SURFACE MICROANALYSIS OF STAINING ON VERTEBRATE FOSSIL BONE FROM THE SANTA FE RIVER, FLORIDA

A Thesis in Geosciences

by Kimberly Foecke

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ABSTRACT

Mammalian fossil bones excavated from the bed of the Santa Fe River in northern Florida exhibit dark black surface stains. Although such stains typically are attributed to secondary mineralization of manganese oxides, our microanalysis revealed little evidence for manganese. Instead, energy dispersive spectroscopy revealed that the surface stains consist of ~1 mm-thick bands of Fe oxide and sulfide. Wavelength dispersive spectroscopy and electron back-scattered diffraction identified the Fe sulfide as pyrite ($\text{FeS}_2$), but the size and concentration of the crystallites of Fe oxide were too small for a definitive mineralogical determination. Fourier transform infrared spectroscopy (FTIR) offered evidence for ferric tannates within the black stains. We hypothesize that an initial stain of iron oxide provided a substrate for the subsequent formation of a ferric tannate complex, which is responsible for the black coloration. In some instances, sulfidation of iron oxide in the reducing micro-environment of interior pores promoted pyrite formation. These results conflict with the presumption that black surface stains on archaeological materials are necessarily attributable to Mn oxides. Further, in light of the instability of pyrite, stains on fossils may pose risks to collections if not properly identified, treated, and curated.
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DEDICATION

Dedicated to Ted, whose unwavering support, love and encouragement has been more vital than he could ever know.
Chapter 1

Background and Project Scope


In some instances, the presumption has proven correct. For example, during human origins research on fossil hominid remains from cave sites in South Africa and Italy, anthropologists have made a concerted effort to remove the black coatings from South African fossils and analyze the mineralogy (Cukrowska et al. 2005, Kuczumow et al. 2010, López-González et al. 2006, Thackeray et al. 2005). In these cases, it was confirmed that the coatings contained manganese. Two other published cases are noteworthy. One case was a study of Pliocene mammals from Argentina, in which manganese oxide was shown to be highly correlated with black staining on bone (Tomassini et al. 2014). The other is a recent study of a Middle Paleolithic faunal assemblage from the Levant, where analysis revealed the presence of insoluble
manganese oxides (Marín-Arroyo et al. 2014). Studies such as these have offered apparent support for the prevalence of manganese oxide stains on fossil bone.

**Project Goals**

We set out to test the assumption that black staining on fossil bone arises from manganese oxide crusts and, if correct, to determine the most common manganese oxide phases within the coatings. A preliminary compositional characterization was performed on a suite of specimens from the Santa Fe River at Ginnie Springs, Florida. However, analyses revealed nearly no manganese in the stains on these specimens. That then led to the following new objectives:

1. Identify the **chemistry and mineralogy** of the fossil bone stains *in situ* using a variety of microanalysis techniques
2. Explore possible **reaction mechanisms** for the origin of the staining
3. Identify short- and long-term **risk to the preservation of specimens** from the staining, and recommend curation practices for this collection

It is known that the formation of certain substances on fossils can result in their degradation. If the staining has the potential to damage specimens, a modified curation regime must be implemented. Additionally, the results have potential taphonomic significance to researchers – staining on bones may indicate re-deposition of specimens from a previous environment or reveal cycles of burial and exposure. Also, an unknown and unstudied compound affecting fossils can cause unforeseen interference with chemical studies unrelated to taphonomic alteration. It is important to take these alterations into account when undertaking research on a fossil collection, and thus the alterations must be documented and understood.

A characterization study was conducted on riverbed samples from Ginnie Springs, FL utilizing multiple analytical techniques, and these results were synthesized with water chemistry data from the United States Geological Survey (USGS).
Chapter 2

Methods

Santa Fe River Samples

The samples for this study come from the bottom of the Santa Fe River near a site called Ginnie Springs, just north of Gainesville, Florida (Fig. 1). At this locality, both fossil bone and rocks are found with black coatings layered upon red coatings (Fig. 2). Most bones at the site derive from Pleistocene vertebrates. Interestingly, modern bone (domesticated pig) was also recovered and found to have the same coating. The specimens in this study were collected over several years by the science diving program operated by Dr. Timothy White through a cross-departmental course within The Pennsylvania State University. Many of the specimens recovered from this site have limited paleontological value, as the displacement from the original points of burial by the Santa Fe River has compromised any provenance, and age information is inaccessible due to extreme collagen degradation. Sample identifications are shown in Table 1. A complete photographic catalogue of samples analyzed can be found in Appendix A.
<table>
<thead>
<tr>
<th>EMS CATALOGUE NUMBER</th>
<th>SAMPLE DESIGNATION</th>
<th>SAMPLE SPECIES</th>
<th>SAMPLE BONE TYPE</th>
<th>SAMPLE LOCALITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMS 425064</td>
<td>1</td>
<td><em>Equus</em> (Horse)</td>
<td>Left calcaneus</td>
<td>Graveyard, Ginnie Springs, Florida</td>
</tr>
<tr>
<td>EMS 425065</td>
<td>2</td>
<td><em>Equus</em> (Horse)</td>
<td>Astragalus</td>
<td>Graveyard, Ginnie Springs, Alachua Co., Florida</td>
</tr>
<tr>
<td>EMS 425066</td>
<td>3</td>
<td><em>Mammathus</em> (Mammoth)</td>
<td>Tooth</td>
<td>Graveyard, Ginnie Springs, Santa Fe River, Florida</td>
</tr>
<tr>
<td>EMS 425067</td>
<td>4</td>
<td><em>Equus</em> (Horse)</td>
<td>Tooth</td>
<td>Graveyard, Ginnie Springs, Alachua Co., Santa Fe River, Florida</td>
</tr>
<tr>
<td>EMS 425068</td>
<td>5</td>
<td>Proboscidean</td>
<td>Tusk</td>
<td>Ginnie Springs, Florida</td>
</tr>
<tr>
<td>EMS 425069</td>
<td>6</td>
<td><em>Odocoileus</em> (Deer)</td>
<td>Antler</td>
<td>Graveyard, Ginnie Springs, Florida</td>
</tr>
<tr>
<td>EMS 425070</td>
<td>7</td>
<td><em>Mammathus</em> (Mammoth)</td>
<td>Tooth plate</td>
<td>Ginnie Springs, Florida</td>
</tr>
<tr>
<td>EMS 425071</td>
<td>8</td>
<td><em>Terrapene Carolina</em> (Tortoise)</td>
<td>Carapace</td>
<td>Newsom home</td>
</tr>
<tr>
<td>EMS 425072</td>
<td>9</td>
<td><em>Sus scrofa</em> (Domestic Pig)</td>
<td>Split canine</td>
<td>Florida – Other provenience unknown</td>
</tr>
</tbody>
</table>
Fig 1. Location of Ginnie Springs Site on the Santa Fe River in northern Florida. Source: Figure modified from the Santa Fe River Turtle Project.

