standards, and reference materials were ablated with a 5 Hz repetition rate, a fluence of 2.1 J/cm², and spot size of 64 μm. Helium carrier gas was mixed with Ar and trace amounts of N₂ via a smooth-flowing device. Oxides monitored on ThO/Th were consistently below 0.3%. Spots were ablated for 40 s after pre-ablation to remove surface contamination, followed by 30 s washout time. Bone analyses were bracketed by analyses of the synthetic silicate glass NIST SRM612, and we used the USGS bone reference material MAPS-4 and NIST SRM610 as quality controls. Iolite v. 3.0 (Patton et al., 2011) extension for the software Igor Pro was used for data reduction. We used 26Ca as the internal standard with a value of 38.2±0.5% (McMillan et al., 2017). The internal (instrumental) precision for low-concentration trace elements (La, Ce, Pb, U) in ‘Perio-spots’ is typically better than 10% 2σ RE and the limit of detections (LODs) for these elements are all <0.09 ppm. The internal precision is typically better than 6% 2σ RE for trace elements present in higher concentration (Cu, Sr) and the LOD for Cu is ≤0.5 ppm and ≤0.05 ppm for Sr. The external precision (among average
ages of multiple analyses) for all elements of interest in NIST SRM610 is typically better than 10% 2RSD, and the external precision for all elements of interest in the USGS MAPS-4 reference material is typically much better than 15% 2RSD. The concentrations of all elements of interest in NIST SRM610 and USGS MAPS-4 were consistently within 5% and 10% of expected values, respectively. To ensure that bone was accurately targeted, we monitored 43Ca counts and other trace element characteristics for each analysis and compared them to analyses of the surrounding epoxy.

3.4. Micro-X-ray fluorescence (μXRF)

Micro-X-ray fluorescence (μXRF) analyses were carried out at the Analytical, Environmental, and Geo-Chemistry (AMGC) research unit of the Vrije Universiteit Brussel (VUB), Belgium to produce ‘Perio-endos’ profiles. A Bruker M4 Tornado μXRF scanner (Bruker nano GmbH, Berlin, Germany) was used for all μXRF measurements. This instrument operates under vacuum conditions (20 mbar), using a Rh source operating under 50 kV and 600 μA without source filters (Winter and Claey, 2017). A polycapillary lens allows the focusing of X-rays onto a 25 μm spot (Mo-Kα). Returning X-rays were detected using two Silicon Shift Detectors and XRF spectra were processed using Bruker’s Esprit software. Quantitative point-by-point XRF line scanning was carried out by letting the X-ray beam to dwell on each 25 μm point for 60 s to allow the ‘Time of Stable Reproducibility and Accuracy’ to be reached (de Winter et al., 2017a, 2017b). Such XRF line scans were positioned directly next to the LA-ICP-MS spots to permit comparison between the results. Spectra from XRF line scans were quantified using the Fundamental Parameters algorithm calibrated for bioapatite matrix effect by the CCB01 bone standard (Bureau of Analyzed Samples, Middlesbrough, UK) in the Bruker Esprit software (de Winter et al., this volume). The resulting elemental concentration data was checked by Ca/P ratios and Ca counts to separate measurements in the bone from those on the resin.

3.5. Raman spectroscopy

Raman ‘Perio-spot’ analyses were also conducted following the procedures of McMillan et al. (2017) with a Horiba XPlora Plus Raman system housed in the LaserSpot facility at the PCIGR. Spectra were collected through a MPlan N 100×/0.90 objective lens with a 532 nm laser. Our instrumental settings were optimised for obtaining a visible
v1-(PO4) peak. We used a slit of 200 μm, a hole of 300–500 μm, 2400 gr/mm, and collected Raman response for a spectral range of 350–1200 cm−1. Acquisition time was 1.5 s per accumulation, and we averaged 20 accumulations per analysis. The analysis locations are situated outside the LA-ICP-MS ‘blanket’ (the area outside the ablation crater covered with redeposited material) for every ‘Perio-spot’. We calibrated the instrument daily on a SiO2 standard at ~520 cm−1. LabSpec 6 software (Horiba) was used for instrumental calibration and to collect the spectra. Mathematical pre-treatment was performed on the raw data in Origin (OriginLab, Northampton, MA) software. Baseline subtraction of each spectrum was carried out with the Asymmetric Least Squares Smoothing function, and each spectrum was normalised to its standard deviation. To accurately identify and fit the ν1-(PO4) peak at approximately 940–960 cm−1, the mathematically pre-treated spectra were clipped to 800–1100 cm−1, and ν1-(PO4) peaks were identified from Savitsky-Golay (quadratic) smoothed 2nd derivatives of each spectrum with 25-point smoothing windows. The peaks were then fitted with Gaussian curves to acquire values for peak centre at maximum intensity (PCMI) and the full-width at half-maximum (FWHM) of the ν1-(PO4) peak.

