EXPERIMENTAL INVESTIGATION INTO THE PRESERVATION AND RECOVERY OF DEGRADED DNA FROM SEDIMENTS

by

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B.Sc. (Hons), Simon Fraser University 2010

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF ARTS

In the
Department of Archaeology
Faculty of Environment

SIMON FRASER UNIVERSITY

Summer 2012

APPROVAL

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Degree: **MA**

Title of Thesis: **Experimental Investigation into the Preservation and Recovery of Degraded DNA from Sediments**

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Date Defended/Approved: August 29, 2012

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Abstract

Controlled experiments were used to recover DNA from sediments in order to understand DNA preservation in sediments and to examine the effectiveness of different DNA recovery methods.

Known quantities of DNA were added to different sediment samples and artificially degraded through heat exposure. DNA extraction techniques included a chloroform/octanol and silica-spin column method. Standard and quantitative PCR were employed to assess the quantity of mtDNA recovered.

The results demonstrate that DNA can be preserved in sediment, with successful DNA detection after exposure to 120ºC for up to 70 hours. It was also shown that the silica-spin column method recovered significantly more DNA than the other method but PCR inhibition was a consistent problem, with at least 25X sample dilution required for successful amplification.

Technical improvements are needed to advance sediment DNA research; however, the data from this study support the notion that degraded DNA can be recovered directly from sediments.

Keywords: *sed*aDNA; sediments; artificial DNA degradation; DNA extraction

Acknowledgements

I would first like to extend my appreciation to my supervisory committee, Dr. Dongya Yang and Dr. Gail Anderson, for their guidance throughout my studies at SFU. Their support at both the undergraduate and graduate level has played a decisive role in shaping my research interests and I am proud to have worked under their direction. Special thanks go to my senior supervisor, Dr. Dongya Yang, for sharing his expertise and spending the time and energy required to train me in (and out) of the lab. I am grateful to Dr. Dean Hildebrand of BCIT for serving as my examiner and providing many helpful comments on my research, as well as to Dr. Eldon Yellowhorn for serving on my examining committee. This study was supported by a SSHRC RDI Grant (Yang), a SSHRC CGS Master's Scholarship and the Department of Archaeology.

In addition, I would like to thank all past and present members of the Yang lab group for their contributions to scientific discussions, lab assistance and general support and friendship. Many thanks go to Andrew Barton and the SFU Department of Archaeology staff (in particular Merrill Farmer, Chris Papaianni, Laura Nielson, Shannon Wood and Peter Locher), all of whom frequently went above and beyond their duties in an effort to help. Dr. Dana Lepofsky and the Department of Archaeology's 2010 cohort also need to be recognized for helping shape this research, as well as for their continued moral support. My gratitude

goes to Joselito Arocena (UNBC) and Chelsey Armstrong for providing soil and sediment samples, to Jodie-Ann Warren for helping me access a humidity chamber, and to Lisa Dyck for helpful editing.

This experience would not have been complete without the friendship and encouragement I have received from my peers, friends and family. I am particularly indebted my parents and brother, whose never wavering belief in me is the single greatest source of support I could have hoped for. Thank you!

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Glossary

Abasic site	A location in DNA that lacks a nitrogenous base, either from spontaneous reaction or due to DNA damage.
Ancient DNA	aDNA; The DNA found in ancient remains. Ancient DNA is typically characterized by low quality and quantity.
Amplicon	A strand of DNA that is produced by PCR amplification. Specific sequence fragments are typically targeted through use of target-specific primers.
'Dirt DNA'	DNA that is recovered from sediments, soil, ice and coprolites.
DNA polymerase	An enzyme involved in DNA replication. It helps catalyze the polymerization of deoxyribonucleotides into a DNA strand.
Gel electrophoresis	A technique that results in the size separation of charged molecules. Electricity is run through an agarose gel causing DNA to migrate in the direction of the current. Shorter fragments migrate faster than larger fragments.
Inhibitor	PCR inhibitor; Any factor that prevents the amplification of DNA. Inhibitors are typically co-extracted alongside DNA and generally exert their effects through direct interaction with DNA or by interfering with DNA polymerases.
Primer	Short synthetic DNA strands that serve as a starting point for DNA synthesis. Primers are used to target specific sequences of DNA during PCR amplification through complementary binding to template DNA.

Probe	TaqMan probe; A dual-labelled specific probe designed to bind to a specific DNA sequence and used in qPCR. They use a reporter fluorophore at the 5' end and a quencher fluorophore at the 3' end. The quencher suppresses fluorescence of the reporter until the probe anneals to a DNA template and is excised by DNA polymerase activity. The amount of fluorescence can be compared to a known standard for sample quantification.
PCR	Polymerase chain reaction; Standard PCR; A molecular technique used to amplify DNA exponentially. PCR relies on thermal cycling to denature, anneal and extend DNA templates during amplification.
Quantitative PCR	qPCR; A molecular technique for the detection and quantification of amplified DNA based on the incorporation of a fluorescent reporter dye. Follows the same principles as standard PCR.
*sed*aDNA	Sedimentary ancient DNA; DNA in ancient sediments and soils. As with other types of aDNA, it is characterized by minute amounts of highly degraded DNA.

Abbreviations

aDNA	Ancient DNA
bp	Base pair
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
eDNA	Extracellular (or environmental) DNA
EDTA	Ethylenediaminetetraacetic acid
iDNA	Intracellular DNA
mtDNA	Mitochondrial DNA
NMWL	Nominal molecular weight limit
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
*sed*aDNA	Sedimentary ancient DNA
SDS	Sodium dodecyl sulphate
TBE	Tris/Borate/EDTA

Chapter 1: Introduction

Sedimentary Ancient DNA

Successful extraction and analysis of ancient DNA (aDNA) from ancient human, faunal and plant remains has provided opportunities for researchers to address important questions in a variety of different fields such as evolutionary biology, conservation, immunology, anthropology and archaeology (e.g. Bos et al. 2011; Speller et al. 2010; Noonan et al. 2006; Shapiro et al. 2004; Bunce et al. 2003; Jaenicke-Despres et al. 2003). aDNA research typically involves the recovery of DNA from physical remains such as bone, teeth, hair, shell and tissues (Gilbert 2003). Thus, until recently, aDNA researchers have relied on the relatively rare discoveries of *in situ* macrofossil remains in order to investigate the past from a molecular perspective. Recent research has shown that aDNA can be recovered directly from archaeological sediments (Hebsgaard et al. 2009), even in the absence of physical remains. This type of DNA is termed 'sedimentary' aDNA (*sed*aDNA) and is sometimes collectively referred to as 'dirt DNA' (a term that encompasses DNA from sediments, soils, ice and coprolites).

*sed*aDNA presents itself as a seemingly limitless source of aDNA that researchers can use to recover previously unavailable genetic information. As a new way to study the past, this newly tapped resource has seen a surge in publications in recent years (e.g. Epp et al. 2012; Jorgensen et al. 2012; Parducci et al. 2012). The study of DNA recovered from sediments and soils can

be broadly divided into two areas: 'intracellular' DNA (iDNA) and 'environmental' or 'extracellular' DNA (eDNA). iDNA is easily the most heavily studied area and involves the study of DNA originating from living soil biota (i.e. microbial and meiofaunal communities). eDNA, which represents a smaller proportion of the DNA present in sediments, is the primary focus of this thesis. It represents the DNA in the environment that originates from organisms and their remains. This type of DNA is subject to both chemical and biological degradation (Pietramellara et al. 2009; Levy-Booth et al. 2007), the extent and cause of which are dependent on environmental conditions (Lindahl 1993).

*sed*aDNA is thought to be of local origin (Jorgensen et al. 2012; Haile et al. 2007; Willerslev et al. 2003) and, although the source of it is unknown, it is speculated to be derived from leaf litter, rootlets, seeds, pollen, faeces, urine, epidermal cells, keratinous tissues or invertebrate exoskeletons (Haile et al. 2007; Lyndolph et al. 2005). As it is possible to age *sed*aDNA through direct dating of the sediments (Arnold et al. 2010; Hebsgaard et al. 2009), this revolutionary concept makes it possible to link past humans, animals and plants in time and space, even in the absence of macroscopic remains. As a result, *sed*aDNA analysis can be a powerful tool which can shed light on and redefine previous interpretations of the past.

The principal focus of the first studies using *sed*aDNA were permafrost samples (e.g. Jorgensen et al. 2012; Haile et al. 2009; Willerslev et al. 2004; Willerslev et al. 2003). This is likely for a variety of reasons, the primary being (1) the extremity of the temperatures promoting good DNA preservation and (2)

minimal leaching between sediment layers (Hofreiter et al. 2001; Greenwood et al. 1999; Lindahl 1993). Since then, this approach has been applied to non-frozen sediments such as those from caves, lakes and archaeological sites (Anderson-Carpenter et al. 2011; Coolen and Gibson 2009; Hebsgaard et al. 2009; Matisoo-Smith et al. 2008; Hofreiter et al. 2003). It should be noted that the non-frozen sediments used are often of noticeably younger age than permafrost samples (probably in an attempt to combat issues of leaching) (Andersen et al. 2012; Haile et al. 2007).

During the last two decades, 'dirt DNA' has been used predominantly as a paleoecological proxy in contexts in which macrofossils are extremely rare or absent. While it is sometimes used stand alone (e.g. Haile et al. 2009; Willerslev et al. 2003), *sed*aDNA analysis has largely been used in conjunction with other macrofossil information (e.g. Jorgensen et al. 2012; Anderson-Carpenter et al. 2011; Hebsgaard et al. 2009). It has been shown to be a compelling line of evidence that can complement other traditional lines of evidence in a non-overlapping way (Jorgensen et al. 2012). Studies using *sed*aDNA have demonstrated that with well controlled circumstances, it can act as a sensitive tool capable of providing otherwise impossible insights from samples up to hundreds of thousands of years old (Willerslev et al. 2003). Notable studies include the revelation of the responses of past species to environmental changes (Haile et al. 2007); reconstruction of paleoecosystems in Siberia and New Zealand (Willerslev et al. 2003); revelation of the youngest evidence of conifer

forest in Greenland (Willerslev et al. 2007); and timing of the last occurrence dates of extinct species (Haile et al. 2009).

While the primary focus of *sed*aDNA studies are paleoecological in nature, to date, one study has utilized the *sed*aDNA approach in an archaeological context. Hebsgaard et al. (2009) used DNA recovered from anthropogenic sediments from a Norse Farm in Greenland to detect population fluctuations of domesticated animals. In accordance with independent studies on conventional macrofossil remains, they were able to use *sed*aDNA to successfully estimate relative changes in livestock through time. Despite this promising study, little analysis of *sed*aDNA has been applied to archaeological sediments.

Challenges in the Study of *sed*aDNA

Secure Dating of DNA

The use of *sed*aDNA in any context is reliant on accurate dating of the DNA. This issue is one of largest concerns in *sed*aDNA research because one cannot actually date the DNA directly; rather, one must date the associated sediments. As a result, one critical assumption in *sed*aDNA research is that the recovered DNA is both autochthonous and synchronous with the sediment sample tested. This is not always the case though, and false positives can result from the analysis of non-primary *sed*aDNA that has been (1) physically transported into the sample via, for example, liquid water or (2) reworked within the sedimentary profile by postdepositional mixing (Arnold et al. 2010).

Although there is compelling evidence that DNA leaching is less of a concern under frozen conditions (Arnold et al. 2011; Willerslev et al. 2007; Hansen et al. 2006), research on the effects of leaching in temperate and wet sediments is less well characterized, with contradictory published results (Andersen et al. 2012; Anderson-Carpenter et al. 2012; Hebsgaard et al. 2009; Rasmussen et al. 2009; Gilbert et al. 2008; Haile et al. 2007). This issue is especially pertinent because non-frozen sediments, such as those from caves, lakes or archaeological sites, are major sources of aDNA. DNA leaching would significantly complicate, possibly invalidate, the interpretation of results in certain contexts (Haile et al. 2007). Arnold et al. (2008; 2010) have proposed that radio carbon (^{14}C) dating and optically stimulated luminescence (OSL) dating of quartz grains associated with *sed*aDNA should be combined in an effort to assess the stratigraphic integrity of *sed*aDNA samples. Nevertheless, the issues surrounding secure dating requires further exploration to ensure the accuracy of *sed*aDNA studies.

Representation Biases

Another major question surrounding the use of *sed*aDNA concerns the extent to which *sed*aDNA reflects the biodiversity present in sediments, both qualitatively and quantitatively. Representation biases may result from numerous different factors such as environmental heterogeneity and taxonomic variance in biomass, density, morphology (e.g. presence of remains such as hair, feathers, etc.), physiology (e.g. production of eggs, faeces, etc.), mobility, behaviour, etc. (Andersen et al. 2012; Andersen 2011; Haile et al. 2007; Willerslev et al. 2003).