Fig 2. a) Bone Sample 1 showing black staining on anterior surface and red staining on posterior surface b) Bone Sample 3 (tooth cross section) showing layers of black and red staining on the tooth surface.
Technique Selection and Sample Preparation

Characterization of River Sediments Associated with Fossil Bones

Sediment samples from the site were analyzed by energy dispersive spectroscopy (EDS) in an environmental scanning electron microscope (ESEM) to identify major element compositions. Sampling locations and sediment descriptions can be found in Table 2:

<table>
<thead>
<tr>
<th>Sample Designation</th>
<th>Sampling Location</th>
<th>Sediment Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SED1</td>
<td>River bottom</td>
<td>Very fine-grained, extremely dark color</td>
</tr>
<tr>
<td>SED2</td>
<td>Side of channel</td>
<td>Coarse-grained, lighter color, sandy</td>
</tr>
<tr>
<td>SED3</td>
<td>Riverbank</td>
<td>Clay-like, dark color, organic inclusions (leaf fragments, etc.) in the matrix</td>
</tr>
</tbody>
</table>

Development of Sample Preparation Protocol for Analysis of Bone at High Vacuum

Quantitative analysis of samples by electron probe microanalysis is optimized when samples are (Goldstein et al. 2014):

1. Able to withstand ultra-high vacuum
2. Anhydrous
3. Able to be polished to 0.05 micron grit
4. Flat
5. Electrically conductive
6. Less than 1 inch in diameter

The fossil bone specimens used in this study violated most of these requirements. Because bones contain a high concentration of volatile organic material, electron beam studies of
bone generally have been conducted by environmental scanning electron microscopy (ESEM). An ESEM has the same functionality as an electron microprobe in terms of EDS, but has two major drawbacks for use in this project – in most instrumental configurations it lacks the capability to perform WDS analysis, and it operates under low vacuum. Consequently, the analytical error is higher than in a high-vacuum system. Very few researchers have attempted ultra-high vacuum analyses on bone, and so a suitable preparation technique was needed.

We found that mechanical fracture of fossil bone preserved the exterior stains with higher fidelity than sawing, and samples therefore were broken into fragments. The small pores in bone tend to retain air and moisture and release it into the microprobe chamber when a vacuum is applied. To combat this, the samples were encapsulated in epoxy using a custom vacuum infiltration system (Fig. 3). The samples were placed in 1-inch molds, which in turn were placed in a vacuum chamber. A high vacuum was drawn for over 1 hour to remove any air and water vapor from even the innermost pore spaces. The custom infiltration system allowed for epoxy to be injected into the molds while the samples were still under vacuum. Subsequently, the vacuum was released, and atmospheric pressure was utilized to drive the epoxy deep into all of the pores. The epoxy was cured for 24 hours and the mounts de-molded. This process successfully eliminated the problem of outgassing when samples were placed within the microprobe, and reinforced the brittle structure of the bone to enable polishing.

A standard metallographic polishing sequence was applied, polishing with abrasives of decreasing size down to a 0.05 micron diamond-colloidal solution. After polishing, the mounts were vacuum-desiccated for another 48 hours to remove any volatiles left from the polishing process. The mounts were carbon-coated to create a conductive surface, and were then ready for analysis. Full penetration of the epoxy was confirmed by light optical microscopy (Fig. 4).
Fig 3. Custom vacuum infiltration system for epoxy mounting of fossilized bone samples.

Fig 4. High magnification micrograph confirming full epoxy penetration into interior pore spaces of bone sample.
High Vacuum Electron Probe Microanalysis – EDS (Energy Dispersive Spectroscopy)

Chemical characterization studies were conducted using a JEOL JXA 8500F Field Emission Electron Microprobe with a 4-crystal silicon drift detector. Energy dispersive spectroscopy (EDS) was utilized for elemental identification. EDS allows for spatial mapping of the location and concentration of elements across the surface of a sample by using characteristic X-rays that are emitted from the sample under an electron beam. All electron probe microanalysis (EPMA) analyses were run at 20 keV accelerating voltage and 25 nA probe current to allow for the excitation of all possible transition metal peaks. Standard spectrometer configuration across all runs was maintained, with maximum pulse throughout at 275 kcps and maximum energy at 40 keV. The electron beam was rastered laterally across the region of interest, collecting a spectrum at each point. These spectra are then compiled to form a hyperspectral data cube, which can be queried to create elemental distribution maps. Cross sections of each specimen were mapped and color overlays generated for each element. In regions of interest revealed by mapping, point spectra were acquired under the same analytical conditions. Regions of interest in this study were defined as areas with high concentrations of elements other than hydroxyapatite (bone material) bulk components.

