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DNA ANALYSIS ON CERAMIC COOKING VESSELS

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BY

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Abstract

Ceramic vessels are a durable material found in abundance throughout the archaeological record. Organic residue analysis has been used to study ceramic vessels utilizing lipid analysis and protein analysis. Lipids and proteins analysis provide limited information at the genus level, leaving out valuable information that species-specific results can tell us such as unique genetic and environmental information. Lipids and proteins analysis provide limited information at the genus and taxonomic levels leading to issues of misclassification of species. If only certain animals or plants of a genus are being used, then unique genetic and environmental information found only at the species level cannot be determined, such as the geographical region of the animal or plant. Studies of DNA analysis on durable archaeological material have been limited to stone tools analysis, using 5%Ammonium hydroxide and PB buffer protocols developed by Shank et al. (2001) for DNA retrieval. There are no current publications on DNA analysis of ceramic vessels.

In this research of DNA extraction using the 5%Ammonium hydroxide and guanidine hydrochloride protocols developed by Shank et al. (2001) for DNA retrieval was used to test 11 archaeological ceramic shards and 16 experimental ceramics shards that were either used to store or cook foodstuff. This paper explores the possibility of DNA extraction and amplification from ceramic shards using the protocols developed by Shanks et al. (2001).

While some DNA was amplified and sequenced from archeological samples, the source of the DNA was due to human and bacterial contamination. No DNA was successfully amplified from the experimental samples. The overall results show that DNA extraction and amplification using the methods developed by Shanks et al. (2001) do not work on ceramic vessels.

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Table of content

List of Figures

List of Tables

CHAPTER 1

INTRODUCTION

1.1 Introduction and Aims

Ceramic shards are a durable material that survive in high frequency in the archaeological record. In the past, the majority of the information obtained from ceramic shards was based on the shape, what they were found with, and where they were found (Eerkens, 2007; Evershed, 1993b; Rice, 1996). Today, organic residue analysis lets us infer the use of said objects, allowing for a better understanding of people's daily behavior through the quantitative and qualitative information gained by looking at absorbed and surface lipids that are preserved on vessels (Eerkens, 2007; Evershed, 1993b; Hall & McGovern, 2016; Rice, 1996). Despite the benefits, organic residue analysis has a limit to the amount of information that can be obtained. Animal and plant identification are limited to the genus level, creating the problem of misclassifying animals and plants that are closely related (Evershed, 1993a, 1993b; Hall & McGovern, 2016; Oudemans & Boon, 2007). For a deeper understanding of how past populations used ceramic pottery, ancient DNA (aDNA) is needed (Hofreiter, Serre, Poinar, Kuch, & Paabo, 2001).

DNA is found in every living organism and holds information on variation, species identification, ancestry, etc., making it useful to reconstruct the environment, seasonal population movement, and diets (Hofreiter et al., 2001; Kaestle & Horsburgh, 2002). This was not possible until the 1980's when the molecular technique known as Polymerase Chain Reaction (PCR) was developed (Kaestle & Horsburgh, 2002). PCR amplifies DNA, making multiple copies of a single fragment of a region of the genome. The ability to make copies of DNA allows us to study DNA, especially after it starts to decay after death, presenting a unique opportunity to

incorporate aDNA into anthropological research (Hofreiter et al., 2001; Kaestle & Horsburgh, 2002).

Two types of DNA are used for genetic studies: mitochondrial (mtDNA), and nuclear, all of which hold a variety of genetic information (Snow 2018; Mielke et al. 2011). Ancient DNA genetic studies often focus on mtDNA. The finite size, lack of recombination, weak selection, high mutation rate, and its abundance in the cell make mtDNA ideal for studying aDNA (Snow 2018; Kimura et al. 2001; Eerkens, 1993).

Ancient DNA studies are not limited to biological remains or organic material, such as osteological material, textiles, and foodstuff. Studies have now branched out to incorporate cultural material, which are found in abundance within an archaeological site (Loy, 1993; Shanks, Kornfeld, & Ream, 2004). Stone tools and ceramic shards are durable materials that tend to be well preserved. DNA has been successfully extracted from stone tools, opening new doorways of examining archeological sites (Hofreiter et al., 2001; Kaestle & Horsburgh, 2002; Paabo, 1989; Shanks et al., 2004; Snow, 2018).

Stone tools, unlike organic material, are more likely to be preserved and tend to be widely found in archaeological sites (Loy, 1993; Rice, 1996). Shanks et al., developed a set of protocols to extract aDNA from the microcracks of stone tools (2004). mtDNA has been successfully extracted and amplified from multiple stone tools from various archaeological sites using these protocols (Shanks et al., 2004). The analysis of the information obtained from DNA found in stone tools allows us to gain a greater understanding of the daily lives and behaviors of past populations that we do not get from organic residue analysis (Evershed, 1993; Hofreiter et al., 2001; Rice, 1996).

Ceramic shards have not been a focus for aDNA analysis. Analysis of ceramic shards primarily relies on organic residue analysis, limiting the type of information that is obtained (Evershed, 1993a; Hall & McGovern, 2016; Rice, 1996). Unglazed ceramics have been shown to preserve absorbed residues, such as lipids and proteins. Absorbed residues are protected within the ceramic vessel's walls from outside contamination and the environment, which presents the possibility that aDNA can also be preserved inside the walls of unglazed ceramics (Eerkens, 2007; Evershed, 1993a; Hall & McGovern, 2016; Oudemans & Boon, 2007; Rice, 1996).

1.2 Research Goals and Significance

This project explores the possibility of aDNA/DNA extraction and sequencing from ceramic cooking vessels, using two protocols developed by Shanks et al. (2001) for stone tools. Analysis will be performed on experimental unglazed ceramic shards fired in a fire pit and kiln and ceramic shards found in the archaeological record obtained from Steven LeBlanc of the Peabody Museum at Harvard. This will test the possibility that DNA can be preserved within the walls of ceramic shards, and whether the protocols developed by Shanks et al. (2001) for stone tools can be used on ceramic shards. The project will also explore the possibility of whether aDNA analysis of ceramic vessels are valuable in studying the daily lives and behaviors of past populations.

Typically, analysis of ceramic vessels is conducted using several types of organic residue analysis on lipids and proteins (Eerkens, 2007; Evershed, 1993b; Rice, 1996). However, organic residue analysis only allows us to look at the genus level of plants and animals. To gain a better understanding of their environments, cultural practices, and migrations patterns, we need to be able to distinguish between plants and animals at the species level (Hofreiter et al., 2001; Kaestle & Horsburgh, 2002). By using aDNA from cooking vessels we can identify what animals and

plants are being consumed at the species level, and thereby reconstruct populations diets, environment, and seasonal population movements. The information obtained from aDNA will provide a more in-depth interpretation of the daily lives and behavior of past populations that we have not been able to obtain through organic residue analysis (Hofreiter et al., 2001; Kaestle & Horsburgh, 2002).

1.3 Hypothesis

The following two hypotheses will be tested in this study: (1) Absorbed aDNA can be extracted and sequenced from archeological ceramic cooking or foodstuff storage vessels using the 5% ammonium hydroxide protocols developed by Shanks et al (2001), and (2) The 5% ammonium hydroxide and guanidine hydrochloride protocols developed by Shanks et al (2001) can be used to extract absorbed DNA from experimental ceramic cooking vessels

1.4 Thesis Outline

This chapter outlines the aim, hypothesis, and significance of the study in order to introduce the basis on which this study is built.

Chapter 2 is a literature review. This chapter evaluates research from literature relevant to aDNA extraction and organic residue analysis involving cultural artifacts. It includes an examination of the various types of absorbed organic residue analysis that have been used to study past populations. This section also examines aDNA analysis on stones tools from various archaeological sites.

Chapter 3 provides an overview of materials and methodologies of the project, including a detailed description of aDNA extraction protocols used and detailed steps on creating experimental samples will be outlined.

Chapter 4 provides a summary of the results of this study, demonstrated in tables and figures.

Chapter 5 is the discussion of the results of the aDNA extraction.

Chapter 6 concludes this thesis. Questions, aims, and conclusions drawn from the results will be revisited. A summary of the project will be drawn, along with future implications and research.

Chapter 2

Literature Review

2.1 Introduction

Molecular analysis of organic residues found on ancient pottery has become an important focus in interpreting the archaeological record. The information obtained through organic residue has become a fundamental source of information on historic consumption practices, economies, and even cultural practices. This analysis method allows us a deeper understanding of the daily lives and behaviors as far back as around 100,000 years (Loy, 1993).

Interpretation of artifacts in the archaeological field has moved past the 'functionalist' theoretical paradigm that was prominent in the 1930s through the 1950s, which focused on the premise that an object must have provided some advantage to those who used it (Oudemans and Boon 2007; Evershed 1993; Loy 1993). Humans are complex creatures that have developed multiple tools for specific tasks. Ceramic vessels have different designs for storage, cooking, hunting, etc., and absorbed residues from the vessel's use can be studied through biomolecular residue analysis to give insight into how past population used these items (Evershed 1993; Barnard, Lanehart, and Malainey 2007; Eerkens 2007; Rice 1996).

Today, organic residue analysis lets us examine minuscule amounts of complex organic material, giving us a higher chance of finding preserved residue within an archaeological site (Evershed 1993; Rice 1996). One of the most significant problems with organic residue analysis is the role the environment plays in its degradation. The environment affects organic residues differently, some of which have higher resistance to degradation making them easier to extract (Oudemans and Boon 2007; Malainey and Figol, 2015; Eerkens, 2007). Lipids are the most

resistant to degradation, followed by proteins and then DNA, with DNA being the most susceptible to damage. Resistance to degradation is why lipids have been the primary compound studied until recently and why they tend to be the most frequently studied organic residues. Methods have been adapted from multiple fields: microscopy, histology, biochemistry, immunochemistry, forensic science, and molecular biology to help retrieve and analyze these residues trapped on archaeological materials (Evershed 1993).