This is further complicated by biases introduced due to differences in DNA extraction and amplification efficiency between different sediment types (Lloyd-Jones and Hunter 2001; Frostegard et al. 1999), differences in DNA amplification efficiency of different species (especially when using general primers) (van Doorn et al. 2009), sequencing technology errors (Quince et al. 2011; Porazinska et al. 2009; Huse et al. 2007) and an incomplete reference database (i.e. information on the natural diversity of species and higher order taxonomic levels may be unavailable) (Andersen 2011). The accumulation of these biases can lead to significant over- and/or underestimations of the organisms recovered from *sed*aDNA samples, both in terms of taxonomic richness and abundance (Quince et al. 2011).

Most likely due to the fact that the absence of any macrofossil remains in *sed*aDNA samples translates to no prior knowledge on diversity being available, this area of concern is largely unexplored. However, one study attempted to evaluate the accuracy of 'dirt DNA' meta-barcoding as an indicator of taxonomic richness by taking advantage of the detailed records and known species composition available from animal holdings in safari parks, zoos and farms. Andersen et al. (2012) demonstrated that the proportional distribution of DNA recovered from soils reflected overall the above ground taxonomic richness. This study also noted that the biomass of the individual populations present acts as a better proxy for 'dirt DNA' deposition rather than the population size, and that animal behaviour was shown to influence DNA deposition rates. Furthermore, the sensitivity of the approach did not increase with the amount of soil tested,

rather sensitivity was related to spatial scale sampling. This important research illustrates the careful consideration one must give to *sed*aDNA studies, as well as the need for further investigation into these issues in order to understand how the *sed*aDNA approach can be further implemented in natural settings.

Despite the high potential of sediments as a source of aDNA, this quickly growing subfield can still be thought of as in its infancy. Due to the relatively unexplored issues and concerns surrounding its implementation into archaeological investigations, *sed*aDNA analysis is still 'hit or miss' in its nature. It can be difficult to determine whether failure to recover *sed*aDNA is the result of an absence of DNA or technical difficulties, such as an inappropriate extraction technique. Before *sed*aDNA analysis can be considered a feasible and reliable research tool for archaeological investigations, the success rate must be improved. It is clear that basic studies are needed to understand how DNA is preserved within sediments and to determine the most effective method to recover DNA from sediments.

Research Objectives

The ultimate goal of this study was to help develop *sed*aDNA analysis into a reliable approach for archaeological investigations by providing some fundamental insights into DNA preservation and recovery from sediments. The specific aims were to use a controlled laboratory setting to:

(1) establish an artificial degradation system in order to simulate ancient DNA degradation within archaeological sediments;

(2) investigate DNA preservation in sediments; and

(3) systematically compare two soil/sediment DNA recovery techniques in order to determine whether sediment type influences methods required for optimal DNA recovery and amplification.

Chapter 2: Background

DNA Degradation

The DNA molecule is a fairly unstable molecule that will degrade with time if it is not repaired. Living cells have an extensive repair system that continuously repairs DNA damage. When an organism dies, its repair systems stop functioning, while spontaneous degradation processes continue. As time goes on, the damage accumulates which can result in the DNA molecule persisting, but only in a highly degraded form (Hoss et al. 1996; Handt et al. 1994; Lindahl 1993; Paabo 1989). This build up of damage over time is central to the methodological problems inherent in aDNA research (Willerslev and Cooper 2005). As damage accumulates, analysis becomes more difficult due to strand breakage, abasic sites, miscoding lesions and DNA crosslinks. These processes result in fewer amplifiable templates, sequencing artefacts and preferential amplification of contaminant (i.e. undamaged) DNA (Alaeddini et al. 2010; Brotherton et al. 2007; Willerslev and Cooper 2005; Paabo et al. 2004; Gilbert et al. 2003; Hoss et al. 1996; Lindahl 1993; Paabo 1989). These amplification artefacts can lead to false positive results and/or misidentifications if proper sequence authentication measures are not followed (Cooper and Poinar 2000).

DNA damage in ancient samples can result from a number of different mechanisms. Enzymatic damage can result from endogenous and exogenous

nucleases cleaving DNA, or bacteria and fungi digesting (i.e. fragmenting) DNA molecules (Lindahl 1993; Eglinton et al. 1991). Under special circumstances such as rapid desiccation, low temperature and/or high salt concentrations, these nucleases and microbes can be inactivated before all genetic information is lost (Paabo et al. 2004; Hofreiter et al. 2001; Smith et al. 2001). Even in such situations, slower but still relentless processes continue to act on DNA. These attacks are the result of chemical reactions like hydrolysis and oxidation, which take place spontaneously in the presence of water and oxygen respectively. DNA hydrolysis causes the breakdown of the N-glycosol sugar-base bonds of DNA, which can create abasic sites (Alaeddini et al. 2010; Hoss et al. 1996; Lindahl 1993). The resulting decrease in the stability of the DNA sugar-phosphate backbone can then lead to strand breakage, cross-linking of DNA strands and incorrect base pair insertions during DNA amplification (Alaeddini et al. 2010; Gilbert et al. 2003; Hoss et al. 1996; Lindahl et al. 1993). Oxidative DNA damage involves the interaction of free oxygen radicals with DNA resulting in abasic sites, base pair alterations, DNA cross-links, sugar modifications and the creation of the PCR inhibitor hydantoin through chemical alterations to nitrogen bases (Alaeddini et al. 2010; Paabo et al. 2004; Hofreiter et al. 2001; Lindahl 1993).

DNA Preservation in Sediments

Little is known about DNA preservation in sediments (Willerslev et al. 2004), especially in regards to the rate at which DNA degrades (Gilbert et al. 2003; Hofreiter et al. 2001). Andersen et al. (2012) claimed that the amount of

DNA retrieved from sediments was influenced by sediment texture. Specifically, they noted that larger amounts of DNA were recovered from samples with larger sediment particle surface areas. This is consistent with current knowledge about DNA preservation in sediments. Previous controlled laboratory experiments have demonstrated that eDNA rapidly adsorbs and binds to the surfaces of mineral grains (Romanowski et al. 1991; Ogram et al. 1998; Lorenz and Wackernagel 1987), clay minerals (Alvarez et al. 1998) and other organic soil particulates such as humic acids (Crecchio and Stotzky 1998; Tsai and Olsen 1992). The formation of these tightly bound organo-mineral complexes significantly reduces the potential for microbial nuclease degradation of adsorbed DNA, thus promoting the long-term preservation of eDNA in sedimentary deposits (Cai et al. 2006; Crecchio and Stotzky 1998; Trevors 1996; Romanowski et al. 1991; Lorenz and Wackernagel 1987). If DNA is likely to adhere to sediments (both particles and particulate organic matter), it is reasonable to presume that more DNA will bind to larger sediment particles. Increased amounts of bound DNA not only results in a greater quantity of DNA present, but leads to enhanced DNA preservation as well. Consequently, in sediment samples composed of larger particles, increased amounts of DNA are available for retrieval.

Unfortunately, extrapolation of the results from controlled studies to natural sedimentary environments is complicated by the number of different factors that affect the adsorption of DNA to sediments. These factors include the mineralogy of the sorbent, the ionic strength of the medium, the pH of the

medium and the length of the DNA polymer (Alvarez et al. 1998). While such studies suggest that *sed*aDNA likely persists as eDNA bound to both particles and organic particulates, one cannot rule out the possibility that DNA survives as free molecules or that cellular DNA is released during the extraction process (Arnold et al. 2010; Haile et al. 2007; Ogram et al. 1998).

Artificial Degradation of DNA

Ideally, one would study DNA preservation and recovery in sediments by enabling DNA to naturally degrade over time; however, this is not practical as degradation reaching aDNA levels would take potentially several hundreds of thousands of years. To speed up the process heat and time were used as controllable factors to artificially degrade modern DNA in order to simulate aDNA found within sediments. Although numerous factors are known to degrade DNA (e.g. UV, humidity, pH), heat was used as temperature is often cited as a key factor in DNA degradation processes (Arnold et al. 2010; Karanth and Yoder 2009; Bollongino and Vigne 2008; Pruvost et al. 2007; Paabo et al. 2004; Capelli et al. 2003). In fact, the temperature (and environment) from which a sample is found is thought to influence DNA degradation significantly more than the chronological age of the remains (Karanth and Yoder 2009; Zhang and Wu 2005; Poinar 2003; Paabo et al. 2004; O'Rourke et al. 2000). Temperature has also been shown to be one environmental variable that is consistently associated with differential aDNA success rates across geographic regions (Bollongino and Vigne 2008; Reed et al. 2003). Furthermore, previous research using this method on bone, teeth and plant samples has demonstrated the usefulness and

convenience of using high temperatures to generate an artificial DNA degradation model in order to study ‘aDNA’ in a controlled laboratory environment (Moore 2011; McGrath 2010; Stagg 2010; Dobberstein et al. 2008; Threadgold and Brown 2003).

Chapter 3: Material and Methods

Sample Selection and Preparation

Control Sediments – Size-based

Archaeological sediments from a shell midden at the O'Connor site located in Port Hardy, British Columbia were chosen for this study as the basic (control) samples to be mixed with DNA tissue samples (Sample E, Table 1). These sediments were in storage for numerous years in the Department of Archaeology at Simon Fraser University and were chosen for the following reasons: 1) the sediments are from an archaeological site on the Northwest Coast, which can potentially provide useful information about *sed*aDNA from sites of this (or similar) regions; 2) the sediments happened to be available for use; and 3) the large quantity available allowed for manipulations (e.g. sorting based on particle size).

In an attempt to control for environmental variables, a single sample of the sediment was manually separated into three sample types based on sediment particle size. The O'Connor Site sediments were sorted by particle size using a graded series of USA Standard sieve screens (Gilson Corporations., Worthington, OH, USA). The sorting was conducted for approximately thirty minutes using a motorized sieve shaker (Model 18480, Cenco Meinzer, Chicago, Illinois, USA). Three samples were selected for testing: ES (<63 μm), EM (212-300 μm) and EL (500-710 μm).

Table 1: Sediment sample information.

Sample Name	Location	Other Information
E	Port Hardy, British Columbia, Canada	Borden number EeSu-5; O'Connor site; shell midden
B	Old Crow River, Yukon Territory, Canada	Site 15; fluvial deposits with incipient soil formation; finely bedded silts and sands; geological; 07/07/79 - Andrews
N	Kentucky, USA	Part of Lower Cumberland Archaeology Project; GBC 80; Nance

Note: Three additional size-based samples were created by separating Sample E by particle size: ES (<63 μm); EM (212-300 μm); EL (500-710 μm).

Control Sediments – Type-based

In order to test the applicability of DNA extraction techniques across a variety of sediment types, three different sediments were selected. In addition to the E samples from shell midden at the O'Connor Site, two different sediment samples were chosen from other sites for testing. These other sediment samples include presumed geological sediments from fluvial deposits with incipient soil formation at Old Crow River located in the Yukon Territory, Canada; and archaeological sediments from a site located in Kentucky, USA that were collected as part of the Lower Cumberland Archaeological Project (LCAP). These samples were designated 'B' and 'N' respectively (Table 1). As with the midden samples, these sediments were in storage for numerous years in the Department of Archaeology at Simon Fraser University. For this study, the selection of the three sediments tested was based primarily on visual

examination (i.e. colour, texture, etc.) in order to ensure three distinct ‘types’ of sediments were chosen (Figure 1).

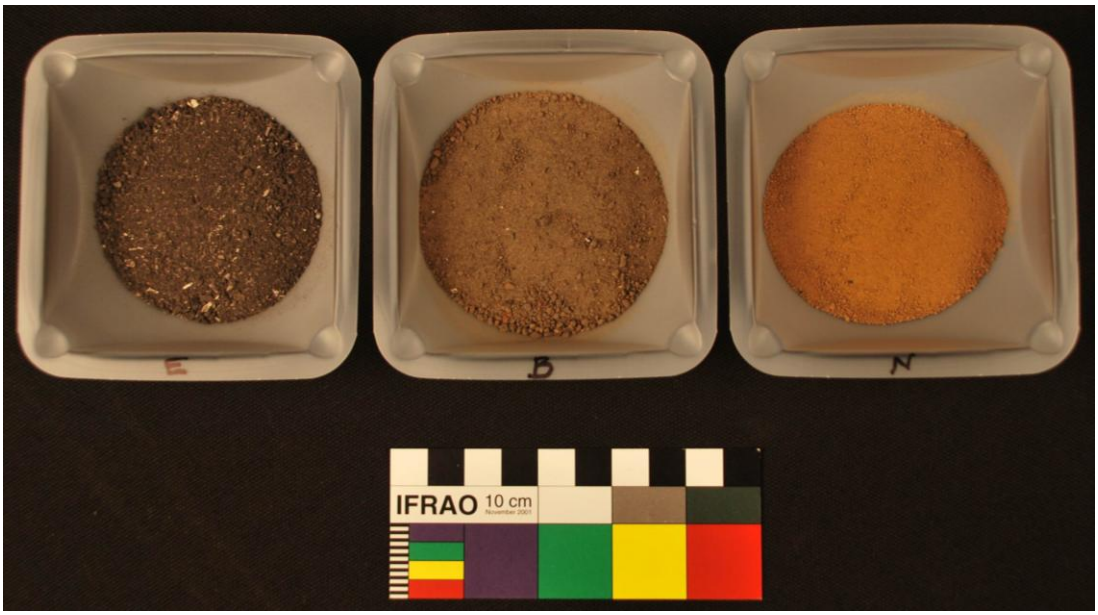


Figure 1: Control sediment samples (type-based). Three sediment samples were chosen based on visual distinctness. L-R: ‘E’ sediments are from shell midden at the O’Connor site; ‘B’ sediments are from fluvial deposits with incipient soil formation at Old Crow River site 15; and ‘N’ sediments are from an unknown site part of the Lower Cumberland Archaeological Project. A sample of the ‘E’ sediments were also separated by particle size for further investigation.

