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THE EFFECTS OF HEAVY METAL CONTAMINATION IN SOIL ON DNA DEGRADATION AFTER DECOMPOSITION

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By

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Abstract

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The application of DNA is an ever-expanding field of research with many implications in the field of forensic investigation. Research into DNA obtained from soil is ongoing and suggests a new avenue in forensic investigation. This research seeks to understand the effects of heavy metal on the preservation of DNA within soil after decomposition of remains. Using pigs as a proxy for human remains, the effects of heavy metal contamination on the microbial environment and endogenous pig DNA was observed. This gave broad insight into the effect of surface burial and Western Montana; summer climate has on DNA degradation.

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Introduction

DNA analysis in forensic cases is a commonly used tool in criminal investigations. Forensic DNA may have come from numerous sources including blood, teeth, bone, hair, tissues, bodily fluids, and fecal material. It can be obtained from many locations including the crime scene, clothing, a victim's body, a suspect's body, and numerous other locations. One of the less commonly used sources in a genetic forensic investigation is soil. When human remains are decomposing, DNA is released into the surrounding environment. Logic then follows that DNA may be preserved within the soil, however, there are few studies that investigate the effects of different types of soil on DNA preservation after decomposition. Notably, in Montana there are many areas that have heavy metal contamination, which may affect the degradation of the DNA within the remains and then how DNA migrates through the soil. It is this that will be investigated through the research presented here.

The rich copper mining and smelting history in Anaconda, Montana, has led the spread of contaminants within the local environment. The smelting process resulted in a harmful waste product known as slag and left much of the area polluted with harmful levels of heavy metal leeching into the environment. The history of copper mining in Anaconda, made it a great location to study the effects of heavy metals on DNA degradation during decomposition.

The goals of this research was to discover how heavy metals effect the spread of DNA within the soil after decompositions, how heavy metals in the soil effects the quality of DNA in terms of the strand length of the DNA both in the decomposing body and in the soil, and the effects heavy metal contamination plays on the total amount of DNA found within a soil sample.

Hypothesis A: High heavy metals concentration within the soil will inhibit the spread of DNA within the soil and will result in shorter DNA strand length, which is one of the ways in which

DNA degrades over time (strand breakage, explained more below). It is expected that amplification (Polymerase Chain Reaction) of longer targeted pieces of DNA will be unsuccessful in soil that has been more heavily contaminated with heavy metals.

Hypothesis B: High heavy metal contamination within a sample would reduce the quantity of the total DNA within a soil sample. It would then be expected that the amount of DNA found in the soil would be quantifiably lower in soil with higher heavy metal (arsenic, lead, etc.) concentrations.

Hypothesis C: High heavy metal concentration within the soil will lead to more degradation (strand breakage) of DNA within the decomposing remains—in this case, pig—resulting in the inability to amplify longer targeted sequences of DNA.

Literature Review

Mining and Superfund History: Anaconda Montana

Anaconda-Deer Lodge County in southwestern Montana has a copper smelting history that lasted almost 100 years (Anaconda Deer Lodge County, 2018). The Anaconda Copper Mining Company began to smelt and process ore in 1882 (Anaconda Deer Lodge County, 2018; EPA, 2016). Ore was mined in Butte and sent to the Anaconda smelter to be processed. The smelter was recognized as one of the leading producers of copper, as well as being the largest free-standing smelter of the time (EPA, 2016). This smelting process led to the dumping of slag and other smelting waste in and around the town of Anaconda (Anaconda Deer Lodge County, 2018). The EPA report states: "Records beginning in 1907 indicate that each day the smelter released over 30 tons of arsenic, copper, lead, sulfur and zinc into the environment" (pg. 2, 2016). Later records from 1978 estimate the average to have increased to 578 tons of contaminants released daily.

The smelter closed in 1980 and by 1983 the EPA listed the 300 square miles around the Anaconda smelter on the Superfund programs National Priorities List (EPA, 2016; Anaconda Deer Lodge County, 2018). Along with the soil contamination, it was also estimated that more than a billion gallons of groundwater were also contaminated (EPA, 2016). Remediation began shortly after Superfund classification, with residential properties housing children given priority (Anaconda Deer Lodge County, 2018).

Remediation Efforts

Remedial efforts for the 300 square mile superfund site were organized by the EPA, the Atlantic Richfield Company (ARCO), and the Montana Department of Environmental Quality (MDEQ) (EPA, n.d.). The EPA has designated the site into numerous operable units (OU's) to better categorize the type of remediation needed or the area within the Superfund site in which

that would take place. The operable units include a Sitewide OU, water, waste and soil, Old Works/East Anaconda, Beryllium Removal, Flue Dust, Arbiter Removal, Smelter Hill, Mill Creek, and Community Soils (EPA, n.d.). Each of the operable units undergo investigation of the site and type of remediation needed, then the EPA makes decisions about type, timeline, and priority level of the remediation process. After remediation, the area is still examined during the five-year review to ensure restoration is maintained (EPA, n.d.).

From 1986 to 2018, 10 million cubic yards of tailings, mining wastes, and contaminated soils have been removed and 500 million cubic yards of waste have been capped in place EPA, n.d.). Other activities include reclamation of 12,500 acres of land and 800 residential and commercial properties have been remediated (Anaconda Deer Lodge County, 2018). Although the area has undergone massive remediation efforts and the health of the land and people within the area have improved significantly, the area still has not been removed from the National Priorities List and the remediation efforts have not been completed (EPA, n.d.).

Heavy Metals and Genotoxicity

Although many heavy metals are found to be dangerous to human health when represented in certain quantities, it is important to identify the effects of these metal on DNA. The EPA has identified five major heavy metal contaminants found within the site: Arsenic, Cadmium, Copper, Lead and Zinc. These metals are known to have negative health effects on humans, wildlife and vegetation (EPA, n.d.).

Arsenic. In living cells both in vitro and in vivo, it has been shown that arsenics has a clastogenic effect on DNA (Chou et al., 2007). Clastogens are substances that create breakages in the chromosome. This can result in the loss, rearrangement, and even addition of some chromosomal segments (Chou et al., 2007). Though studies are controversial, The CDC

identified several effects that inorganic arsenic has on human DNA, including chromosomal aberrations, sister chromatid exchange, DNA adducts, mutation, and over expression of p53, micronuclei, DNA repair inhibition, and it is classified as a human carcinogen (Chou et al., 2007). Unfortunately, no studies have been found on the effects of inorganic arsenic on human genes after death or during decomposition.

Cadmium. Cadmium is also a clastogenic agent. The effects include DNA damage, micronuclei, sister chromatid exchange, and chromosomal aberrations (Obaid et al. 2012). The studies on cadmium's effect on human DNA focused mainly on lymphocytes (Obaid et al 2012). These studies are limited also focus on samples taken from living humans, so again the effects of this metal are unknown in DNA after death.

Copper. Copper's effect on DNA is not known. No studies were found in which copper effects DNA, but there are studies on its effects on fly larvae, white leghorn chicken, mice, and aquatic life (Dorsey & Swarts, 2004). These studies identified recessive lethal alleles, chromosomal aberration, micronuclei, and sperm abnormalities (Dorsey & Swarts, 2004). These studies however do not allow us to make any distinctions into the effects on human DNA.

Lead. Most of the lead genotoxicity studies have been done on small populations of industrial workers. Lead has been associated with gene mutation, DNA damage, sister chromatid exchange, micronuclei formation, and DNA methylation (Abadin et al., 2020). These studies were done on workers, so it is possible that they have co-exposures to other heavy metals (Abadin et al., 2020). Therefore, some of the damage seen in their DNA maybe due to exposure to genotoxic heavy metals (Abadin et al., 2020).

Zinc. There is very little indication that zinc causes damage to DNA in humans (Roney et al, 2005). There has been evidence of chromosomal aberrations in mice that had a low calcium

diet (Roney et al., 2005). This may be due to the fact the zinc can displace calcium within the body. This calcium displacement can lead to chromosome breaks and impairing the repair process (Roney et al., 2005). However, there is not enough evidence to determine that it may damage human DNA.

Pigs as Proxy for Human Remains

Pigs and other mammals have long been used as replacements for human cadavers and human tissue in decomposition studies (Dautartas, 2018). The use of nonhuman mammals is a direct result of the difficulty in obtaining human cadavers, regulations on location of human burials, and the ethics in using human remains for this type of study (Daurtartas, 2018; Stokes, 2013). There are concerns about the use of pigs as proxies for human remains regarding whether results obtained from pig decomposition can be applied to human decomposition. A study comparing decomposition in pigs, sheep, and cows to humans attempted to determine whether studies using nonhuman mammals were applicable to humans (Stokes, 2013). An entomology study found that of the insects captured on pig and human cadavers, 99.67% of the insects were found in both species and only rare insects were found on only one or the other (Stokes, 2013; Schoenly et al, 2007; Haskell et al, 2002). Microbial activity in the soil during decomposition were discovered to be higher in porcine and bovine than in humans (Stokes, 2013). However, they did find that the overall patterns of nutrients and chemical changes were similar between nonhumans and human skeletal muscle decomposition (Stokes, 2013).

It has been suggested that pigs make the best proxies because their size, skin thickness, hair coverage and other factors are remarkably similar between humans and pigs (Dautartas et al., 2018; Catts, 1992; Byrd & Castner, 2001; Schoenly et al., 2006). One of the studies that examines the use of pigs as proxies is by Schoenly et al., which found that pigs can be used as a

substitute for human cadavers, as there were limited differences found in the decomposition process (2007). The drawback of this study was that only two pigs and one human cadaver were used (Schoenly et al, 2007). Dautartas et al. found that human remains are less predictable than animals, and the use of animals introduces a higher rate of error because they do not account for the variability found in humans (2018). They concluded that pigs can still be useful to get baseline information about the decomposition rates etc. but more specific studies about time since death, etc., need to use human cadavers (Dautartas et al, 2018).

Research conducted at the Forensic Investigation Research Station (FIRS) in Colorado, found that pigs as proxies may be more suitable for studies on individual variables, such as trends in taphonomy (Connor et al., 2018), but not for larger scale studies comparing multiple variables. Pigs are fit for studies on individual variables due to populations tending to be more homogenous than human samples (Connor et al., 2018). Keough et al. conducted a study on the effectiveness of pigs as proxies, and their findings also suggest that pigs are ineffective to be used in decomposition studies especially in the early stages of decomposition (2016). They suggest a model that can adjust for differences found between pigs and humans (Keough et al., 2016).

A study was also done to determine whether cadaver dogs would be able to alert on the presence of volatile inorganic compounds (VOCs), in the soil after the remains were removed (Perrault et al., 2015). These VOC's are produced as the body changes during decomposition and can be detected by cadaver dogs when human remains are or were present at the dog's alert location (Perrault et al., 2015). They used pig remains to deposit the VOC's because the major compounds are found to be similar and pigs are often used to train cadaver dogs as well (Vass, 2012; Stadler et al., 2013; Cablk et al., 2012; Perrault et al., 2015). Overall studies indicate that

though there some differences, pigs can be proxies for human cadavers if human cadavers cannot be used.