2.2 Organic Residues

Organic residue is a broad term that has been used by archaeologists to describe the features of a biological material that have morphological characteristics that are amorphous to the naked eye. Currently there are three main types of organic residue are used in archaeological sciences: DNA, proteins, and lipids. There are other types of organic residues, such as collagen, that are not as widely studied within the archeological world (Barnard, Lanehart, and Malainey 2007; Eerkens, 2007; Evershed,1993; Rice 1996). The three categories mentioned above can be found in either visible or absorbed residues on nonorganic material.

Visible residues were the original focus of organic residue analysis (Evershed 1993). They are classified as material that is visible on the surface of an object, whether it is a residue, deposit, or encrustation (Rice 1996; Eerkens, 2007). Visible residues tend to take the form of either resins, waxes, soot, or charring on tools. Distinct types of chemical analyses are used to identify the organic component and biopolymers on tools so that we can interpret their role in the daily lives of the population (Evershed 1993). Absorbed organic residues are a mixture of organic components and a finite number of biopolymers within an inorganic matrix. This is the result of organic material coming in contact or through consequent absorption of organic material within the wall of an object. They are invisible to the naked eye and are the most

common. 80% of all organic residues retrieved are those that have been absorbed into the walls of the implement. Absorbed residues have been successfully extracted from ceramic vessels in lipid and protein studies and via stone tools in aDNA studies (Shanks, Kornfeld, and Ream 2004; Oudemans and Boon 2007; Copleya et al. 2005; Loy 1993).

2.2.a Lipids

Lipids are loosely defined as any organic compound class that has derivatives from fatty acids, are hydrophobic, and soluble in organic solvents (Evershed 1993; Oudemans and Boon 2007; Eerkens, 2007; Rice 1996). Amino acids, waxes, cholesterol, fatty acids, natural oils, waxes, and steroids all fall within the lipid classification. They are medium sized molecules that are produced by living organisms and tend to be the main focus of organic analysis within archaeological sites (Evershed 1993). Lipids' hydrophobic nature and biomolecular makeup make them stable over an extended period and resistant to decomposition and degradation, especially when compared to other organic residues that have higher rates of degradation, such as aDNA, proteins, or carbohydrates (Evershed 1993; Barnard, Lanehart, and Malainey 2007; Eerkens, 2007; Malainey and Figol, 2015; McGovern and Hall 2016; Rice 1996).

In biomolecular archaeology, the foremost area of interest is the source of the lipids (Evershed 1993). Lipid compounds from plants and animals are often found in association with artifacts. the sources of lipids are determined by comparing components or mixtures of the compounds from either animals or plants through the chemical analysis methods of chromatography and isotope analysis (Evershed 1993; Hall and McGovern 2016; Malainey and Figol, 2015; Evershed, 1993; Eerkens, 2007; Rice 1996). The components and mixtures found on artifacts are created through human modification and intervention, such as through culinary activities and lipids are left over from the refining and mixing of organic material that is used in

food (Loy 1993; Eerkens, 2007; Malainey and Figol, 2015). Preservation of lipids are found within and on the ceramic wall. Contamination from fatty acids found in the clay the ceramics vessels are created from is unlikely. Additionally, the heat from firing most preindustrial ceramics is high enough to degrade any fatty acids that are found in the clay naturally; heat between 400-600 degrees Celsius in oxidizing and non-oxidizing environments removes all of the fatty acids and hydrocarbons (Evershed 1993; Eerkens, 2007).

Lipids tell us the generic origin of the plant and animal species by breaking down the lipid's classes: triglycerides, phospholipids, and sterols. Sterols are most commonly from cholesterol found in animal or plant sources, while phospholipids are predominantly found in plants. Triglycerides are three fatty acid chains that are found in fats and oils in human diet (Dunne 2017; Evershed 1993; Rice 1996; McGovern and Hall 2016; Evershed 1993). To help determine the animal and/or plant genus of what is being cooked, stable isotope analysis is needed. Stable isotope analysis lets us distinguish between fatty acids using metabolic differences in different animals and plants by looking at stable carbon isotope values. The number of $C_{16,}$ C_{18} and the number of double bonds found in the samples determine the type fatty acid present (Oudemans and Boon 2007; Evershed 1993; McGovern and Hall 2016).

2.2.b Molecular analysis of lipids in the archaeological record

By using organic residue analysis, archaeological studies involving ceramic vessels have been used to interpret culinary practices, domestication of ruminants, and much more (Eerkens 2007; Rice 1996; Copleya et al. 2005). The largest lipid analysis study involved 438 ceramic vessels from six Neolithic sites in southern Britain. Organic residue analysis was used to determine the domestication of ruminants and whether Neolithic agricultural practices had reached Britain. This was determined by evidence of dairying from lipids consistent with dairy

fat, though the use of gas chromatography to examine the total lipid extraction before being submitted to gas chromatography combustion isotope ratio mass spectrometry to determine their stable carbon isotope values (Copleya et al. 2005).

Dairying can be implied based on traditional lines of evidence; by relying on animal bone profiles consisting of a species' age and sex. The site showed a high number of mature females, a low number of mature males, and a high number of young animals, suggesting the possibility of dairying (Copleya et al. 2005). Traditional lines of archaeological analysis of osteological material have problems when interpreting necessity or how they disposed of deceased animal remains. They could have separate disposal methods for different animals, or we are missing the larger picture, due to loss of osteological remains.

Organic residue analysis using lipids helped interpret Neolithic agricultural practices to a greater extent than traditional methods (Copley et al. 2005). Ceramic shards found at the 6 Neolithic sites showed evidence of dairy using lipid and isotope analysis. The lipids found were consistent with dairy fat 50-60% of the time on ceramic sherds from all the Neolithic sites (Copleya et al. 2005). This evidence confirms that dairying was part of the Neolithic agricultural practices in the 5th millennium B.C. (Copleya et al. 2005).

2.5 Molecular analysis

Today, lipids are not the only organic residues used for analysis in the archaeological record. Protein and aDNA analysis have become prominent areas of research using organic residue analysis (Shanks, Kornfeld, and Ream 2004). In the past, it was believed that protein and DNA were too susceptible to degradation to be retrieved from artifacts from an archaeological site (Evershed 1993b).

Advances in organic residue analysis like immunological analysis, PCR, and Next Generation Sequencing have made it possible to retrieve detailed information about artifact use. These analyses help us to determine patterns across diet, such as seasonal diets, the geographical origin of the food being prepared, and what specific species are being prepared. This information allows for a more accurate interpretation of the behaviors and lives of past populations (Loy 1993; Barnard, Lanehart, and Malainey 2007; Newman et al. 1993; Shanks, Kornfeld, and Ream 2004).

2.5a Proteins

Proteins are found in a variety of different forms. They are large molecules that play many vital roles within an organism; they help regulate the structure and function of the body's tissues and organs (Mielke et al. 2011). Proteins are created from 20 different amino acids combinations to create different 3-dimensional structures. Proteins are more susceptible to degradation than lipids but have a higher resistance than DNA. This makes protein samples harder to retrieve than lipids (Mielke et al. 2011).

2.5b Molecular analysis of Proteins in the archaeological record

Protein analysis identifies species at the taxonomic family level relationship using the blood origins on stone tools and ceramic vessels (Tuller and Saunders 2012). Blood proteins can handle harsh conditions when absorbed into micro-cracks and, within the walls of ceramic vessels', retain their antigenicity and biological activity (Shanks, Kornfeld, and Ream 2004; Newman et al. 1993). Proteins have been successfully retrieved in the form of antigens, demonstrating that the amount and condition retained on the artifacts can be successfully preserved and used for identification

Immunological analysis uses techniques that analyze antigen-antibody reactions (Gerlach, NewMan, and Hall 1996). These techniques are based on past work involving classic precipitin tests. Antigen-antibody reactions are specific mostly at the taxonomic level, but under the right conditions more species-specific information can be obtained (Gerlach, NewMan, and Hall 1996; Shanks, Kornfeld, and Ream 2004; Newman et al. 1993; Tuller and Saunders 2012). Antigen-antibody reaction is the basis for all the tests involving blood protein identification. Immunological response determines what species the blood originates from based on species-specific antigen reactions (Gerlach, NewMan, and Hall 1996; Shanks, Kornfeld, and Ream 2004; Newman et al. 1993; Tuller and Saunders 2012). This technique has been used for years to analyze blood stains in the medico-legal and serology fields before being adopted into the archaeological world (Gerlach, NewMan, and Hall 1996).

Multiple methods have been developed for protein analysis in archaeological studies, including cross-over immunoelectrophoresis (CIEP), enzyme-linked immunosorbent assay (ELISA), radioimmune assay (RIA), liquid chromatography-tandem mass spectrometry (LC-MS/MS), in addition to other protein analysis methods. (Paabo 1989; Seeman et al. 2008; Hardy and Ra 1997; Newman et al. 1993; Lowenstein et al. 2006a). LC-MS/MS is the only method that has successfully retrieved protein residues from ceramic vessels found in the archaeological record.

LC-MS/MS uses a “shotgun” proteomic approach that allows detection of both fragmented and denatured proteins, which can show a wide range of proteins within the sample. This method has successfully identified proteins associated with ruminant adipose fats and dairy from ceramic vessels in Çatalhöyük (Hendy Jessica et al. 2018). Five vessels at Çatalhöyük detected more than one foodstuff. The majority of the proteins found at the site were derived

from milk of *Ovis capra* and even *Bovinae*. Hemoglobin proteins form *Caprinae*, *Cervidea* and *Bovina* were also detected from the ceramic matrix (Hendy Jessica et al. 2018). Protein residues were also found on the outside of vessels that were painted red, suggesting that animal fat could have been used for post-firing treatment. The protein analysis on ceramic vessels from Çatalhöyük gives us a more detailed picture of pottery found in early farming societies. Analysis of ceramic vessels showed that cereals and legumes were being processed in the pottery found in the site (Hendy Jessica et al. 2018).