3.6. Fourier transform infrared spectroscopy (FTIR) Infrared analyses of the elemental and structural characteristics of bone were carried out on at AMGG-VUB on a Bruker Vertex 70v infrared spectrometer coupled with a Hyperion 3000 infrared microscope, to which a germanium ATR plate was attached. For each sample, 2 or 3 line scans of 7 to 11 points were measured next to the LA-ICP-MS profiles, totaling 32 line scans. Only measurements with sufficient infrared absorbance, showing good contact between the germanium crystal and the bone sample, were included. For each spectrum, several infrared indices were measured. Unfortunately, the use of the germanium crystal prevents the measurement of wavenumbers below 600 cm−1, where the infrared splitting factor, an indicator of crystallinity, is usually measured (Weiner and Bar-Yosef, 1990). Instead, we used the 1060/1075 ratio (Lebon et al., 2010) that also provided information about the crystallinity of the samples. The WAMPI (Water-Amide Phosphate Index; Roche et al., 2010) provides information about the amount of organic matter and structural water present in the bone. It is usually measured by comparing the infrared band at 1650 cm−1 to the phosphate band measured at 605 cm−1, although that band is not available here. Instead, the WAMPI* was calculated by the 1650/1030 ratio using the major phosphate peak at 1030 cm−1. For the same reason, the BPI* was measured instead of the BPI (LeGeros and Legeros, 1984; Sponheimer and Lee-Thorp, 1999) to evaluate the amount of type B carbonates present in the samples before and after pre-treatment. The indices calculated throughout the different line scans of the same sample were reproducible, varied minimally along ‘Perios-ends’ profiles, and were thus averaged prior to interpretation.

4. Results

4.1. Trace element characteristics before and after pre-treatment

4.1.1. LA-ICP-MS ‘Perio-spots’
The trace element concentrations of ‘Perio-spot’ analyses from both pre-treated and untreated bone samples are presented in Table 1. Trace element variations observed between the ‘Perio-spots’ of untreated and treated aliquots of the cremated bone (BR06) were small, not systematic, and likely related to intra-bone heterogeneity. Concentrations of Sr, La, and Ce were similar before and after pre-treatment of the youngest bone from Scladina (OSGRO-1), and concentrations of Cu, Pb, and U increased after pre-treatment (Fig. 2). In the second oldest bone from Scladina (OSGRO-24), concentrations of Cu, Sr, and U were comparable before and after pre-treatment and concentrations of La, Ce, and Pb decreased after pre-treatment. Concentrations of Cu, Sr, Pb, and U were comparable before and after pre-treatment of the oldest bone from Scladina (OSGRO-14), and La and Ce concentrations decreased after pre-treatment.

4.1.2. LA-ICP-MS ‘Perio-ends’ profiles
The two mirrored ~250-micron ‘Perios-ends’ profiles on the cremated bone, BR06, show no systematic change as a result of pre-treatment (Figs. 3 and 4; Table 2). After pre-treatment, the youngest bone from Scladina Cave, OSGRO-1, is characterized by higher or relatively unchanged Cu, Sr, Pb, and U concentrations at the periestal surface, lower concentration of La and Ce at the periestal surface, and much lower concentrations of all elements except for Sr and U farther into the bone. The concentration gradients in the second-oldest bone from Scladina Cave, OSGRO-24, and the oldest bone, OSGRO-14, both show similar trends: the pre-treatment resulted in a decrease of all elemental concentrations along the periestal surface, and the impacts of pre-treatment typically decrease farther into the bone (except U in OSGRO-24). In general, the effectiveness of pre-treatment at removing exogenous trace elements from bone correlates with the age of the samples from Scladina Cave.

4.1.3. μXRF ‘Perios-ends’ profiles
The pre-treatment procedure systematically modified the Sr/Ca and Pb/Ca of all ‘Perios-ends’ profiles measured by μXRF (Supplemental Information; Fig. 5). The Sr/Ca and Pb/Ca near the periestal surface of the two younger bones from Scladina Cave, OSGRO-1 and OSGRO-24, decreased as a result of pre-treatment. Both the cremated bone (BR06) and the oldest bone from Scladina Cave (OSGRO-14) have consistently higher Sr/Ca and Pb/Ca along the periestal surface after pre-treatment. The Mn/Ca and Fe/Ca of ‘Perios-ends’ profiles were also modified by the pre-treatment procedure in all bones (Fig. 5). The pre-treatment procedure resulted in a higher Mn/Ca and Fe/Ca near the periestal surface of the oldest bone from Scladina Cave (OSGRO-14) and lower Mn/Ca and Fe/Ca in the youngest bone from Scladina (OSGRO-1). Both the second oldest bone from Scladina Cave (OSGRO-24) and the cremated bone (BR06) showed no systematic change in Fe/Ca, and Mn/Ca in OSGRO-24 decreased as a result of pre-treatment.
4.2. Structural characteristics pre- and post-treatment