DNA Degradation

There are many changes that occur within the body after death. These changes affect the rate of decomposition of the body as well as the degradation of the DNA. One of the first changes to occur in the body postmortem is autolysis in the cell membrane (Burns 2013, Dent et al. 2003). Autolysis leads to proteins, lipids, and carbohydrates being released into the extracellular environment (Burn 2013, Dent et al., 2003). As autolysis occurs, DNA is released freely into the body (Johnson & Ferris, 2002; Bogas et al., 2009). As this damage progresses, stranded breakages occur to the DNA. The speed of this degradation is dependent upon the organ the sample is taken from (Johnson & Ferris, 2002). Johnson & Ferris found that fragmentation in DNA occurs more rapidly from three hours post-mortem to 56 hours post-mortem than at later points post-mortem (2002). After autolysis occurs, significant DNA degradation occurs to segments of 180 base pairs or greater through endogenous endonucleases (Alaeddini et al., 2010). As cells become necrotic they create random patterns of degradation along the chromosome. The rate of necrosis is generally affected by environment and temperature, with damp, warm environments accelerating necrosis (Alaeddini et al., 2010). Enzymes and proteases are released via cell death, the lysosomal proteases are responsible for breaking down the histone protein resulting in DNA cleavage (Alaeddini et al., 2010). These fragments of DNA may be degraded further by nucleases in the cell or from the environment (Alaeddini et al., 2010). The presence of microorganisms increase as the nutrient rich fluid spills from the necrotic cells, and this leads to further break down of macromolecules in the host (Alaeddini et al., 2010). The DNA may also be damaged further by hydrolysis and oxidation at a slower rate (Alaeddini et al.

2010). Hydrolysis leads to base loss and then breakage (Alaeddini et al. 2010). "Oxidative damage mostly includes modification of sugar residues, conversion of cytosine and thymine to hydantions, removal of bases and crosslinkages" (Alaeddini et al., pg 4, 2010). As DNA spills out of the body during the liquefaction processes, microorganism found in the soil may further degrade DNA.

Extraction and Analysis of DNA from Soil

Soil is an area of interest for DNA extraction, as it may give investigators the ability to determine if a body has been moved or if remains were present at a specific location. However much of the research has been focused on ancient DNA. aDNA can be obtained from many different sources including bone, teeth, faeces, sediment, and ice (Hebsgaard et al., 2009). Although often researchers may not be aware that remains do not need to be present in order to obtain aDNA from a site (Hebsgaard et al., 2009). One of the most prevalent concerns when extracting DNA is whether leaching will be a factor—wherein DNA will move through the soil to lead to stratigraphic layers that were not occupied by individuals may still carry their DNA, leading to misinterpretations of occupation of an archaeological site. Many ancient DNA studies have extracted aDNA from permafrost regions (Hansen et al. 2001; Willerslev et al. 2003; Willerslev, Hansen Poinar 2004; Willerslev, Hansen, Ronn et al., 2004; Lydolph et al., 2005; Mitchell et al. 2005; Haile et al., 2007). Willerslev et al. and Lydolph et al. found that in permafrost environments, leaching and redeposition did not seem to occur frequently (2003;2005; Haile et al. 2007). Haile et al. conducted a study to examine to cave sites in New Zealand to determine whether leaching and redeposition is a problem in non-permafrost areas (Haile et al., 2007). They found that DNA preservation does follow the histories of the site, but they did observe downward movement of the DNA, which could create inaccuracies in creating a

chronology of the area (Haile et al. 2007). Hebsgaard et al found that aDNA from a historic site could be preserved in sediment even if left unprotected in a nonfrozen environment (2009). Research conducted on aDNA in both permafrost and unfrozen sediment have shown relatively good preservation of aDNA.

When we look at DNA exacted from soil samples in forensic cases, there has been a lot of research into soil microbiome and very little into extracting endogenous DNA from the soil (Thomas et al. 2018). As a cadaver begins to decompose, the DNA also begins the decay processes. The first step of the process being autolysis of the cell membrane, which allows the DNA to be released from its cellular matrix and into environment around it (Bogas, 2009; Emmons et al., 2007). DNA preservation rates differ depending upon the environment that it is released into. DNA is able to survive in the soil by binding to the soil matrix which allows the DNA to avoid being degraded or destroyed by DNase and other enzymes (Emmons et al., 2007). In the instance of a burial, soil pH, moisture percentage, concentration of humic substances, mineral content, cation concentration, and organo-mineral complexes are the major factors that need to be considered (Bogas, 2009). Emmons et al. conducted a study on DNA preservation in the soil; they found that nuclear DNA was unrecoverable in most cases but that mtDNA could be detected consistently throughout the decomposition process (2007). This study also shows that bacteria association with the human microbiome was positively correlated to the amount of mtDNA that can be found in the environment (Emmons et al., 2007).

DNA degradation in the soil is dependent upon soil type (Bogas, 2009). Bogas found that sand, clay, and marshy soil all showed high levels of DNA degradation, but marshy soil showed the most degradation (2009). A study that is particular importance to this project was done by Thomas et al. in northeastern Montana, on the migration of mitochondrial DNA. The

study found that DNA of buried remains migrates radially from the burial site and that in proper conditions may survive more than five months (2018). This study also suggests that Sanger Sequencing method may only be sufficient for a short time in this environment, but that even basic and inexpensive techniques can allow for DNA extraction and amplification from this environment, though the DNA may be degraded or damaged (Thomas et al., 2018).

Methods

Pig processing

On August 7th, 2020, two pigs weighing between 160-180lbs were purchased from the Hutterite colony located outside Great Falls, MT. These pigs were euthanized upon purchase and internal organs were removed. The pigs were put on ice until they were butchered on August 8th, 2020. The pork haunch was removed with the skin still attached for use in this project. After removal, each shoulder was cut into pieces weighing between 240 and 250 grams. Six pieces were removed from each shoulder for a total of 24 pieces. Then the four samples that were as close to equal size, thickness, and skin to muscle ratio as possible from each shoulder were chosen for use in this research project. The rest were saved for backups. Off each of the 16 pieces of pig shoulder, two roughly equal portions of the remains were sliced off each sample, which filled a 2mL microcentrifuge tube, for use as control in the DNA portion of the project. The first portion contained skin, hair and subcutaneous fat and the other portion was muscle tissue. All portions of the pig were then frozen.

Procedure for soil samples

On August 9, 2020, 16 five-gallon buckets were purchased, and three holes were drilled into the bottom for drainage during the experiment. The buckets were then sprayed with a bleach solution to avoid contamination that may have happened before they were purchased. After the bleach was wiped out, they were then rinsed with tap water. Eight glass jars were also purchased

and decontaminated using the same processes. These buckets were then taken to the field to be filled with dirt from the designated sites (Table 1). The sites included: The wetlands area near the airport outside of Anaconda, Jaycee park, 1804 Hamburg St., the red sands area near the Old Works walking trail, in the Mill creek area behind the gun range, and the railroad track on Madison St., all of which are located in Anaconda-Deer Lodge County (Figure 1). Soil was also collected from Lubrecht forest near Ovando, Montana (MT), and from bagged potting soil purchased at Walmart shopping center in Missoula MT (Figure 2).

Table 3.1 Provenience of Soil for each Bucket.

Bucket number	**Soil Provenance**
1	Lubrecht Experimental Forest, Greenough, MT
2	Lubrecht Experimental Forest, Greenough, MT
3	Potting Soil - Walmart, Missoula ,MT
4	Potting Soil - Walmart, Missoula ,MT
5	1804 Hamburg St., Anaconda, MT
6	1804 Hamburg St., Anaconda, MT
7	Bowman Field, Anaconda,MT
8	Bowman Field, Anaconda,MT
9	Corner of Madison St. and East Third St. , Anaconda, MT
10	Corner of Madison St. and East Third St. , Anaconda, MT
11	Old Works walking trail- red sands, Anaconda, MT
12	Old Works walking trail- red sands, Anaconda, MT
13	Jaycee Park, Anaconda, MT
14	Jaycee Park, Anaconda, MT
15	Millcreek Road, Anaconda MT
16	Millcreek Road, Anaconda MT