High Vacuum Electron Probe Microanalysis – WDS (Wavelength Dispersive Spectroscopy)

WDS analyses also were conducted with a JEOL JXA 8500F Field Emission Electron Microprobe. The advantage of WDS is that it allows for increased peak resolution and decreases background radiation substantially. This makes for far more accurate quantitative results (Goldstein et al. 2014). A new application of WDS for determining the oxidation state of elements in bone material has been assessed as part of the scope of this project. For WDS analyses, the electron beam diameter was adjusted to between 0 µm (focused) and 10 µm depending upon run-time to mitigate beam damage to the sample. Beam damage can affect peak
positions in a WDS spectrum (Goldstein et al. 2014), and preventing peak drift was an important consideration for these experiments. Beam size in all oxidation state analytical runs was 10 μm, mitigating peak drift due to beam damage. Beam damage can also cause redox transformations, which would affect any oxidation state measurements. Through a calibration process, the offset limits for all spectrometers were set to [0.625, –0.675], which enabled peak position comparison between spectrometers. For mapping using WDS, analysis was conducted at an accelerating voltage of 13 keV, probe current of 50 nA, 1400x magnification, and working distance of 11.145 mm. Spot size was 1 μm, and grid spacing was set at 1 μm (actual collection spacing ~2.5 μm x 2.5 μm accounting for interaction volume).

**X-Ray Diffraction (XRD)**

X-Ray diffraction (XRD) experiments were conducted on a Bruker D8 Diffractometer with a Cu-$K_{\alpha}$ source and Vantec 500 detector for mineralogical characterization of the stained and unstained bone. XRD was performed on both the intact surfaces (un-modified) and cross-sections (using mounts created for microprobe work) of all samples. Experiment parameters were as follows: 1 mm slit, 40 keV accelerating voltage, 40 mA beam current, beam size 1 mm x 3 mm, scanning range of 15° - 75° 2θ, 20 cm working distance. XRD was performed on whole samples that were rotated.

**Electron Back-Scatter Diffraction (EBSD)**

Electron back-scatter diffraction (EBSD) experiments were carried out on a JEOL JSM 7100F Scanning Electron Microscope equipped with an EBSD detector in order to identify microscale inclusions within the bone. The same epoxy vacuum infiltration protocol was applied, with standard epoxy substituted by a conductive epoxy containing nickel powder. This substitution was necessary to control charging artifacts in the scanning electron microscope.
(SEM), as coating the sample with carbon would have interfered with EBSD data collection.

Thorough surface preparation is essential to collecting high quality EBSD data. The sample was polished in a metallographic polishing sequence from 240-µm grit to 0.05-µm grit. The sample was left in a vibratory polisher on 0.05-µm grit colloidal silica solution for 18 hours to ensure the highest quality surface polish possible. The sample was plasma cleaned before insertion into the SEM. Analysis was conducted at 20 keV and 300 pA.

**Fourier Transform Infrared Spectroscopy (FTIR)**

Fourier Transform Infrared Spectroscopy (FTIR) was used in this study in order to identify organic compounds (Harwood and Moody 1989). FTIR experiments were conducted using a Nexus 670 ThermoNicolet Fourier Transform Infrared Spectrometer continuously purged with dried CO$_2$-free air. Samples of stained bone were powdered with a mortar and pestle, and mixed with KBr beam splitter following the protocol presented in Jaén and Navarro (2009).
Chapter 3

Results

Mineralogy and Composition of River Sediment

Energy dispersive spectroscopy of three Santa Fe River sediments revealed strong peaks for Si, Al, and O, presumably as the result of quartz particles and aluminosilicate clays. However, the samples also contained detectable concentrations of iron. Additionally, two of the sediment samples (SED1 and SED2) contained some sulfur, with SED1 showing the highest intensity sulfur signal (Fig. 5).

The chemistry of the Santa Fe River in this region has been well-documented for many years, due to interest in the preservation of spring environments and concerns about nitrate pollution (USGS 2015). The United States Geological Survey (USGS) maintains an open-source database for their measurements of water quality around the country, and has a large amount of data for the Santa Fe River. We draw from this database for a characterization of water chemistry in the area of our site of interest, as a USGS measurement point is less than 4 miles from the fossil collection site.

Data from the last 10 years show high organic matter content, high nitrate levels, and relatively high sulfur concentrations, and an average pH of between 5 and 7 depending upon the season of measurement (USGS 2015). According to the USGS water resources database and other surveys of the chemistry of the hydrological system in northern Florida, the Santa Fe River is classified as a “blackwater” river (Florida Department of Environmental Protection 2015). A blackwater river is formed by the decay of vegetation into slow-moving water in wetlands or treed swamps (Janzen 1974). As the vegetation decays, tannins leach into the water, turning it a dark reddish brown color. Tannins represent a large class of compounds with polyphenolic
moieties (Mole 1993) and play important roles in the ripening of fruit. They also contribute to the bitter taste of many leaves, and assist in plant growth regulation.