There are problems when it comes to interpreting methods that rely on visual changes for the reaction strength, such as precipitin tests, and human error (Tuller and Saunders 2012). Other problems with protein analysis interpretations can occur. Other problems with protein analysis interpretations can occur when looking at species that are closely related. Cross-reactivity is one such example and is the result of antisera from one species reacting with the blood proteins of another, closely related species. For example, antisera from a dog will react to a wolf or coyote blood (Gerlach, NewMan, and Hall 1996; Shanks, Kornfeld, and Ream 2004; Newman et al. 1993; Barnard, Lanehart, and Malainey 2007; Tuller and Saunders 2012). To distinguish between closely related species, DNA analysis is required.

2.5c DNA

DNA codes for every living organism and holds valuable information about variation, ancestry, etc. There are multiple areas of genetic studies: mitochondrial (mtDNA), nuclear, and Y-chromosome DNA, all of which hold a variety of genetic information (Snow 2018; Mielke et al. 2011). Mitochondrial DNA was one of the primary focuses in aDNA analysis because it is abundant in the cell. This, coupled with the other characteristics of mtDNA, make it ideal for aDNA retrieval from artifacts (Snow 2018; Kimura et al. 2001; Eerkens, 2007).

DNA begins to degrade soon after death; strands start to break, and bases break down. The older the DNA, the less there is a chance of preservation. Other factors affect DNA preservation, such as the environment (Hofreiter et al. 2001; Mielke et al. 2011; Paabo 1989). There has been a correlation found between DNA preservation and the environment: DNA tends to be better preserved in the cold. Meanwhile the hotter the climate, the less likely it is that the DNA will be preserved, unless located in a cool and dry cave (Hofreiter et al. 2001; Mielke et al. 2011; Paabo 1989). It was initially believed that aDNA revival could only go back as far as 100kya. Due to advancement in the field of DNA through next-generation sequencing technology we now can retrieve DNA as far back as 800kya (Hofreiter et al. 2001; Mielke et al. 2011; Paabo 1989).

Polymerase chain reaction (PCR) is used to amplify DNA. This allows us to make thousands of copies of a particular region of minute or degraded DNA (Snow 2018). Once the DNA has been amplified it must then go through a sequencing process. Sanger sequencing, or dideoxy sequencing, is one of the standard methods used for sequencing the DNA from stone tools (Paabo 1989; Knapp and Hofreiter 2010; Schuster 2008). This method sequences base pairs that have been amplified by PCR so that we can read them. When applied to stone tool analysis, we are able to learn about what the object was being used on (Poinar et al. 1996; Paabo 1989; Evershed 1993; Shanks, Kornfeld, and Ream 2004; Knapp and Hofreiter 2010; Schuster 2008).

DNA gives detailed information on past populations' environment, life, and behavior by allowing us to distinguish between closely related species, making it a valuable tool for studying archaeological sites (Evershed,1993.; Rice 1996; Shanks, Kornfeld, and Ream 2004). DNA does have some drawbacks. DNA is highly susceptible to degradation, more than proteins and lipids (Evershed 1993b; Rice 1996; Eerkens 2007; Barnard, Lanehart, and Malainey 2007; Evershed

1993a). This makes DNA retrieval harder in the archaeological record, but possible. We can overcome this to some degree using sequencing and PCR, but there will always be a limitation due to time and environment. DNA will not always be recoverable in an archaeological site, but that does not mean we should not try for it (Hofreiter et al. 2001; Evershed 1993a; Barnard, Lanehart, and Malainey 2007; Eerkens 2007).

Degradation is not the biggest problem with DNA analysis: contamination is (Hofreiter et al. 2001; Kimura et al. 2001; Evershed 1993a; Mielke et al. 2011). Contamination is the number one enemy when working aDNA or DNA in general. Contamination comes from all sorts of places: from the researcher, bacteria, leftover DNA from past amplification processes, excavation, and the list goes on (Poinar et al. 1996; Hofreiter et al. 2001; Paabo 1989; Mielke et al. 2011). Contamination creates issues when trying to interpret DNA results. Contamination can distort distributions and frequencies affecting the reliability of the results (Flickinge, 2015). Precautions are taken to help prevent contamination. For example, aDNA extraction takes place in a clean room in full body anti-contamination suits. Even though contamination can be a problem, it does not mean the aDNA analysis is not possible (Poinar et al. 1996; Kimura et al. 2001; Paabo 1989).

2.5d Molecular analysis of DNA in the archaeological record

Ancient DNA extractions has been successfully performed on stone tools using two methods developed by Orin Shanks: (1) 5% ammonium hydroxide and (2) guanidine hydrochloride. Studies have shown that 60-80% DNA can be retrieved after introducing fluorescently labelled blood protein and DNA to experimental stone tools. The fluorescently labeled DNA allowed researches to measure how much DNA was left within the microcrack (Shanks et. al., 2001). DNA, using the methods mentioned above, showed that 60- 80% of DNA

could be extracted. 5% ammonium hydroxide typically has a greater success rate with 78% of DNA trapped in the microcracks of the 15 stone tools tested (Shanks et al.,2001). Guanidine hydrochloride had a slightly lower success rate, with 61% of all DNA trapped being released (Shanks et. al., 2001). Out of the 60-80% of DNA extracted only 10% of the samples produce DNA results that could be sequenced (Kimura et al. 2001). Even though stone tools have a low success rate, they have successfully been used to interpret the use of stone tools found in multiple archaeological sites, such as the La Quina Charentes (Kimura et al. 2001; Hardy and Ra 1997; Hofreiter et al. 2001).

La Quina Charentes is located in south-west France. This site is from the Middle Paleolithic era, dating between 35,000 to 65,000 years ago, and consists of well-preserved animal bones, Neanderthal remains, and Mousterian stone tools (Hardy and Ra 1997). Eight Mousterian stone tools and soil samples, collected 2-3mm from the stone tools, were selected for aDNA testing. DNA extraction was done using the guanidine hydrochloride method, while analysis was performed using PCR amplification and SEQUENASE protocols developed in the United States for sequencing (Hardy and Ra 1997). Five out of the eight stone tools yielded aDNA. The DNA analysis focused on a single region of the mitogenome, cytochrome b, because of the extensive database of known animal species and accompanying diagnostic markers (Hardy and Ra 1997). Four sequences were carried out: the first sequence yielded aDNA from boar/pig and rhinoceros; the second sequence yielded an unknown mammalian DNA that is closest to that of a sheep; the third sequence was unknown mammalian DNA that was only found in the soil sample SQM5–4950; the fourth sequence was primarily from modern humans, more than likely due to contamination. The information obtained from DNA analysis gave archaeologists specific identification of the species of origin, which they would not have been able to obtain with other organic analysis methods (Hardy and Ra 1997).

2.6 Conclusion

Organic residues found on objects from archaeological sites have a complex mixture of biological material found on them, created by humans (Hardy and Ra 1997; Evershed 1993a; Rice 1996; Eerkens 2007; Evershed 1993b). Ceramic shards and stone tools have been used for multiple tasks throughout their life. The information from the organic residue found gives us a look into the behavior of individuals within the sites (Rice 1996). By using mass spectrometry (MS) and gas chromatography (GC), we are able to distinguish the different lipid compounds found on archaeological material to gain more in-depth information than we could with spectroscopic methods (Evershed 1993a; Eerkens 2007; Evershed 1993b; Rice 1996). Cross over electrophoreses allows us to distinguish species at the taxonomic family levels by looking at proteins (Shanks, Kornfeld, and Ream 2004; Gerlach et al. 1996; Newman et al. 1993). Organic residue analysis gives us the tools needed to gain viable information on durable artifacts (Evershed 1993a; Eerkens 2007; Evershed 1993b). Unlike biological material such as textiles, food, remains, etc., stone tools and ceramic vessels are durable material that is found in abundance in archaeological sites. Therefore, we can pull trace evidence off the biological material that came in contact with them and use that information to better determine their use, and the behavior of the past population in general, with greater detail (Evershed 1993a; Eerkens 2007; Evershed 1993b; Rice 1996).

This literature review on organic residue analysis on ceramic vessels and aDNA analysis on stone tools provides insight as too whether or not DNA can be extracted from ceramic vessels. As demonstrated above, unglazed ceramic vessels are able to absorb and protect lipids and proteins, indicating that there is a low possibility of DNA preservation. Despite the low

possibility of preservation, stone tool and protein analysis of ceramic vessels shows that DNA can be successfully extracted from durable archaeological material and not just organic material.

Chapter Three

Materials and Methods

3.1 Experimental ceramic cooking vessels

The experimental ceramic vessels used in this project were created using two firing methods: (1) Kiln fired to create porous immature clay vessels known as Bisquware, and (2) Pit fired to create a denser clay vessel. Ceramic descriptions can be found in table 3a.

Shards	Description
15-17, and 30- 32	Ceramic vessels fired in a kiln used to cook pork chops with ¼ cup of water
18 and 33	Control Ceramic vessels fired in a kiln
20-23 and 25-27	Ceramic vessels fired in a fire pits used to cook pork chops with ¼ cup of water
24 and 28	Control Ceramic vessels fired in a fire pit

Table 3a: description of experimental kiln and fire pit samples used in this project

3.2 Archaeological ceramic samples

18 ceramic shards were acquired from Steven LeBlanc of the Peabody Museum at Harvard University. Steven LeBlanc selected the shards based on the following criteria: the objects must have been used to prepare or store foodstuff. The samples sent showed evidence of being used for food preparation, storage, or serving of food; there were darkened or burned marks on the exterior of the vessels, as well as residue visible on the interior. The ceramic descriptions can be found in 3b.