Control DNA

Control DNA is defined as the DNA (or tissue) of known species and quantity that is added to the test sediments. Earlier pilot studies using liquid forms of DNA (both ‘naked’ and cellular DNA) in a controlled laboratory setting saw aggregates forming, resulting in non-homogenous mixtures of DNA and

sediment (data not shown). To avoid this problem, powdered tissue was chosen as the source of DNA to be added to sediment samples. *Ovis aries* (sheep) was the chosen mammalian species because it is both easy to obtain and unlikely to be already present in the sediments used. 100% pure dehydrated sheep lungs (N/A-Nothing Added, Cambridge, Ontario, Canada) were purchased from a local pet store and blended into powder form using a commercial blender (Model BL10450HB, Black and Decker Corporation, Towson, Maryland, USA) (Figure 2). Tissue powder was stored in glass containers at room temperature until further use.

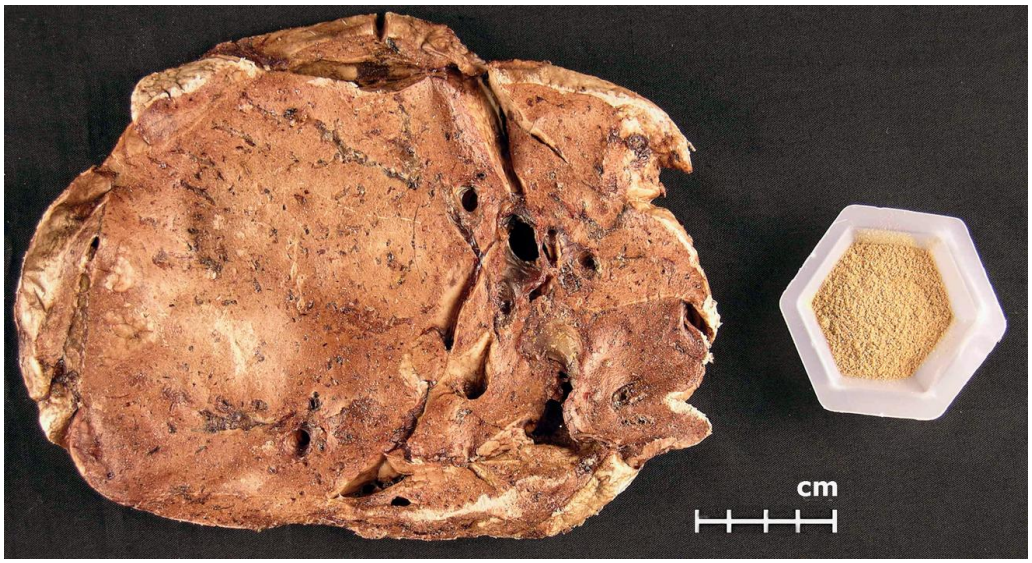


Figure 2: Control DNA samples. Dehydrated sheep lungs (L) were blended into a powder (R) to serve as a controlled source of DNA. The sheep lung powder tissue was chosen due to the ease in which it is mixed with sediments.

Temperature-Induced DNA Degradation

For the size-based samples, 25 g of sediment was weighed out and transferred to a 50 mL tube. 2 g of powdered sheep tissue was added to each tube and mixed thoroughly for homogenization. 2 g aliquots of each sediment-DNA sample were then placed in an aluminium weigh boat and subjected to 95ºC heat treatment using a laboratory oven (Model 19200, Barnstead/Thermolyne, Dubuque, Iowa, USA) for various incubation times (0 hours, 8 hours, 16 hours, 24 hours).

Preliminary results of the size-based samples suggested DNA degradation was slower than anticipated. In an attempt to degrade DNA at a faster rate, for the type-based samples, the same steps were repeated with a few exceptions: the ratio of sediment to DNA was 30 g to 0.5 g; the incubation temperatures and times were increased to 120ºC and 0 hours, 24 hours, 48 hours and 70 hours respectively.

As the uniformity of the temperature within the oven used is unknown, the placement of the samples within the oven may influence the levels of sample heat exposure. In order to remove any biases that may affect heat exposure, the heating space was broken into nine numbered grids (Figure 3). A random sample generator (Stat Trek, http://stattrek.com/Tables/Random.aspx) was used to randomly assign a number between one and nine to each sediment-DNA sample that was to be heated (Appendix 1). Samples were placed in the area of the oven where the grid number corresponded to the assigned number.

Using a top loading balance (Model VIC-123, Acculab, Norwood, New Jersey, USA), samples were weighed before and after heat treatment to document percentage weight loss.

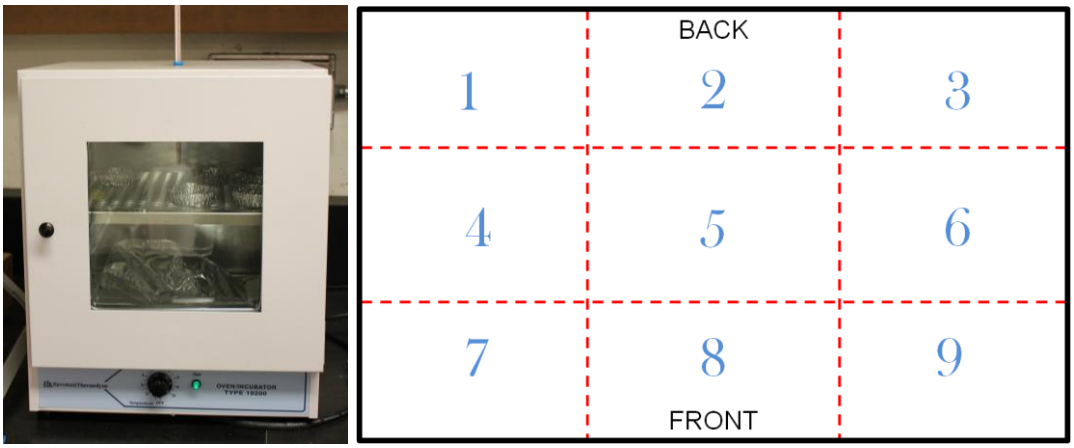


Figure 3: (L) A laboratory oven was used to degrade DNA via extreme heat treatment; (R) The top shelf of the oven was divided into a nine square grid. To remove any biases related to placement of sample in the oven, a random sample generator was used to determine sample placement within the grid.

DNA Extraction

This study was carried out in two phases. The first involved the use of the size-based sediment-DNA samples (ES, EM, EL); the second, the type-based sediment-DNA samples (E, B, N) (Table 1). As both followed the same protocols, the following passages describe the extractions of the type-based sediment-DNA experiments. Any discrepancies between protocols are listed in Appendix 2.

A positive control of sheep powder was also included with each extraction. Sediment blank controls were included with each extraction step in order to ensure that sediment samples did not contain any sheep DNA prior to the addition of the sheep lung powder. For each extraction, blank extraction controls were also included.

Organic Extraction: Chloroform/Octanol Method

Chloroform/octanol extractions were based on the protocol of Haile (2012). After manually shaking each sample to ensure homogenization, 0.45 g of sediment was transferred to Lysing Matrix E tubes (MP Biomedicals, Santa Ana, California, USA) containing 600 µL of Bulat buffer (0.02 g/mL N-lauroyl sarcosine; 50 mM Tris-HCl (pH 8.0); 2 mM EDTA; 0.15M NaCl; 1 mg/mL proteinase K; 3.5% 2-mercaptoethanol; 50 mM DTT) (Andersen 2011; Bulat et al. 2000). The samples were then vortexed to mix at maximum speed (3,400 rpm) for thirty minutes using a VX-200 Vortex Mixer (Labnet, Woodbridge, New Jersey, USA), followed by incubation overnight at 65ºC in a rotating hybridization oven (Model 6243, Thermo Scientific, Nepean, Ontario, Canada).

After incubation, samples were adjusted to 1.15 M NaCl. 300 µL of chloroform/octanol (24:1) were added to each sample, followed by incubation in a rotating hybridization oven at room temperature for ten minutes. Samples were then centrifuged for two minutes at 13,000 rpm. The supernatant (approximately 500 µL on average) was transferred to a clean 2 mL tube and PB Buffer (QIAGEN, Hilden, Germany) was added to one volume of supernatant in a 5:1 ratio. The resulting mixture was transferred to a QIAquick spin column (QIAGEN,

Hilden, Germany) and centrifuged for one minute at 13,000 rpm. As QIAquick spin columns hold a maximum of 800 µL, this step was carried out multiple times, with replacement of the collection tubes after each centrifugation step. 500 µL of Salton Wash Buffer 1 (MP Biomedicals, Santa Ana, Californica, USA) was added to each spin column, followed by centrifugation for one minute at 13,000 rpm. After placing columns in new collection tubes, 500 µL of Salton Wash Buffer 2 (MP Biomedicals, Santa Ana, Californica, USA) was added to each sample. Spin columns were centrifuged as before and new collection tubes used. 500 µL Buffer AWI (QIAGEN, Hilden, Germany) was added to each spin column, followed by centrifugation at 13,000 rpm for one minute and subsequent replacement of the collection tubes. To elute the DNA, 200 µL of EB Buffer was added to each spin column and the columns were then incubated at 65ºC for approximately five minutes, or until membranes began to drip. Samples were centrifuged for one minute at 13,000 rpm and the resulting elute was transferred to a 0.5 mL tube. The elution process was then repeated and the second elution added to the same 0.5 mL tube holding the first elution. The resulting samples were stored at -20ºC for further use.

Non-organic Extraction: Silica-spin Column Method

Silica-spin column extractions followed a modified protocol based on Yang et al. (1998). After manually shaking each sample to ensure homogenization, 0.45 g of sediment was transferred to a 15 mL tube containing 3 mL lysis buffer (10% EDTA; 0.5% SDS; 0.5 mg/mL proteinase K; 10 mg/mL DTT). The samples were then vortexed to mix at maximum speed (3,400 rpm) for fifteen minutes

using a VX-200 Vortex Mixer (Labnet, Woodbridge, New Jersey, USA). Samples were incubated overnight at 50ºC in a rotating hybridization oven (Model 6243, Thermo Scientific, Nepean, Ontario, Canada).

Following incubation, samples were centrifuged at 4,400 rpm for thirty minutes and 2 mL of supernatant was transferred to an Amicon Ultra-4 centrifugal filter device (30,000 NMWL, Millipore, Billerica, Massachusetts, USA). The Amicon devices were then centrifuged at 4,400 rpm until samples were down to a concentration of ≤100 µL (approximately eighty minutes). 500 µL of Buffer PB (QIAGEN, Hilden, Germany) was combined with the concentrated supernatant and the resulting mixture transferred to a QIAquick spin column (QIAGEN, Hilden, Germany) for purification. To bind DNA to the silica membrane, the spin columns were centrifuged for one minute at 13,000 rpm. After transferring the spin columns to new 2 mL collection tubes, each spin column was washed with 400 µL Buffer PE (QIAGEN, Hilden, Germany) and centrifuged for one minute at 13,000 rpm. This step was then repeated with an additional two minutes of centrifugation time. Spin columns were placed in new collection tubes after each wash. 100 µL of EB Buffer (QIAGEN, Hilden, Germany) was added to each spin column, followed by incubation in a heat block at 68ºC for approximately eight minutes, or until membrane began to drip. Samples were then centrifuged for one minute at 13,000 rpm and the resulting elute transferred to a 0.5 mL tube. This process was then repeated and the resulting (second) elution was transferred to a separate 0.5 mL tube. Both the first and second elutions were stored at -20ºC for further use.

Assessment of DNA Recovery

Since the DNA recovered from the test sediments is most likely composed of a majority of unwanted background DNA (e.g. environmental DNA, microbial DNA), PCR amplification of targeted, specific DNA markers becomes the only way for this study to detect and quantify recovered control DNA.

Standard PCR was used first to gain a rough idea of whether DNA could be recovered and amplified from sediments, as well as of the level of PCR inhibition in any recovered DNA. Quantitative PCR (qPCR) was then used to more accurately assess the quantity of DNA recovered.

Short fragments of mitochondrial DNA (mtDNA) were targeted for amplification for two reasons: (1) the high copy number of mtDNA compared to nuclear DNA (1000's of copies per cell vs. 2 copies per cell) and (2) the fragmented nature of degraded DNA means short fragments are more abundant and, therefore, easier to detect (Paabo et al. 2004).

Primers were designed to amplify a 139 bp fragment of the D-loop region of the sheep mitochondrial genome (Table 2). These primers were modified from McGrath (2010), analyzed using the online software Netprimer (PREMIER Biosoft International, Palo Alto, California, USA) and optimal annealing temperature determined using a gradient PCR (data not shown).