Fig 5. EDS spectra of a) surface sediment, b) channel side sediment, and c) bottom sediment from the site showing concentrations of iron and sulfur.
XRD Results

XRD was performed on both the intact surfaces and cross-sections of all samples (results from Bone Sample 3 shown as representative). The collected spectra were background-subtracted and $K\alpha_2$-stripped (Fig. 6). The resultant spectra from all analyses exhibited peaks matching only hydroxyapatite (Fig. 7), which is the primary crystalline phase in bone. The absence of any diffraction peaks not belonging to hydroxyapatite could indicate that either the staining compounds were not crystalline, or that crystalline phases occur in such low concentrations that they were not detectable using conventional XRD. Therefore, other methods were required to identify these unknown compounds.
Fig 6. Process of background subtraction and k-stripping of the raw XRD data. Bone Sample 3, 10 hour run.

Fig 7. Hydroxyapatite peak lines from the International Center for Diffraction Data (ICDD) database fitted to the processed XRD spectrum from Bone Sample 3. All peaks are accounted for - there are no extraneous peaks indicating any crystalline material other than hydroxyapatite.
High Vacuum EPMA EDS Results

The EDS hyperspectral mapping technique described above was performed on cross-sections of all bone samples (Appendix A) at varying magnifications. Trends discussed in this section were characteristic of all specimens, and the images shown are representative examples of these trends.

In compositional maps of the stained areas that were selective for Mn (e.g., Fig. 8), Mn was detected at intensities below 1 count per second (cps). By contrast, the major elements in bone (calcium and phosphorus) were detected at intensities of 340 – 450 cps. Other minor elements yielded intensities between 30 and 80 cps. These results indicate that concentrations of Mn in these samples were negligible, strongly suggesting that the surface staining cannot be attributed to manganese oxides.

The only elements observed at high concentrations in the stained areas of these samples that are not usual components in bone were iron and sulfur, which occurred within layered bands in the stained regions in each specimen (Fig. 8, Fig. 9). These observations indicate the presence of two distinct compounds – one that contains iron but no sulfur, and one that is an iron-sulfur compound.

The occurrence of Fe- and Fe-S-compounds within the hydroxyapatite matrix suggests that the staining process was diffusive and that the stain did not precipitate as a crust on the surface of the bone. The absence of Ca and P where Fe and S occur suggests that the Fe-rich phases crystallized within macropores in the bone structure.
Fig 8. EDS mapping of Bone Sample 3. Magnification 140x. Maximum x-ray intensities are shown in counts per second (cps) and correspond to the relative amount of each element present in the sample. Note iron-sulfur correlations in two bands, and iron band in between with no sulfur.
Fig 9. EDS mapping of Bone Sample 3. Magnification 270x. Maximum x-ray intensities are shown in counts per second (cps) and correspond to the relative amount of each element present in the sample. Note pockets of high intensity iron-sulfur correlation, and the lack of calcium and phosphorous in these pockets.
High Vacuum EPMA WDS Results

As part of this project, a new application for WDS was assessed to determine elemental oxidation state in bone material. If it were possible to determine the oxidation state of an element by using WDS, it could potentially enable phase identification without need of a separate technique or instrument. This is very appealing to many researchers because it is a cost-effective option that also enables highly resolved spatial sampling.

To test this idea and to guide identification of the compounds in the stained areas of our samples, we focused on the sulfur in the iron-sulfur phase identified by the EDS maps (e.g. Fig. 9). Atoms of the same element in different oxidation states have slightly different activation energies. Two of the oxidation states of sulfur are very far apart energetically: $S^{2-}$ and $S^{6+}$. It has been shown that by using WDS to analyze the activation energy range of sulfur, it may be possible to discriminate between these two states by observing a small peak shift (Pérez et al. 2012, Carroll and Rutherford 1988). In a technique assessment study outlined in Appendix B, we tested whether a peak shift in sulfur was observable in sulfide ($S^{2-}$) and sulfate ($S^{6+}$) standards and whether the sulfur oxidation state in bone material (the “unknown”) could then be identified based upon this shift. When spectra from Fe-S inclusions within the bone were compared with these standards, the Fe-S inclusion spectra aligned with the wavescan of the $S^{2-}$ standard (shown in Fig. 10). This result indicated that the sulfur in the observed iron-sulfur compound is in the reduced state (a sulfide), which narrowed the list of possible compounds substantially and helped to direct further analysis. These results also indicate that for sulfur, oxidation state analysis by WDS is successful in bone material.

The oxidation state experiments conducted were point analyses, and thus the results only demonstrate that at a given point the sulfur is in the reduced state. To obtain a better picture of sulfur across the entire area of the staining, we developed a WDS mapping technique to determine the oxidation state of sulfur to constrain the distribution of sulfides and sulfates. A total
of 300+ WDS spots were collected in a grid across the section, and the data processed to create a “ratio map” of sulfide vs. sulfate character. Results show that the staining does not have exclusively sulfide character – rather, a gradient exists between sulfate and sulfide character (Fig. 11), although a strongly positive correlation exists between the concentration of sulfur and the amount of sulfur in the reduced (S²⁻) rather than oxidized (S⁶⁺) state. When these data are integrated with iron concentration (Fig. 12), we see that sulfur with high sulfide character is most strongly associated with higher concentrations of iron. The relationship between the concentrations and oxidation states of S may indicate that the iron-sulfur compound within the stain is oxidizing and diffusing through the bone over time.

