OPTIMISATION OF DNA EXTRACTION FROM TEETH SUBMERGED IN FALSE BAY, SOUTH AFRICA

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(17 166)

Presented in partial fulfillment of the requirements for an

M.Phil. Biomedical Forensic Science

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Abstract

Extracting forensically useable DNA from human remains recovered from an open marine environment is problematic, and in some cases, impossible. The reason is unclear given the lack of research on marine decomposition, DNA survival in seawater, and possible methods to optimise the DNA extraction workflow. Compounding this problem is the fact that South Africa experiences a high number of unidentified human bodies entering its mortuaries each year, and these individuals often remain unidentified. The aim of the study was to extract forensically useable DNA from pig (*Sus scrofa*) teeth submerged *in-situ* in an open marine environment, by a process of optimisation and implementation. Detailed environmental information was available for this study. A DNA extraction technique was developed and optimised on "fresh" control pig teeth (n = 13). The developed methods for decontamination, tooth sampling, and the optimised DNA extraction protocol were successfully performed on these, with forensically useable DNA obtained. However, this was not the case for the sub-sample of experimental pig teeth (n = 6) tested. Implementation of the developed method on a larger sample of experimental teeth (n = 28) was warranted to assess the recovery of nDNA and mtDNA. Amplification of nDNA by qPCR was successful in 60% (17/28) of samples for a 96 bp fragment, and in 46% (13/28) for 200 bp. By comparison, mtDNA showed a detection rate of 57% (16/28) for a 486 bp fragment via PCR amplification. In seven samples mtDNA was detected where nDNA was not, demonstrating improved survivability in seawater. Colder and more stable seawater temperatures is hypothesised to have preserved molecular elements. DNA hydrolysis and the possibility of DNAase activity from marine bacteria, may have contributed to poor DNA preservation in the other samples. Recovery of DNA from teeth submerged in an open marine environment is complex and requires further investigation in human samples to improve the identification process for individuals who have died at sea.

Acknowledgements

Venturing into post-graduate studies is not possible without the help and guidance from many different players. First and foremost, I would like to acknowledge my supervisors, Dr. Victoria Gibbon and Laura Heathfield. They both invested time and effort into growing me as an independent researcher. I have learnt so much from them and I value the knowledge and experience I have gained.

I would also like to acknowledge those who provided research and personal funding. Dr. Victoria Gibbon (Research Development Grant (2016 & 2017), the Division of Forensic Medicine and Toxicology, Professor Alan Morris (NRF – Western Cape decomposition studies), and the National Research Foundation (Scarce Skills M & D Scholar), all made financial contributions and without this, the research would not have been possible. The financial assistance of the National Research Foundation towards this research is acknowledged. Opinions expressed in this thesis and the conclusions arrived at, are those of the author, and are not necessarily to be attributed to the National Research Foundation.

I would like to acknowledge Charles Harris, Tyronne Dewet, Michael Cassar, Deon Abrahams, AK Samuels, and Bradley Hendricks from the Department of Human Biology. These gentlemen assisted in several aspects of the project by obtaining supplies and space in order to do the project. I express my thanks to Roelcol Company, Malmesbury for suppling the fresh pig carcasses. Furthermore, the opportunity to study in a marine setting would not have been possible without the valuable contributions from Professor Alan Morris and Belinda Speed, who started research in marine decomposition, obtained all samples, and provided the environmental data. In the same token, I wish to acknowledge the Research Diving Unit, who went down to collect the pig teeth.

Thank you to my colleagues and friends who provided support, lots of fun and plenty of great memories over the past two years of my masters. Finally, I express my gratitude to my family, my parents Howard and Ingrid Longden-Thurgood, my sister Jayde, and my fiancé Devin Finaughty. Without these people in my life I would never have been able to do this. I am

extremely blessed to have parents who provide never ending love, support and guidance. They have been a wonderful example of how hard work pays and for this reason they are my greatest role models in life. My sister who always comes to check on me while I am writing and reminds me to take breaks and eat chocolate. My fiancé who in the past couple of months has been my coffee bearer, my cook, my proof-reader, my consoler, my cheerleader, and my rock. Thank you for still wanting to marry me after the writing frustrations.