The same primers were used for qPCR, in conjunction with the probe designed by McGrath (2010) (Table 2). The design of the primers and probe used for qPCR were analyzed using the online software Beacon Designer (PREMIER Biosoft International, Palo Alto, California, USA).

Table 2: Primers and probes used for standard and quantitative PCR analysis.

Name	5'-3' Sequence
F596 (Primer)	ATGCGTATCCTGTCCATTA
R735 (Primer)	AGATGCCTGTTAAAGTTCATT
OA-638 (Probe)	CCGCGTGAAACCAACAAC

Note: The targeted fragment was 139 bp of the Dloop region of sheep mtDNA. The probe used a 5' 56-FAM label.

Standard PCR

In addition to 3 µL of DNA, the final 30 µL reaction mixtures contained 1x PCR Gold Buffer (Applied Biosystems, Foster City, California, USA), 2.5 mM $MgCl_2$ (Applied Biosystems, Foster City, California, USA), 0.2 mM dNTPs (Fermentas, Hanover, Maryland, USA), 0.3 µM of forward primer F596, 0.3 µM of reverse primer R735, 0.75-1.875 U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, Carlsbad, USA) and 1.0 mg/mL BSA. Blank controls created during DNA extraction were also used in lieu of template DNA for each reaction. For each PCR reaction, a negative control was also created. Either a Thermal Cycler Personal or a Mastercycler Gradient (Eppendorf, Hamburg, Germany) was used to cycle samples as follows: initial denaturation at 95°C for twelve minutes; sixty cycles consisting of denaturation at 95°C for thirty seconds, primer annealing at 55°C for thirty seconds and primer elongation at 70°C for forty-five seconds; and a final elongation step for seven minutes at 72°C.

In a separate laboratory, 5 µL of each PCR product was stained with SybrGreen (Invitrogen Life Technologies, Carlsbad, California, USA) and

separated by electrophoresis (100V for thirty minutes) on a 2% agarose gel immersed in 0.5x TBE loading buffer. For each gel, 5 µL of 100 bp DNA ladder (Invitrogen Life Technologies, Carlsbad, California) was also stained and size fractionated. The resultant fingerprints were visualized under a dark reader (Clare Chemical Research, Dolores, Colorado, USA).

Quantitative PCR

This study employed the TaqMan chemistry-based approach of DNA detection. This was the choice method because TaqMan-based DNA detection uses a fluorogenic probe specific to the target sequence in order to detect the target as it accumulates during PCR. This allows for increased specificity and sensitivity in DNA quantification in comparison to other chemistries (e.g. Sybr-based DNA detection) which use dyes that bind to double stranded DNA in order to detect amplicons as they accumulate. The qPCR assay used here was originally developed and described by McGrath (2010).

The final volume for qPCR was 20 µL and contained the following: 2 µL DNA sample, PerfeCta qPCR Supermix with ROX (Quanta Bioscience, Gaithersburg, Maryland, USA), 0.3 µM of forward primer F596, 0.3 µM of reverse primer R735 and 0.25 µM fluorescent 5' nuclease hydrolysis probe (5' 56-FAM labelled) with a ZEN quencher (Integrated DNA Technologies, Coralville, Iowa, USA). qPCR was carried out using an StepOne Real-Time PCR system (Applied Biosystems, Foster City, California, USA). Cycling parameters included an initial denaturation at 95˚C for three minutes followed by fifty-five cycles of 95˚C for

thirty seconds, 55˚C for thirty seconds, and 70˚C for forty-five seconds. The final extension step was at 72˚C for seven minutes.

To obtain a DNA standard for qPCR, an artificial DNA custom minigene with a similar sequence to the targeted sheep Dloop sequence was designed (Integrated DNA Technologies, Coralville, Iowa, USA). UV absorption was used to determine the concentration of this artificial DNA (Nanodrop 1000, Nanodrop Technologies, Wilmington, Delaware, USA). In order to avoid supercoiling of the circular DNA standard (Hou et al. 2010), the artificial DNA was linearized through cleavage by the restriction enzyme APaI (New England Biolabs, Ipswich, Massachusetts, USA). For each qPCR, new dilutions of the artificial DNA were prepared. Standards and unknown samples were amplified in duplicate for each qPCR.

Inhibition Countermeasures

Organic inhibitors such as humic acids and fulvic acids are typically co-extracted alongside target DNA in *sed*aDNA studies and act to inhibit PCR amplification (Alaeddini 2012; Andersen 2011; Willerslev et al. 2003). To counteract such effects, a combination of strategies were used to overcome (presumed) inhibition. This involved amplifying a dilution series of each sample [to determine the point where the potential inhibitors no longer affect PCR amplification] (Moore 2011; King et al. 2009; Kemp et al. 2006); adding BSA to the reaction [to bind to inhibitors and/or stimulate the DNA polymerase] (King et al. 2009; Kemp et al. 2006; Cooper 1994); and, in some instances, increasing the

amount of DNA polymerase used to 2.5X (or 1.875 U) [to overwhelm PCR inhibitors which deactivate polymerases] (Kemp et al. 2006).

Contamination Controls

This study presented a new challenge for contamination control. Experiments dealt with modern DNA from soft tissues that were artificially degraded to mimic naturally degraded DNA. For this reason, contamination was less of a concern compared to experiments using 'authentic' ancient samples. However, as experiments dealt with various levels of DNA degradation, ample consideration was still given to contamination issues in order to minimize its effects in experiments. As a result, this study followed protocols and principles commonly used in the aDNA field (Kemp and Smith 2010; Gilbert et al. 2005; Cooper and Poinar 2000).

All sample preparation was carried out in a general use laboratory in the Department of Archaeology at Simon Fraser University. This lab was in a separate building from where any molecular bench work was being carried out. All molecular work was carried out following strict contamination control measures typical of aDNA research (Kemp and Smith 2010; Cooper and Poinar 2000). This includes the physical separation of pre- and post-PCR activities. Pre-PCR activities were carried out in the dedicated Forensic DNA Laboratory at Simon Fraser University. This lab houses dedicated rooms for each step of sample analysis (sample preparation, DNA extraction and PCR setup) (Figure 4A) and each of those rooms uses dedicated equipment and reagents chosen with contamination prevention in mind (e.g. filtered pipette tips, sterilized

disposables, laboratory-grade reagents, etc.). Strict protocols regarding unidirectional workflow are vigorously enforced. The laboratory is regularly decontaminated with bleach and workers must wear dedicated clothing and shoes underneath full body coveralls, gloves and masks (Figure 4B). Positive controls were included in DNA extractions and PCR setups. Sediment blank controls and multiple extraction blank controls were included during each extraction experiment in order to monitor levels of contamination. In addition to the blank controls, for each PCR (standard and quantitative), negative amplification controls were also included in an effort to detect any potential contamination.

Figure 4: (A) The Forensic DNA Laboratory at Simon Fraser University contains three dedicated work spaces surrounding a common room; (B) Work in the Forensic DNA Laboratory follows strict contamination controls including the use of full body coveralls, gloves, masks among others.

Chapter 4: Results

Size-based Samples

Sample Preparation and Processing

To assess the potential role of sediment particle size in *sed*aDNA preservation and recovery, sediments from shell midden were separated by particle size to generate three separate size-based samples: ES (0-63 µm), EM (212-300 µm) and EL (500-710 µm). While these newly created samples came from a singular source, upon size separation they became visually and texturally distinct (Figure 5).

Figure 5: Control sediment samples (size-based). Shell midden sediments were separated by particle size. L-R: ES sediments were 0-63 µM; EM 212-300 µM; EL 500-710 µM.

After heat treatment at 95ºC for various amounts of time (0-24 hours in 8 hour intervals), sediment-DNA samples lost an average of 2.9% of their starting weight (Appendix 1). Because these samples are from highly organic shell midden, this is most likely caused by the loss of moisture. While, the average percentage of weight loss increased with particle size, the differences appear negligible (ES: 2.4% vs. EM: 2.9% vs. EL: 3.4%). There was no visible difference in sediment appearance after heat treatment at any of the time points.

Recovered DNA

Samples from each time point were extracted following both a non-organic (chloroform/octanol) and organic protocol (silica-spin column) (Haile 2012 and Yang et al. 1998 respectively). The resulting DNA extracts varied greatly in appearance between protocols. For both methods, the final steps involve elution of the DNA from a QIAquick spin column. The chloroform/octanol method produced DNA that was clear to faintly yellow (but still translucent). The silica-spin column method produced DNA that was blackish in colour and opaque (Figure 6).

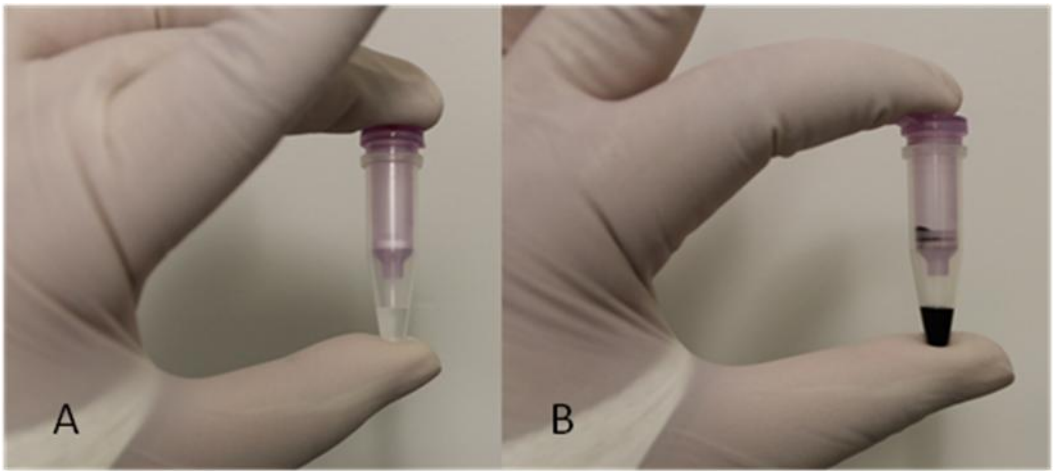


Figure 6: Eluted DNA from size-based samples varied in appearance between the chloroform/octanol method (A) and the silica-spin column method (B). Sample ES-8 hours pictured here.

PCR Amplification

Non-organic Method Extracts

As the dark colour of the DNA recovered using the silica-spin column protocol is likely an indicator of the presence of PCR inhibitors (Kemp et al. 2006), our strategy of sample dilution, addition of BSA and increased amount of DNA polymerase were successful in overcoming PCR inhibition.

Following these strategies, the 0 hour time point for each of the three sample types was amplified in a dilution series as follows: 1X (undiluted), 5X diluted, 25X diluted and 125X diluted. A summary of the results is shown in Table 3. Despite 125X dilution with ultra-pure water, the samples were still discoloured (although not completely opaque). After sixty cycles of PCR, 'smears' were present upon visualization of the 1X and 10X amplicons; however,

clear bands did not appear until after 25X dilution for all three sediment samples (Figure 7). Samples diluted 125X also generated clear bands of the expected size. Further attempts to amplify the remaining time points using 20X and 25X dilutions failed (e.g. lanes 2-5, Figure 8). Positive controls used in these subsequent reactions successfully amplified, indicating the overall PCR was successful.

Table 3: A summary of a dilution series of 0 hour size-based silica-spin column sample extracts.

Sample	Undiluted	5X Dilution	25X Dilution	125X Dilution
ES	- (dark smear)	- (faint smear)	+	+
EM	- (dark smear)	- (faint smear)	+	+
EL	- (dark smear)	- (faint smear)	+	+

Note: Sixty cycles of PCR were used to target a 139 bp fragment of sheep mtDNA. Reactions included BSA and 2X DNA polymerase. X represents the amount the sample was diluted by using ultra-pure water to test levels of PCR inhibition. (-) and (+) indicate PCR amplification was negative (no band) or positive (band) respectively.