Fig 10. Normalized sulfur oxidation state WDS data showing sulfur peaks for troilite (standard - blue), SrSO₄ (standard - green), and Bone Sample 3 (unknown - orange). A clear peak shift is visible between the sulfide and sulfate standards. The sulfur peak taken from the bone sample aligns very well with the troilite peak.
Fig 11. Sulfate/Sulfide concentration and gradient map. Warm colors represent high sulfide character, cool colors represent high sulfate character. Large point sizes indicate large concentrations and small point sizes indicate low concentrations. WDS experiment performed on Bone Sample 3.

Fig 12. Sulfate/Sulfide gradient map correlated with iron concentration. Warm colors represent high sulfide character, cool colors represent high sulfate character. Large point sizes indicate large concentrations of iron and small point sizes indicate low concentrations of iron. WDS experiment performed on Bone Sample 3.
**EBSD Results**

To determine the identity of the Fe sulfide phase, EBSD was performed on all samples (Bone Sample 4 shown as representative). A small area was mapped using EDS in order to locate an area of iron-sulfur correlation. The region surrounding the Fe sulfide inclusions yielded no electron diffraction lines indicative of a crystalline phase, even though XRD and EDS had confirmed the presence of crystalline hydroxyapatite. This result may be attributed to the large size of the hydroxyapatite unit cell, rendering the hydroxyapatite outside the detection of EBSD. Consequently, EBSD patterns of the iron sulfide phase exhibited no interference from accessory minerals (Fig. 13a). A search for matches of iron-sulfur phases within the EBSD database yielded an identification of pyrite (Fig. 13b). Yellow lines have been superimposed onto our collected EBSD pattern, and blue lines represent the calculated EBSD lines for pyrite. The closeness of the match offers convincing evidence for our identification.
Fig 13. a) EBSD diffraction pattern from Bone Sample 4 b) matched to standards database diffraction pattern for pyrite.
FTIR Results

An FTIR experiment was performed after the technique presented in Jaén and Navarro 2009 in order to identify any ferric tannate complexes in the specimens. Their study examined the differences in FTIR spectra between tannic acid (“pure tannins”) and artificially created ferric tannate complexes in order to characterize unique expressions of the ferric tannates in the spectra at various pH levels. Their data from pH 7 has been aggregated and displayed in Fig. 14. This pH most closely matches the average pH of the Santa Fe River in the area where these samples were collected (USGS water quality database, 2013-2015). Fig. 14b shows FTIR spectra of tannic acids from two different plant types, and Fig. 14a shows the ferric tannate complexes that resulted from the reactions of these same acids with aqueous iron nitrate. The authors note several differences between the two, most notably the sharpening of the peak at ~1500 cm$^{-1}$, and the loss in intensity of the peaks below 1500 cm$^{-1}$ (Jaén and Navarro 2009). At 1600 cm$^{-1}$ and below, the spectra collected from the bone (Fig. 15) samples appear similar in peak number, location, and shape to those ferric tannate spectra collected by Jaén and Navarro 2009. Subtle differences in this region are likely due to the variability in spectra caused by differential tannin structures in different species of plant. Tannins are generally known to be somewhat variable between species in terms of molecular structure. The basic structure is the same (Fig. 16), but positions and numbers of hydroxyl groups can vary. This variation very likely explains one major discrepancy between the spectra from the bone samples and the spectra from Jaén and Navarro 2009. Their data show an extremely broad peak in the 3500 cm$^{-1}$ region. This region is reflective of the C-OH bonds in some of the hydroxyl groups (Jaén and Navarro 2009). The bone spectra also show a peak in this region, but it is much less broad. Hydroxyl groups, as noted above, vary in position and number between different types of tannin molecules. These changes would be reflected in the FTIR spectrum as differences in peak breadth and number in the 3500 cm$^{-1}$ region. It is hypothesized...
that this observed difference between the bone measurements and measurements from Jaén and Navarro 2009 is due to the ferric tannate complexes being derived from different species of tannin molecules. It is even possible that multiple species of tannin molecules contribute to the signal from the bone, due to the dense and varied vegetation surrounding the Santa Fe River. As such, it is concluded that a ferric tannate complex is likely present on these bone samples, and is most likely the compound responsible for the black staining visible on the surface of the specimens.
Fig 14. Data from Jaén and Navarro 2009 showing a) FTIR spectra of laboratory created ferric tannate complexes and b) FTIR spectra of their parent tannic acids ("pure tannins") at pH 7. Source: Figure modified from Jaén and Navarro 2009.

Fig 15. FTIR spectra collected from a) Bone Sample 4 and b) Bone Sample 6.
Fig 16. Generalized base structure of tannic acid. Source: Figure modified from National Institute of Standards and Technology (NIST) open access database.
Chapter 4

Discussion

Compound Identification and Possible Reaction Mechanisms

It is clear from the EDS hyperspectral mapping data and the optical microscope images that this staining process is likely a multi-phasic reaction, suggesting that the stain developed in different stages. We propose that Fe oxide, presumably hematite (Fig. 17a), was deposited within the surface layers of the bone first. Next, tannins within the river water bonded with the Fe oxide to generate ferric tannates (Fig. 17b). Finally, reducing bacteria within the pores of the fossil bone transformed some of the hematite to pyrite (Fig. 17c). The rationale for this interpretation follows.
Iron Oxide Reaction Mechanism

It is known that iron oxide coatings (particularly hematite) frequently form on fossil bone (Bao et al. 1998). Iron oxide formation mechanisms in fossil bone are well studied (Pfretzschner 2001). If the sediment is rich in iron, this reaction is more likely to take place. Additionally, decomposition of organic matter “may enhance the weathering of iron-bearing minerals in soils surrounding carcasses, while [...] bones might provide favorable sites for iron accumulation” (Bao et al. 1998). Iron is well-known to accumulate in lateritic soils in tropical regions, and our EDS analyses of sediments from the collection site confirmed the presence of iron. This, combined...
with the high organic matter content of the Santa Fe River (USGS 2015), would provide conditions favorable for the formation of iron oxide on these fossil specimens.