Figure 3.1 Map of Soil Provenience in Anaconda- Deer Lodge County, MT

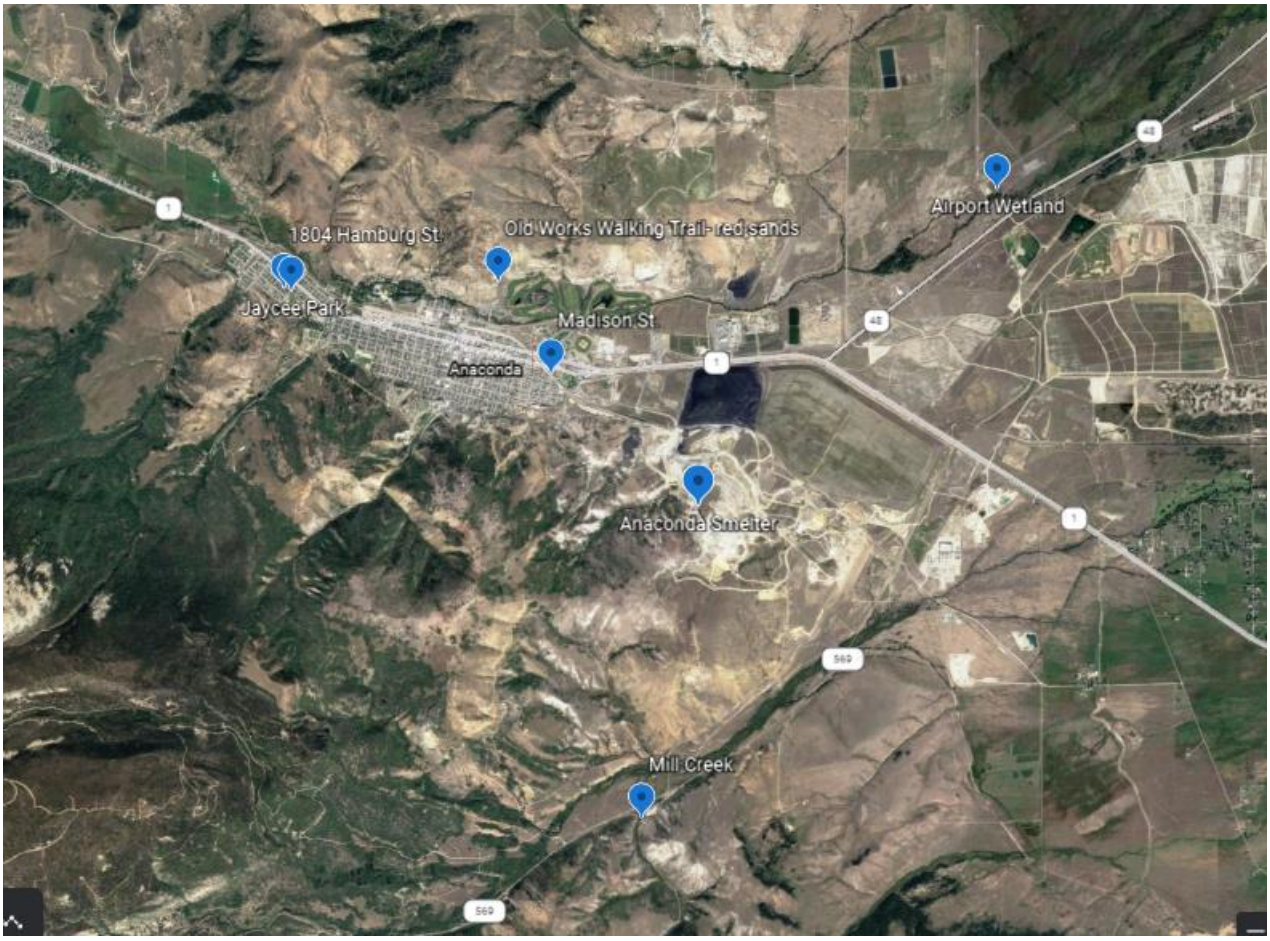
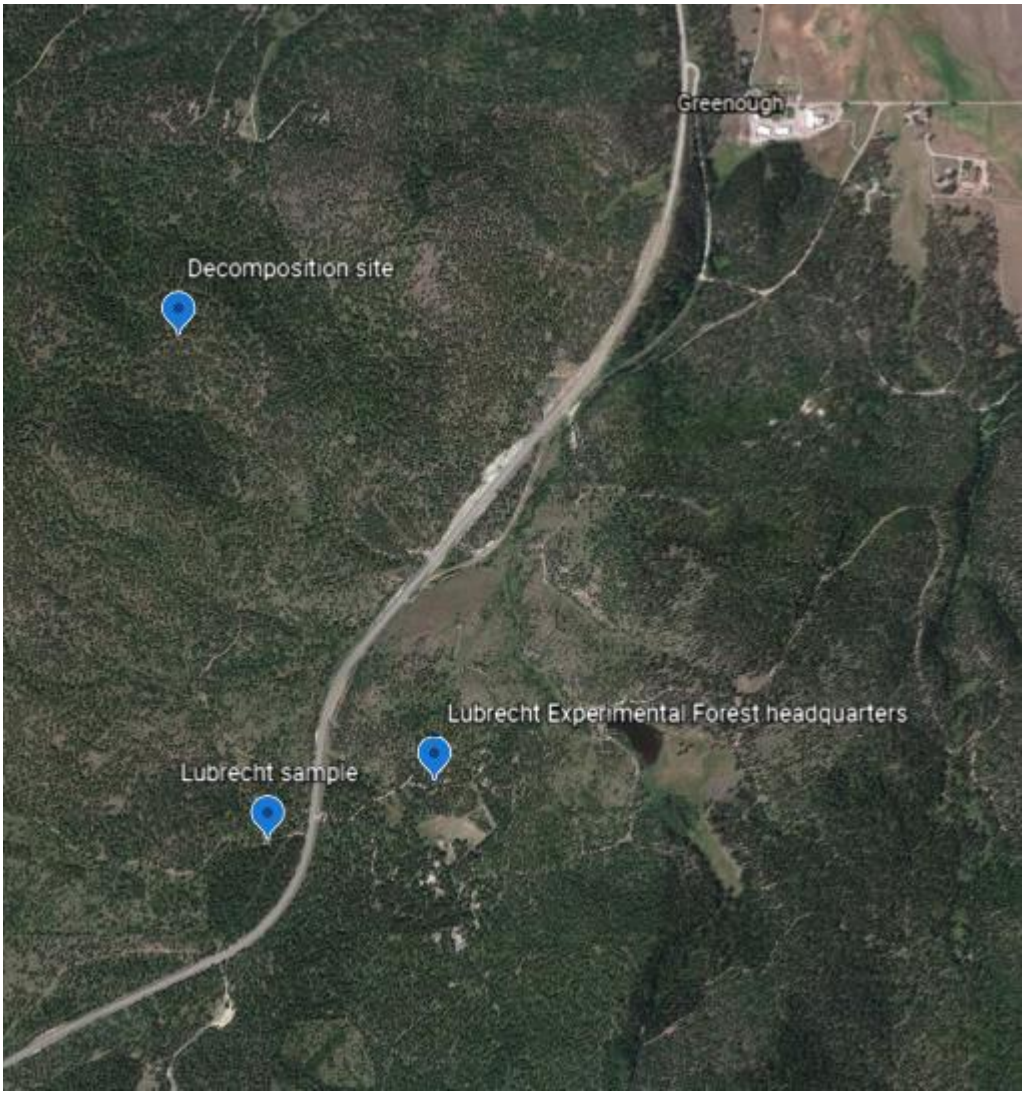


Figure 3.2. Project Location and Soil Sample Location in Lubrecht Experimental Forest, Greenough, MT.



The buckets were filled until they were about two inches from the lip of the bucket. The equipment was cleaned with the bleach spray and then rinsed with water between each bucket, and nitrile gloves were also changed between each bucket. At each site, two buckets were filled with a sum of 16 buckets, and one Ziplock bag was filled for use in testing for heavy metal contamination, and a glass jar was filled for use as a control before the pig is introduced to the soil. After all samples were obtained, the buckets were covered with sterilized tin foil to avoid

contamination and for transportation to the experiment site. The jars were frozen to ensure preservation of any DNA within the sample and the Ziplock bags were kept at room temperature until testing could take place.

Site Set-Up

Soil and pigs were transported to Lubrecht Experimental Forest on August 17th, 2020. Pigs were kept frozen until site set-up was completed. The site of the project contained a chain-link fenced in area which was held in place by four T-posts. The inside of the chain link was lined with chicken wire, and the roof was made of rebar and lined with chicken wire. For the purposes of this project the enclosure was electrified using a solar powered energizer to avoid predation, as wildlife including bears and mountain lions were present in the area. Insulators were attached to the chain link in four rows to avoid a disruption in the electrical conduction. The rows alternated a ground wire and a live wire on the inside of the fence, a tarp was used to cover the ground to avoid leakage of contaminated soils into the area. Buckets containing the soil samples were placed inside the enclosure. Two in each row and eight in each column (Figure 3 and 4). After all the buckets were placed then the pig samples were placed into the buckets and were left to thaw into the dirt. After the pig was placed inside the bucket, the bucket opening was then wired closed with chicken wire to avoid small predators dragging off the samples. The buckets were then left exposed to the elements until active decomposition was completed. Observation from the site were recorded and photographed on a weekly basis for six weeks, as it took six weeks until the pig samples were no longer actively decaying.

Figure 3.3 Overview of Site.

Figure 3.4 Buckets Before Decomposition (First Week).

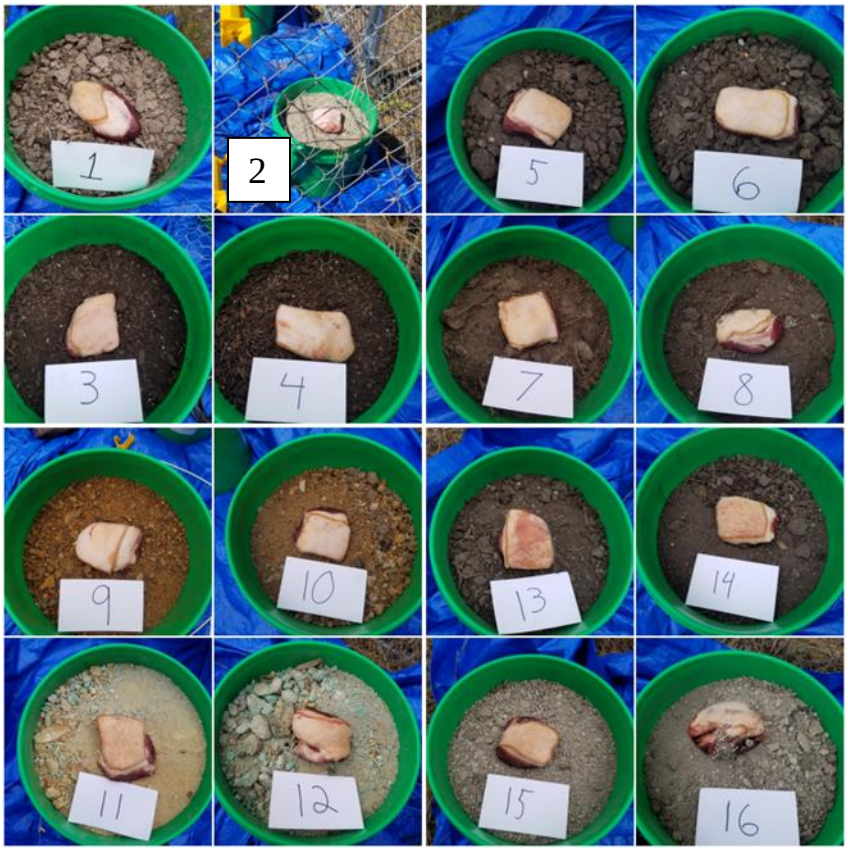
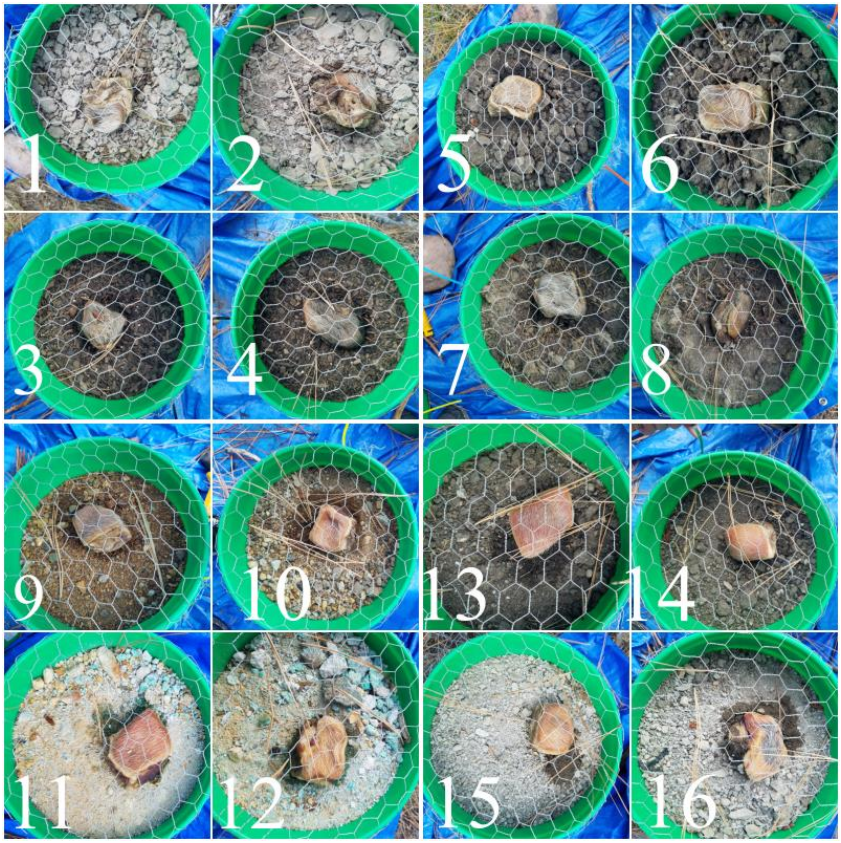


Figure 3.5 Bucket After Six weeks of Decomposition.



Site Clean-up and Sampling

At the end of the sixth week, on September 27th, 2020, the final observations were made and the samples were removed from the area. What remained of the pig samples were placed into individual Ziplock bags and labeled with a number as well as the location of the soil sample. Fresh nitrile gloves were used for each sample. They were kept on ice until they could be put into the freezer. The buckets containing the soil samples were loaded and removed from the site for excavation at a separate location.

The buckets were excavated outside my residence on the patio, using a standard archaeology trowel. The buckets were excavated and sampled in three, four-inch layers. Each sample contained enough soil to fill a 2mL microcentrifuge tube. Between each layer all tools

were sanitized and gloves were worn and sanitized throughout the process to reduce contamination between the layers. Contaminated soil was then transported and disposed of at the depository in Anaconda, MT.