Shards	Description
1	A large bowl with black burned interior that may have been used for special food processing function.
2	The bottom or near bottom of a possible water jug or dry storage jar.
3	From near the bottom of a serving bowl.
4	From near the bottom and was broken into two pieces with an obvious rim from a Mimbres bowl.
5	A large jar with black on the interior. The black coloration found on the jar is possibly from cooking or from firing.
6	Another Mimbres bowl that was used for serving food and as burial goods. Black stains created from touching human skin and hair.
7	Bowl with unknown use.
8	A bowl that is black from possibly being burned.
9	Bowl that has been burned.
10	Probable large water bowl.
11	A corrugated jar that held either water, dry storage, or cooking.
12 and 12A	From the same bowl, smaller pieces show signs of fire use.
13	Large corrugated jar. The black staining in the shard is probably due to use.
14	From near the bottom of a small jar that has not been cleaned.
15	From the midpoint of a jar. The exterior's black discoloration suggest that it had been used over a fire.
16	A large plate like vessel. Has an unknown black discoloration on the exterior.
17	A rim from the side of a jar. The shard has a black discoloration that is possibly soot.

Table 3b: Description of archaeological samples used in this study

3.3 Pre-analysis

Eight experimental ceramic bowls were created from clay powder mined in Missoula, Montana by the University of Montana's ceramics department. Clay powder was added to water until the clay reached a dough-like consistency. The clay was then spread across a canvas top table to remove excess water. This was done on each side for ten minutes.

Once the excess water was removed, the clay was then folded 20 times, readying the clay for shaping. Water was added as needed to keep the clay from drying out. After folding the clay,

the clay was shaped using a pinching method to create eight bowls: four small, two large, and two small, deep bowls. The clay bowls were left to dry on a shelf in the University of Montana's ceramic firing room, as they waited to be bisqued.

3.4 Firing

The eight-ceramic bowls were bisqued at 1828 degrees Fahrenheit for 2 days in the kiln (Figure 3c). Bisqueing is when the clay is baked until all chemically bonded water molecules are removed. From there, four ceramic bowls were fired in a 31.5" L x 15" H x 25.5" W fire pit, built from cinderblocks as seen in figure 3d. One inch of sawdust was placed in the bottom of the firing pit, the ceramic bowls were then placed inside, and a thick layer of tightly packed sawdust was placed on top of the ceramic bowls so that they were completely covered. By tightly packing the sawdust, a slow burn was created to ensure that the ceramic vessels cooked for the appropriate amount of time. A layer of newspapers was placed over the sawdust and the corners were lit using a blow torch. A ceramic board was placed on top of the fire pit to prevent any flames from escaping. The ceramics were left in the fire pit for 10 hours and were removed once they were cold to the touch. The firing methods used in this project were not the same methods used in the archaeological record. The methods used in this project were the best that could be done with the resources at the University of Montana.

Figure 3c: Cross electric kiln. Left front view, and right-angled view of the inside and control panel

Figure 3d: Fire pit. Left sideview, and right aerial view

3.5 Labels

Extraction 1 consisted of archaeological shards 5, 7, 9, 11, 12, and a control. Each shard was assigned a number from 1-6. The extraction samples will be referred to as 1-6. Extraction 2 consists of shards 1, 3, 14, 15, 16 and a control. Each shard in extraction 2 was assigned a number from 7-12. The extracted samples will be referred to as samples 7-12. Experimental kiln samples used in protocol 1 are labeled 15-17 and 18 for the control ceramic shard. Experimental firepit samples are labeled 20-24 and 25 for a control. Experimental firepit samples are labeled

F25-F28 and 29 for a control. Experimental kiln samples used in protocol 2 are labeled 30- 33 and 34 for control.

3.6 DNA Extraction

The methods used in this project to extract DNA from the ceramic vessels were primarily based on the procedure that Shanks et al. (2001) developed for stone tools. The DNA extractions were performed in the UMT Snow Ancient Lab. Full anti-contamination gear was used in all processes, including extraction and amplification, to reduce contamination risks.

3.6a Extractions Protocol 1: 5% Ammonium hydroxide: Archaeological Ceramic Samples

To reduce the risk of surface contamination from the excavation, archaeologists, or lab technicians, the ceramic vessels were subjected to three washes using ultra-pure water with soaks of 2-3 minutes. Following, they were sealed in individual polypropylene bags with 4ml of 5% ammonia hydroxide. An extra bag was filled with 5ml of 5% ammonia hydroxide for a negative control, to be subjected to the same steps as those with possible DNA in order to help determine if contamination was introduced during the extraction. Once sealed, the samples were left to soak for two days. The 5% ammonia hydroxide created an advantageous pH level for absorbed DNA removal from any crevices it may have been stored in.

After 48 hours, the polypropylene bags were subjected to sonication using a water bath at room temperature at 50 Hz for 30 minutes. After removal from the ultrasonic, the samples were air dried to prevent contamination from the water. An incision was made in each bag using scissors and the liquid content was drained into UV irradiated 50μl tubes. DNAway was used to prevent cross-contamination from the scissors by cleaning and allowing drying between each sample.

5% ammonium hydroxide interferes with DNA amplification and must be removed. Glass test tubes were filled with the 5% ammonium hydroxide from the samples and placed in a vacuufuge set to low heat to evaporate the 5% ammonium hydroxide. They were checked every 30 minutes. The pH levels were checked every hour with a buffer solution and if any of the samples indicated high pH they were placed back into the vacuufuge. This process was repeated until all pH level tests indicated neutral pH levels.

Due to complications with the vacuum pump during the first day, the vacuufuge was set to high temperature to compensate for the lowered suction until a new pump arrived. The entire process of the removal of 5% ammonium hydroxide took four days throughout two weeks. The samples were stored in the freezer and sealed with parafilm when they were not in the centrifuge, causing possible DNA loss due to the freeze/thaw cycles.

1ml of PB buffer (Qiagen)a purified pre-made guanidine hydrochloride base solution, was added to the samples once the pH was at an acceptable level, and they were vortexed to mix. 13ml of PB buffer was added to 15ml tubes, followed by a combination of the 1ml PB buffer and DNA mix. Once mixed, the samples were placed in the centrifuge for approximately 7 minutes at 1500 RPMs. DNA samples were then added to filtered funnels connected within a 50ml tube and placed in the centrifuge for 8 minutes and 37 seconds. The filters are designed to catch and isolate DNA while removing any residual extraction buffers and impurities. The filters were then placed onto 2ml collection tubes. 600µl of PE buffer (Qiagen) were added and spun through using a smaller centrifuge for 1 minute at 6000 RPMs. The flow-through gathered at the bottom of the tubes was discarded. A dry run was performed for 1 minute at 6000 RPMs to remove any remaining liquids. 50µl of H2O was added to the filters, allowed to incubate at room temperature for a minute, and spun through for 1 minute at 6000rpm to remove

DNA from the filter and be collected into new low-bind 2ml collection tubes. The steps were repeated to make sure that all DNA was cleaned off the filter. The samples were stored in the freezer when not in use.

3.6b PCR and sequencing: Archaeological Ceramic Samples

The same PCR protocol was used for all DNA sample extractions and is outlined in Table 3a . 13.37µl of the PCR master mix was aliquoted into 0.2µl tubes, along with 5µl extract from each sample, for a reaction volume of 18.37µl. The samples were placed in the thermal cycler for 60 cycles with 52°C annealing temperature (94°C for 5 minutes, followed by 60 cycles of 92°C 30 seconds, 52 °C for 30 seconds, 52°C for 30 seconds 72°C for 30 seconds, then a final hold of 72°C for 5 minutes) in the post-PCR room. PCR was run twice on each extracted sample, using two different primers to amplify regions of cytochrome b (cytb) and (2) 16 rRNA (16s). The primers used were L15684 (cytb forward) 5'-CTCCACACATCCAAACAACG-'3, and H1576 (cytb reveres) 5'-TGTTCGACTGGTTGTCCTCC-'3 to target the116 bp fragments of the cytochrome b region of the mitochondria and L16s04 (16s forward) 5'TCTCTTACTTCCAATCCGTG-'3 and H16s06 (16s reverse) 5'CGGAGGTTGTTYTGTTCTCC-'3 to target the191 bp fragments 16 rRNA region of the mitochondria. The 16s primers are designed to amplify a region of the mitochondrial that is species specific and can be identified by using BLAST in Genebank post-sequencing. Cytb primers distinguish between most species and rarely amplify human DNA, making them the best likelihood of determining the source of any amplified DNA (Shanks, 2005).

N=	H20	Buffer	Taq	MgCL2	dNTP	forward Primer	Revers primer	Template DNA	Master mix total	Reaction total
1	8.76µl	1.5µl	.08µl	.45µl	2.4µl	.18µl	.18µl	5µl	13.37µl	18.37µl

Table 3a: PCR master mix protocol for N=1 samples.

Electrophoresis with 2% agarose gel was used to determine the presence of amplified DNA within the samples. If the PCR product produced a band in any of the control samples (which shouldn't amplify if there is no PCR contamination), or no bands were found in any of the samples, a second PCR attempt was completed. The PCRs were considered successful if the agarose gel produced any bands as evidence of successful amplification. The successful sample was then prepared for sequencing to determine the source of the bands.