**Ferric Tannate Reaction Mechanism**

Surfaces of bone that were in direct contact with river sediment exhibited only iron oxide stains. When surface regions of bone were exposed to river water, however, black ferric tannate layers were also observed. In all of these cases, the black tannate layering was observed only as a secondary coating on top of the red iron oxide layers (as shown in Fig. 17). The iron oxide and the ferric tannate bands often occurred in repeating layers, perhaps indicating cycles of burial and exposure in the taphonomic history of the specimen. These patterns may offer some use in reconstructing the history of a site. The attachment of the tannates to underlying Fe oxide suggests these compounds reacted with iron to form a ferric tannate complex on the surface of the bone. Ferric tannates are black complex compounds that are “highly insoluble, corrosion protective, [and] hav[e] a structure that depends on the pH of the solution” (Jaén and Navarro 2009). They are known to adsorb to surfaces where either a) there exists a reactive layer of iron oxide, or b) there is iron nitrate in solution and a surface in contact with the solution provides nucleation sites (Jaén and Navarro 2009). Both of these conditions prevailed in the Santa Fe River at the site of interest. USGS water quality monitoring for many years has shown elevated nitrate levels in the spring regions that feed the Santa Fe River (USGS 2015).

It therefore seems likely that ferric tannate formed by seepage through the pore spaces in the bone specimens, adsorbing in microscopic layers to the surfaces of the pores. In highly porous specimens (e.g., Fig. 18a), the staining penetrated the specimen fully. In less porous specimens such as teeth (Fig. 18b), the diffusion of the ferric tannate was limited by pore size changes in the specimen. Teeth have an exterior layer of enamel coating a core of dentin. Enamel is more porous than dentin, and we hypothesize that the sharp interface between stained and un-stained tooth
observed in these specimens is related to restricted flow due to a pore size change at the enamel-dentin boundary.
Fig 18. a) Extremely porous bone sample in which staining was able to penetrate fully b) Less porous tooth sample in which the staining was not able to penetrate as far.
**Pyrite Reaction Mechanism**

We propose that the formation of pyrite in these samples occurred when sulfidation of iron oxides in a reducing environment triggered the formation of pyrite. The extent and speed of pyrite formation presumably was dependent upon several factors, including type and amount of iron oxide, pH, concentration of sulfur anions in aqueous solution, and the presence of Fe- and S-reducing bacteria (Peiffer et al. 2015, Kraal et al. 2013). The pore spaces of the fossil bone were likely anoxic due to the decomposition of the organic matter. The oxic-anoxic interface is often associated with the formation of pyrite in geological materials (Herbert Jr. et al. 1998). Additionally, hydrogen sulfide penetrates bone quite easily (Locke 2004) and could have promoted sulfidation of the iron oxide layer, as hydrogen sulfide is a product of anaerobic respiration that often occurs at depth in slow-moving or stagnant water bodies such as the Santa Fe River. These crystals would likely be in too low concentrations for detection by XRD, but were within detection limits by EBSD.

**Bone Staining as a Potential Taphonomic Indicator**

Based on the results of this study and studies in which manganese was implicated in staining of bone specimens, it appears that bone staining is an extremely environmentally dependent process that produces nearly identical visible effects with very different chemistry. Literature demonstrating manganese staining reveals a common trend – arid depositional environments. This is in contrast to the depositional environment of the bones in this study, in which a hydrological system is integral to the formation of the staining compounds. These patterns show potential for reconstruction of taphonomic environments affecting stained specimens over time. Depending upon the chemistry of staining on bone, it may be possible to
draw conclusions about the depositional environmental history of specimens based upon the mechanisms driving the formation of different types of stains.

**Implications for Fossil Collections**

It is well established in museum literature that pyrite oxidation in fossil specimens is a continuous process that is extremely damaging to the structural integrity of the specimen (Cornish and Doyle 1984). This damage is well documented in shelled fossil specimens such as ammonites (Moore and Lieberman 2009). In high humidity storage conditions, fossils can develop what is known as “pyrite disease.” This occurs when existing pyrite oxidizes to form iron sulfate, which expands beyond the volume the original pyrite had occupied. This causes fracturing within the specimen and can result in disintegration of the material. The presence of some sulfate character in the fossil specimens studied here, as revealed by WDS, indicates that this process may already be underway. As the pyrite crystals exist in the interior of the specimen, expansion stresses from pyrite oxidation increases the risk of fracture substantially. If the size of the pyrite crystals is small, risk of catastrophic fracture is decreased but the oxidation reaction rate is increased. Decisions regarding relative risk to a collection from pyrite staining should consider this factor in addition to the overall amount of pyrite present in the specimens.

To prevent further damage to collections affected by pyrite formation, it is proposed that the humidity should be controlled in the storage environment. If storage conditions are not airtight, the collection may be subjected to substantial humidity, especially in certain climates. To mitigate this problem in a cost-effective way, it is generally recommended that de-humidifiers be placed in the laboratory or sample storage space. Additionally, specimens should be placed in airtight containers when not being used. Target relative humidity in the storage environment
should be under 30% (per the American Museum of Natural History guidelines). This should slow the oxidation substantially and preserve the collection.