4.2.1. Raman crystallinity of ‘Perio-spots’

The Raman characteristics of all ‘Perio-spots’ are presented in Table 1 and Fig. 6. The Raman PMI of the $v_1'(PO_4)$ peak for all samples range between 942.4 and 959.9 cm$^{-1}$ and the FWHM of the $v_1'(PO_4)$ peak for all samples range from 9.0 to 25.0 cm$^{-1}$. The Raman characteristics of bones of different ages from Scladina Cave all responded differently to pre-treatment, and the cremated bone (BR06) was only minimally affected (Figs. 6 and 7). On average, the $v_1'(PO_4)$ FWHM of OSGRO-1 did not change, but the $v_1'(PO_4)$ PMI increased; both the $v_1'(PO_4)$ FWHM and the $v_1'(PO_4)$ PMI of OSGRO-24, the second oldest bone from Scladina Cave, increased; and the $v_1'(PO_4)$ PMI of OSGRO-14, the oldest bone from Scladina Cave, decreased and the $v_1'(PO_4)$ FWHM increased.

4.2.2. Fourier transform infrared spectroscopy (FTIR)

There was no systematic variation in BPI* or WAMI* observed among the FTIR analyses situated along ‘Perios-endos’ profiles on the same bone. Averages of the analyses from each bone show that BPI* and WAMI* are inversely correlated and vary systematically with age prior to pre-treatment (Table 4). The cremated bone (BR06) did not contain any measurable organic matter nor water at any point, and, as a result of pre-treatment, the BPI* of BR06 remained within uncertainty (Fig. 8). Pre-treatment of the youngest bone from Scladina Cave (OSGRO-1) caused a decrease in both WAMI* and BPI*. Pre-treatment of the second oldest bone from Scladina Cave (OSGRO-24) resulted in an increase in WAMI* and BPI*. The WAMI* of the oldest bone from Scladina Cave (OSGRO-14) did not change and the BPI* decreased as a result of pre-treatment.

4.3. Pre-treatment leachate weights

The measured weights for the leachates from ‘old’ bones produced during each pre-treatment step are provided in Table 5 and plotted down Scladina Cave stratigraphy in Fig. 9. The cremated bone (BR06) lost no measurable amount of mass during all stages of the chemical pre-treatment and thus is not included in Table 5 or Fig. 9. On average, the youngest bone from Scladina Cave (OSGRO-1) lost the least mass (~5%) as a result of chemical pre-treatment, whereas the two older bones, OSGRO-24 and OSGRO-14, responded similarly to each other in
terms of total mass loss (~12%) but to a greater extent than OSGRO-1. The two youngest bones from Scladina Cave, OSGRO-1 and OSGRO-24, each lost mass during both the water and the acid steps; the oldest Scladina bone lost no mass during the water step and only lost mass during the acid step.

5. Discussion

5.1. Characterizing diagenetic alteration of the trace element content and structural characteristics of bone at high spatial resolution

As cation exchange occurs in calcium phosphates, Lanfranco et al. (2003) have demonstrated that the Raman ν₁-(PO₄)³⁻ peak location (quantified as PCMI) decreases during the substitution of Ca with both Sr and Pb. They also suggest that the width of the ν₁-(PO₄)³⁻ peak (quantified as FWHM) is influenced by cation substitution and increases during the substitution of Ca with up to ~90% Sr and ~50% Pb. Similar trends have been documented by Thomas et al. (2011), who showed increasing ν₁-(PO₄)³⁻ FWHM and decreasing ν₁-(PO₄)³⁻ PCMI with replacement of Ca²⁺ with Sr²⁺ in synthetic apatite, and they were able to readily differentiate among synthetic, geological, and biogenic apatite with Raman spectroscopy alone. Although the ν₁-(PO₄)³⁻ FWHM of bioapatite can also increase as a result of short-range disorder due to the lack of large crystallites and the presence of organic matter (Wopenka and Pasteris, 2005), as well as by increased CO₃²⁻ concentrations (Thomas et al., 2011), the combination of lower peak location (ν₁-(PO₄)³⁻) PCMI, greater peak width (ν₁-(PO₄)³⁻ FWHM), and higher trace element concentrations or ratios to Ca provides valuable evidence for diagenetic cation replacement. Using these empirical observations, we combine structural analyses by Raman spectroscopy and FTIR as well as in-situ trace element analyses by μXRF and LA-ICP-MS to investigate the diagenetic states of each bone analyzed in this study and, importantly, attempt to differentiate between trace element adsorption and diagenetic cation replacement in each specimen.

The low concentrations of exogenous trace elements (<5 ppm of La, Ce, Pb, and U; Table 1) and the minimal intra-bone variation among ‘Perio-spots’ and ‘Peri-ends’ profiles in the cremated bone (BR06) prior to pre-treatment show that little to no diagenetic alteration occurred after burning. Only slightly increased concentrations of La, Ce, Pb, and U are present near the periosteal surface compared to the bone interior in the untreated halves (Table 2). This concentration gradient may relate to the minimal diagenetic alteration potentially induced during post-burial contamination of the bone surface from surrounding sediments or contamination from the surrounding epoxym. The Raman ν₁-(PO₄)³⁻ PCMI of BR06 are similar to those of unaltered bone (Lanfranco et al., 2003; Thomas et al., 2011), showing that any exogenous trace elements in the cremated bone were likely adsorbed and not incorporated into the bioapatite crystallites. Additionally, increased concentrations of exogenous trace elements are limited to well within
250 μm from the periosteal surface and thus would likely not greatly influence life history analyses.