Extraction Procedure: Pig

The pig controls were prepared by cutting the sample into approximately 0.10 grams pieces for extraction, using a razor blade. The samples were cut on top of a disposable cutting board in the Modern DNA Laboratory in the Anthropology Department of the University of Montana. Then the decomposition samples were prepared in the same way. All samples had their DNA isolated using the Charge Switch Forensic DNA Purification kit from Invitrogen. The extraction procedure begins by adding 1mL of lysis buffer and 10uL of Proteinase K (both from the kit) to a microcentrifuge tube, and then the sample was added to the microcentrifuge tube as well. The samples were incubated in a water bath at 55 degrees C for 30 minutes. After incubation, the supernatant was transferred into a fresh microcentrifuge tube. 200uL of Purification Buffer was added to the tube containing the supernatant. Then the Charge Switch Magnetic beads were vortexed and 20uL were added to the tube. The beads were pipetted gently to mix with the supernatant. After mixing the sample was left to incubate at room temperature for one minute and then moved to the Magna Rack for one minute. The supernatant was removed and discarded. The beads were washed with 500uL of wash buffer by pipetting up and down twice, then placed back into the Magna Rack for one minute. The supernatant was removed and discarded again then the tube was removed from the rack and the washing procedure was repeated. After the wash was completed, the sample was then eluted by adding 150uL elution buffer to the tube. The supernatant was mixed by pipetting until the beads were full resuspended within the mixture and then incubated for one minute. The samples were then placed into the

Magna Rack for one minute. Then the eluted DNA was transferred to new tubes, leaving the beads behind. This procedure was completed on all 64 samples of pig remains.

Extraction Procedure: Soil

The DNeasy PowerSoil Pro Kit (Qiagen) was used for extraction of DNA from soil samples. The kit contained Power Bead Pro Tubes, CD1 solution, CD2 solution, CD3 solution, EA solution, C5 solution, and C6 solution. Along with these solutions the kit also contained microcentrifuge tubes, MB spin columns, elution tubes, and collection tubes. 250mg of soil and 800uL of CD1 was added to the Power Bead Pro Tubes. Then an alternative lysis method (alternative lysis method A., pg 18, DNeasy PowerSoil Pro Kit Handbook) was used due to the inability to access a vortex adapter. The alternative lysis method required the Power Bead Pro tubes with the supernatant to be vortexed then heated to 70 degrees C for five minutes and then the procedure was repeated. Next the samples were centrifuged a 15,000 x g for one minute and the supernatant was then moved to a new microcentrifuge tube, and 200uL of CD2 was added. The tubes were vortexed to mix and then centrifuges again at 15,000 x g for one minute. The rest of the soil was then pelleted against the side of the tube and 700uL of the supernatant was transferred to a clean tube. 600uL of CD3 was added to each tube and the lysate was then vortexed and added to the MB Spin Columns. The MB spin column was then centrifuged at 15,000 x g for one minute and the flow-through was discarded and the rest of the lysate from the previous step was placed into the MB Spin Columns and centrifuged again until all of the lysate had passed through the spin column. The spin column was placed into a clean collection tube and 500 uL of EA was added to the spin column. The column was then centrifuged again at 15,000 x g for one minute. The flow-through was discarded and then 500uL of C5 was added to the spin column and a centrifuged at the same settings as above. The flow-through was again discarded

and then the spin column was centrifuged at 16,000 x g for two minutes to ensure all flow-through had moved through the column. The columns were placed into clean elution tubes and 100uL of C6 was placed into the MB Spin Column. Then the samples underwent a final centrifuge at 15,000 x g for one minute, after which the DNA was ready to undergo preparation for Polymerase Chain Reaction (PCR).

PCR Procedure and Gel Electrophoresis

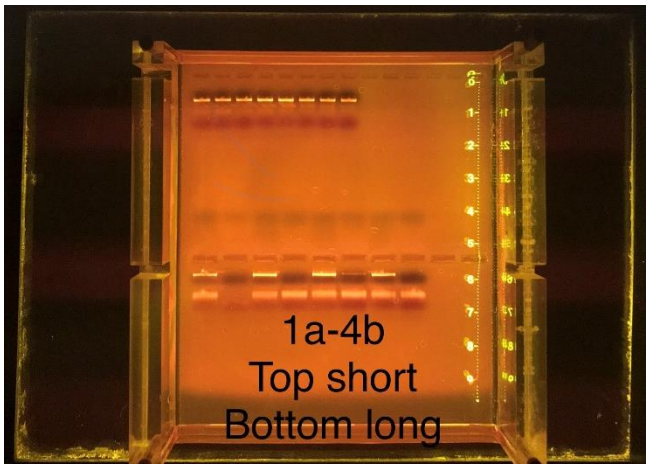
Before PCR both soil and pig samples were analyzed using Qubit technology, on a Qubit4 machine. Qubit dsDNA HS Assay Kit by Invitrogen was used to determine the quantity of DNA within all of the samples. This procedure required 195uL of the Qubit solution and 5uL of the DNA elute. First the kit standards were run in order to get a top range of DNA within a sample and the bottom range of DNA within the samples. Qubit only gives information on the amount of DNA within the sample, not the type of DNA present.

To prepare samples for PCR, short and long forward and reverse pig primers were purchased from Invitrogen. The short primers were CO2susF2 (5'-GCC TAA ATC TCC CCT CAA TGG TA- 3') and CO2susR2 (5'- AGA AAG AGG CAA ATA GAT TTT CG- 3') (Thomas, 2016; Pangallo et al. 2010). This set of primers targets and amplifies a 212 base-pair fragment of the pig mitogenome. The long primers were CytB PorkF (5'- AAC CCT ATG TAC GTC GTG CAT -3') and CytB PorkR (5' ACC ATT GAC TGA ATA GCA CCT- 3') (Sahilah et al. 2012; Monteil-Sosa et al., 2000). This set of primers targets and amplifies a 531 base-pair fragment of the pig mitogenome. The primers need to be diluted into a working concentration (10uM) by putting 10uL of concentrated primers into a microcentrifuge tube and then adding 90uL of water.

PCR mixture was prepared for testing samples. The mixture was created using 8.76uL of water, 2.4uL of 10uM dNTPS, 1.5uL of 10x PCR buffer, 0.45uL of MgCl2, 0.18uL of the 10uM forward and reverse primers, and 0.08uL of Platinum Taq (Invitrogen). Then 3uL of the DNA was added to the cocktail and the samples were vortexed to homogenize the samples. This process was done on all eluted samples for both long and short primers separately. After the DNA has been added to the PCR solution, they underwent PCR. PCR was run for 40 cycles. The samples were heated to 95˚C. Then samples then underwent three stages per cycle: the denaturing process which occurs at 95˚C for 30 seconds, the annealing process occurring at 55˚C for 30 seconds, and the extension process at 72˚C for 30 seconds; at which point the temperature stays at 72 ˚C for and 5 minutes.

After PCR, the samples then underwent gel electrophoresis. A 2% gel was made using one gram of Agarose gel powder combined with 50mL of Tris-borate-EDTA (TBE) Buffer. The mixture was then heated until boiling, and once cooled, 2uL of ethidium bromide was added to the gel mixture. The gel solution was mixed well and poured into a gel tray and the comb was inserted to create the wells. Once the gel had solidified the comb was removed and TBE was poured into the gel rig until the gel was covered and buffer tanks on both sides were filled. The samples were then prepped for the gel by adding 5uL of the DNA to 2uL of loading dye. Next the prepared samples were added to the wells, the electrodes were attached, and the machine was allowed to run for 10 minutes. Then the gel and tray were removed from the apparatus and the gel was placed under UV light. The DNA that amplified glows under UV light and a picture was captured of each gel run (Figure 6).

Figure 3.6 Gel electrophoresis run. Samples 1a-4b were amplified using both primer sets (targeting both long and short segments of the pig mitogenome). As can be seen in the image, all of the short fragments amplified, whereas only some of the longer fragments successfully amplified.



X-ray Fluorescence Testing

X-ray Fluorescence (XRF) testing is used to determine the amount and type of heavy metals found within a soil sample. This project utilized the XRF machine from the Anaconda Deer Lodge County Superfund office with the help and instruction of Neal Schranz, staff engineer at Water & Environmental Technologies. The XRF first had to be calibrated to ensure a proper reading. The system was connected to the computer and then clipped into its benchtop stand. The program was opened on the computer so data and calibration information could be recorded. Then calibration Standard 1 was analyzed in a 30 second scan, the calibration requires the recovery to be between 80-120%. Standard 2 was analyzed in 30 second scan and required the arsenic level to be below 10parts per million (ppm) and the lead to be below 20ppm. Standard 3 and 4 were analyzed in 30 seconds scans and also had to have a recovery level to be between 80-120%. All standards for arsenic, lead, copper, cadmium and zinc were recorded. Next roughly a handful of soil collected from the Airport wetland, the Red Sands on the Old

Works walking trail, the Millcreek shooting range, Jaycee Park, 1804 Hamburg St., and Madison St. railroad tracks in Anaconda Deer Lodge County, as well as soil from Lubrecht Experimental Forest near Ovando Montana, and bagged potting soil from Walmart in Missoula, were placed into clear, quart sized Ziplock bags. The bags were folded over to keep the soil packed together. Then each bag underwent a 30 second scan with the XRF machine in three sections: the right side of the bag, the middle, and the left side. The data for arsenic, copper, lead, cadmium, and zinc were recorded in ppm and automatically exported into an excel file. Next closeout calibrations are completed to make sure the machine remained calibrated throughout all testing. Only the 1st and 2nd standard are used in the closeout. The Standards must follow the same calibrations procedure as before the testing began and the numbers for lead and arsenic must remain similar in both calibration test.

Data Analysis

Next statistical analysis of the data collected throughout the experiment was conducted using IBM's Statistical Package for Social Sciences software (SPSS) version 25. All statistical analyses were conducted with a 95% confidence interval. First, a comparison of means was completed on the pig muscle tissue before decomposition and after decomposition using a paired sample T-test. The variables used to conduct this test were the muscle before decomposition qubit results and the muscle after decomposition qubit result. Then significance value was examined to determined whether the change between the qubit scores before and after decomposition were statistically significant. A paired sample T-test was used to compare the qubit results for skin before and after decomposition as well.

Finally, a One-Way Analysis of Variance test (ANOVA) was conducted on the Qubit results for the different soil layers. This test analyzed the statistical significance of the Qubit

result between each layer based upon the sample number. In this test the groups were the soils layer post-exposure qubit results for layer 1, 2, and 3. The factor in this case was the sample number, as it could be used to only compare the layers for each sample. These statistical analyses will allow for further explanation of the data obtained from the Qubit results.

Results

Observation During Decomposition

The decomposition of the pigs resulted in full mummification of the remains after six weeks of exposure to the elements. Mummification is the point at which the tissue desiccates, becoming hard and containing no or little moisture. The mummification, instead of full decomposition, was likely a result of the hot, dry summers experienced in the region. Full decomposition for the purposes of this project is the point at which remains are no longer visible due to tissue breakdown. The samples were removed from the site once there were no longer any signs of active decay in any of the samples.

Each week the samples were examined for insect activity. All of the buckets experienced maggot colonization except for buckets 11 and 12, which exhibited dead flies but no other sign of fly or maggot activity. Buckets 11 and 12 also showed the slowest rate of observable decomposition. Buckets 1-4 were the first samples to mummify. All observable insect activity had ended by the 4^{th} week, and the samples stopped exhibiting sign of active decay by the 5^{th} week. Active decay is defined as the point in which the remains are undergoing putrefaction and autolysis. Meaning that the enzymes and bacteria are working together to break down the tissues and cells within the remains (DiMaio & DiMaio, 2001).