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Abbreviations

A	Adenine
aDNA	Ancient DNA
bp	Base pair
C	Canine
C	Cytosine
CTD	Conductivity, temperature, and depth
Cyt B	*Cytochrome B*
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FPS	Forensic Pathology Service
FSL	Forensic Science Laboratory
G	Guanine
HV	Hypervariable
I	Incisors
kg	Kilogram
MBG	Molecular biology grade water
m	Meters
mm	Millimetre
M	Molar
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
ng	Nanograms
pg	Picograms
PCR	Polymerase chain reaction
Pm	Pre-molar
qPCR	Quantitative real-time PCR
RNA	Ribonucleic acid
SAPS	South African Police Service
SNPs	Single nucleotide polymorphisms

STR	Short tandem repeats
TLR4	*Toll-like receptor 4*
TBE	Tris-Borate-EDTA
T	Thymine
V	Version

Chapter 1: Introduction and Literature Review

1.1 Background

A serious issue faced in South Africa is the resolution of crime (Wild, 2017). In a country that is crippled by ever-increasing caseloads, the search for solutions to improve forensic processes in the South African Police Service (SAPS) is continuous (Wild, 2017). One matter arising is the identification of deceased individuals (Evert, 2011; Wild, 2017). Considering that in 2017, over one million serious crimes were reported in South Africa, of which 20 000 were murders, it is no surprise that the burden of unidentified human remains persists (Evert, 2011; South African Police Service, 2017; Wild, 2017). The challenges faced in identifying human remains are not only attributed to excessive caseloads, but also due to high volumes of decomposed, burnt, and skeletonised remains entering mortuaries, often lacking formal identification (Evert, 2011; Wild, 2017). The importance of identifying these remains lies in both social and criminal justice, thus, is an integral component in the field of forensics. According to crime statistics, the Western Cape province has the second highest crime rate in South Africa (South African Police Service, 2017), and murder accounts for nearly 50% of the unnatural deaths occurring in Cape Town (Crime Statistics South Africa, 2017). Due to this, forensic practitioners have been put under strain in recent years, particularly in the identification of human remains. The purpose of this introduction is to highlight issues that surround victim identification and focus on the advantages DNA poses in particularly challenging situations, such as remains washing up from a marine environment.

1.2 Forensic Human Identification

In South Africa, Forensic Pathology Services (FPS) are responsible for individuals that have died of unnatural causes, as defined by the *Inquests Act* (Act 58 of 1959). The forensic pathologist, a medical doctor with specialist training in anatomical and clinical pathology, aims to determine the cause of death and aid the court in establishing the manner of death. When decomposed, skeletonised, or burnt remains are encountered, the assistance of specially trained individuals can be requested (e.g. forensic odontologists and forensic anthropologists). Several

limitations are encountered when building a demographic profile from skeletal remains, *e.g*. if the remains are fragmented (Hughes-Stamm *et al*., 2011). In such scenarios, deoxyribose nucleic acid (DNA) may be the only method to facilitate identification (Sweet *et al*., 1999; Gaytmenn & Sweet, 2003). Bones and teeth have been extensively researched as sources of DNA, since these elements are long-surviving after prolonged post-mortem periods, and, provide a protective environment from external conditions (Ginther *et al*., 1992; Sullivan *et al*., 1992; Prinz *et al*., 2007; Ohira *et al*., 2009). However, the process of identification using DNA becomes challenging when remains are exposed to harsh environmental conditions for long periods. In these cases, the protective cellular environment is compromised leading to degradation of DNA (Alaeddini *et al*., 2010), thus, complicating identification using molecular markers (Fondevila *et al*., 2008; Alvarez-Cubero *et al*., 2012).

In Cape Town, South Africa, there has been an increase in the number of human remains washing up from the sea each year (Speed, personal communication, 2017). Identifying these individuals via traditional anthropological methods proves especially difficult, as remains can be extensively decomposed, skeletonised, or fragmented (Speed, personal communication, 2016). In Cape Town, Belinda Speed (University of Cape Town) recently started an investigation into the decomposition sequence and timing in the open marine environment of False Bay. The establishment of the research gave an opportunity to investigate DNA extraction from teeth submerged in a cold-water open marine environment and attempt to develop and optimise a method to successfully extract DNA from teeth. To initiate this research, an understanding of teeth as a source of DNA and preservation of DNA is required.