Prior to pre-treatment, the ‘old’ bones from Scladina Cave all exhibit higher average concentrations of ex-vivo trace elements along their periosteal surface than the cremated bone (Table 1). The youngest Scladina Cave bone (OSGRO-1) has much more variance in trace element concentrations among ‘Perio-spots’ than the two older bones (OSGRO-24 and OSGRO-14). For example, the concentration of La in OSGRO-1 ranges from 0.863 to 14.24 ppm, whereas the concentration of La ranges from 1.91 to 3.54 ppm in OSGRO-24 and from 18.34 to 23.8 ppm in OSGRO-14 (Table 1). OSGRO-1 also exhibits a wide range of Raman ν₁-(PO₄)²⁻ PMI, no correlation between Raman ν₁-(PO₄)²⁻ PMI and La/Ca, and localised correlation of Sr/Ca and Pb/Ca with Raman ν₁-(PO₄)²⁻ PMI (Fig. 10). The Raman ν₁-(PO₄)²⁻ FWHM is also locally correlated with increasing Sr/Ca and Pb/Ca and exhibits a large variance, indicating that a range of concurrent processes was affecting OSGRO-1 at the time of discovery, some of which increase short-range disorder, and some of which likely relate to the recrystallization of bioapatite. These observations suggest that, at the time of discovery, OSGRO-1 was likely undergoing numerous early diagenetic processes (e.g., loss of organic matter, permineralization, recrystallization) and was thus more diagenetically reactive than the two older bones from

Fig. 10. The Raman ν₁-(PO₄)²⁻ Peak Centre at Maximum Intensity (PMI; left column) and the ν₁-(PO₄)²⁻ Full Width at Half Maximum (FWHM; right column) plotted against Sr/Ca, La/Ca, and Pb/Ca collected by LA-ICP-MS for each ‘Perio-spot’ analysis from the untreated halves of each bone analyzed in this study. Linear regressions among ‘Perio-spot’ analyses from OSGRO-24 and -14 are represented by solid lines that are the same color as the marker fills, and the outlying ‘Perio-spot’ analysis from OSGRO-14 is identified in each pane.
Table 3
Treated/untreated trace element concentrations for each ‘Perios-endos’ profile collected with LA-ICP-MS. Bolded values indicate the first spot in the profile, or ‘Perio-spot’, and * or ** indicate profiles in mirrored locations.

<table>
<thead>
<tr>
<th>Treated/Untreated</th>
<th>Cu</th>
<th>Sr</th>
<th>La</th>
<th>Ce</th>
<th>Pb</th>
<th>U</th>
<th>Distance From Perioseal Surface (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSGRO-1</td>
<td>2.3</td>
<td>1.06</td>
<td>0.81</td>
<td>0.943</td>
<td>1.5</td>
<td>2.3</td>
<td>0-64</td>
</tr>
<tr>
<td>OSGRO-1</td>
<td>0.79</td>
<td>1.02</td>
<td>0.03</td>
<td>0.084</td>
<td>0.31</td>
<td>1.1</td>
<td>64-128</td>
</tr>
<tr>
<td>OSGRO-1</td>
<td>0.42</td>
<td>1.07</td>
<td>0.007</td>
<td>0.02</td>
<td>0.090</td>
<td>0.71</td>
<td>128-192</td>
</tr>
<tr>
<td>OSGRO-1</td>
<td>0.70</td>
<td>1.09</td>
<td>0.1</td>
<td>0.57</td>
<td>0.3</td>
<td>1.1</td>
<td>192-256</td>
</tr>
<tr>
<td>OSGRO-24</td>
<td>0.80</td>
<td>0.908</td>
<td>0.25</td>
<td>0.2</td>
<td>0.38</td>
<td>1.0</td>
<td>0-64</td>
</tr>
<tr>
<td>OSGRO-24</td>
<td>0.92</td>
<td>1.00</td>
<td>1.0</td>
<td>0.5</td>
<td>0.54</td>
<td>0.9</td>
<td>64-128</td>
</tr>
<tr>
<td>OSGRO-24</td>
<td>1.1</td>
<td>0.955</td>
<td>0.8</td>
<td>0.5</td>
<td>0.33</td>
<td>0.89</td>
<td>128-192</td>
</tr>
<tr>
<td>OSGRO-14</td>
<td>0.80</td>
<td>0.935</td>
<td>0.34</td>
<td>0.14</td>
<td>0.6</td>
<td>0.82</td>
<td>0-64</td>
</tr>
<tr>
<td>OSGRO-14</td>
<td>1.2</td>
<td>0.956</td>
<td>0.48</td>
<td>0.7</td>
<td>1.9</td>
<td>1</td>
<td>64-128</td>
</tr>
<tr>
<td>OSGRO-14</td>
<td>0.91</td>
<td>1.00</td>
<td>0.6</td>
<td>1.2</td>
<td>2.0</td>
<td>1.2</td>
<td>128-192</td>
</tr>
<tr>
<td>OSGRO-14</td>
<td>0.63</td>
<td>1.02</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>1.0</td>
<td>192-256</td>
</tr>
<tr>
<td>BR06**</td>
<td>0.99</td>
<td>0.869</td>
<td>2.18</td>
<td>1.8</td>
<td>0.79</td>
<td>0.8</td>
<td>0-64</td>
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<tr>
<td>BR06**</td>
<td>0.88</td>
<td>1.11</td>
<td>1</td>
<td>1.1</td>
<td>1.1</td>
<td>3</td>
<td>64-128</td>
</tr>
<tr>
<td>BR06**</td>
<td>0.91</td>
<td>0.883</td>
<td>0.75</td>
<td>0.84</td>
<td>1.3</td>
<td>0.8</td>
<td>128-192</td>
</tr>
<tr>
<td>BR06**</td>
<td>0.79</td>
<td>0.703</td>
<td>0.9</td>
<td>0.85</td>
<td>0.71</td>
<td>0.8</td>
<td>192-256</td>
</tr>
<tr>
<td>BR06*</td>
<td>1.0</td>
<td>1.20</td>
<td>0.91</td>
<td>0.77</td>
<td>0.53</td>
<td>1.0</td>
<td>0-64</td>
</tr>
<tr>
<td>BR06*</td>
<td>0.85</td>
<td>0.938</td>
<td>0.94</td>
<td>0.8</td>
<td>0.26</td>
<td>0.6</td>
<td>64-128</td>
</tr>
<tr>
<td>BR06*</td>
<td>1.2</td>
<td>1.07</td>
<td>0.99</td>
<td>0.85</td>
<td>0.63</td>
<td>1</td>
<td>128-192</td>
</tr>
<tr>
<td>BR06*</td>
<td>1.0</td>
<td>1.03</td>
<td>1</td>
<td>1</td>
<td>0.56</td>
<td>0.3</td>
<td>192-256</td>
</tr>
</tbody>
</table>