All amplified DNA samples that produced a band were prepped for Sanger sequencing using the sing USB® ExoSAP-IT® PCR Product Cleanup protocol. The protocol used can be found in Table 3b. Once the samples were prepared, they were taken to the University of Montana's Genomics Core for DNA sequencing. Following this, the sequence was run against GenBank using the BLAST tool to determine which sequence it aligned most closely with.

samples	Amplified DNA	USB® ExoSAP-IT®	H20	cytb Forward Primer	Mixture total	Thermal cycler setting
4	5µl	2µl	3µl	5µl	15µl	exosap
	Amplified DNA	USB® ExoSAP-IT®	H20	cytb reverses Primer	Mixture total	Thermal cycler setting
4	5µl	2µl	3µl	5µl	15µl	exosap
samples	Amplified DNA	USB® ExoSAP-IT®	H20	cytb Forward Primer	Mixture total	Thermal cycler setting
7	5µl	2µl	3µl	5µl	15µl	exosap
samples	Amplified DNA	USB® ExoSAP-IT®	H20	cytb reverses Primer	Mixture total	Thermal cycler setting
7	5µl	2µl	3µl	5µl	15µl	exosap
samples	Amplified DNA	USB® ExoSAP-IT®	H20	16s Forward Primer	Mixture total	Thermal cycler setting
7	5µl	2µl	3µl	5µl	15µl	exosap

samples	Amplified DNA	USB® ExoSAP-IT®	H20	16s Reveres Primer	Mixture total	Thermal cycler setting
7	5µl	2µl	3µl	5µl	15µl	exosap
samples	Amplified DNA	USB® ExoSAP-IT®	H20	16s Forward Primer	Mixture total	Thermal cycler setting
8	5µl	2µl	3µl	5µl	15µl	exosap
samples	Amplified DNA	USB® ExoSAP-IT®	H20	16s Reveres Primer	Mixture total	Thermal cycler setting
8	5µl	2µl	3µl	5µl	15µl	exosap

Table 3b: sequencing preparation protocol for archaeological samples 4, 7 and 8.

3.7 Experimental ceramic samples

Two protocols developed by Shanks et al. were used on the experimental samples (2001). Protocol 1 uses 5% ammonium hydroxide to abstract DNA from the samples. This is the same protocol used on the archaeological ceramic shards with a few changes that will be mentioned later. Protocol 2 uses guanidine hydrochloride or PB Buffer.

3.8 Protocol 1: 5% ammonium hydroxide: Experimental ceramic samples

To remove the risk of surface contamination from the samples, the shards were soaked in a 10-20% diluted bleach solution for 3 minutes. The 10-20% bleach solution was used to remove any DNA left on the surface of the shards so that any DNA retrieved what was absorbed into the ceramic vessels' walls. The samples were then soaked in ultra-pure water twice for 2 minutes to remove any leftover 10-20% bleach solution. Extraction was the same as stated above in 3.6a. Due to complications with vacuufuge, the samples were diluted by adding 13ml PE buffer to the 15ml tubs containing the samples to lower their PH level. PB Buffer was used to test that PH levels were at the correct levels.

3.8a PCR and Sequencing: Protocol 1: 5% Ammonium Hydroxide Experimental Ceramic Samples

The PCR protocols used can be found in Table 3a. Primers used were CO2sus F2 (Pig Forward) (5'GCCTAAATCTCTCCCCTCATGGTA -3) and CO2susR2 (Pig reverse) (5'AGAAAGAGGCAAATAGATTTTCG -3) to target the 212 bp fragment of mitochondrial DNA from *Sus scrofa domesticus* (Thomas 2016). Pig specific primers were used to amplify only pig DNA from the samples. After the samples are amplified using the thermocycler for 65 degrees Celsius (94°C for 5 minuets, followed by 94°C 30 seconds, 65 °C for 30 seconds, 72°C for 30 seconds then a final hold of 72°C for 7minutes) for the pig primers in the post-PCR room.

3.9 Protocol 2: PB Buffer Experimental Ceramic Samples

The modern ceramic samples used in this analysis were recently used to cook pork, leaving residue on the surface. To remove the risk of surface contamination from the sample, they were soaked in 10-20% diluted bleach solution for 30 seconds. The 10-20% bleach solution to remove any DNA left on the surface of the shards so that any DNA retrieved would be that absorbed into the ceramic vessels. The samples were then soaked in ultra-pure water twice for 2 minutes to remove any leftover 10-20% bleach solution.

After cleaning, the shards were sealed in polypropylene bags with 10ml of PB Buffer (Qiagen), a purified guanidine hydrochloride-based solution. PB buffer breaks open cells, allowing DNA to be freed from the pores into the solution (Broom et. al, 1990)**.** The control samples were subjected to the same steps as the samples with possible DNA. The control sample is used to help determine if contamination is present, by indicating whether DNA is present or not, once amplified and ran through electrophoresis. The bags were sealed and left to soak in the soaking drawers for one week.

After soaking, the DNA samples were added to the filtered funnels connected to 50ml tubes and placed in the centrifuge for approximately 8 minutes. This is done to catch DNA in the

filter and to remove any impurities. The filters were placed in 2ml tubes. 600µl of PE buffer (ethanol wash buffer; Qiagen) were added and spun through using a smaller centrifuge for 1 minute at 6000rpm. The liquid gathered at the bottom of the tubes were dumped into the waste disposal jar. A dry spin was performed for 1 minute at 6000rpm to remove the remaining liquids (mostly ethanol). The filter was placed into low-bind 2ml final tubes. H2O was then added and spun through for 1 minute to remove DNA from the filter. The steps were repeated to make sure that all DNA was cleaned off the filter. The samples were stored in the freezer when not in use.

3.9a PCR and Sequencing: Experimental Ceramic Samples

The PCR protocols used can be found in Table 3a. Primers used were CO2sus F2 (Pig Forward) 5'GCCTAAATCTCTCCCCTCATGGTA -3 and CO2susR2 (Pig reverse) 5'AGAAAGAGGCAAATAGATTTTCG -3, L15684 (cytb forward) 5'CTCCACACATCCAAACAACG-'3, H1576 (cytb reveres) 5'TGTTCGACTGGTTGTCCTCC-'3, 16S04 (16s forward) 5'-TCTCTTACTTCCAATCCGTG-'3 and H16S06 (16s reverse) 5'-CGGAGGTTGTTYTGTTCTCC-'3The PCR protocols used can be found in table 3.a. Electrophoresis was used to determine the presence of amplified DNA within the samples. If the 2% agarose gel produced bands in any of the control samples or no bands, the PCR was re-run. The PCR was considered successful if the 2% agarose gel produced any bands that were not in the controls.

3.9.1 Qubit Quantification Analysis: For all samples

Analysis of the quantification of DNA concentration present in samples extracted from ceramic shards was done using high sensitivity assay Qubit® before PCR amplification. 195µl of the qubit working solution was added to special qubit tubes with 5µl of DNA. The qubit

quantitates the amount of all DNA from the samples, whether it is bacterial or from an unknown source, to determine the total concentration of DNA within the samples. Qubit assays uses target dyes that fluoresce when bound to DNA, similar to what is done with ethidium bromide. The qubit fluorometer reads the fluorescence and calculates the DNA concentration based on the volume of DNA added to the reaction.

3.9.2 Inhibition Test for All Samples

An inhibitor test was performed to gauge whether any inhibitory substance that prevents DNA from amplifying was also extracted from the sample. Modern human and pig DNA were introduced to the PCR sample to determine if an unknown inhibitor was present in the sample, preventing amplification, as seen in table 3d. If inhibitors were found, samples were diluted in an attempt to reduce the inhibition. Dilution protocol can be found in table 3e.

N=	H20	Buffer	Taq	MgCL2	dNTP	forward Primer	Revers primer	Template DNA	Master mix total	Modern Pig DNA	Reaction total
1	8.76µl	1.5µl	.08µl	.45µl	2.4µl	.18µl	.18µl	5µl	13.37µl	2µl	8.76µl

Table 3d 1: Inhibitor test protocol for archaeological samples.

N=	Template DNA	H20	Mix total
1	2µl	18µl	20µl

Table 3e: dilution protocol for archaeological samples

Chapter 4

Results

4.1 Eliminating contamination

As a precaution against contamination, full anti-contamination gear was worn during extraction methods and PCR preparation. Latex or nitrile gloves were worn during all steps in the post PCR room. Controls were established to detect contamination and, if contamination was detected, PCRs were re-run with new primers and H2O.

An inhibitor of an unknown substance inhibited amplification of the DNA figures 4a and 4b. Two methods were used in an attempt to remove or reduce inhibitors: (1) samples were re-run through DNA purification steps. (2) If purification failed to remove inhibitors the samples were diluted.

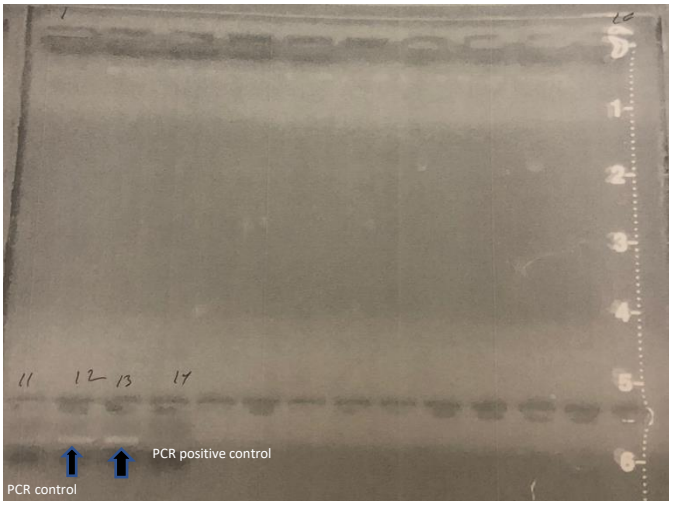

Figure 4a: Agarose gel for inhibitor test archaeological sample 16s primers : the samples are loaded left to right: top 1-10 bottom 11-14: 1-6 extraction 1 : Template DNA and modern pig DNA,7-12 extraction 2: Template DNA and modern pig DNA,,12: band in sample negative control,13: band in PCR positive control with only modern pig DNA, 14: PCR negative control no DNA.