Museums and research institutions of many types may possess collections that are at risk from pyrite oxidation damage. In contrast, manganese oxides have not been shown to pose any risk of damage to fossils, and the present study has shown that pyrite-rich coatings may be mistaken for Mn oxide stains. Whereas Mn oxide stains are removable if research needs require it, ferric tannate staining is not removable from a highly porous material like bone, even though it can be separated from non-porous materials like metallic iron (Binnie et al. 1995). Clearly, misidentification of the compounds that cause stains can have consequences for collections and also research conducted on those collections. Collection curators should fully assess the chemistry of post-depositional alterations on fossil specimens whenever possible in order to implement the best practices for preservation and future research endeavors.
References


### Appendix A

#### Sample Catalogue

Sample Designation Chart (exact copy of specimen labels, Penn State University)

<table>
<thead>
<tr>
<th>EMS CATALOGUE NUMBER</th>
<th>SAMPLE DESIGNATION</th>
<th>SAMPLE SPECIES</th>
<th>SAMPLE BONE TYPE</th>
<th>SAMPLE LOCALITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMS 425064</td>
<td>1</td>
<td><em>Equus</em> (Horse)</td>
<td>Left calcaneus</td>
<td>Graveyard, Ginnie Springs, Florida</td>
</tr>
<tr>
<td>EMS 425065</td>
<td>2</td>
<td><em>Equus</em> (Horse)</td>
<td>Astragalus</td>
<td>Graveyard, Ginnie Springs, Alachua Co., Florida</td>
</tr>
<tr>
<td>EMS 425066</td>
<td>3</td>
<td><em>Mammathus</em> (Mammoth)</td>
<td>Tooth</td>
<td>Graveyard, Ginnie Springs, Santa Fe River, Florida</td>
</tr>
<tr>
<td>EMS 425067</td>
<td>4</td>
<td><em>Equus</em> (Horse)</td>
<td>Tooth</td>
<td>Graveyard, Ginnie Springs, Alachua Co., Santa Fe River, Florida</td>
</tr>
<tr>
<td>EMS 425068</td>
<td>5</td>
<td>Proboscidean</td>
<td>Tusk</td>
<td>Ginnie Springs, Florida</td>
</tr>
<tr>
<td>EMS 425069</td>
<td>6</td>
<td><em>Odocoileus</em> (Deer)</td>
<td>Antler</td>
<td>Graveyard, Ginnie Springs, Florida</td>
</tr>
<tr>
<td>EMS 425070</td>
<td>7</td>
<td><em>Mammathus</em> (Mammoth)</td>
<td>Tooth plate</td>
<td>Ginnie Springs, Florida</td>
</tr>
<tr>
<td>EMS 425071</td>
<td>8</td>
<td><em>Terrapene Carolina</em> (Tortoise)</td>
<td>Carapace</td>
<td>Newsom home</td>
</tr>
<tr>
<td>EMS 425072</td>
<td>9</td>
<td><em>Sus scrofa</em> (Domestic Pig)</td>
<td>Split canine</td>
<td>Florida – Other provenience unknown</td>
</tr>
</tbody>
</table>
Appendix B

WDS Oxidation State Method Development

Determination of S oxidation state

WDS Peak Shift Experiment: Stage 1

In order to test whether the sulfur-containing inclusions in the fossil bone were sulfides or sulfates, we compared the sulfur activation states of the inclusions with two NIST standards: a sulfate (SrSO₄) in which sulfur is in the S⁶⁺ state, and a sulfide (FeS – troilite variety) in which sulfur is in the S²⁻ state. The orientation of the PET crystal was optimized for acquisition of the spectrum for sulfur, and scans were taken of both standards to determine whether a peak shift was observable. With the beam size increased to 5 µm, a peak shift was clearly present (Fig. A). We then obtained a WDS spectrum for Bone Sample 3. When the resulting spectrum was plotted against the standards, the bone wavescan clearly aligned with the wavescan of the troilite standard (FeS) (Fig. A).
Next, we attempted to test the limitations of the peak shift in reliably discriminating oxidation states of sulfur, and additionally tested if it is possible to do the same with iron. It was hypothesized that the closer the oxidation states were energetically, the more difficult it would be to de-convolve the peaks. A previous study involving iron had concluded that quantitatively it is not reliable to discriminate between Fe$^{2+}$ and Fe$^{3+}$ (the two most relevant states for natural systems) (Quintiliani et al. 2015). However, the present experiment was designed to test discrimination only in a qualitative context.

Fig A. Normalized sulfur oxidation state WDS data showing sulfur peaks for troilite (standard - blue), SrS04 (standard-green), and Bone Sample 3 (unknown-orange). A clear peak shift is visible between the sulfide and sulfate standards. The sulfur peak taken from the bone sample aligns very well with the troilite peak.

**WDS Peak Shift Experiment: Stage 2**
The experimental setup was essentially the same as the stage 1 experiment, with the addition of more standards that span the oxidation state ranges of sulfur and iron common in natural systems. Standard choices are laid out in Table A.