Table 4
FTIR results averaged per bone. Errors are 1SD external precision of the bone averages.

<table>
<thead>
<tr>
<th>Sample</th>
<th>State</th>
<th>BPI* ± 0.01</th>
<th>WAMPI* ± 0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR06</td>
<td>Treated</td>
<td>0.05 ± 0.02</td>
<td>0</td>
</tr>
<tr>
<td>BR06</td>
<td>Untreated</td>
<td>0.04 ± 0.01</td>
<td>0</td>
</tr>
<tr>
<td>OSGRO-1</td>
<td>Treated</td>
<td>0.14 ± 0.01</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>OSGRO-1</td>
<td>Untreated</td>
<td>0.17 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>OSGRO-24</td>
<td>Treated</td>
<td>0.17 ± 0.01</td>
<td>0.1 ± 0.01</td>
</tr>
<tr>
<td>OSGRO-24</td>
<td>Untreated</td>
<td>0.16 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>OSGRO-14</td>
<td>Treated</td>
<td>0.12 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>OSGRO-14</td>
<td>Untreated</td>
<td>0.13 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
</tbody>
</table>

The oldest bone from Scaldina Cave (OSGRO-14) typically has the highest concentrations of exogenous trace elements among the three ‘old’ bones analyzed in this study and exhibits a variance similar to OSGRO-24 in ‘Perio-spot’ trace element concentrations in the untreated aliquots. The FTIR results show that organic matter could still be present in the bone, and, with the exception of one ‘Perio-spot’, the Raman ν1-(PO4)3⁻ FWHM consistently decreases with increasing Sr/Ca (R² = 0.996, with the outlier removed), La/Ca (R² = 0.982, with the outlier removed), and Pb/Ca (R² = 0.497, with the outlier removed) in the untreated aliquot. The Raman ν1-(PO4)3⁻ FWHM also increases with increasing Sr/Ca (R² = 0.949, with the outlier removed), La/Ca (R² = 0.975, with the outlier removed), and less so for Pb/Ca (R² = 0.223, with the outlier removed). Without the removal of the uncorrelated ‘Perio-spot’, the R² values are typically 3–5 orders of magnitude lower among all variables. Additionally, the Raman characteristics for ‘Perio-spots’ from OSGRO-14 typically have greater absolute ν1-(PO4)3⁻ FWHM values than OSGRO-24, which is consistent with the replacement of Ca by Sr and Pb in calcium phosphates (Lanfranco et al., 2003; Thomas et al., 2011). Due to the high concentrations of trace elements and the strong evidence for diagenetic cation replacement of Ca with ex-vivo trace elements such as La, we propose that OSGRO-14 was the least diagenetically reactive at the time of discovery and has potentially entered stages of later diagenesis (i.e., it had already reached a relatively diagenetically stable state by the time of discovery).