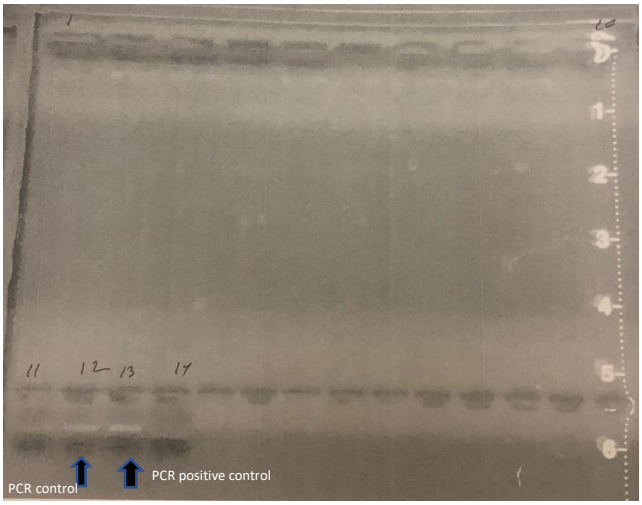

Figure 4b Agarose gel for inhibitor test archaeological sample cytb primers. the samples are loaded left to right: top 1-10 bottom 11-14: 1-6 extraction 1 : Template DNA and modern pig DNA,7-12 extraction 2: Template DNA and modern pig DNA,12: band in sample negative control,13: band in PCR positive control with only modern pig DNA, 14: PCR negative control no DNA.

4.2 DNA results: Archaeological ceramic samples

Preliminary data confirmed the presence of DNA after quantification of DNA concentration present in the samples. Results are depicted in tables 4a and 4b. Agarose gel electrophoresis was run to determine if DNA amplification was possible. Two 2% agarose gels were run (1) for the PCRs containing 16s primers and (2) PCR containing cytb primers. The PCRs ran contained 12 samples: 1-6 (extraction 1) and 7-11 (extraction 2), and 12 (PCR control). 4 faint bands were present: 2 in samples containing 16s primers and 2 in samples with cytb primers. The first PCR attempt showed the 16s and cytb primers did not anneal or amplify due to an unknown inhibitor. After dilution, the 4 samples that were successfully amplified (Figure 4c and 4d) were sent off for Sanger sequencing and then run against GenBank using the BLAST tool. The results revealed two clear human sequences and one clear bacterial sequence of *Gemmatirosa kalamazoonesis*. The fourth sample sent out for sequencing was unsuccessful.

1	2	3	4	5	6 (Control)
.0968 ng/µl	Out of Range	Out of Range	1.12 ng/µl	0.0 512 ng/µl	Out of Range

*Table **4a**: extraction 1 Qubit quantification of DNA level of archaeological ceramic samples*

7	8	9	10	11	12 (control)
0.274 ng/µl	0.788 ng/µl	0.0200 ng/µl	0.0628 ng/µ	Out of range	Out of Range

*Table **4b**: Extraction 2 Qubit quantification of DNA level of archaeological ceramic samples*

4.3 Avoiding inhibition: Experimental ceramic samples

An inhibitor test was run on both samples from protocols 1 and 2 to determine if an inhibitory substance was present in the sample. The inhibitor test was run on samples from

protocols 1 and 2 the results are depicted in figure 4e and 4f. The fire pit sample from protocol 1 inhibitor tested was run after dilution. Results are depicted in figure 4g.

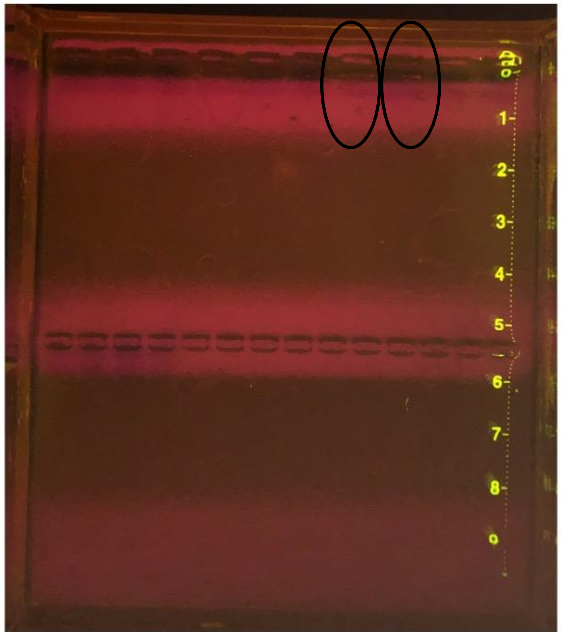

Figure 4c: Agarose gel for 16s diluted archaeological samples: the samples are loaded left to right: top 1-10 bottom 11-13 2 bands one for sample 7 and one for sample 8 of extraction 2

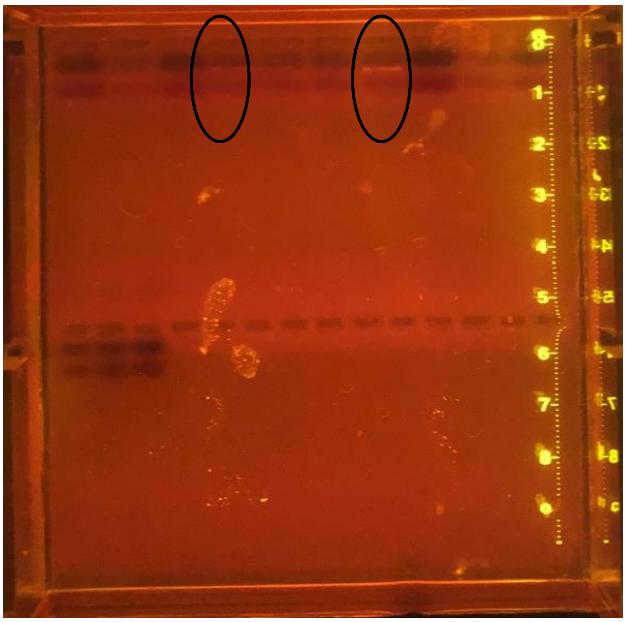

Figure 4d: Agarose gel for cytb diluted archaeological samples the samples are loaded left to right: top 1-10 bottom 11-13: 2 bands one for sample 4 and one for sample 7 of extraction 2

4.3 Avoiding inhibition: Experimental ceramic samples

An inhibitor test was run on both samples from protocols 1 and 2 to determine if an inhibitory substance was present in the sample. The inhibitor test was run on samples from protocols 1 and 2 the results are depicted in figure 4e and 4f. The fire pit sample from protocol 1 inhibitor tested was run after dilution. Results are depicted in figure 4g.

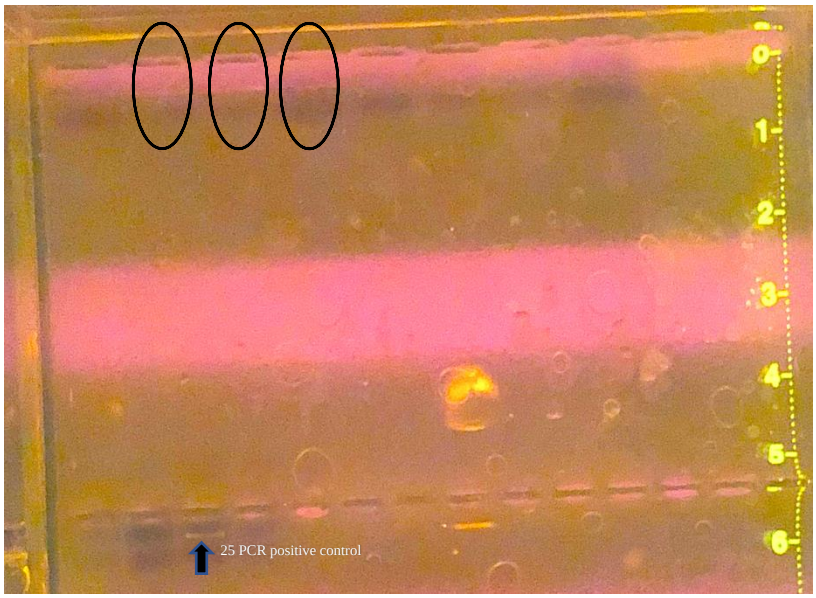

Figure 4e: Agarose gel of inhibitor test protocol 1 experimental samples. The samples are loaded left to right: top 15-21, bottom22-26- 15-19 kiln fired samples and 20-24 fire pit samples. Kiln samples produced faint bands in 16,17 and 18. fire pit samples produced no bands. 25 PCR positive control produced a band.