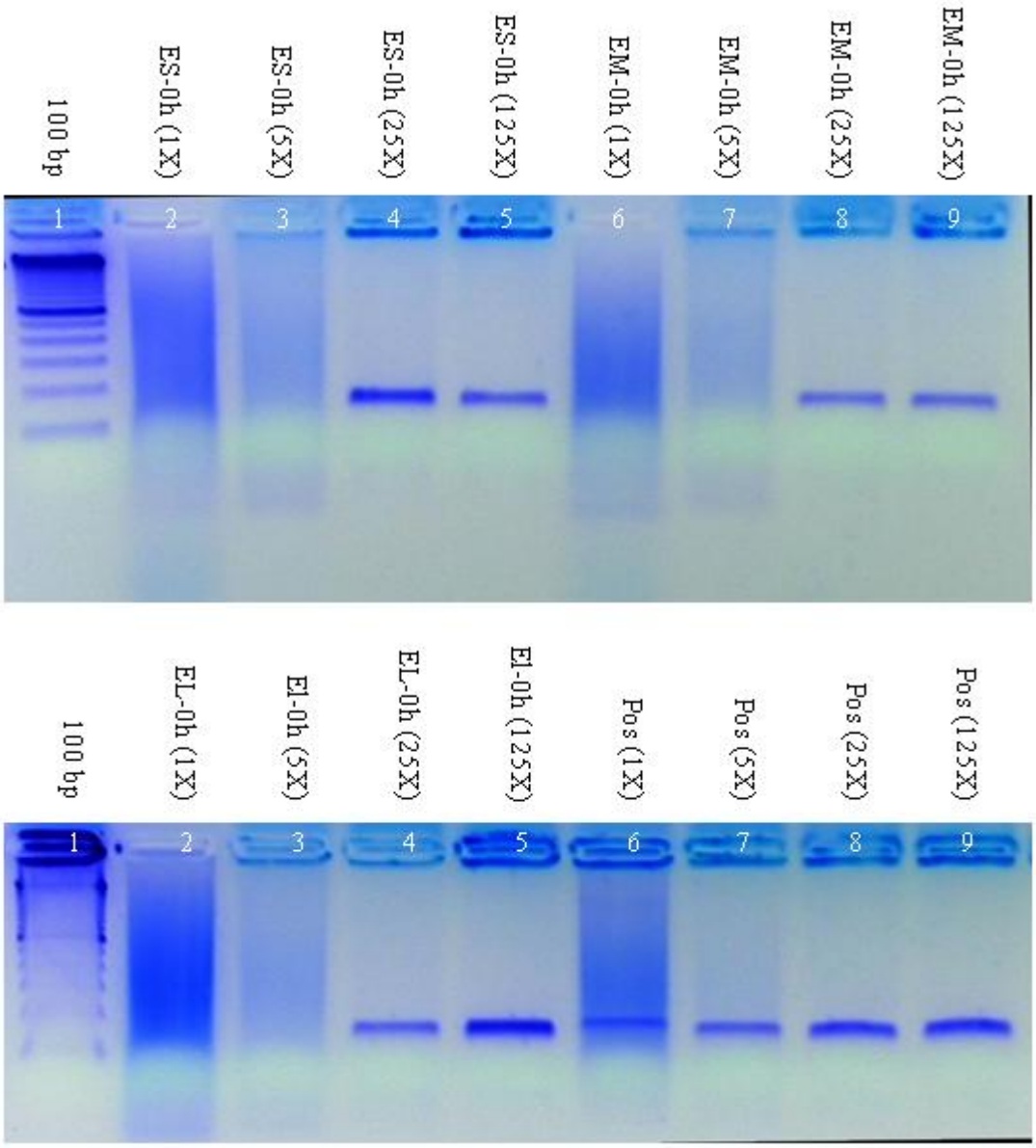


Figure 7: Dilution series of 0 hour samples ES, EM and EL extracted following the modified silica-spin column method (Yang et al. 1998). Primers were designed to target a 139 bp fragment of sheep mtDNA. After sixty cycles of PCR, amplicons were visualized with SybrGreen on a 2% agarose gel. 100 bp represents 100 bp ladder; Pos represents a sheep positive control; X represents the amount the sample was diluted by using ultra-pure water. A negative control was included in PCR setup (no positive amplification) but was not included in this gel image.

Organic Method Extracts

ES samples from each time point were successfully amplified in the same manner as the non-organic (silica-spin column) extracts; however, the ES DNA samples were not diluted (Figure 8). The ES sediment blank also generated a positive amplification band. This sample represents the sediments prior to the addition of the sheep lung tissue powder.

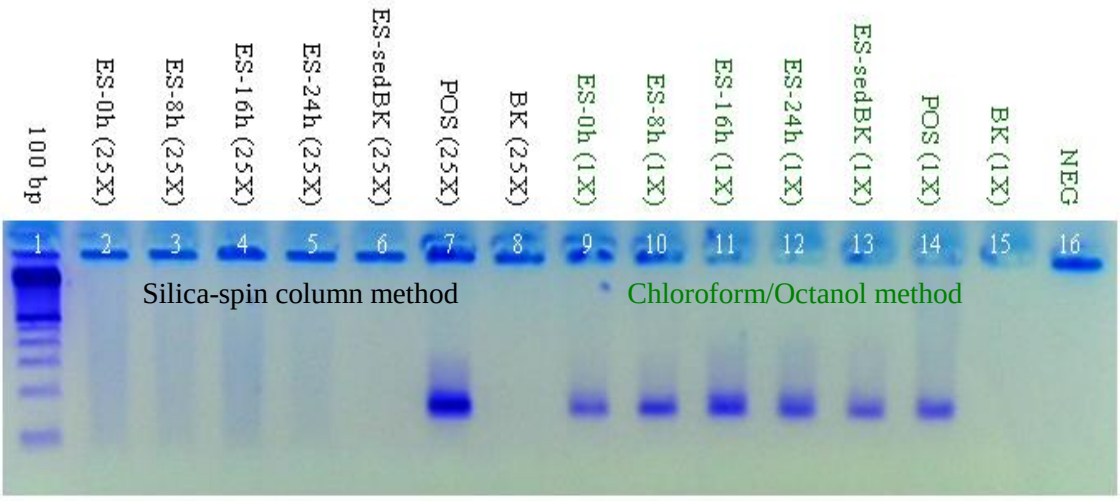


Figure 8: Successful amplification of all ES sample time points extracted following the chloroform/octanol method (Haile 2012) (Lanes 9-12). Primers were designed to target a 139 bp fragment of sheep mtDNA. After sixty cycles of PCR, amplicons were visualized with SybrGreen on a 2% agarose gel. Samples were undiluted. 100 bp is 100 bp ladder; POS is a sheep positive control; sedBK is a sediment blank control; BK is a blank extraction control; NEG is a PCR negative control. Positive amplification of ES-sedBK (lane 13) indicates the presence of sheep DNA (either a result of sheep DNA being present in the sediments prior to the addition of control DNA or from contamination during the DNA extraction process). [Lanes 2-8 are from a different experiment (silica-spin column exacts of ES diluted 25X – no amplification in the sediment-DNA samples was detected].

Type-based Samples

Sample Preparation and Processing

Due to the high copy number from the size-based samples, the ratio of sediment to sheep tissue powder was increased from 25:2 g to 30:0.5 g for the type-based samples. Heat treatment temperatures was also increased to 120ºC and incubation times were raised to extend from 0-70 hours (in 24 hour intervals).

After heat treatment, the E samples (shell midden) lost an average of 2.2% of their starting weight, while the B samples (geological, fluvial deposits) and N samples (unknown, archaeological) lost an average of 0.3% and 0.2% respectively (Appendix 1). As weight loss is likely a reflection of moisture loss, it makes sense that the high organic content of shell midden would mean those samples would see a higher loss in weight. There was no visible difference in sediment appearance after heat treatment at any of the time points.

Recovered DNA

Samples from each time point were extracted with both a organic (chloroform/octanol) and non-organic protocol (silica-spin column method) (Haile 2012 and Yang et al. 1998 respectively). Unlike with the size-based samples, the resulting DNA products generated did not vary greatly in appearance between protocols. The final steps of both methods involve elution of the DNA from a QIAquick spin column. The chloroform/octanol method produced clear, translucent DNA across all three sediment types. The silica-spin column method produced DNA that was translucent across all three sediment types; however,

while both the E and N samples were clear, the elutes of the B samples ranged from brownish-yellow in appearance to clear (Figure 9).

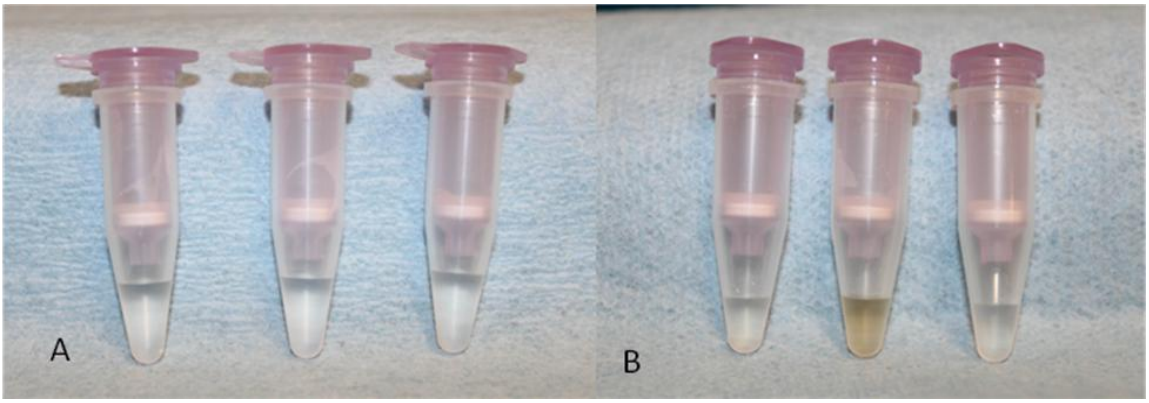


Figure 9: Physical appearance of eluted DNA from type-based samples extracted using the chloroform/octanol method (A) and the modified silica-spin column method (B). Only the B samples extracted with the non-organic method exhibited any discoloration. Samples E-48hours, B-48hours, N-48hours pictured here (L-R).

PCR Amplification

Initial attempts to amplify all time points from each sediment sample type extracted by the modified silica-spin column method were moderately successful (sixty cycles of PCR; undiluted DNA) (Figure 10). While clear bands of the expected size were present for only two of the N samples (24 hours and 70 hours), faint bands were visualized for the remaining N samples and all of the E samples. Faint smears that concentrated around the expected fragment size were present in the B samples (Figure 10). No positive amplification was detectable in both sediment and extraction blank controls; however, the negative

control generated a positive band approximately 200 bp larger than the targeted fragment size.

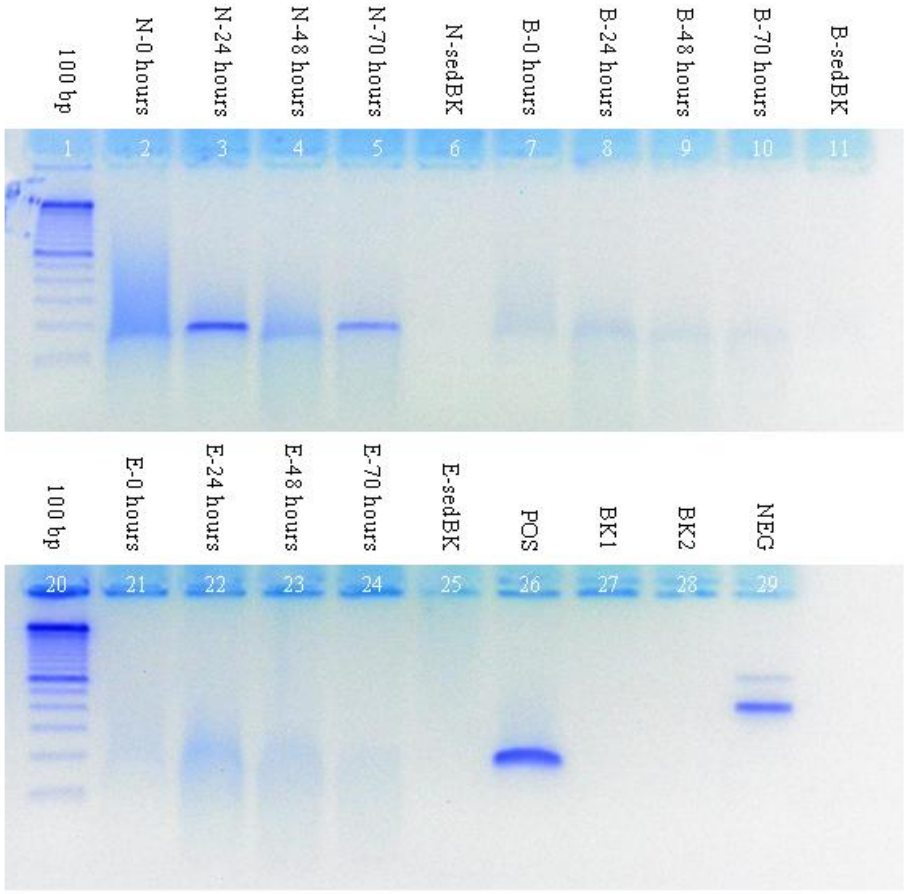


Figure 10: Amplification of type-based sediment samples extracted using a silica-spin column method. Primers were designed to target a 139 bp fragment of sheep mtDNA. After sixty cycles of PCR, undiluted amplicons were visualized with SybrGreen on a 2% agarose gel. 100 bp represents 100 bp ladder; sedBK represents sediment blank controls; BK represents blank extraction controls; Neg represents negative control.

Sample dilution was then used to overcome PCR inhibition. To determine the appropriate dilution factor, a dilution series was created using the E-48 hour sample from both extraction methods. The results are summarized in Table 4. The two samples were diluted with ultra-pure water as follows: 1X (undiluted), 2X diluted, 8X diluted, 32X diluted, 128X diluted. Sixty cycles of PCR were used and amplification of the chloroform/octanol extracts were successful in the 1X, 2X and 128X samples. Amplification of the silica-spin column extracts resulted in clear, positive bands only after 32X dilution (Appendix 3).

Table 4: A summary of a dilution series of E-48 hours samples extracted with the silica-spin column and chloroform/octanol methods.