XRF Results

Samples of soil were examined for the presence of the main heavy metals attributed to the smelting waste. The soil samples were varied in the levels of heavy metals present within

them. In Anaconda, the EPA has ordered residential soil must be remediated if it contains more than 250 parts per million (ppm) of arsenic and/ or 400 or more ppm of lead. In commercial spaces, the remediation threshold for arsenic is 500ppm and there is no determination of the lead threshold identified (Anaconda Deer Lodge County, 2018).

Table 4.1 Average Levels of Heavy Metals within Soil Samples. Level of Detection is abbreviated LOD.

Sample	**Avg. Arsenic Level (ppm)**	**Avg. Lead Level (ppm)**	**Avg. Copper Level (ppm)**	**Avg. Zinc Level (ppm)**	**Avg. Cadmium Level (ppm)**
Potting Soil	<LOD	20.9	110.21	198.97	<LOD
Airport Wetlands	767.95	674.56	5,624.92	2,531.03	<LOD
Madison St.	646.99	238.16	3,505.22	1,797.07	<LOD
Old Works	1527.59	798.32	36,866.93	3,949.06	<LOD
Lubrecht	4.08	5.1	<LOD	84.44	<LOD
Jaycee Park	69.96	75.15	462.89	278.32	<LOD
1804 Hamburg St.	389.15	247.47	2,117.84	933.52	<LOD
Millcreek	78.4	57.44	184.59	229.23	<LOD

Based upon these designations by the EPA, the Airport wetland area, Madison St., and the Old Works walking trail meet the commercial arsenic guidelines for remediation, and 1804 Hamburg St. meets the residential arsenic guidelines for remediation. Interestingly, the Old Works walking trail has drastically higher levels of copper than any of the other samples. As expected, the potting soil and the soil from Lubrecht Experimental Forest had very low levels of heavy metals as they did not come from Anaconda- Deer Lodge County. Also, Millcreek shooting range and Jaycee Park had relatively low levels of heavy metals considering their location within in Anaconda- Deer Lodge County. All samples showed lower than the level of detection (>LOD) of

cadmium in the samples. Overall, the XRF results show that a good spectrum of soil samples was presented for use in this project, allowing for a variety of tests to demonstrate whether or not DNA preservation would occur in any of these conditions.

Soil and Pig Qubit Quantification

The data obtained by the Qubit tests show that double-stranded DNA is present in all samples, however none of the DNA analyzed using Qubit can specifically be identified as pig DNA, as the Qubit merely quantified all DNA regardless of origin. It can be assumed that DNA obtained from pig samples themselves are likely pig DNA, but may also be attributed to other micro-organisms on or within the samples.

Table 4.2 Qubit results for pig samples from before and after the samples had been left in the respective buckets. Samples represent pieces of the pig taken from different parts of the mummified pig.

Sample Number	**Soil Pig was Sampled From**	**Qubit for Muscle (ng/uL)**	**Qubit for Skin/Hair (ng/uL)**
1	Pre-exposure: Lubrecht	1.04	1.12
2	Pre-exposure: Lubrecht	6.52	1.03
3	Pre-exposure: Potting soil	8.8	4.4
4	Pre-exposure: Potting soil	2.81	0.532
5	Pre-exposure: 1804 Hamburg St.	5.96	10.9
6	Pre-exposure: 1804 Hamburg St.	3.4	0.105
7	Pre-exposure: Airport Wetlands	0.0272	4.8
8	Pre-exposure: Airport Wetlands	1.38	1.59
9	Pre-exposure: Madison St.	3.91	2.22
10	Pre-exposure: Madison St.	0.389	1.2
11	Pre-exposure: Old Works	2.56	14.6
12	Pre-exposure: Old Works	1.48	0.492
13	Pre-exposure: Jaycee Park	2.49	10.1
14	Pre-exposure: Jaycee Park	9.64	7.08
15	Pre-exposure: Millcreek	5.36	2

16	Pre-exposure: Millcreek	3.81	13.8
17	Lubrecht	6.64	3.49
18	Lubrecht	7.84	5.24
19	Potting soil	0.199	5.32
20	Potting soil	2.5	1.64
21	1804 Hamburg St	0.944	0.246
22	1804 Hamburg St	1.48	0.52
23	Airport Wetlands	1.55	0.03
24	Airport Wetlands	0.0548	0.0688
25	Madison St	0.038	4.56
26	Madison St	3.33	1.36
27	Old Works- red sand	4.68	0.397
28	Old Works- red sand	1.61	3.72
29	Jaycee Park	0.201	1.42
30	Jaycee Park	0.106	6.96
31	Millcreek	12.6	1.3
32	Millcreek	2.83	5.16

The pre-exposure samples in the case of the pig data represent samples taken from each individual portion of the pig before they were introduced to the respective soil samples. The Qubit analyses show that results vary between the quantity of DNA detected in the muscle tissue samples versus the skin and hair samples. Also, quantities of DNA vary significantly depending on the soil the pig remains were sampled from. Some samples vary greatly even within tissue type and the soil it was sampled from. The statistical analysis used to compare the means of the pre-exposure qubit results to the post-exposure qubit results for muscle indicated no statistical significance between the pre-exposure and the post-exposure qubit results, meaning that it is unlikely that the changes that occurred in the post- exposure significantly attributed to the difference in values of total DNA found within the samples. This indicates that overall changes during decomposition did not significantly affect the amount of total DNA within the remains after decomposition.

Table 4.3 Comparison of Means in Muscle Qubit Results

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
					95% Confidence Interval of the Difference				
		Mean	Std. Deviation	Std. Error Mean	Lower	Upper			
Pair 1	Muscle Before Decomp. - Muscle After Decomp.	.8108375	4.5258219	1.1314555	-1.6008027	3.2224777	.717	15	.485

The pre- and post- exposure skin samples were also compared, demonstrating that was no statistical significance found between the samples, again demonstrating that the changes that occurred in the post- exposure significantly attributed to the difference in values of total DNA found within the samples.

Table 4.4 Comparison of means in pre- and post- exposure skin qubit results

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
					95% Confidence Interval of the Difference				
		Mean	Std. Deviation	Std. Error Mean	Lower	Upper			
Pair 1	Skin Before Decomp. - Skin After Decomp.	2.1585750	5.5242110	1.3810528	-.7850693	5.1022193	1.563	15	.139

The Qubit result on the soil samples are likely more representative of microorganism populations within the soil than that of the DNA left behind by the pigs themselves, as, again, the Qubit is quantifying the total amount of double-stranded DNA present. The pre-exposure soil samples were used to ensure that pig DNA was not present within the soil before the project began, which could only be determined by amplification and a gel electrophoresis. Additionally, only a

single sample was extracted for each of the pre-exposure soil samples, as they were not placed in buckets like the rest.

Table 4.5 Soil Qubit results. Layer 1 is the top layer taken from the sample, with layers 2 and 3 below it (layers taken at 4inch intervals).

Bucket Number	**Location of Sample**	**Sample Number within Layer**	**Layer 1 Qubit Results (ng/uL)**	**Layer 2 Qubit Results (ng/uL)**	**Level 3 Qubit Results (ng/uL)**
1	Lubrecht	1	1.84	1.13	1.9
1*	Lubrecht	2	1.84	3.57	1.76
2	Lubrecht	1	3.09	3.21	8.12
2	Lubrecht	2	2.53	3.88	5.88
3	Potting Soil	1	2.52	5.52	6.28
3	Potting Soil	2	1.44	5.28	9.44
4	Potting Soil	1	1.85	1.34	8.16
4	Potting Soil	2	1.68	3.98	4.8
5*	1804 Hamburg St.	1	3.64	1.44	3.28
5	1804 Hamburg St.	2	1.86	1.68	3.76
6	1804 Hamburg St.	1	2.73	2.88	3.2
6	1804 Hamburg St.	2	2.12	2.2	4.56
7	Airport Wetlands	1	0.908	1.44	4.28
7	Airport Wetlands	2	1.79	1.71	4.48
8	Airport Wetlands	1	0.844	2.18	4.12
8	Airport Wetlands	2	1.59	2.03	5.64
9*	Madison St.	1	0.0556	0.192	0.0864
9*	Madison St.	2	0.134	0.185	0.0992
10*	Madison St.	1	0.113	0.118	0.0997
10*	Madison St.	2	0.226	0.174	0.119
11*	Old Works	1	0.11	0.0632	0.0428
11	Old Works	2	0.034	0.0364	0.0372
12	Old Works	1	0.0352	0.0524	0.0864
12*	Old Works	2	0.0316	0.0812	0.0268
13	Jaycee Park	1	0.848	1.98	9.48
13	Jaycee Park	2	2.5	5.48	6.6

14	Jaycee Park	1	2.14	3.18	4.72
14*	Jaycee Park	2	1.88	9.28	5.36
15	Millcreek	1	0.348	0.416	0.52
15	Millcreek	2	0.46	0.295	0.809
16	Millcreek	1	0.327	0.327	1.1
16	Millcreek	2	0.764	0.105	1.79
Pre-exposure 1	Lubrecht	1	5.08	N/A	N/A
Pre-exposure 2	Potting Soil	1	6.28	N/A	N/A
Pre-exposure 3	1804 Hamburg St.	1	2.45	N/A	N/A
Pre-exposure 4	Airport Wetlands	1	3.85	N/A	N/A
Pre-exposure 5	Madison St.	1	0.292	N/A	N/A
Pre-exposure 6	Old Works	1	0.616	N/A	N/A
Pre-exposure 7	Jaycee Park	1	2.18	N/A	N/A
Pre-exposure 8	Millcreek	1	0.58	N/A	N/A

The pre-exposure samples give an interesting insight into the microbial levels within the soil before the pig was introduced when they were quantified using the Qubit. The microbial levels within the soil maybe of interest to researchers as well as to investigators because the types and levels of microbial DNA found within the soil can indicate not only the biodiversity of the area but may also inform investigator to the possible location of a burial.

In most cases the third layer of the soil from the non-control samples tended to have the highest concentration of total DNA. However, nine of the 32 non-control samples did not follow this trend—these samples are indicated by an asterisk. Overall, these Qubit results give us an idea of the total amount of DNA present in the soil both before and after decomposition of the pig remains occurred.

Statistical analysis of the Qubit results obtained from the post-exposure soil layer samples showed that there is a statistical significance in the difference of the amount of average total DNA found within each layer within each sample. Meaning that there is statistical significance in the

change in the amount of total DNA between the layers, which demonstrates that the amount of DNA varied significantly between the layers of soil taken from each of the buckets.

Table 4.6 Comparison of means between soil layer qubit results.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Layer 1 Qubit Results (ng/uL)	Between Groups	28.959	15	1.931	6.566	.000
	Within Groups	4.704	16	.294		
	Total	33.663	31			
Layer 2 Qubit Results (ng/uL)	Between Groups	112.364	15	7.491	3.771	.006
	Within Groups	31.787	16	1.987		
	Total	144.151	31			
Level 3 Qubit Results (ng/uL)	Between Groups	255.808	15	17.054	13.639	.000
	Within Groups	20.005	16	1.250		
	Total	275.813	31			

Amplification

This project also utilized PCR followed by gel electrophoresis for both the soil and pig samples after exposure to six weeks of the Montana late summer. Each sample underwent amplification of both long and short strands of DNA. This data did not produce information about the differences in DNA degradation among pigs decomposed in the different soil types. Unfortunately, no amplifiable pig DNA was recovered from either the soil or the pig samples after decomposition had occurred.