Table 5
Measured weights of untreated bones and leachates from each pre-treatment step. All weights are reported in grams. External precision on all measurements is better than 0.002 g, 2SD.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Context</th>
<th>Untreated Dry Weight (g)</th>
<th>H2O Leachate Weight</th>
<th>H2O Leachate %</th>
<th>Acid Leachate Weight</th>
<th>Acid Leachate %</th>
<th>Total Leachate Weight (g)</th>
<th>Total Leachate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSGRO-1</td>
<td>Complex</td>
<td>0.7193</td>
<td>0.0047</td>
<td>0.6534</td>
<td>0.0306</td>
<td>4.2541</td>
<td>0.0353</td>
<td>4.9075</td>
</tr>
<tr>
<td>OSGRO-24</td>
<td>Unit</td>
<td>0.3267</td>
<td>0.0056</td>
<td>1.7141</td>
<td>0.0322</td>
<td>9.8561</td>
<td>0.0378</td>
<td>11.5702</td>
</tr>
<tr>
<td>OSGRO-14</td>
<td>Unit 6a</td>
<td>0.2273</td>
<td>0</td>
<td>0.0000</td>
<td>0.0283</td>
<td>12.4505</td>
<td>0.0283</td>
<td>12.4505</td>
</tr>
</tbody>
</table>
5.2. Impact of acetic acid pre-treatment on the trace element and structural characteristics of cremated and ‘old’ bones

The pre-treatment procedure had minimal impact on the chemical composition and structure of the cremated bone (BR06). This demonstrates the resilience of such bones to diagenetic alteration and supports their value as analytes for life history investigations (e.g., Snoek et al., 2016), assuming that they were not intensively ‘contaminated’ during the cremation process. Albeit minimally, the pre-treatment of BR06 typically increased the concentrations of Sr, La, Ce, and Pd along the periosteal surface (profile BR06*; Figs. 3 and 5). Although contamination resulting from the pre-treatment could explain these observations, increases in the concentrations of Sr and Pb as well as Sr/Ca and Pb/Ca after pre-treatment are more likely caused by the dissolution and removal of more soluble and reactive bioapatite and/or calcium carbonate and thus a decrease in Ca concentrations (as shown by the µXRF results), rather than the addition of exogenous trace elements. This was not visible in the Raman \( V_1(PO_4^-) \) PCMI and \( V_1(PO_4^-) \) FWHM of ‘Peri-os-spots’ likely due to the very small area within which the dissolution of bioapatite occurred adjacent to the periosteal surface. As a result, we consider the minimal impact of pre-treatment and potential removal of more reactive bioapatite (and/or calcium carbonate) from cremated bone as beneficial to investigations of in-vivo Sr and Pb isotopic signatures.

Among the Pleistocene bones from Scaldna Cave, the impact of the pre-treatment procedure correlates with their age and hypothetical degree of diagenetic alteration. The trace element content was modified most in the youngest, most diagenetically reactive bone (OSGRO-1), for which the pre-treatment procedure was most effective at removing trace elements from the bone interior (i.e., > 100 μm into the bone from the periosteal surface; Figs. 3 and 4). After pre-treatment, OSGRO-1 appears to still be ‘contaminated’ with higher or unchanged concentrations of Ca, Sr, La, Pb, and U adjacent to the periosteal surface (Table 3; Figs. 3 and 4). Both older bones from Scaldna Cave (OSGRO-24 and OSGRO-14) typically exhibited lower concentrations of exogenous trace elements (such as La and Ce) in the pre-treated halves compared to the untreated halves, although the diagenetic concentration gradients in ‘Perios-ends’ profiles were still present after pre-treatment, and the impacts were overall less significant on OSGRO-14 than OSGRO-24 (e.g., Fig. 4). The increase in Sr/Ca and Pb/Ca along the outer bone surface after pre-treatment identified in BR06 was also observed for the oldest bone from Scaldna Cave (OSGRO-14; Fig. 5).

For the youngest bone from Scaldna Cave (OSGRO-1), we suspect that the increased or unchanged trace element concentrations at the periosteal surface and the marked decrease of concentrations farther into the bone after pre-treatment are related to two possible mechanisms: 1) the inability of the procedure to remove Fe—Mn periosteal surface coatings or 2) the redistribution of trace elements initially removed from farther within the bone. For the oldest bone (OSGRO-14), we suggest that the increased relative concentrations along the periosteal surface are a result of the preferential removal of less stable bioapatite and the resilience to pre-treatment of the recrystallized biominal component that has likely undergone ex-vivo cation replacement. This indicates that acetic acid chemical pre-treatment may increase the relative abundance of exogenous trace elements in old, diagenetically altered and recrystallized bone.