	Undiluted	2X Dilution	8X Dilution	32X Dilution	128X Dilution
Silica-spin Column Method	-	-	-	+	+
Chloroform/Octanol Method	+	+	-	-	+

Note: Sixty cycles of PCR were used to target a 139 bp fragment of sheep mtDNA. X represents the amount the sample was diluted by using ultra-pure water to test levels of PCR inhibition. (-) and (+) indicate PCR amplification was negative (no band) or positive (band) respectively.

All samples from all time points (both extraction methods) were diluted 100X. Amplification of these samples using sixty cycles of PCR resulted in a 100% success rate (Figure 11). No positive amplification was detectable in both sediment blank controls, extraction blank controls and PCR negative controls.

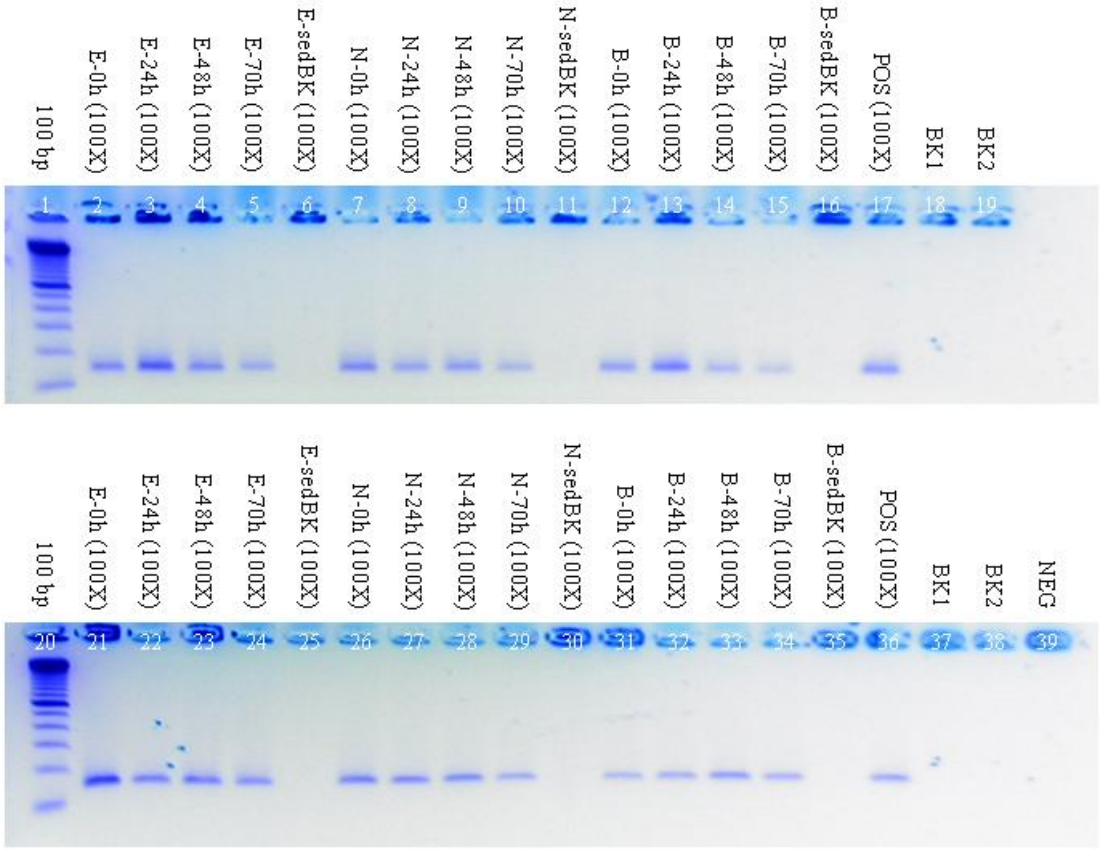


Figure 11: Successful amplification of DNA from all heat treated samples across three sediment types (E, B, N) regardless of extraction method (Top row: chloroform/octanol extraction method; bottom row: silica-spin column extraction method). Primers were designed to target a 139 bp fragment of sheep mtDNA. After sixty cycles of PCR, amplicons were visualized with SybrGreen on a 2% agarose gel. All samples were diluted 100X with ultra-pure water to reduce potential PCR inhibition. 100 bp represents 100 bp ladder; POS represents a sheep positive control; sedBK represents a sediment blank control; BK represents a blank extraction control; NEG represents a PCR negative control. No contamination was detected. Lanes 2-19 represent samples extracted following Haile (2012); lanes 21-38 represent samples extracted following a modified version of Yang et al. (1998).

Quantitative PCR

Due to time constraints, limited qPCR experiments were carried out. Only those sediments from shell midden have been tested so far. For the size-based samples, ES samples were tested; for the type-based samples, E samples were tested.

qPCR of the ES samples were carried out with undiluted and 50X diluted extracts for the organic (chloroform/octanol) (Haile 2012) and non-organic (silica-spin column) (modified Yang et al. 1998) methods respectively. Despite heating at 95ºC for up to 24 hours, the copy numbers of the samples were quite high (Table 3). The samples subjected to the most heat treatment (24 hours) still presented an average of 10,176 and 4,542,722 copies of DNA in extracts from the organic and non-organic methods respectively (Table 3). All samples extracted following Haile (2012) successfully amplified, whereas Yang et al. (1998)'s method resulted in failed amplification of the early time points (0 hours and 8 hours) but ES-16 hours and ES-24 hours were successful (Table 5). One sediment blank control yielded seven copies of DNA, indicative of background contamination (data not shown).

qPCR of the E samples diluted 100X was successful for all samples and showed a pattern of degradation (Table 5; Figure 12). Despite increasing both temperature and incubation time, the average template quantity was 11,468 and 924,938 for the most degraded samples (E-70 hours, adjusted to account for 100X dilution) (non-organic and organic methods respectively). No contamination was detected.

Table 5: Mean DNA quantities obtained from quantitative PCR.

Sample	Non-organic extraction method (Mean DNA Quantity)	Organic extraction method (Mean DNA Quantity)
Sample ES		
0 hours	Undetermined	106,983
8 hours	Undetermined	138,898
16 hours	24,562,628	75,452
24 hours	4,542,722	10,176
Sample E		
0 hours	313,072	216,575
24 hours	159,640	12,049
48 hours	100,762	6,812
70 hours	92,494	1,147

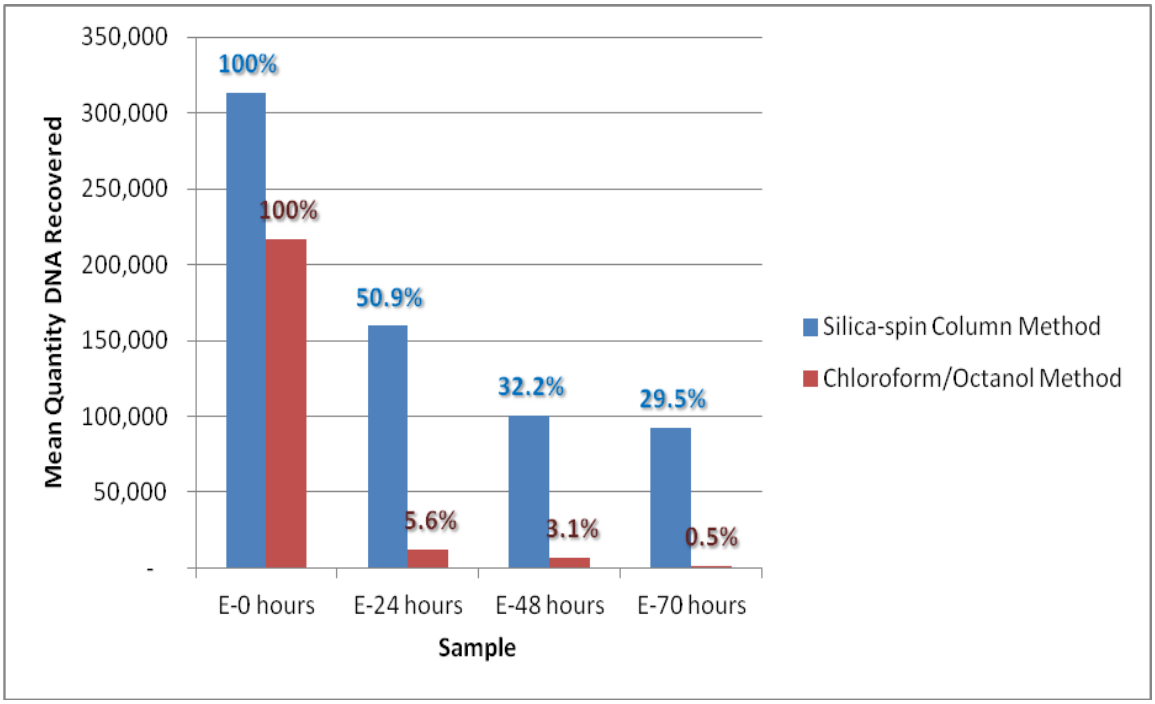


Figure 12: Comparison of sample E DNA degradation patterns after extraction with an organic and non-organic DNA extraction method. Average mean quantities shown. The number of hours is the amount of time exposed to 120ºC heat. Percentages shown are the percentage of each 0 hour sample remaining.

Chapter 5: Discussion

Authenticity of DNA Data

Although this study does not deal with aDNA, vigorous and strict contamination control measures originally developed for aDNA research were followed whenever applicable. As aDNA research involves analyzing minute amounts of low quality DNA, it is particularly susceptible to false positive results stemming from contamination. Consequently, the field of aDNA has dedicated much of its energy to emphasizing the need for authentication of aDNA studies (Kemp and Smith 2010; Gilbert et al. 2005; Gilbert 2003; Cooper and Poinar 2000). While there are many debates regarding the best approaches to authenticate aDNA (Kemp and Smith 2010; Cooper and Poinar 2000), some key steps are unanimously crucial.

This study used dedicated laboratory facilities to reduce the chances of contamination. Extensive steps were taken to minimize contamination in order to ensure that both the DNA degradation patterns and comparisons of DNA extraction methods were as trustworthy as possible. As described earlier, contamination control measures followed those most commonly used in the field of aDNA. This included the use of access-restricted facilities to ensure physical separation of pre- and post-PCR work. An appropriate pre-PCR laboratory based on sample type was also used. This means that experiments were carried out in a forensic DNA laboratory that handles a range of degraded DNA, rather

than an ancient or modern DNA laboratory (specialized for highly degraded and non-degraded samples respectively).

The results from the multiple blank and negative controls embedded in this study suggests that the majority of the data obtained are authentic. However, unexpected PCR amplification was observed in one sediment blank control (ES-sedBK). Subsequent qPCR analysis determined that the level of contamination was insignificant when compared to the DNA quantities recovered from the 24 hour heat treated samples (seven copies vs 4.5 million copies). This type of sporadic, background amplification is not unexpected since a 0 hour sample (i.e. modern DNA) was included in all experiment steps (sample preparation, DNA extractions and PCR set ups). Just a few molecules of DNA could easily be transferred from this sample at any stage and the high number of PCR cycles employed could potentially amplify them.

Additionally, one PCR negative control yielded a positive amplification band. This band was over 200 bp larger than the target fragment indicating sporadic, non-specific amplification and is likely a reflection of the taxonomic richness present in sediment DNA extracts.

In addition to contamination prevention, numerous other criteria can be used to assess the authenticity of this study (Cooper and Poinar 2000). This includes the recognition of appropriate molecular behaviour – as heat exposure increases we expect DNA quantity to decrease, a pattern that was observed. Although amplicons have yet to be sequenced in order to confirm the species identity of the recovered DNA, a sheep-specific qPCR assay with a tested and

validated probe ensured that PCR products generated were the target fragment and not the by-product of unintentional amplification (McGrath 2010).

While the findings support the authenticity of the obtained data, additional work can be conducted to further increase confidence in my results. This includes the repetition of all lab work by an independent researcher at a different facility than the one used. While this task may have some logistic challenges, reproducibility tests can be a strong argument that helps further authenticate my findings.

DNA Preservation in Sediments

The mixing of control sediments with powdered sheep tissue was found to be a feasible controlled experiment for investigating DNA preservation and degradation in sediments.

Careful consideration and effort was given to determining the ratio of sediment to DNA required to make the system work in both a sensitive and effective manner. McGrath (2010) found that heating 1 g of sheep bone powder at 85ºC resulted in an average of just three copies of starting mtDNA after only 12 hours. Based on this observation, it was decided that 2 g samples would be heated at 95ºC for 24 hours, in 8 hour intervals. As each sample had a sediment to DNA ratio of 25:2, approximately 0.08 g of sheep tissue powder was in each 2 g sample heating.

Despite using a small amount of starting source DNA, a high temperature and long incubation times, the samples subjected to the most heat treatment (24

hours) yielded an average of 10,176 and 4,542,722 copies of DNA (chloroform/octanol and silica-spin column methods respectively) (Table 5). While the DNA template number appeared to decrease with time, degradation advanced much slower than originally anticipated. Not reaching levels anywhere near those seen in aDNA, the DNA was found to be well preserved in the sediments.