These unexpected results may have other factors at play, as amplification of the control samples of the pig revealed mixed results even though they were never exposed to the elements.

Table 4.7 Amplification of DNA from Pig Pre- exposure samples prior to samples being placed in buckets.

Sample Number	Destination After Sampling for Control Variables	Tissue Type	Amplification of Short Strands of DNA	Amplification of Long Strands of DNA
1a	Lubrecht-Bucket 1	Muscle	Yes	Yes
1b	Lubrecht-Bucket 1	Skin/Hair	Yes	No
2a	Lubrecht-Bucket 2	Muscle	Yes	Yes
2b	Lubrecht-Bucket 2	Skin/Hair	Yes	No
3a	Potting soil-Bucket 3	Muscle	Yes	Yes
3b	Potting soil-Bucket 3	Skin/Hair	Yes	No
4a	Potting soil-Bucket 4	Muscle	Yes	Yes
4b	Potting soil- Bucket 4	Skin/Hair	Yes	No
5a	1804 Hamburg St- Bucket 5	Muscle	Yes	Yes
5b	1804 Hamburg St. -Bucket 5	Skin/Hair	Yes	No
6a	1804 Hamburg St. -Bucket 6	Muscle	Yes	Yes
6b	1804 Hamburg St. -Bucket 6	Skin/Hair	Yes	Yes
7a	Airport-Bucket 7	Muscle	Yes	Yes
7b	Airport-Bucket 7	Skin/Hair	Yes	Yes
8a	Airport-Bucket 8	Muscle	Yes	Yes
8b	Airport-Bucket 8	Skin/Hair	Yes	No
9a	Madison St.-Bucket 9	Muscle	Yes	Yes
9b	Madison St.-Bucket 9	Skin/Hair	Yes	Yes
10a	Madison St.-Bucket 10	Muscle	Yes	Yes
10b	Madison-Bucket 10	Skin/Hair	Yes	Yes
11a	Old Works-Bucket 11	Muscle	Yes	Yes
11b	Old Works-Bucket 11	Skin/Hair	Yes	Yes
12a	Old Works-Bucket 12	Muscle	Yes	Yes
12b	Old Works-Bucket 12	Skin/Hair	Yes	Yes
13a	Jaycee Park-Bucket 13	Muscle	Yes	Yes
13b	Jaycee Park- Bucket 13	Skin/Hair	Yes	Yes
14a	Jaycee Park- Bucket 14	Muscle	No	Yes
14b	Jaycee Park-Bucket 14	Skin/Hair	Yes	Yes
15a	Millcreek-Bucket 15	Muscle	Yes	Yes
15b	Millcreek-Bucket 15	Skin/Hair	Yes	Yes
16a	Millcreek-Bucket 16	Muscle	Yes	Yes
16b	Millcreek-Bucket 16	Skin/Hair	Yes	Yes

Any control samples not amplifying (a “No” in the last two columns) means that when these pigs were placed into the soil samples, there was reduced chance of amplification in the samples taken after decomposition, as the DNA present was either damaged or in too low an amount to allow for amplification even prior to mummification. The majority of samples that did not amplify were long strands of DNA from the skin and hair samples. However, one of the samples of muscle tissue from Jaycee Park did not amplify, but the sample did have amplification of long strands. The amplification process did not give much insight into the effect of soil on DNA degradation due to the lack of amplification from all samples except the pre-exposure pig samples.

Discussion

The goal of this project was to evaluate and begin a discussion and research into DNA degradation during decomposition in Montana. This research was a preliminary study into the effects of soil type, pollution, and decomposition has on the preservation of DNA within soil, as well as within the pig sample left on top of it. This research also provides information about the microbial community within different soil samples and opens many avenues for new research.

Hypothesis A

Hypothesis A stated that high heavy metals concentration within the soil will inhibit the spread of DNA within the soil and will result in shorter DNA strand length. It was expected that amplification (Polymerase Chain Reaction) of longer targeted pieces of DNA will be unsuccessful in soil that has been more heavily contaminated with heavy metals. This hypothesis could not be tested in in this framework, as no pig DNA within the soil or within the post-exposure pig sample amplified. It is likely that changes to variables such as length of environmental exposure, burial type, size of pig samples, season, or exposure to elements may influence the ability for amplification of the DNA, and therefore ability to prove or disprove this hypothesis.

Hypothesis B

Hypothesis B states that high heavy metal contamination within a sample would reduce the quantity of the total DNA within a soil sample. It would then be expected that the amount of DNA found in the soil would be quantifiably lower in soil with higher heavy metal (arsenic, lead, etc.) concentrations. This hypothesis appears to be supported based on the data we have observed. The data shows that Madison St. and Old Works walking trail which had high levels of heavy metal detect within the soil, tended to have significantly lower amounts of total DNA within the soil both pre- and post- exposure to the pig samples. In contrast, Lubrecht

Experimental Forest and the potting soil sample had low levels of heavy metals detect and tended to contain larger quantities of total DNA, especially the samples taken from the bottom layer of the buckets However, two outliers exist within this data. Millcreek which had significantly lower level of heavy metals within the sample, also shows a low yield of total DNA. This may be due to this sample being obtained near a gun range and tended to contain the most amount of gravel within the soil. The other outlier was the Airport samples: the heavy metal levels were high in this sample, but the soil tended to have a slightly elevated level of DNA found within the layers of the soil. This may be due to plant material, as the qubit detected the higher levels of total DNA in the bottom bucket layer, and this layer corresponds to the top layer of soil at the site. In this area there was a great deal of vegetation as it was near the Warm Spring creek.

Hypothesis C

Hypothesis C predicted high heavy metal concentration within the soil will lead to more degradation (strand breakage) of DNA within the decomposing pig remains, resulting in the inability to amplify longer targeted sequences of DNA. Based upon the results of this study, this hypothesis was also not able to be examined as none of the decomposed pig samples' DNA amplified. This suggests that other factors caused all post- exposure sample to degrade beyond the point of detection in PCR, causing amplification failure. As stated previously, this may mean that a change in the variables suggested in Hypothesis A may allow amplification of the pig DNA and allow this hypothesis to be accepted or rejected.

Amplification Failure

One of the most interesting and puzzling outcomes that resulted from this experiment was the inability to amplify pig DNA from the soil or the remains after the mummification.

It is unclear why the samples did not amplify and maybe a result of faulty primers (although very unlikely), problems within the protocol, researcher error, or an actual lack of pig DNA within the samples, possibly due to mummification and/or the decision to leave the remains on the surface of the soil. Although it is possible that a mistake was made, all soil samples, unamplified, pre-exposure pig samples, and post-exposure pig DNA were all re-analyzed using PCR and gel electrophoresis. Also, it is unlikely that researcher error or faulty primers played a role in the inability to amplify the DNA from pigs or soil after decomposition, as the pig pre-exposure samples amplified using the same primers as well as the same protocol. This suggests that it is more likely that mummification and the surface burial played a role in the amplification failure.

Arning and Wilson researched bacterial aDNA preservation within calcified lung pluera, ice cores, preserved internal organs, coprolites, mummified remains, skeletal lesions, dental calculus, and dental pulp (2020). They found that mummified remains were unable to preserve the full representation of the human microbiome. The changes during decomposition and the mummification process lead to a shift in the types of bacteria able to survive within the remains, as well as take-over by exogenous bacterial colonies as a result of the decomposition process (Arning & Wilson, 2020). This research suggests that the endogenous pig DNA was potentially swamped out by bacterial DNA. Also, it is likely that as the remains underwent putrefaction as well as autolysis, and DNA was degraded severely after decomposition. Had the sample been buried there may have been a greater possibility of amplification, as it may have had less environmental exposure and may have fully decomposed allowing more DNA to be released and bound to elements within the soil.

Another possible factor in amplification failure may have been the size of the sample leading to lower DNA levels to begin with. Additionally, during butchering the animal is drained

of its blood and internal organs shortly after death, resulting in less genetic material overall (Thomas, 2019).

Soil layers Qubit Results and XRF Observations

This project did obtain data on the measurement of total DNA within the soil and the mummified pig remains. Interestingly, it is possible to see difference in the amount total DNA within the layers of soil in comparison to the location. Old Works walking trail, Madison St., and Millcreek all show much lower qubit results in all layers compared to the other samples. Madison St. and Old Works walking trail also had very high signatures of all five of the heavy metals found in the area. However, the airport had higher levels of heavy metals than what was found at Madison St., yet the total DNA yield did not exhibit the same trend as Madison St. with the soil qubit results.

Upon analysis of each sample at all three layers, in 23 of the 32 cases the highest total soil DNA qubit yields occurred in the third (bottom) layer. This suggest that the DNA maybe migrating down as well as experiencing different levels of exposure between the layers. The top layer of soil would have been in contact with sunlight and would dry out faster than the darker, cooler bottom layer. This area within the buckets were generally more conducive to the microbial community within the buckets, the effects of which on DNA preservation remain to be explored.

Relationship Between Pre- and Post-exposure Pig Samples

When the average pre-exposure pig Qubit quantifications were compared to the post-exposure pig Qubit quantification, the paired T-test found that the change observed within the quantifications was not statistically significant. Meaning that the amount of DNA quantified by the Qubit machine is not significantly different after the pig underwent decomposition. However,

the pre-exposure pigs show amplification of pig DNA in most cases, whereas the post-exposure pigs show no amplification. Logically, this shows that DNA from the remains had to have been overrun by microbial DNA, or we likely would have seen a statistical significance between the amount of DNA obtained pre- and post- exposure in both skin and muscle tissues. Also, according to Young et al. samples need to be stored at a -80˚C or else microorganisms and fungus may continue to degrade DNA (2014; Thomas, 2019). The samples in this case were stored at a common freezer temperature of about -20˚C, which would not completely stop microbes from degrading the sample. This may be the reason that some of the pre-exposure, could not be amplified.

Overall, this study has given some insights into DNA degradation of surface burials in soil types from Western- Montana. This research currently indicates that there is poor preservation of DNA in muscle and skin tissue in the context of surface burials, likely due to environmental causes.

Limitations

This study has several limitations that may impact future research. The most major limitation is the use of pig remains as a proxy for human remains. Despite many similarities between pigs and humans, there are still distinct and important differences. This research assumes that the pig DNA will behave similarly to human DNA if exposed to the same environments.

It another limitation was that decomposition occurred in such a confined space that it may have changed the behaviors and degradation of the DNA from what would have occurred in more natural environment. The bucket may have played a larger role in the outcome of the experiment. For the purposes of the experiment, the confinement of the sample as well as the role

of the bucket were not considered as variables in the experiment, as they remained constant for each sample.

Degradation after sampling was not considered and samples were not stored at the proper temperature of -80˚C (as the Snow Lab does not have access to this expensive piece of equipment), nor were they analyzed on the same day (Young et al, 2014; Thomas 2019). Multiple time gaps occurred in the analysis of samples. Again, for the purposes of this project this was not factored into the project.

Future Research

This study only gave preliminary findings of the possibilities of researching DNA degradation and the effect soil type has on this process. There are numerous possible ways that this research can be adapted or expanded upon. DNA degradation processes could be compared in remains that are buried, submerged in water, within different structures, etc. This research could be expanded by doing an analysis of DNA degradation across time, by collecting DNA samples at specific intervals for a period of time.