Small changes in the FTIR BPI* suggest that little or no carbonates or bioapatite was removed from the cremated bone (BR06) during pre-treatment, and no organic matter was present in either the treated or untreated halves (Fig. 8). The youngest Scaldna Cave bone, OSGRO-1, showed a decrease in BPI* related to the removal of more carbonates than bioapatite during pre-treatment, as well as a decrease in the organic matter and water content. The organic matter and water content of OSGRO-24, the second-oldest bone from Scaldna Cave, increased dramatically, likely as a result of the removal of sediments from pore spaces as well as carbonates (either primary or secondary) and bioapatite. This suggests that the relative amount of organic matter and water increased drastically in OSGRO-24. The organic matter content and BPI* of the oldest bone from Scaldna, OSGRO-14, did not significantly change as a result of pre-treatment.

The Raman results provide a key piece of evidence to support the potential dissolution of more soluble bioapatite as a result of the pre-treatment of old bone. As with the FTIR, the Raman results indicate that the cleaning procedure had little to no impact on BR06. The \( V_1(PO_4^-) \) PCMI of OSGRO-1 and of OSGRO-24 typically increased to slightly below typical calcium phosphate values (Lanfranco et al., 2003; Thomas et al., 2011) as a result of pre-treatment (Figs. 6 and 7), suggesting that altered bioapatite and adsorbed trace elements were preferentially removed during pre-treatment (i.e., the pre-treatment was ‘successful’). Although the average \( V_1(PO_4^-) \) FWHM of OSGRO-24 increased during pre-treatment, this is likely a result of a relative increase in the abundance of amorphous organic matter and the exposure of very small bioapatite crystallites (increasing short-range disorder; Wopenka and Pasteris, 2005) and not an increase in the abundance of diagenetically altered apatite crystallites, which is supported by the FTIR results. However, the \( V_1(PO_4^-) \) PCMI of the oldest Scaldna bone (OSGRO-14) decreased as a result of pre-treatment and the \( V_1(PO_4^-) \) FWHM greatly increased as a result of pre-treatment (Figs. 6, 7, and 10); both characteristics indicate a relative increase in the abundance of altered, recrystallized bioapatite. Considering that there was no observed change in organic matter content between the treated and untreated aliquots of OSGRO-14 by FTIR, this suggests that unstable, more soluble, and less altered bioapatite crystallites may have been preferentially removed from OSGRO-14 as opposed to the more stable, less soluble recrystallized diagenetic apatite crystallites that have undergone cation exchange (i.e., the pre-treatment was not ‘successful’).

These outcomes support the hypothesis that elements which become lattice-bound during diagenesis (e.g., strontium; Budd et al., 2002) cannot be removed by chemical pre-treatment without completely dissolving bioapatite crystallites (e.g., Bentley, 2006 and references therein). Once dissolved, differentiating between in- and ex-vivo geochemical signatures in solution would also pose a major, perhaps greater, challenge to researchers than doing so in situ. Evaluating the effectiveness of pre-treatment by comparing the trace element and structural characteristics of pre-treated and untreated aliquots of the same bone in situ can be used to investigate how well adsorbed and permineralized exogenous elements are removed as well as the potential effects of recrystallization. Based on our analyses, very reactive bones still undergoing early diagenetic processes appear to respond positively to chemical pre-treatment with acetic acid, and, for less reactive older bones, avoiding diagenetically altered areas could be accomplished by mapping diagenetic indicators in situ and microsampling potentially unaltered regions.

5.3. Identifying the diagenetic state of bones via chemical leaching

With the stratigraphic, temporal, and taphonomic control afforded by the Scaldna Cave collections, we have identified another potentially valuable proxy for evaluating the diagenetic state of archaeological and palaeontological bones. It follows a similar logic as the ‘solubility profiles’ described by Sillen (1986, 1989) but notably without the requirement of elemental analysis. The two youngest and most reactive bones analyzed from Scaldna, OSGRO-1 and OSGRO-24, lost significant mass during both the water and acid pre-treatment steps, whereas the bone from the Later Diagenesis period (OSGRO-14) did not; rather, it lost all of its leached mass in the subsequent acid step. The total amount of material lost during each leaching step correlates with the diagenetic
state of the bone, with more material removed from older, more altered bones than from younger bones (Fig. 9; Table 5). This suggests that the water step primarily removes sediments incorporated into pore spaces and degraded organic matter, and thus it is most effective on bones undergoing early diagenesis that are more porous than recrystallized/diagenetically altered bone. The acid step likely removes permineralized carbonates and other inorganic diagenetic impacts, as well as more reactive bioapatite crystallites. Although the viability of this approach needs to be much more thoroughly investigated with a larger sample set, our results indicate that researchers may be able to tentatively identify the diagenetic state of a bone, and thus estimate the success of chemical pre-treatment, by measuring the fraction of the sample that is removed in each step of the procedure. If corroborated by future analyses, and ideally when combined with observations of the evolution of trace element and structural characteristics before and after pre-treatment, this approach may prove to be extremely useful for identifying the likelihood of collecting in-vivo signatures from a bone sample.