Based on these results, the type-based sediment-DNA samples were created using a smaller ratio of sediment:powder (60:1), increased temperature (120ºC) and increased incubation time (70 hours, in 24 hour intervals). Again, 2 g of sediment-DNA mixture was heated (approximately 0.03 g sheep tissue powder per sample). qPCR revealed high copy numbers of DNA (relative to aDNA) in all E samples (Table 5), reiterating the same pattern of DNA preservation observed earlier. While the levels of DNA in these samples are closer, they still do not mimic the trace levels found in aDNA samples. Nevertheless, a degradation pattern is clearly illustrated through qPCR analysis (Figure 12). Furthermore, standard PCR indicated that this pattern of good preservation holds regardless of sample type and extraction method – DNA was detectable in the most degraded samples from all 3 sediment types (E, B, N) and using both recovery methods (Figure 11).

The finding that relatively high quantities of DNA persisted under the conditions of the heat treatment are quite surprising. Based on McGrath (2010), it was expected that the DNA would degrade at a much faster rate. This is due to a variety of considerations, the primary being McGrath's use of 1 g of freshly

ground bone powder. It has been argued that DNA is better preserved in bone than in soft tissue (Hagelberg 1994; Hagelberg and Clegg 1991), although in this instance that would not necessarily play a role as the bone samples were powdered prior to heat treatment (any 'protection' that the hard tissues of bone may provide will be eliminated upon powdering). Rather, the increased amount of powder heated by McGrath (1 vs ≤0.8 g) likely affords some of the interior, unexposed powder some protection. Furthermore, the bone powder used was freshly ground (i.e. at maximal DNA levels). The sheep lungs purchased had been dehydrated and one can assume that results in some levels of DNA degradation. For these reasons, it was anticipated that the conditions selected would be more than sufficient to degrade the sediment-DNA samples to aDNA levels (i.e. trace amounts).

One possible reason for the discrepancy with McGrath (2012) is the use of randomized sample placement in the oven in this study. McGrath's study did not do this step. While sample placement of that study is unknown, when using an oven to artificially degrade samples in a time lapsed manner, it would be natural to place those samples remaining in the oven longest at the back, while those to be removed first at the front. This can potentially bias results, as those samples in the back are less prone to temperature fluctuations that may result from the oven door seal cracks or even the oven door being opened to remove samples. Samples in the back may also be closer to the heat source and, if oven temperatures are not uniform throughout, exposed to increased temperature compared to those samples at the front. While such considerations are important

for any controlled laboratory study; the impact from any related discrepancies are not likely to impact the experiment to the level observed here.

Another potential reason for the persistence of a large amount of DNA in the sediments despite exposure to extreme heat is that 'storage' within sediments is an excellent means of DNA preservation. While it is suspected that sediment-DNA complexes serve to protect DNA (Cai et al. 2006; Crecchio and Stotzky 1998; Trevors 1996; Romanowski et al. 1991; Lorenz and Wackernagel 1987), maybe its impact is larger than previously thought. The coupling of soft tissues as a source of *sed*aDNA with this suggestion is particularly intriguing. Much of the literature on *sed*aDNA postulates that its main sources are high volume wastes such as urine and faeces (Andersen et al. 2012; Hebsgaard et al. 2009; Haile et al. 2007). There is acknowledgement that skin cell, hair cells, etc. contribute to *sed*aDNA but have not been suggested as primary sources. One reason for this is that waste products are generated and deposited all year round and sometimes in concentrated areas (e.g. territorial markings). While some behaviours such as shedding occur continuously, the main source of soft tissue deposition would be upon death, via decomposition of a cadaver (Hebsgaard et al. 2009). As such, it is a common thought that as waste is continuously deposited, it 'saturates' the sediment/soil in a sense (Andersen et al. 2012; Haile et al. 2007). As the amount of DNA deposited increases, so do the number of sediment-DNA complexes that form. This increases the likelihood of DNA preservation and, ultimately, DNA detection and analysis by *sed*aDNA researchers. However, the finding that sediments may preserve DNA in soft

tissues in a meaningful way despite exposure to conditions that are known to highly degrade DNA is an interesting one. Perhaps, sediment-DNA complexes do not simply offer 'some' protection to DNA that is abundant enough for detection, rather they offer 'great' protection to even small amounts of DNA enabling those small amounts to be detected. This may be the reason why the oldest known (relatively well accepted) DNA recovered to date is *sed*aDNA (Willerslev et al. 2003). It would be interesting to explore how DNA preservation in sediments is influenced by the starting source material.

This study has successfully shown that DNA can be degraded in sediments using an artificial heat treatment model, laying down a solid foundation to examine DNA preservation in different sediments and to explore other factors that would affect DNA preservation and degradation. I intend to continue to pursue this line of research and move forward to recover and quantify DNA from the three size-based sediment-DNA samples. Based on what I have learnt from these studies, a couple of alterations will be made for future work. First, increased time and temperature will be used to degrade DNA to the low levels typically dealt with in the field. Second, I will proceed using the optimal DNA recovery technique (in this case, the silica-spin column method, see following passages). This is because the determined DNA template quantities reported do not reflect the actual levels of DNA present in the samples, rather the ability of the selected extraction method to recover DNA from those samples. This is evident by the observed differences in the severity of DNA degradation between extraction methods (Figure 12). As a result, I want to choose the most effective

and efficient DNA method to develop the most realistic DNA degradation patterns possible. Once I have these degradation curves for all three size-based samples, direct comparisons will offer insight into whether sediment particle size has any influence on DNA preservation. This type of information can help field researchers select the most appropriate sediment samples for *sed*aDNA analysis. For example, if results are consistent with Andersen et al. (2012)'s observations and it is found that larger sediment particles (e.g. sand) preserve DNA better than smaller sediment particles (e.g. silt and clay), researchers can focus their resources (both time and monetary) on sediment samples composed primarily of sand in order to optimize the likelihood of successful results. On the other hand, if it is found that sediment particle size does not affect DNA preservation, researchers can confidently select the best samples for their research question without having to consider the particle composition of the samples.

Comparison of DNA Extraction Techniques

One key issue in *sed*aDNA research revolves around recovering DNA from sediments. This can be challenging for a variety of reasons: (1) the rarity of samples available for testing; (2) theoretically, only trace amounts of DNA are preserved; (3) the low quality and quantity of DNA present is extremely sensitive to contamination by modern DNA sources; and (4) co-extracted PCR inhibitors can result in false negative results. One practical remedy to such problems is to maximize the amount of DNA recovered, in terms of both quantity and quality (with as few inhibitors as possible).

Much careful consideration has been given to the efficiency of extraction techniques used on faunal remains, easily the most studied remains in the aDNA field (Rohland and Hofreiter 2007; Bouwman and Brown 2002; Prado et al. 2002; Yang et al. 1998). This is followed by a few comparisons of plant aDNA extraction methods (Moore 2011; Giles and Brown 2008). To our knowledge, no comparisons – limited or comprehensive in nature – have been made in regards to *sed*aDNA recovery techniques.

This study attempted to compare the effectiveness of two DNA extractions techniques on small amounts (<5 g) of sediments. A (organic) chloroform/octanol method (Haile 2012) was chosen as it was deemed to be the primary method of extraction in research studying *sed*aDNA from small amounts of sediment/soil (Hebsgaard et al. 2009; Haile et al. 2007; Willerslev et al. 2004; Willerslev et al. 2003). Because nearly every study to date has used some form of organic extraction, the second method chosen to test was a (non-organic) silica-spin column method (modified from Yang et al. 1998). This was chosen because silica-based extraction methods have been consistently shown to be optimal for recovering DNA from ancient faunal remains (e.g. Rohland and Hofreiter 2007; MacHugh et al. 2000; Yang et al. 1998).

DNA Recovery

Although research is ongoing and results are limited, the current data are compelling. qPCR of both ES samples and E samples suggest that the modified silica-spin column method (Yang et al. 1998) is clearly and markedly better at recovering DNA than the chloroform/octanol method (Haile 2012). Analysis of

ES samples was prevented by PCR inhibition but an interesting observation is that the most heated sample (95ºC for 24 hours) yielded 42X more DNA than the octanol/chloroform method did when used on the non-treated, 0 hour time point (4,542,722 vs. 106,983 respectively) (Table 5).

The E samples showed that the non-organic (silica-spin column) method consistently yielded more DNA than the organic (chloroform/octanol) method, with the differences becoming more meaningful with increased heat exposure (Table 6). For example, for the 0 hour samples there was a 1.4X difference in quantity of DNA recovered; for the 70 hour sample this difference leapt to 80.7X. This striking observation highlights the advantage of the silica-spin column method when working with degraded samples.

Table 6: Quantity of DNA recovered using the non-organic DNA extraction method relative to the organic extraction method.

Sample	Relative Quantity of DNA (Non-organic method/Organic method)
E-0 hours	1.4
E-24 hours	13.2
E-48 hours	14.8
E-70 hours	80.7

Note: Non-organic extractions followed Haile (2012); Organic extractions followed a modified version of Yang et al. (1998). Relative quantities were calculated from the mean DNA template number.

Another way to illustrate the importance of selecting the most appropriate DNA extraction technique is by looking at the degradation patterns discussed above (Figure 12). Comparison of DNA degradation from the starting quantity (E-0 hours) to the first (and least) degraded sample (E-24 hours) show a 0.5% and 29.5% recovery of initial template amount for the organic and non-organic extraction methods respectively. If you think of the 0 hour time point as the total 'pool' of starting DNA, then it is clear that the silica-spin column method is the more effective method as the amount of starting DNA is clearly higher. If the two methods tested differed in effectiveness, but were equally as efficient, then you would expect the degradation rate to be relatively equal between the two methods (relative to their respective 0 hour time point). This is clearly not the case – the silica-spin column method is much more efficient, recovering 30% of the initial amount of DNA compared to the 0.5% recovered using the chloroform/octanol method. If these samples had been from authentic sediments and/or reached degradation levels typically seen in aDNA research, it is very likely that end result of the organic extractions would have been a false negative one. This underscores how an inefficient and ineffective extraction method can hinder *sed*aDNA research, leading to underestimates of DNA levels and potentially false negative results.

The dominating success of the modified silica-spin column method over the chloroform/octanol method is likely due to a combination of reasons. While both methods included an overnight lysis step, as well as DNA isolation and purification via a silica-spin column, the ratio of lysis:powder was significantly

higher (66.7 vs 1.3 µL/mg) in Yang et al. (1998)'s method. This would favour increased DNA recovery by ensuring that a sufficient amount of buffer is available to lyse cells. Furthermore, DNA yield was likely sacrificed in the organic method through use of a chloroform/octanol step to remove impurities (as opposed to the size-exclusion filtration method used in the non-organic extractions).

PCR Inhibition

Nearly all silica-spin column method extracts showed evidence of inhibitor co-extraction while DNA extracts recovered using the chloroform/octanol method revealed PCR inhibition was not a serious problem. It is clear that the silica-spin column method, while extracting more DNA, also co-extracts more inhibitors.

It also appears that the there was no difference in the amount of inhibitors co-extracted among the size-based sediment samples. This study used standard PCR of the size-based samples for a 'quick and dirty' assessment of PCR inhibition levels. No difference in amount of dilution required to overcome PCR inhibition was found (Table 3). Regardless of particle size, all samples exhibited the same levels of PCR inhibition, successfully amplifying only after 25X and 100X dilution with water.

The finding that the level of inhibition was the same for all three sediment sizes is interesting because particle size is thought to affect DNA recovery rates, with DNA recovery increasing with particle size (Andersen et al. 2012). The reasoning is that the rapid adsorption and binding of DNA to sediment particles

enable the formation of sediment-DNA complexes that protect the DNA from degradation by nucleases (Crecchio and Stotzky 1998; Tsai and Olsen 1992). Larger particles mean more surface area per particle, which allows more complexes to form. One mechanism in which PCR inhibitors are thought to work is through direct binding to DNA (Alaeddini 2012). Following this, if particle size influences the amount of DNA available, then one would assume that particle size also influences the amount of PCR inhibitors that are co-extracted alongside said DNA. The finding that particle size did not appear to have any impact on the dilution factor required to overcome PCR inhibition contradicts this hypothesis and suggests that PCR inhibition may be more a product of sediment chemical composition rather than physical composition.

While multiple factors likely play a role in the observed difference in DNA extraction technique efficiency, the key difference maker is most probably the method in which proteins and lipids are removed. Haile (2012) uses chloroform/octanol (24:1), while the silica-spin column method uses size-exclusion filtration. The chloroform/octanol approach is excellent for removing impurities from mediums such as sediments/soils, which are brimming with complex mixtures of (unknown) biochemicals. This is presumably why it appears to be the 'go to' method in *sed*aDNA research. In this study, it was effective at removing impurities, as evidenced by the clear nature of all extracts with this method (Figure 6; Figure 9). [Note: discolouration is only an indicator of the presence of PCR inhibitors (Kemp et al. 2006) and an internal positive control qPCR assay would be beneficial in this instance as it would enable us to

quantitatively assess the amount of inhibition present in the samples for the two DNA extraction methods tested (Moore 2011; King et al. 2009).] Furthermore, unlike the silica-spin column method, all samples were successfully amplified without requiring any dilutions to overcome PCR inhibition. However, while able to remove impurities much more successfully, one has to wonder if the trade off of significantly less DNA being recovered is worth it, especially in light of the fact that, in all instances, a simple dilution series was capable of overcoming PCR inhibition (Table 3; Table 4).