There are also many ways to examine the microbial environment of a burial. Microbial composition in a burial could be examined over time, which could expand on the effects of soil type or contaminants on the microbial community. Using shotgun sequencing to identify and compare the microbe species found with the burials or remains would allow for this kind of research. The possibilities and advancing technologies are leading to an expansion in microbiome research, identification of individuals using DNA obtained from many sources as well as many other forensic and archaeological avenues for investigating death and it processes.

Conclusion

This study was unable to determine whether heavy metal play a role on DNA degradation in decomposed remains. It is also unknown as to whether exposure to heavy metal will result in short strand length in the DNA. The limited data collected in this study does however show that less DNA tends to be quantified in soils containing high levels of heavy metals but several other factors including soil type and pH may be factors in this trend. Overall, the main observation in this study shows that surface burial and mummification of remains will likely lead to poor preservation of DNA from the remains. Further research needs to be done in order to understand factors effecting DNA preservation in different soil types.

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Appendix 1: Decomposition Progress Photos

Photo A.1 Decomposition photos week 2

Photo A.2 Decomposition photos week 3

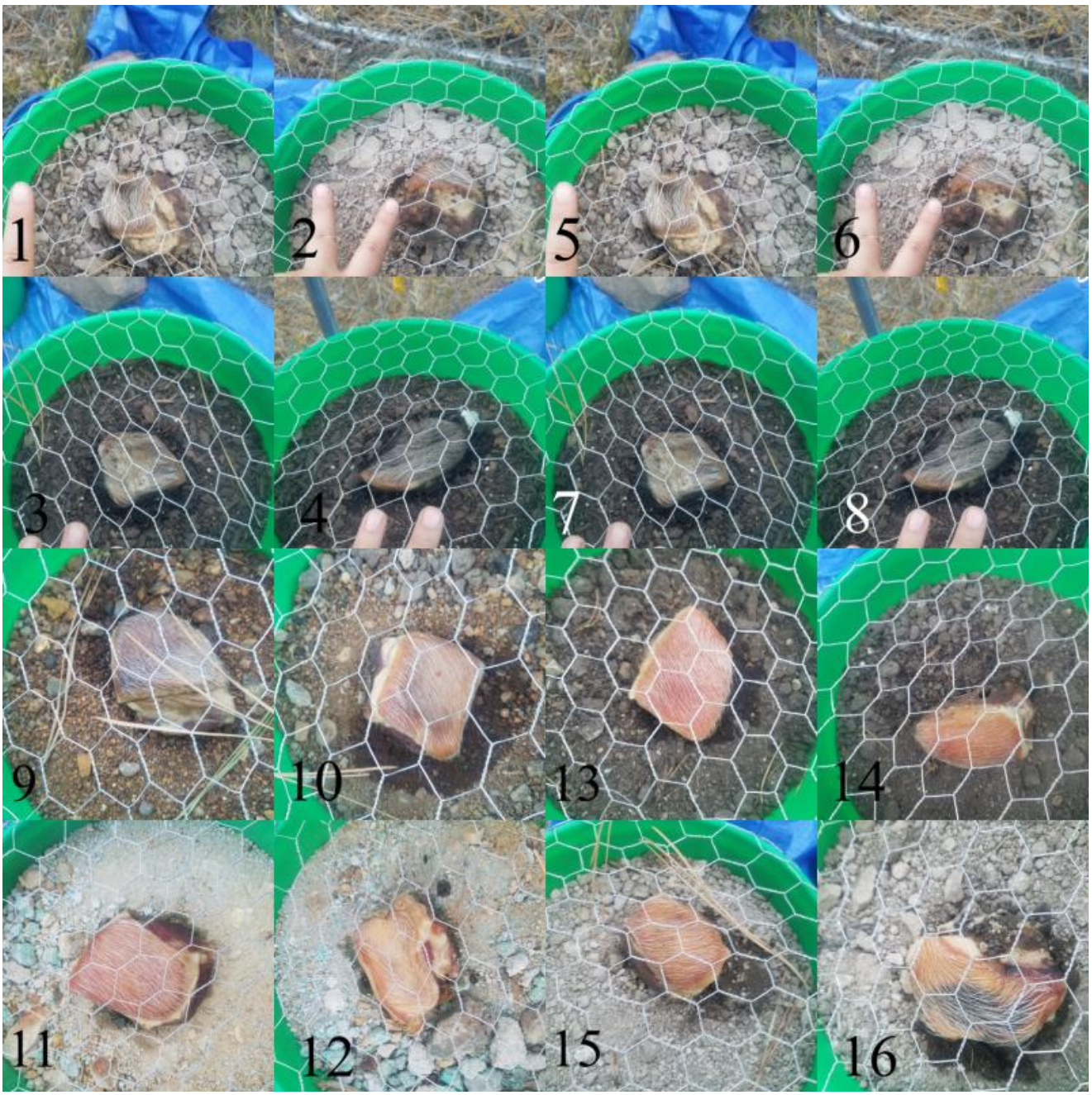
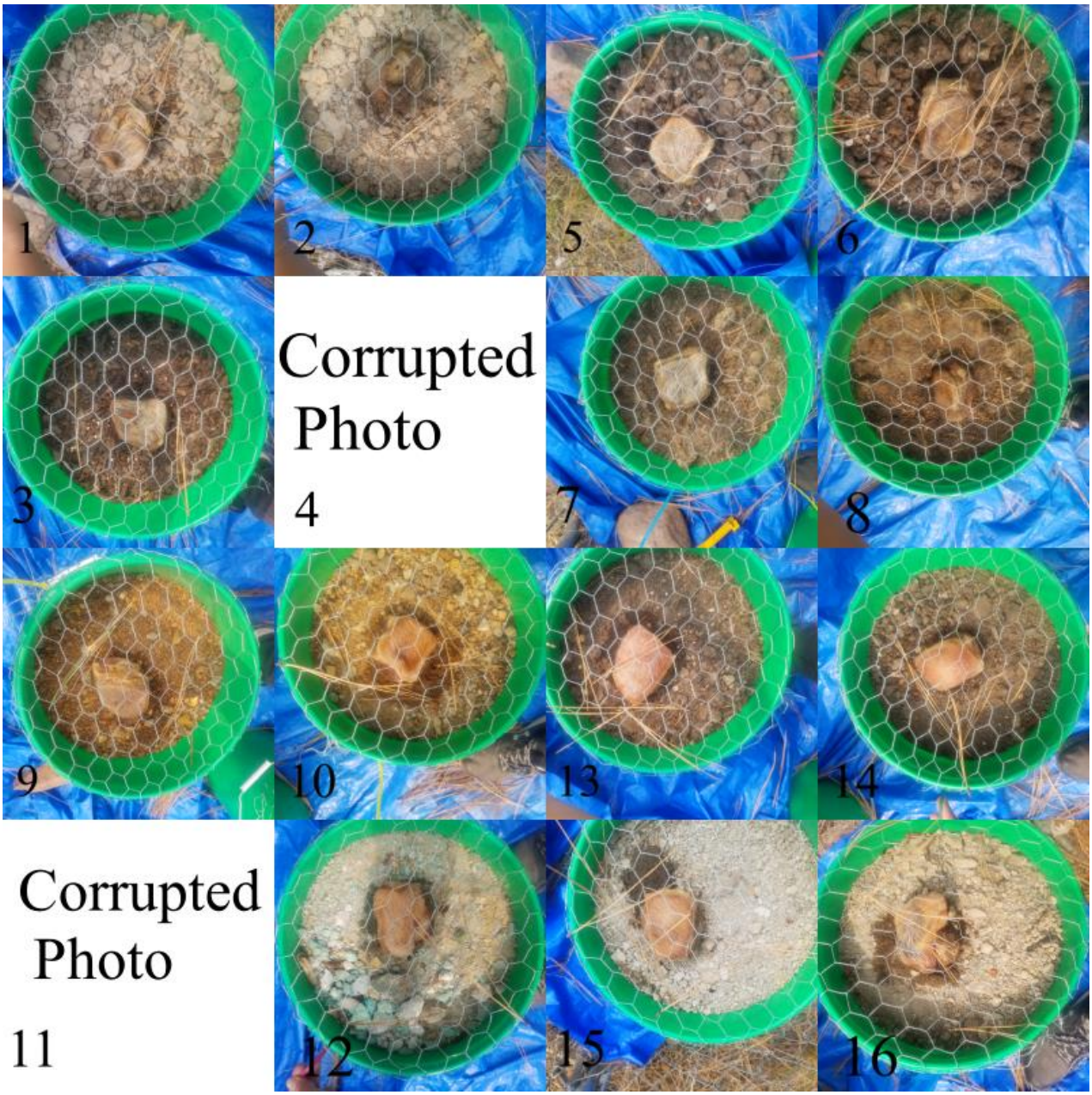
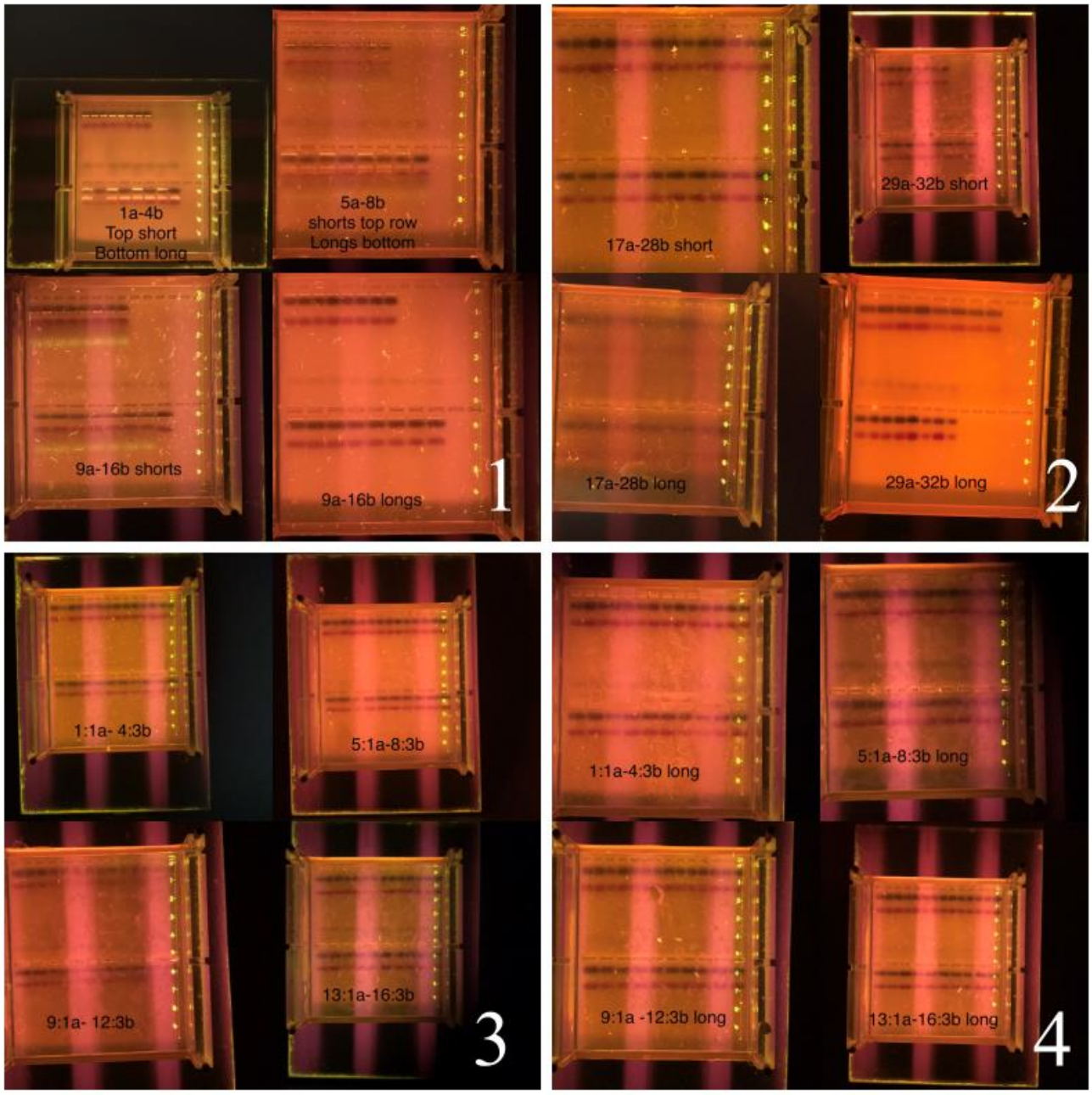