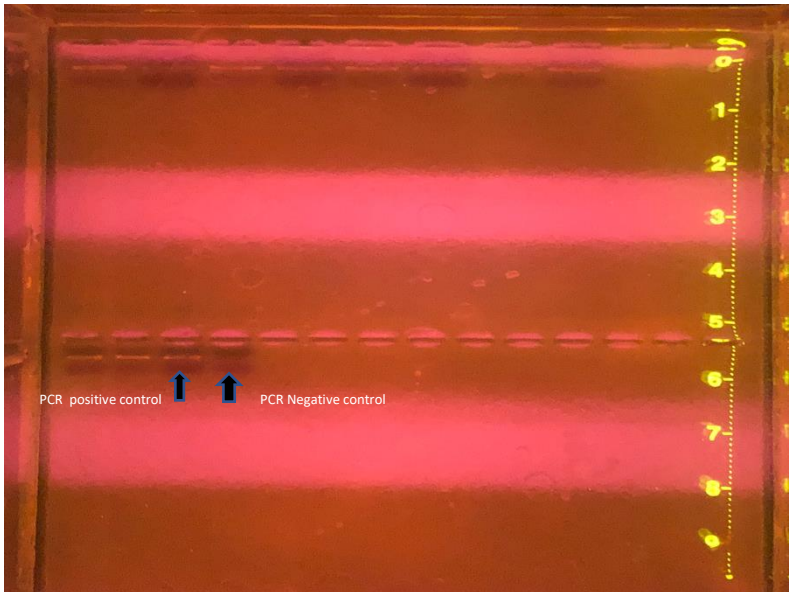

Figure 4f: Agarose gel of inhibitor test protocol 2 experimental samples. the samples are loaded left to right: top F25-K31, bottom K32-36- 15Bands in F25-F29 kiln fired samples. K30-K34 fire pit samples and 35 PCR positive control.

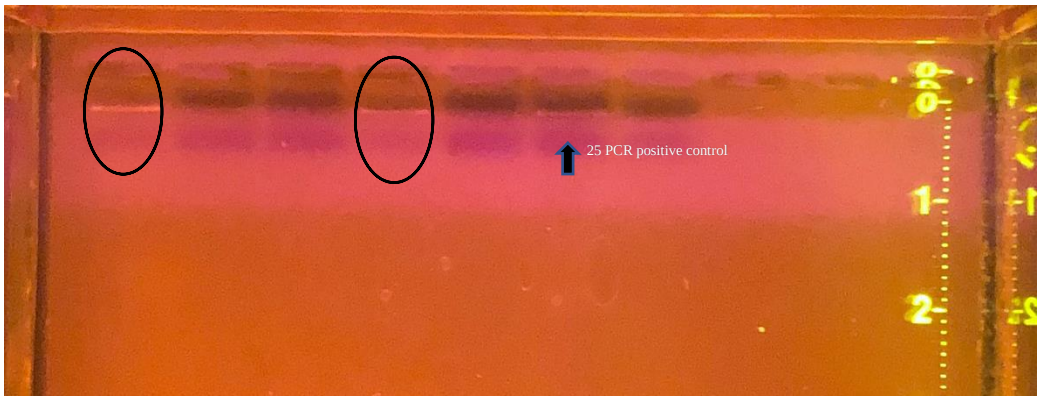

Figure 4g: Agarose gel of: Protocol 1 Diluted firepit experimental samples with pig primer: gel well loaded left to right as followed top 20-27. Bands in 20,23 and 25 PCR positive control

4.4 DNA Results: Experimental samples

Preliminary data from protocols 1 and 2 experimental samples confirmed the presence of DNA after quantification of DNA concentration present in the samples. Results can be seen in

Table 4c, 4d, 4e, and 4f. 2% agarose gels were run to determine if DNA could be amplified for sequencing. The PCRs ran with pig primers were tested using agarose gel to determine if amplification was successful for both protocols. The PCR containing pig primers samples from protocol 1 showed a single band. The results were re-run to rule out contamination as depicted in figures 4h and 4i. The results of the first gel could not be repeated. The PCR for protocol 2 containing pig, 16s and cytb primers were unable to be successfully annealed and amplified as depicted in figures 4j, 4k and 4l.

15	16	17	18	19
Out of Range	Out of Range	.0280 ng/µl	Out of Range	Out of Range

Table 4c: Extraction 3 protocol 1 Qubit quantification of DNA level of experimental kiln ceramic samples

20	21	22	23	24
Out of Range	Out of Range	0.0480 ng/µ	Out of Range	Out of Range

Table 4d: Extraction 3 protocol 1 Qubit quantification of DNA level of experimental fire pit ceramic samples.

F25	F26	F27	F28	F29
.154 ng/µl	.080 ng/µl	.112 ng/µl	.100 ng/µl	.0850 ng/µl

Table 4e: Extraction 1 protocol 2 Qubit quantification of DNA levels of fire pit experimental samples

K30	K31	K32	K33	K34
0850 ng/µl	.0760 ng/µl	.0650 ng/µl	Out of Range	Out of Range

Table 4f: Extraction protocol 2 extraction: Qubit quantification of DNA levels of kiln experimental samples

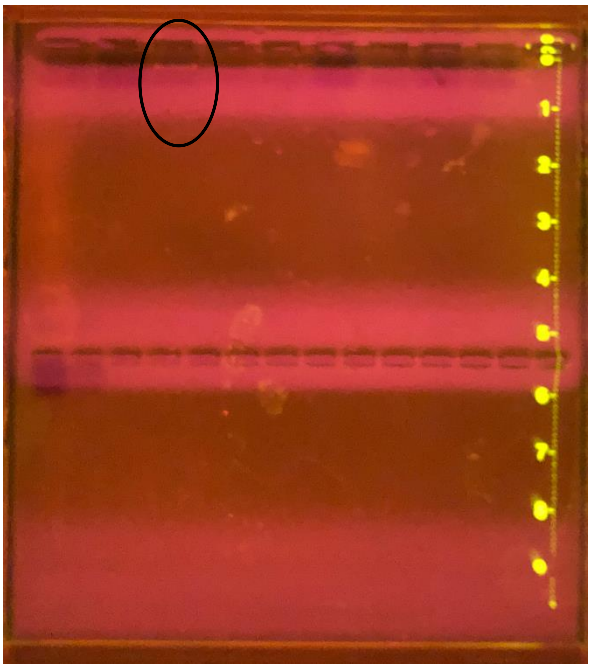

Figure 4h: Agarose gel of: Protocol 1 experimental samples with pig primers: gel well loaded left to right as followed top 15-22, - bottom 23-25. one band in sample 17.

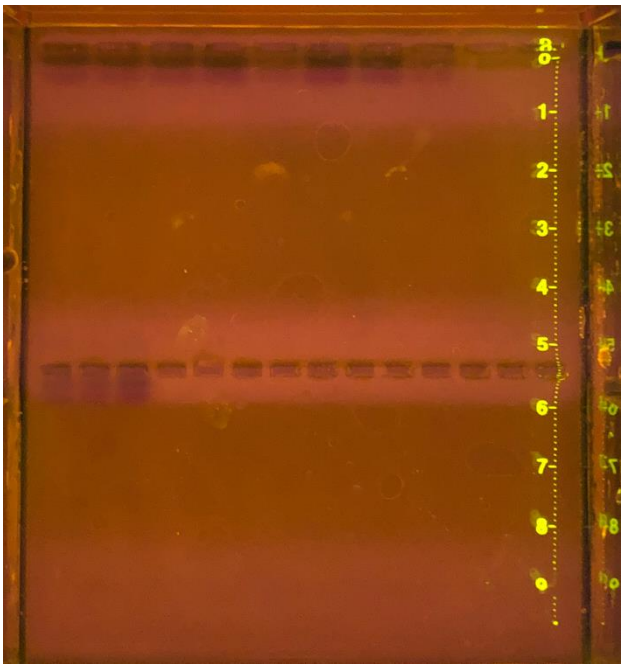

Figure 4i: Agarose gel of: Protocol 1 experimental samples with pig primers PCR rerun: gel wells loaded left to right as followed top 15-21, - bottom 22-25.

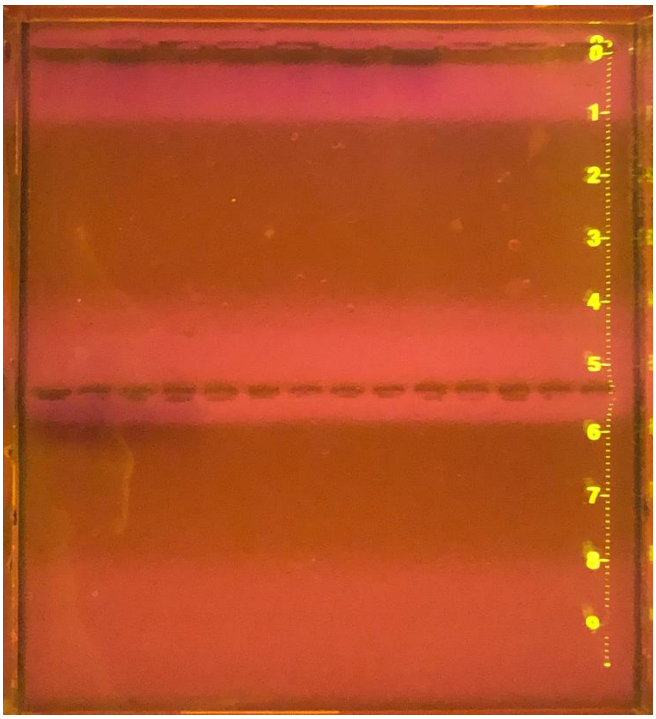

Figure 4j: Agarose gel of: Protocol 2 experimental samples with pig primers: gel well loaded left to right as followed top 25-31, bottom 32-35.