1.3 Teeth: Source of DNA

Teeth have been demonstrated as good sources of DNA in challenging cases, as evidenced by previous successes in human identification (Sweet & Sweet, 1995; Primorac *et al*., 1996; Sweet *et al*., 1999). Despite the vast literature on DNA extraction techniques from teeth, research in this field has mostly been limited to terrestrial studies on ancient DNA (aDNA) (Höss *et al*., 1996; Stone *et al*., 1996; Burger *et al*., 1999; Adler *et al*., 2011), or using contemporary human remains for laboratory-based studies (Schwartz *et al*., 1991; Rubio *et al*., 2009; Hughes-Stamm *et al*., 2011).

To understand DNA extraction from teeth, a basic knowledge of tooth anatomy is required (Figure 1.1), and valuable when deciding on a method (Hervella *et al*., 2015). Anatomically, teeth are made up of two distinct regions, one being the crown covered by enamel and the second being the root(s), covered by cementum (Tilotta *et al*., 2010). Enamel has a high mineral content, predominantly made up of calcium, phosphate, and magnesium (Armstrong & Brekhuis, 1937). Due to this, enamel is regarded as avascular and not considered a useful source of DNA (Higgins & Austin, 2013). Despite the lack of DNA in this component, the properties of enamel, such as its strength and reduced permeability, provide a valuable physical barrier between the environment and the DNA inside the tooth (Newbrun & Pigman, 1960; Woodward *et al*., 1994; Cuy *et al*., 2002; Malaver & Yunis, 2003; Alakoç & Aka, 2009; Pinchi *et al*., 2011). DNA-rich regions within teeth include pulp, cementum, and dentine (Corte-Real *et al*., 2006; Adler *et al*., 2011; Higgins & Austin, 2013; Hervella *et al*., 2015), owing to the vascular supply and cellular components comprising these tissues, these regions are targeted as sources of DNA from teeth.

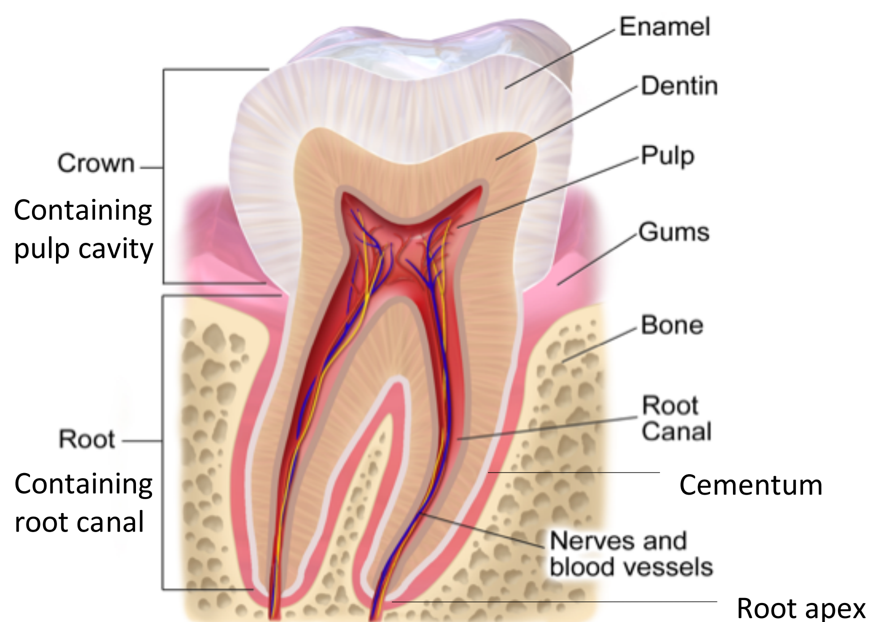

Figure 1.1: Basic anatomy of the tooth (BruceBlaus, 2014). The pulp, cementum, and dentine are considered as DNA-rich regions within the tooth, while the enamel is avascular providing a physical barrier between the inner tooth and external environment.