5.4. Comparison of LA-ICP-MS, μXRF, Raman spectroscopy, and FTIR for evaluating the diagenetic alteration of bone

5.4.1. Trace element analysis by LA-ICP-MS and μXRF

We analyzed the trace element content of bones with two complementary techniques, LA-ICP-MS and μXRF. Both techniques have unique benefits for identifying diagenetic alteration. LA-ICP-MS has lower detection limits than μXRF, making this technique better suited for measuring REE and other low-concentration elements at high spatial resolution. The low concentration trace elements measurements with μXRF are also less precise than LA-ICP-MS, but LA-ICP-MS is minimally destructive to the bone surface. μXRF has several additional advantages: it permits more accurate measurements of diagenetic indicators that are more challenging to measure with ICP-MS due to technical issues such as polyatomic interferences (e.g., Mn, Fe), can be used to quantify Ca concentrations (LA-ICP-MS of bone typically requires the use of Ca as an internal standard), is completely non-destructive to the bone surface, and can map the elemental distribution within a sample at high spatial resolution more efficiently and less destructively than LA-ICP-MS.

5.4.2. Structural analysis by Raman spectroscopy and FTIR

One of the major outcomes of this study is the identification of the possible preferential removal of unaltered bioapatite during the pre-treatment of OSGRO-14, our interpretations of which were made more confident due to the combination of Raman spectroscopy and FTIR. The applicability of Raman spectroscopy to identifying cation replacement in calcium phosphates is somewhat limited if organic matter content is not also measured by FTIR. The \( \nu_1(PO_4) \) peak broadening observed in Raman spectra during cation replacement can also occur due to increases in short-range disorder related to the exposure of very small unaltered bioapatite crystallites, which are only stable when surrounded by organic matter. For example, if the Raman \( \nu_1(PO_4) \) peak broadens as a result of pre-treatment and the organic matter content does not change (as in the case of OSGRO-14), then changes in the abundance of altered biomineral components are much more likely than an increase in short-range disorder due to the degradation of organic matter that surrounds the bioapatite crystallites. Combining two independent lines of evidence to support the same conclusions also greatly improves the confidence we have in our interpretations of the impacts of pre-treatment on bones of different ages or those that have been treated differently ex-vivo.

5.5. Future work

Some notable directions of future work exist that would further quantify the impact of chemical pre-treatment on the trace element characteristics of both diagenetically and anthropogenically altered bone. Measuring the trace element content of leachates removed from each bone similar to Sillen (1986, 1989) would aid in confirming the impacts of pre-treatment we have identified in situ, and also would facilitate identifying which pre-treatment step removed most of the exogenous trace element content. The correlation of removed leachate weights and the diagenetic state of bones should also be characterized in greater depth with a much larger sample size. More samples should be characterized before and after chemical pre-treatment both from Scladina Cave and Broechem, as well as from different archaeological and palaeontological contexts to strengthen the interpretations of the trends we have identified. Additionally, evaluating the modifications to the Sr, and potentially Pb, isotopic composition with LA-(MC/HR)-ICP-MS in situ before and after chemical pre-treatment with the ‘Perio-spot’ technique and ‘Perio-end’ profiles would quantify the relationship among the differences we observe in trace element characteristics and changes in radiogenic and stable isotope composition.

6. Conclusions

We characterized both the diagenetic alteration and the impact of acetic acid pre-treatment on the trace element and structural characteristics of 1) cremated bone and 2) ‘old’ bones of different Pleistocene ages that were not cremated. We found that the cremated bone exhibits little to no diagenetic alteration with regard to Cu, Sr, La, Ce, Pb, and U, and the diagenetic alteration of ‘old’ bones from Scladina Cave increases with increasing age. Although the trace element concentrations and their ratios to Ca in the cremated bone increased slightly after pre-treatment, it is most likely due to the preferential removal of more soluble and reactive bioapatite or calcium carbonate. The youngest and most reactive bone from Scladina Cave showed the most dramatic change in trace element content as a result of pre-treatment, although the trace elements removed from the bone interior may have been redistributed along the periostral surface. The second oldest bone from Scladina responded relatively well to pre-treatment, with a reduction of exogenous trace element concentrations and structural changes that indicate a greater potential to obtain in-vivo signatures after pre-treatment. The trace element content of the oldest bone from Scladina Cave responded similarly to the second oldest bone, although the observed structural changes and Sr/Ca and Pb/Ca show that the pre-treatment process may have preferentially removed less stable, more soluble, and less altered bioapatite, increasing the potential for contamination of in-vivo signatures during life history analyses. The effectiveness of chemical pre-treatment for removing exogenous trace elements from diagenetically altered bone thus varies greatly depending on their diagenetic state and if they have been cremated prior to burial. We also observed large differences in the leaching characteristics of each bone that strongly correlate with their hypothetical diagenetic states. Comparing the amount of material removed by each pre-treatment step by weighing dried-down leachates may therefore be a valuable approach to estimating the diagenetic state of a bone and the success of pre-treatment procedures. Before conducting life history investigations, we suggest that researchers pre-treat cremated bone to remove sediments and calcium carbonates and characterize the diagenetic state of ‘old’ bones by measuring their trace element and structural characteristics, as well as the amount of bone material leached during chemical pre-treatment, to evaluate the probability of obtaining in-vivo signatures.