Implications

Technical Context

Use of Heat Treatment for Artificial Degradation

The variable nature of the composition and chemistry of sediments make direct comparisons of *sed*aDNA results difficult. Due to environmental heterogeneity and representation biases, direct comparisons are even challenging when working with sediment samples from the same site. This is problematic because *sed*aDNA analysis is an ever-growing research area that requires basic, fundamental studies in order to overcome low success rates. Efforts to transform *sed*aDNA analysis into a standard approach in archaeological investigations are dependent on it being an accurate and reliable tool. This means that success rates need to be improved. One way to do this is through comprehensive studies aiming to systematically compare and/or optimize DNA recovery techniques, both in terms of DNA recovery and PCR inhibition removal. However, these comparisons are dependent on sample

homogeneity, which is problematic when dealing with ancient sediments. As a result, an artificial degradation model like the one used here can be an innovative alternative to authentic *sed*aDNA samples.

This study demonstrates that heat can be successfully used as a proxy for time in controlled laboratory experiments aiming to artificially degrade DNA in sediments. Although the levels of degradation reached in this study were not typical of those seen in aDNA research, it is clear from the degradation patterns that continued exposure to high temperature (or an increase in temperature) would have enabled successful mimicking of aDNA levels. This approach will enable researchers to conduct experiments (e.g. direct comparisons of techniques) that would otherwise be impossible and, in doing so, will help push the field forward from a technical perspective.

Furthermore, this style of experimentation has the potential to reveal insights that may be otherwise overlooked. For example, the fact that sediment DNA requires significantly more time and heat to degrade than bone powder was surprising. Such an insight can potentially explain the successful recovery of *sed*aDNA from samples hundreds of thousands of years old (Willerslev et al. 2003), an age from which no aDNA from physical remains has been recovered to date. Furthermore, it has the potential to direct future *sed*aDNA studies. Current studies have primarily targeted permafrost samples, partially due to the sub-freezing temperatures which act to help preserve DNA. If the finding of excellent DNA perseveration in sediments holds up to further testing, then a *sed*aDNA approach may be a promising means to retrieve genetic information from those

sites where aDNA recovery from macrofossil remains have failed due to unfavourable preservation conditions. Without a controlled laboratory setting and controlled degradation factors, this insight may have gone unnoticed.

Determining the Most Appropriate DNA Extraction Technique

The two DNA extraction methods used in this study were designed specifically for those circumstances when only small amounts of sediments (< 5 g) are available for DNA testing. Most *sed*aDNA research arises from situations in which huge amounts of sediments are available, enabling multiple extractions, each from 10+ g of starting material. Although one should always aim for maximal DNA recovery when carrying out aDNA research, such circumstances allow for increased opportunities to detect rare species. When only a small amount of starting material is available, those opportunities disappear. Especially when taking into consideration the issue of representative biases in *sed*aDNA samples, one must be confident that DNA recovery is maximal in order to ensure the accuracy of any results. The results of this study strongly suggest that the silica-spin column method is superior for recovering DNA from sediments. Consequently, in order to increase *sed*aDNA recovery success rates, future work should focus on developing techniques to optimize this method. Most notably, this would include developing ways to minimize and prevent PCR inhibition. Some potential modifications include the use of multiple 'clean up' steps. Kemp et al. (2006) found that repeating silica extractions (the last step of the silica-spin column method) resulted in the removal of PCR inhibitors from extracts of ancient human remains. As *sed*aDNA samples are often diluted in

order to ensure successful PCR amplification, this ‘repeated silica extraction’ method may prove more effective at removing those inhibitors co-extracted alongside the DNA.

As the silica-spin column method allows for easy manipulation of protocols, DNA extractions can also be modified for testing the effectiveness of incorporating various chemical reagents such as InhibitEX tablets (Qiagen, Hilden, Germany). These tablets are designed to remove impurities during the extraction of difficult samples like stool and soil.

Attempts to minimize PCR inhibition should also extend beyond the extraction step to the PCR amplification step. While it is known that dilutions are an easy and successful way to overcome PCR inhibition in *sed*aDNA samples (Andersen 2011), PCR amplification can also potentially be improved by the use of engineered DNA polymerases. These polymerases are often engineered with broad resistance to common PCR inhibitors (e.g. humic acids, fulvic acids, polyphenol complexes) and can be used to catalyze PCR amplifications despite the presence of inhibitors (Baar et al. 2011; Zhang et al. 2010; Kermekchiev et al. 2009; Shapiro 2008).

It is noted that the current findings involve recovering and quantifying DNA from samples of the same shell midden. Although Andersen et al. (2012) reported that the amount of DNA recovered was more closely related to the amount of starting material than soil chemistry, the applicability of the results to other types of sediments is unknown and is an issue I am continuing to explore.

Natural Field Setting

Despite the fact that research is still ongoing, this study uses a controlled laboratory setting to lay down the foundation for meaningful results that offer some fundamental knowledge to the study and use of *sed*aDNA in an investigative context. Such research will eventually enable researchers to select sediment samples most likely to contain *sed*aDNA and also quickly determine the most appropriate technique to recover *sed*aDNA from those samples. As a result, the likelihood of recovery will be optimized and the authenticity of *sed*aDNA studies will be given more credence. With increased reliability and an improved success rate, the technique can then be used confidently in archaeological investigations. Although *sed*aDNA has been used in an archaeological context just once to date (Hebsgaard et al. 2009), the possible insights it could offer are seemingly endless. Among other things, *sed*aDNA could be used to potentially link occupation layers with genetic groups, investigate the diet of past cultures and determine the genetic composition of inhabitants, even when no physical remains are preserved. By freeing aDNA researchers from the requirement of obtaining physical remains, *sed*aDNA is truly a revolutionary concept and tool.

Furthermore, the exciting possibility of detecting the presence of a species even when no macroscopically identifiable remains exist not only enhances our ability for archaeological research but also sheds new light on numerous other fields. While aDNA researchers are already taking advantage of the ability to use *sed*aDNA to reconstruct paleoecosystems and investigate

species evolution and extinction dynamics, some possible extensions of this technique remain unexplored. For example, the closely related field of forensic DNA could benefit from a reliable means to recover highly degraded DNA from sediments. A *sed*aDNA approach could be a powerful line of evidence when dealing with mass (or other) graves, in which bodies are typically buried and then later moved. In these types of circumstances, this method could serve as a link between a body and a site when no other evidence is available.

Chapter 6: Conclusion

This study has successfully used a series of controlled laboratory experiments: mixing ancient sediments with modern sheep tissues; exposing the sediment-DNA samples to high temperatures for various time periods; recovering DNA from the sediments using two DNA extraction techniques; and using standard and quantitative PCR of a mtDNA marker to assess recovered DNA and to compare the recovery efficiency of methods.

It was found that when heated in an oven, sediment samples mixed with powdered soft tissue can serve as excellent test materials that can aid in the understanding of DNA preservation in sediments. This study also demonstrated that heat treatment can be used to artificially degrade DNA in order to simulate *sed*aDNA found in a natural setting. Additionally, sediment-DNA samples required more exposure to degradation factors than previously thought. This suggests that sediments may act as an excellent shield to protect DNA from degradation factors and, consequently, may be a great source material when studying aDNA.

It was shown that the control DNA can be well preserved in sediments. DNA was detected from multiple sediments after many hours of exposure to high temperatures (up to 120ºC for 70 hours). When compared with McGrath (2010), it is surprising to notice that DNA appears to preserve better in sediments than in

bone tissues. The reason behind this observation deserves a more thorough study in the future.

This study has also revealed a dramatic difference in DNA extraction efficiency between two commonly used methods. Results clearly indicate that the silica-spin column method (modified from Yang et al. 1998) is the superior method at recovering artificially degraded DNA from sediments. However, results also show that this method is heavily plagued by an inability to remove PCR inhibitors effectively. While a simple dilution of 20X or more may help overcome the problem, this is not the ideal approach for the problem. Consequently, more research is called for to reduce the co-extraction of inhibitors with the silica-spin column method. To my knowledge, this was the first time that a comparison of extraction methods was attempted on degraded sedimentary DNA.

The current study has laid down a solid foundation for lab-based sediment DNA research. It can be expected that more fundamental questions can be adequately addressed through the study of more sediment types under different environmental conditions.

Appendices

Appendix 1: Sample oven placement information and weight loss as a result of heat treatment.

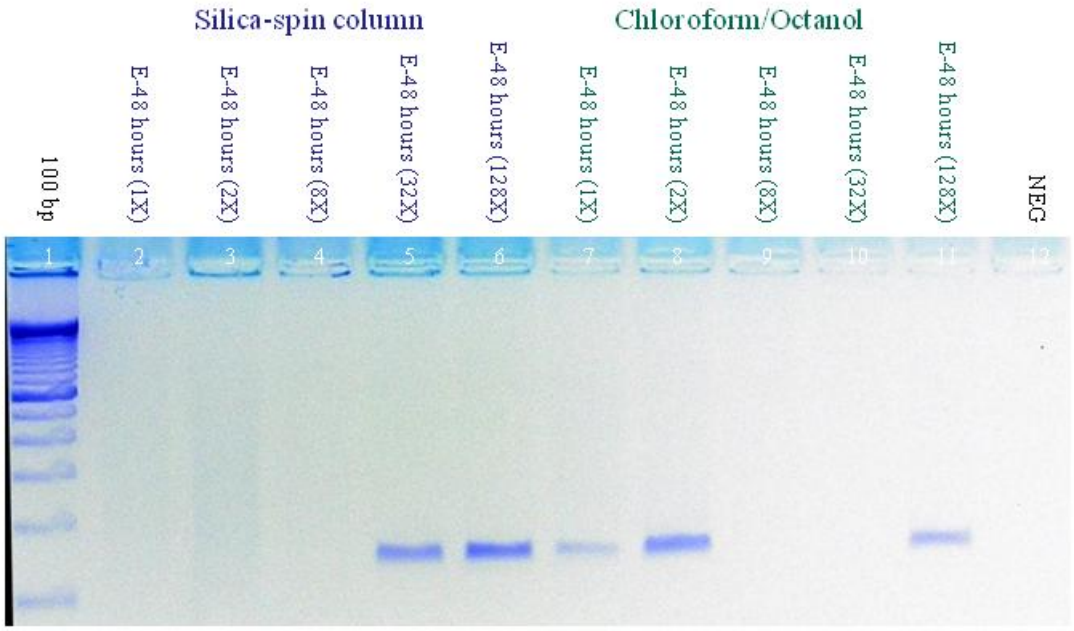
Sample	Randomly Assigned Number for Placement in Oven	Sample Weight Prior to Heat Exposure (g)	Sample Weight After Heat Exposure (g)	Sample Weight Loss After Heat Exposure (%)
ES-8 hours	5	1.97	1.91	3.00
ES-16 hours	1	1.98	1.92	3.33
ES-24 hours	8	1.99	1.91	3.92
EM-8 hours	7	1.98	1.93	2.32
EM-16 hours	9	1.97	1.91	2.99
EM-24 hours	1	1.98	1.91	3.34
EL-8 hours	4	1.98	1.93	2.13
EL-16 hours	6	1.98	1.92	2.73
EL-24 hours	3	1.99	1.94	2.36
E-24 hours	5	2.00	1.97	1.90
E-48 hours	7	2.00	1.95	2.40
E-70 hours	4	2.00	1.96	2.30
B-24 hours	6	2.01	2.01	0.05
B-48 hours	9	2.01	2.00	0.45
B-70 hours	2	2.01	2.00	0.30
N-24 hours	8	2.00	2.00	0.20
N-48 hours	1	2.01	2.00	0.25
N-70 hours	3	2.00	1.99	0.25

*Note: Samples ES, EM, EL exposed to 95ºC; samples E, B, N 120ºC.

Appendix 2: Discrepancies in DNA extraction protocols.

DNA Extraction Steps	Size-based Sediment Samples	Type-based Sediment Samples
Silica-spin Column Method (modified from Yang et al. 1998)		
Amount of sample used	0.40 g	0.45 g
EDTA in lysis buffer	0.5 M	10%
Amount of lysis buffer	4 mL	3 mL
DNA binding buffer	PN Buffer (Qiagen)	PB Buffer (Qiagen)
Chloroform/Octanol Method (Haile 2012)		
First and second DNA elutions	Separated (200 µL each)	Combined (Total of 400 µL)

Appendix 3: Dilution series of ES-48 extracts from two extraction methods. Primers were designed to target a 139 bp fragment of sheep mtDNA. After sixty cycles of PCR, amplicons were visualized with SybrGreen on a 2% agarose gel. 100 bp represents 100 bp ladder; Neg represents the negative control. X represents the amount the sample was diluted by using ultra-pure water in order to overcome inhibition. Lanes 2-6 are silica-spin column extracts; lanes 7-11 chloroform/octanol extracts.



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