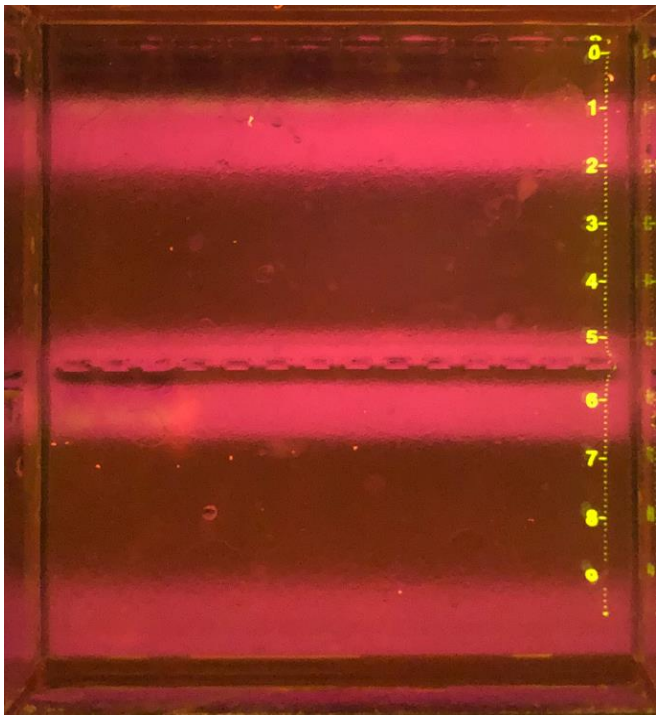

Figure 4k: Agarose gel of: Protocol 2 experimental samples with cytb primers: gel well loaded left to right as followed top 15-22, bottom 23-25.

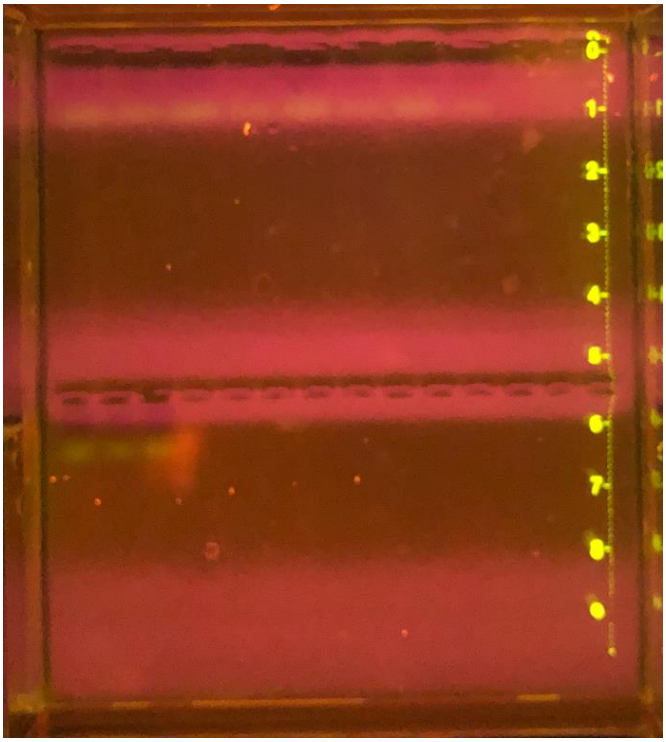

Figure 4l: Agarose gel of: Protocol 2 experimental samples with 16s primers: gel well loaded left to right as followed top 15-22, - bottom 23-25.

Chapter 5

Discussion

5.1 DNA results

The analysis described above provides information regarding DNA extraction from both archaeological and experimental ceramic material using 5% ammonium hydroxide and PB buffer using PCR and Sanger sequencing to establish the presence of any viable DNA. The results suggest that DNA cannot be successfully extracted using the methods mentioned in this thesis.

Archaeological and experimental samples from protocol 2 DNA quantification analysis produced readable results for the overall majority of all samples tested as depicted in tables 4a, 4b, 4c, and 4d. The experimental samples from protocol 1 only produced readable results for two samples between protocol 1 kiln and fire pit samples as depicted in tables 4a, and 4b. DNA quantification calculates all DNA within the sample, whether it is contamination, bacterial or from the use of the ceramic vessel. The preliminary results suggested that DNA was present in the extraction samples. These results do not alone prove that the DNA extraction protocols were a success. To test the successfulness of the extractions, PCR and sequencing was performed.

Only four archaeological samples were successfully annealed, amplified, and sent for sequencing. Sequencing the results determined that DNA was primarily due to human contamination. Contamination is always a possibility when working with DNA, especially aDNA. One sequencing result was consistent with the bacteria *Gemmatirosa kalamazoonesis*.

The experimental samples from protocols 1 and 2 did not successfully produce DNA amplification. One band was present in the first PCR attempt of protocol 1 but it could not be reproduced, suggesting that it was due to possible contamination. The protocol 2 samples were

first run using pig primers and, when no bands were present, samples were then re-run with new pig primers. However, bands continued to be missing in the gel, suggesting amplification was not successful for both the endogenous DNA (if present), nor contamination. Samples were re-run using 16s and cytb primers to determine the source of the high read of DNA quantification results, but both PCRs did not produce any bands as depicted in figures 4j and 4k. An inhibition test was run on samples after the PCRs from both protocols 1 and 2 to determine if an inhibitor was present, as depicted in figures 4e and 4f. The inhibition test showed that only the fire pit samples from protocol 1 were severely inhibited. The samples were diluted to reduce the unknown inhibitor effect on amplification but was unsuccessful for half of the samples as depicted in figure 4g preventing any further attempt of amplification.

The overall results indicate that DNA extraction from ceramic vessels using 5% ammonium hydroxide or PB buffer as depicted in the protocols developed by Shanks et al. (2001) is not currently possible. Ceramic vessel absorbed residue analysis should rely on already proven methods, as it has been shown that the preservation ability for ceramic vessels is excellent for organic residues, such as lipids and proteins, which have been successfully extracted from vessels containing foodstuff and cooking in past studies (Oudemans and Boon 2007; Evershed 1993; McGovern and Hall 2016).

5.3 Unexpected results

Gemmatirosa kalamazoonesis also known as KBS708 was sequenced from archaeological sample 4. KBS708 was first identified in 2013 and is the only species of the genus *Gemmatirosa* of the phylum *Gemmatimonadetes* (Radosevich et al. 2013; Debruyn et al. 2014). *Gemmatimonadetes* are frequently detected in a variety of environments and can be found primarily in soil (Neal et al. 2017; Radosevich et al. 2013; Zeng et al. 2015; Debruyn et al.

2014). Not much is known on this bacterium's ecological importance and characterization due to the difficulties in cultivating it (Neal et al. 2017).

These results do not provide information on the use of the ceramic vessel from which the DNA was extracted. The source of the DNA is probably from the soil, but whether it is from the shards' place of origin or if it was cross contaminated from another source is unknown.

5.4 Limitations

When interpreting the results of the DNA analysis of both archaeological and modern samples, it is necessary to address biases and complications inherent to the DNA extractions from ceramic vessels used in this project that can have a possible effect on the results. (1) The small sample size of a total of 27 ceramic shards were used:11 archaeological, 8 kiln and 8 fire pit experimental ceramic shards were used in this project. The 16 experimental shards came from the same 8 experimental ceramic vessels. The samples size was affected by shard size. The archaeological ceramic vessel shards selected was limited to what could fit onto the cleaning boats and the polypropylene bags. Experimental ceramic vessel's samples size was limited what could be sculpted form for a small bucket of clay. (2) Only one type of clay and two firing methods were used. There is a large variety in clay types and firing techniques that are unique to where they are found (Balkansky, Feinman & Linda, and Nicholas 1997; Rye 1976; Tite and Maniatis 1981; Smith 2007). It is unknown how clay types and firing methods affect DNA preservations and amplification. Future research should take clay type and firing methods into account. (3) Mechanical complications were a factor in this project. During the first and second extraction of the archaeological samples, the pump went out. The heat of the vacuumfuge was turned to high to increase evaporation. Subjecting the samples to heat for a prolonged time can cause DNA degradation. While in the process of extraction for the experimental samples of

protocol 1, the vaccufuge broke during the evaporation phase of the 5% ammonium hydroxide. To reach the correct pH balance, PB buffer was used to dilute the samples until the correct pH was reached. (4) During the soaking process of the extraction phase for the DNA from protocol 2's ceramic shards, the PB buffer used to extract the DNA partially leaked out of F25, F28, F29, K32, and K34 polypropylene bags. 650µl of PB buffer was re-added to the bags to try and salvage any remaining DNA in the bags. This has the possibility of changing the results due to DNA loss. Future studies should consider the possibility of bag leakage and tightly seal the bottom of the bags.

5.5 Significance and future research

This project is only an introduction to DNA analysis of ceramic vessels and only two extraction methods used were designed for stone tool analysis. Currently, DNA analysis of ceramic vessels is not a viable tool for studying their use. This research shows that methods developed for lipid and protein analysis are better suited for absorbed organic residue studies at this time. Methods for DNA extraction from ceramic vessels are not currently available, but this does not rule out the possibility for their successful development in the future. This project did not incorporate all types of firing methods, clay types, or clay preparation, which are all factors that can affect the success of DNA extraction. Future research is needed to determine if firing methods could affect DNA preservation and or amplification. The inhibitor test on the experimental samples of protocol 1 showed that over half the kiln fired samples were uninhibited. The fire pit samples of mature and dense clay were all inhibited. This possibly suggests that the process used to fire these samples produced an inhibitor substance. Research is also needed to determine if different clay types affect DNA preservation, amplification, and/or sequencing. Much is unknown about the relationship between DNA analysis of ceramic vessels,

and more research is needed to determine if DNA can be successfully abstracted from ceramic vessels to infer their use.

Chapter 6

Conclusion

The overall results indicate that the methods developed by Orin Shanks do not work for extracting DNA from ceramic vessels. The DNA that was successfully amplified was from human and bacterial contamination. The bacteria amplified was from *Gemmatirosa kalamazoonesis*, from an unknown source. This study is only one step in determining if ceramic cooking vessels can preserve absorbed DNA and these results do not eliminate future possibilities that DNA can be extracted from ceramic shards. More research is needed to determine if clay type and/or firing methods affect absorbed DNA preservation or amplification. For now, ceramic vessel analysis should be done using organic residue analysis that has been proven successful in studying absorbed residues.

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