1.4 DNA Preservation Post-mortem

DNA is regarded as a relatively stable molecule, yet long post-mortem intervals complicate the process of DNA preservation in teeth (Burger *et al.*, 1999). DNA is inherently susceptible to hydrolytic cleavage, oxidation, and microbial enzymes (Lindahl, 1993; Alaeddini *et al.*, 2010). The major action of post-mortem DNA degradation occurs within a few hours or days after death, as a result of cellular autolysis (Burger *et al.*, 1999). Once an organism dies, cells undergo metabolic collapse and homeostatic breakdown, and since DNA repair mechanisms no longer exist, DNA becomes increasingly susceptible to these "attacks" (Alaeddini *et al.*, 2010). Necrosis of cells accelerate this process, where the cells expand and eventually rupture, releasing multiple enzymes that damage DNA (Alaeddini *et al.*, 2010). In addition, the breakdown of cells exposes DNA leaving it susceptible to microbial nucleases, particularly those from microorganisms that colonise the body and proliferate post-mortem. Once DNA has fragmented to less than 300 base pairs (bp), there is a loss of genetic information reducing the likelihood of performing successful DNA identification (Polymerase chain reaction and short tandem repeat analyses) (Bender *et al.*, 2004).

Despite the natural degradation of DNA post-mortem, there are certain processes and conditions that act to preserve DNA. It has been demonstrated that in dry and cold conditions DNA can be extracted successfully from teeth, even after long periods (Burger *et al.*, 1999; Gotherstrom *et al.*, 2002). In dry conditions, the pulp within teeth desiccates into a fibrous like tissue (Duffy *et al.*, 1991) providing protection against environmental insult (Pötsch *et al.*, 1992). A process known as diagenesis, where the mineralised component of teeth undergoes chemical changes, is also believed to protect DNA (Kohn *et al.*, 1999; Hedges, 2002). However, the presence of water affects both the persistence of pulp within teeth and the process of diagenesis. Pulp degrades more rapidly in wet environments (Duffy *et al.*, 1991), thus, reducing the chance of successfully extracting DNA from this element. In addition, water-logged dental tissue displays increased damage to the mineralised component post-mortem (Gotherstrom *et al.*, 2002). Therefore, in immersion conditions there would be loss of protection from the mineral phase. Based on this, it is suspected that a marine environment poses sub-optimal preservation conditions for DNA. Furthermore, difficulties in extracting DNA from teeth exposed to immersed environments have been previously reported (Alvarez García *et al.*, 1996; Graw *et al.*, 2000; Drake, 2014; Hughes-Stamm *et al.*, 2016). However, the

oceanic environment of False Bay is a cold-water open marine habitat, thus, possibly providing some level of protection to DNA, and considering the issues faced with identification, investigation into DNA recovery from teeth is necessary.

1.5 Marine Decomposition and Molecular Preservation

Conducting marine research comes with far greater challenges than on land, as a result of its remoteness, the required specialised equipment, and the costs involved (Anderson & Bell, 2016). The need for it in South Africa has increased in recent years considering the difficulties faced when identifying human remains retrieved from this environment (Speed, personal communication, 2017). Marine decomposition studies thus far have used *Sus scrofa* (pig) as an animal proxy for human bodies, because of the anatomical similarities they share with humans. These include specific parameters important for decomposition studies, including fat-to-skin ratio, trunk dimension, and intestinal microbiome (Catts & Goff, 1992). Dentally, pigs share morphological similarities in the gross anatomy of their molars and mandibular shape, and possess similar radiodensity of enamel to that of humans (Herring, 1976; Fonseca *et al*., 2004; Popowics *et al*., 2004). Genetically, pigs share a common evolutionary ancestor with humans (O'Brien *et al*., 1999) and have previously been used as models for aDNA and molecular anthropological studies (Duffy *et al*., 1991; Gotherstrom *et al*., 2002; Foran, 2006; Adler *et al*., 2011; Drake, 2014). Furthermore, the advantages of using porcine tissue in such research are: for their ease and cost-efficiency of procurement, capacity to standardise samples, less public opposition to their use compared to donated human bodies, and finally, the ability to study teeth in their natural state within the jaw.

1.5.1 DNA Preservation

Little research is available on DNA survival in a marine environment, or if current techniques used in the DNA extraction workflow can be optimised to increase the success rate in obtaining DNA. The main drivers theorised to reduce preservation of DNA within teeth in this environment, include: increased hydrolytic cleavage, and the dilution effect the presence of water may have on DNA (Alvarez García *et al*., 1996; Burger *et al*., 1999).