Photo A.3 Decomposition photos week 4

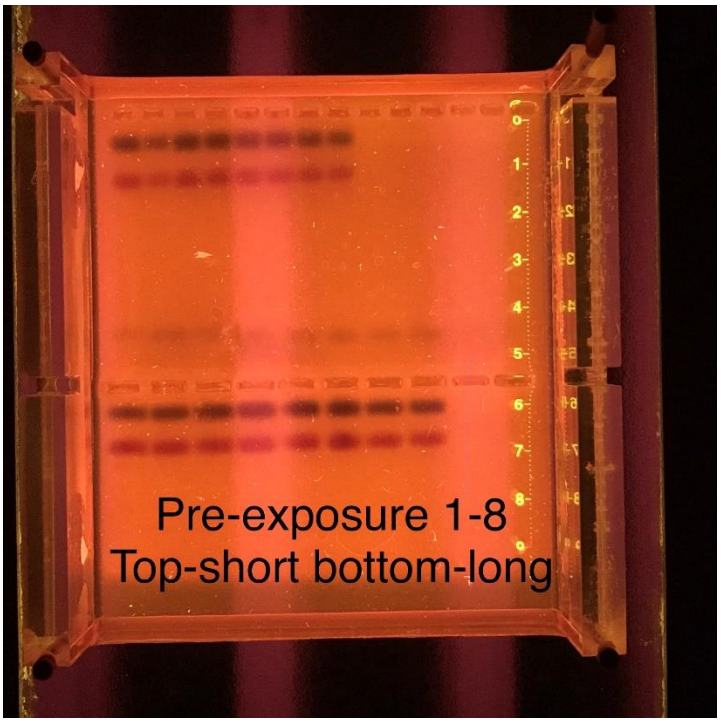
Photo A.4 Decomposition photos week 5



A.5 Gels: Box 1 depicts pre-exposure pig samples with amplification. Box 2 depicts post-exposure pig samples with no amplification. Box 3 and 4 depict post-exposure soil samples with no amplification.



A.6 Gel for pre-exposure pig with no amplification


Pre-exposure 1-8
Top-short bottom-long

References

Abadin H, Taylor J, Buser M, Scinicariello F, Przybyla J, Klotzbach J, Diamond G, Citra M, Chappell LL, Mcllroy LA. Toxicology Profile for Lead. U.S. Department of Health and Human Services. 2020; 256-262.

Alaeddini, Reza, Walsh, Simon J, & Abbas, Ali. (2009). Forensic implications of genetic analyses from degraded DNA—A review. Forensic Science International: Genetics, 4(3), 148-157.

Bogas V, Carvalho M, Anjos MJ, Pinheiro MF, Corte-Real F. Genetic identification of degraded DNA samples buried in different types of soil. Forensic Science International: Genetics Supplement Series 2009;2(1):169–71. https://doi.org/10.1016/j.fsigss.2009.08.031.

Burns KR. Forensic anthropology training manual. Boston:Pearson Education, Inc., 2013.

Byrd, J.H. & Castner, J.L. (2001). Forensic Entomology: The Utility of Arthropods in Legal Investigations. Boca Raton, FL: CRC Press.

Catts, E.P. (1992). Problems in estimating the postmortem interval in death investigations. Journal of Agricultural Entomology, 9, 245-255

Cablk ME, Szelagowski EE, Sagebiel JC. Characterization of the volatile organic compounds present in the headspace of decomposing animal remains, and compared with human remains. Forensic Sci Int. 2012;220:118–25.

Chou S, Harper C, Ingerman L, Llados F, Colman J, Chapell L, Osier M, Odin M, Sage G. Toxicological Profile for Arsenic. U.S. Department of Health and Human Services. 2007; 199-210.

Connor M, Baigent C, Hansen ES. Testing the Use of Pigs as Human Proxies in Decomposition Studies. Journal of Forensic Sciences 2018;63(5):1350–5. https://doi.org/10.1111/1556-4029.13727.

Dent BB, Forbes SL, Stuart BH. Review of human decomposition processes in soil. Env Geol 2004;45(4):576–85. https://doi.org/10.1007/s00254-003-0913-z.

DiMaio, V.J., DiMaio D. 2001. Forensic Pathology, 2nd ed. Boca Raton, FL: CRC Press.

Dorsey A, Ingerman L, Swarts S. Toxicological Profile for Copper. U.S Department of Heath and Human Services. 2004; 66-69

Emmons, Alexandra L, DeBruyn, Jennifer M, Mundorff, Amy Z, Cobaugh, Kelly L, & Cabana, Graciela S. (2017). The persistence of human DNA in soil following surface decomposition. Science & Justice, 57(5), 341-348.

Forbes SL, Perrault KA. Decomposition odour profiling in the air and soil surrounding vertebrate carrion. PLoS ONE. 2014;9:e95107.

Haile, James, Holdaway, Richard, Oliver, Karen, Bunce, Michael, Gilbert, M. Thomas P, Nielsen, Rasmus, . . . Willerslev, Eske. (2007). Ancient DNA Chronology within Sediment Deposits: Are Paleobiological Reconstructions Possible and Is DNA Leaching a Factor? Molecular Biology and Evolution, 24(4), 982-989.

Hansen A, Willerslev E, Wiuf C, Mourier T, Arctander P. 2001. Statistical evidence for miscoding lesions in ancient DNA templates. Mol Biol Evol. 18:262–265.

Haskell NH, Schoenly K, Hall RD. Testing reliability of animal models in research and training programs in forensic entomology, part II, final report. Washington, DC: U.S. Department of Justice, National Criminal Justice Reference Service (NCJRS), 2002

Hebsgaard, Martin B, Gilbert, M. Thomas P, Arneborg, Jette, Heyn, Patricia, Allentoft, Morten E, Bunce, Michael, . . . Willerslev, Eske. (2009). 'The Farm Beneath the Sand' – an archaeological case study on ancient 'dirt' DNA. Antiquity, 83(320), 430-444.

IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.

Johnson LA, Ferris JAJ. Analysis of postmortem DNA degradation by single-cell gel electrophoresis. Forensic Science International 2002;126(1):43–7. https://doi.org/10.1016/S0379-0738(02)00027-0.

Kenneth G. Schoenly, Neal H. Haskell, David K. Mills, Carine Bieme-Ndi, Kristle Larsen, & Yer Lee. (2006). Recreating Death's Acre in the School Yard: Using Pig Carcasses as Model Corpses, to Teach Concepts of Forensic Entomology & Ecological Succession. The American Biology Teacher, 68(7), 402-410. doi:10.2307/4452028

Keough N, Myburgh J, Steyn M. Scoring of Decomposition: A Proposed Amendment to the Method When Using a Pig Model for Human Studies. Journal of Forensic Sciences 2017;62(4):986–93. https://doi.org/10.1111/1556-4029.13390.

Lydolph M, Jacobsen J, Arctander P, Gilbert M, Gilichinsky D, Hansen A, Willerslev E, Lange L. 2005. Beringian paleoecology inferred from permafrost-preserved fungal DNA. Appl Environ Microbiol. 71:1012–1017.

Mitchell D, Willerslev E, Hansen A. 2005. Damage and repair of ancient DNA. Mut Res. 571:265–276.

Monteil-Sosa J.F., Ruiz-Pesini, E., Montoya, J., Roncales, P., Lopez-Perez, M.J. & Perez-

Martos, A. 2000. Direct and highly species-specific detection of pork meat and fat in meat products by PCR amplification of mitchondrial DNA. Journal of Agricultural and Food Chemistry 48: 2829-2832.

Obaid F, Ashizawa A, Wright S, Tucker P, Jenkins K, Ingerman L, Rudisill C. Toxicological Profile for Cadmium. U.S. Department of Health and Human Services. 2012; 184-191.

Pangallo D, Chovanova K, Makova A. Identification of animal skins of historical parchments by polymerase chain reaction (PCR)-based methods. Journal of Archaeological Science Dec. 2010;37:1202-1206.

Perrault, K.A., Stefanuto, PH., Stuart, B.H. et al. Detection of decomposition volatile organic compounds in soil following removal of remains from a surface deposition site. Forensic Sci Med Pathol 11, 376–387 (2015). https://doi-org.weblib.lib.umt.edu:2443/10.1007/s12024-015-9693-5

Roney N, Smith CV, Williams M, Osier M, Paikoff SJ. Toxicological Profile for Zinc. U.S. Department of Health and Human Services. 2005; 73-75

Rosier E, Cuypers E, Dekens M, Verplaetse R, Develter W, Van de Voorde W, et al. Development and validation of a new TD-GC/MS method and its applicability in the search for human and animal decomposition products. Anal Bioanal Chem. 2014;406:3611–9.

Sahilah, A.M. & Nazri, Wan & Shahimi, Safiyyah & Yaakob, Norhayati & Abdullah Sani, Norrakiah & Abdullah, Aminah & Babji, Abdul & Ghani, Maaruf. (2012). Comparison Between Pork and Wild Boar Meat (Sus scrofa) by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). Sains Malaysiana. 41. 199-204.

Schoenly KG, Haskell NH, Hall RD, Gbur JR. Comparative performance and complementarity of four sampling methods and arthropod preference tests from human and porcine remains at the forensic anthropology center in Knoxville, Tennessee. J Med Entomology 2007; 44: 881– 94

Stadler S, Stefanuto PH, Brokl M, Forbes SL, Focant JF. Characterization of volatile organic compounds from human analogue decomposition using thermal desorption coupled to comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry. Anal Chem. 2013;85:998–1005.

Steadman DW, Dautartas AM, Kenyhercz MW, Vidoli GM, Jantz LM, Mundorff AZ. Differential scavenging activity among pig, rabbit and human subjects. J Forensic Sci. In press.

Vass AA. Odor mortis. Forensic Sci Int. 2012;222:234–41.

Willerslev E, Hansen A, Binladen J, Brand T, Gilbert M, Shapiro B, Bunce M, Wiuf C, Gilichinsky D, Cooper A. 2003. Diverse plant and animal genetic records from Holocene and Pleistocene sediments. Science. 300:791–795.

Willerslev E, Hansen A, Poinar H. 2004. Isolation of nucleic acids and cultures from fossil ice and permafrost. Trends Ecol Evol. 19:141–147.

Willerslev E, Hansen A, Rønn R, Brand T, Barnes I, Wiuf C, Gilichinsky D, Mitchell D, Cooper A. 2004. Long-term persistence of bacterial DNA. Curr Biol. 14:R9–R10.