Table A. WDS oxidation state standard selections

<table>
<thead>
<tr>
<th>Standard Name</th>
<th>Chemical Composition</th>
<th>Oxidation State(s) of Interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brimstone (native sulfur)</td>
<td>S</td>
<td>S(^0)</td>
</tr>
<tr>
<td>Native metallic iron</td>
<td>Fe</td>
<td>Fe(^0)</td>
</tr>
<tr>
<td>Gypsum (var. Selenite)</td>
<td>CaSO(_4)</td>
<td>S(^{2+}) (theoretical S(^{6+}), actual is roughly S(^{2+}))</td>
</tr>
<tr>
<td>Troilite</td>
<td>FeS</td>
<td>S(^{2+}), Fe(^{2+})</td>
</tr>
<tr>
<td>NIST K-412 (glass)</td>
<td>Many included, all known</td>
<td>Fe(^{2+})</td>
</tr>
<tr>
<td>NIST K-411 (glass)</td>
<td>Many included, all known</td>
<td>Fe(^{2+})</td>
</tr>
<tr>
<td>NIST K-409 (glass)</td>
<td>Fe(_2)O(_3) beaded inclusions</td>
<td>Fe(^{3+})</td>
</tr>
</tbody>
</table>

Standards were mounted and an Au/Pd alloy coating was deposited at a thickness of 2 nm. Bone Sample 3 was used as the “unknown” sample for both iron and sulfur in this experiment, and the pre-existing carbon coat was milled off and replaced with 2 nm of Au/Pd alloy. Au/Pd coating was chosen to mitigate the effects of carbon buildup during analysis, ensuring extreme sensitivity to the iron peak shift while still maintaining conductivity requirements. A South Bay Technology (SBT) Ion Beam Sputtering/Etching System with a KRI Kaufmann Source Controller was used for this process.

Parameters

Beam size in all runs (with the exception of NIST K-409) was 10 µm, mitigating peak drift due to beam damage. Beam damage can also cause oxidation, which could affect any oxidation state measurements. Beam size was focused to 0 µm for analysis of NIST K-409 due to a need for spatial resolution in analysis of the small hematite inclusions. Through a calibration process, the offset limits for all spectrometers were set to [0.625, – 0.675], which enabled peak
position comparison between spectrometers. One spectrometer was optimized for iron L-lines. Assessing peak shifts using iron L-lines was selected because K-line shells are not active in chemical bonding of iron and thus a peak shift would not be expected. A TAP crystal failed to detect iron L-lines, so an LDE pseudo-crystal was selected for these measurements. Previous optimizations for sulfur on a PET crystal were repeated in this experiment.

Scans were collected from each standard on elements of interest from Table A. An overnight scan was conducted on Bone Sample 3, collecting both iron and sulfur peaks across the surface of the bone (cross-section of the specimen). The beam size for the bone scan was again increased to 10 µm and the linescan contained 17 points at a spacing of 50 µm. Results indicate that discrimination of the oxidation state of sulfur is reliable, but is not for iron.

**WDS Peak Shift Experiment: Stage 3**

This experiment had two components:

1. EDS mapping of sulfur + iron–rich areas at incrementally decreasing energy (10 keV, 5 keV, and 3 keV) to determine detection limit and spatial/energy resolution, along with spatial/depth correlation of the two elements.

2. Creating a “ratio map” for oxidation states of sulfur to show the distribution of sulfate vs. sulfide character through the layers of staining. Calcium and phosphate were also included in this analysis.

   a. **Parameters:** Analysis was conducted at an accelerating voltage of 13 keV (informed by the results of component 1, discussed below), probe current of 50 nA, 1400x magnification, and working distance of 11.145 mm. Spot size was 1 µm, and grid spacing was set at 1 µm (actual collection spacing ~2.5 µm x 2.5 µm accounting for interaction volume). A total of 300+ spots were collected for
the grid. The standards used are detailed in Table B, and Bone Sample 3 was the chosen specimen for the grid.

<table>
<thead>
<tr>
<th>Component Standardized</th>
<th>Standard Name</th>
<th>Chemical Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>Fluorapatite</td>
<td>Ca(_5)(PO(_4))_3\text{F}</td>
</tr>
<tr>
<td>Ca</td>
<td>Fluorapatite</td>
<td>Ca(_5)(PO(_4))_3\text{F}</td>
</tr>
<tr>
<td>S (Sulfide)</td>
<td>Pyrite</td>
<td>FeS(_2)</td>
</tr>
<tr>
<td>Fe</td>
<td>Hematite</td>
<td>Fe(_2)O(_3)</td>
</tr>
<tr>
<td>O</td>
<td>Hematite</td>
<td>Fe(_2)O(_3)</td>
</tr>
<tr>
<td>SO(_4) (Sulfate)</td>
<td>Anhydrite</td>
<td>CaSO(_4)</td>
</tr>
</tbody>
</table>

Results from the first component of the experiment indicate that the energy detection limit for correlated resolution is as low as 3 - 5 keV. Optimum detection is achieved at around 13 keV. This accelerating voltage was then applied to the second stage of this experiment for best results. Results from the second stage are displayed in Figs B and C, and show that the staining does not have exclusively sulfide character - rather, a gradient exists between sulfate and sulfide character.
Fig B. Sulfate/Sulfide concentration and gradient map. Warm colors represent high sulfide character, cool colors represent high sulfate character. Large point sizes indicate large concentrations and small point sizes indicate low concentrations. WDS experiment performed on Bone Sample 3.

Fig C. Sulfate/Sulfide gradient map correlated with iron concentration. Warm colors represent high sulfide character, cool colors represent high sulfate character. Large point sizes indicate large concentrations of iron and small point sizes indicate low concentrations of iron. WDS experiment performed on Bone Sample 3.