Previous investigation into DNA survival in seawater has been done in a laboratory setting (Schwartz *et al*., 1991), using fish tanks (Hughes-Stamm, 2012), or placing samples in a semi-protected marine environment, *i.e*. a harbour (Drake, 2014). The above studies have reported contradicting results with regard to DNA yields and the preservation of status of DNA within teeth. Schwartz *et al*. (1991) demonstrated that high molecular weight DNA could be extracted from human teeth after one month of submersion in 25°C seawater, and after two weeks at 37°C. Hughes-Stamm (2012), also studying human teeth, showed that reasonable DNA preservation was observed in teeth after a period of six months. However, both studies were conducted on human dental extracts in artificial marine environments, meaning the effect of environmental factors, other than salt content and temperature, were not considered.

Studies conducted in more realistic marine settings also have contrasting results with regards to DNA recovery from teeth exposed to this environment (Alvarez García *et al*., 1996; Drake, 2014). Alvarez García *et al*. (1996) demonstrated that amplifiable DNA of 200 bp in length was extractable from teeth after two weeks of submersion, and after 6 months a 600 bp fragment was successfully amplified. In contrast, Drake (2014) demonstrated that negligible DNA was present after eight weeks of submersion in human and pig teeth samples.

The contradicting results between studies could be explained by differences in seawater environments studied, the source of the teeth (*i.e*. dental patients versus pig teeth), or the use of different methodologies to assess DNA quantity and quality. For example, many studies focus primarily on amplification-based methods for DNA assessment. Although this is valuable in assessing both quantity and quality, it can underestimate the amount of DNA within the sample, depending on the targeted region of interest, and the extent of DNA fragmentation (Sedlackova *et al*., 2013). Therefore, employing multiple methods of DNA assessment would provide a more comprehensive analysis of DNA degradation in a marine environment. In addition, to understand DNA preservation in a marine environment, it is imperative that studies are conducted in the context of the local environment, as environmental variables influence DNA preservation and degradation (Hughes-Stamm, 2012; Alvarez García *et al*., 1996; Burger *et al*., 1999; Rollo *et al*., 2002; Schwartz *et al*., 1991).

DNA preservation studies conducted in marine environments have primarily focused on nuclear DNA (nDNA). This is likely due to the use of nDNA in the generation of a "forensic

DNA profile" where short tandem repeats (STRs) are targeted for their high discriminatory value. Terrestrial and aDNA studies have demonstrated that mitochondrial DNA (mtDNA) has improved preservation over nDNA (Pfeiffer *et al*., 1999; Corte-Real *et al*., 2006; Foran, 2006; Adler *et al*., 2011; Bolnick *et al*., 2012; Amer *et al*., 2017). Reasons for improved mtDNA preservation include its circular nature, higher copy numbers within cells, and the protective environment the mitochondrion provides, meaning mtDNA is less susceptible to degradation (Robin & Wong, 1988; Sullivan *et al*., 1992; Bär *et al*., 2000; Foran, 2006; Adler *et al*., 2011). Therefore, it is worth investigating mtDNA preservation in a marine environment if nDNA is unobtainable.

1.6 Rationale

Taking these factors into consideration, conducting research in the local marine environment to assess the recovery of DNA from teeth is important. Moreover, the many failures in victim identification experienced in South Africa – particularly in marine wash-up cases – reinforces the necessity of investigating the retrieval of DNA from other environments to further improve this process.

1.7 Research Aims and Objectives

The aim of this project was to assess the recovery and forensic usability of DNA extracted from teeth submerged in the cold-water marine environment of False Bay. A large component of the project involved method development of a DNA extraction workflow to address whether current techniques could be optimised to enhance DNA yields from porcine teeth samples. Optimisation was conducted prior to the assessment of DNA survival, therefore, a separate chapter devoted to this aspect of the project is presented. The specific aims and objectives for the two different components of the minor dissertation were:

1. Optimise a DNA extraction technique for teeth removed from domestic porcine carcasses (*Sus scrofa*) submerged in a natural sea environment in False Bay, Cape Town.

Objectives:

- Establish a DNA extraction technique on control teeth removed from fresh porcine heads.
- Evaluate extracted DNA for forensic use.
- Test protocol adjustments on control teeth and a subset of experimental teeth derived from pig carcasses used in a marine decomposition study.
- Confirm DNA extracted from teeth is of pig origin.

2. Assess DNA survival in teeth exposed to seawater in False Bay, Cape Town for different lengths of time and in all seasons (summer, spring, winter, autumn).

   Objectives:

   - Apply the optimised DNA extraction procedure to a larger sample of experimental teeth.
   - Measure DNA quantity and quality using multiple methods.
   - Evaluate DNA quality and survivability of both nDNA and mtDNA by assessing quantity and quality results.

Chapter 2: Method Development and Optimisation

2.1 Strategy

The development of a DNA extraction workflow for teeth was approached through an optimisation process to maximise the yield and quality of DNA. Since DNA extraction from teeth was not yet a standardised method at the University of Cape Town, the workflow was developed as a new protocol. The aim was to use the commercial QIAamp® DNA Investigator kit (QIAGEN, Germany), which the South African Police Service's (SAPS) Forensic Science Laboratory (FSL) currently employs, as a basis of optimisation. However, initial testing was performed on the Quick g-DNA™ MiniPrep kit (ZymoResearch, USA).

Optimisation of the DNA extraction workflow was an iterative process, whereby, one variable was changed at a time and the results informed the next steps of optimisation. Broadly, method development and optimisation took place on control teeth at three stages within the DNA extraction workflow: 1) decontamination of tooth surfaces, 2) sampling of individual teeth, and 3) DNA extraction. DNA samples generated from this process were quantified and their quality was also assessed. Porcine teeth were used in this study, and in the remainder of the chapters porcine teeth will simply be referred to as teeth, unless required for description purposes.

DNA extracted from teeth needed to meet criteria based on what is considered 'forensically useable'. Ideally, this would translate into sufficient yield and quality of DNA to generate a forensic DNA profile. Since porcine teeth were being used for this study, the commercial DNA profiling kits for humans would have been uninformative, as primers are specific to higher primates. While STR kits for pig species have been developed, their cost was not warranted in this study, and therefore, criteria were developed based on the quality parameters for conventional DNA profiling. In this study, forensically useable was defined as:

1. DNA yield preferably greater than 0.5 nanograms (ng), but at least 1 picogram (pg) as quantified by quantitative real-time polymerase chain reaction (qPCR) (QIAGEN, 2016)

2. Intact DNA of at least 600 bp for a traditional STR profile (QIAGEN, 2016) **OR**
3. Intact DNA of at least 300 bp for a mini-STR profile (Applied Biosystems®, 2012)

While mtDNA is useful in forensic investigations (Templeton *et al*., 2013; Zhang *et al*., 2016; Amer *et al*., 2017; Bär *et al*., 2000), it was not included in this definition. Regardless, mtDNA was explored in this study as a possible alternative to nDNA in samples exposed to an open marine environment. This will be further discussed in the summary.

Method development and optimisation was initially conducted on control teeth and once DNA results met these criteria, the method was implemented on experimental teeth. A subset of experimental teeth was included in the optimisation process to investigate what protocol adjustments would improve DNA results for teeth that had been exposed to the open marine environment of False Bay. These were also included to gauge to what extent a marine environment would affect DNA recovery from teeth. The schematic diagram below summarises the process of optimisation (Figure 2.1).

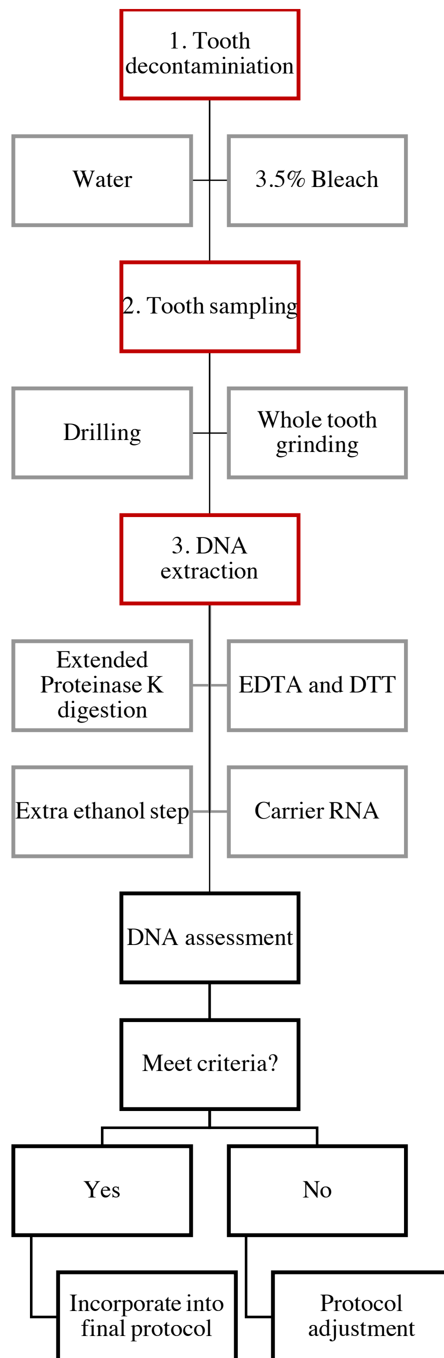

Figure 2.1. Flow diagram summarising the optimisation stages and steps within method development. There were three stages that comprised the DNA extraction workflow, including tooth decontamination, tooth sampling, and DNA extraction. Each protocol adjustment was tested, and, if extracted DNA met the criteria of forensically useable, it was added to the final protocol.

2.2 DNA Assessment

Technical methods pertaining to DNA assessment methodology is provided first, followed by the materials and optimisation experiments. The specific stages of the DNA extraction workflow that were developed and optimised are presented separately with an introduction, results, and motivations for the methods chosen. A discussion into the process of method development and optimisation is presented in Chapter 5. This format has been chosen so that the technical background of the DNA assessment contextualises the evaluation of the DNA results generated from this component of the study.

2.2.1 Overview

During optimisation, DNA samples were assessed for DNA quantity and quality via three methods: (i) Nanodrop™ 2000 Spectrophotometry to measure gross nucleic acid concentration and purity; (ii) Qubit® fluorometry to measure the amount of double-stranded (ds) DNA within the sample; and (iii) qPCR with primers designed specifically to amplify pig DNA to measure the concentration of amplifiable pig-specific DNA within the sample. Primers were designed to target different sized amplicons, to assess the degradation status of DNA. An evaluation into PCR inhibition was also performed by a process of diluting DNA samples during qPCR analysis. The culmination of these methods served as a holistic assessment of the forensic usability of DNA extracted from pig teeth, without the need to generate a pig-specific STR profile. Detailed methodology for these quantification techniques is provided below.

2.2.2 Nanodrop™ Spectrophotometry

The Nanodrop™ 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) instrument was blanked before use, by loading 2 µl of the appropriate elution buffer. For each sample, 2 µl of DNA was loaded onto the pedestal. The Nanodrop™ Spectrophotometer measured the absorbance of the solution at 260 nm, 280 nm, and 230 nm wavelengths, which measured the amount of nucleic acid, proteins, and chaotropic salts, including organic compounds, respectively. Proteins, chaotropic salts, and organic compounds, such as phenol, constitute impurities if present within the DNA sample. Absorbance ratios of 260/280 and 260/230 above 1.8 are considered to be relatively pure DNA (Thermo Fisher Scientific, USA;

Loughrey & Matlock, 2016). The concentration of DNA and the purity ratios were recorded in triplicate and an average was calculated for each sample.

2.2.3 Qubit® Fluorometry

A Qubit® 3.0 fluorometer (Thermo Fisher Scientific, USA) was used as a second quantification method to measure the concentration of dsDNA within the sample. The instrument was calibrated and the samples were prepared according to manufacturer's instructions (Qubit® dsDNA HS Assay kits, handbook, p.2-15) using the High Sensitivity assay kit (Life technologies, USA). DNA concentrations were measured in duplicate for each sample and results were recorded.

2.2.4 qPCR

Two sets of primers, targeting two different amplicon lengths (96 bp and 200 bp) within the *Toll-like receptor 4* (*TLR4*) gene on exon 1 of the pig genome sequence (Downloaded from: http://ensembl.org/index.html, date accessed 23rd January 2017) where used to quantify amplifiable DNA and assess DNA degradation. Primers were designed to be between 18 – 23 bp in length, have a GC content of between 50 – 60%, and a melting temperature (T_M) between 50 – 60°C. These properties, as well as primer dimer formations, were assessed on OligoAnalyzer Version 3.1 (Integrated DNA Technologies Inc., USA, https://eu.idtdna.com/calc/analyser). A ΔG above -4 was deemed acceptable for primer dimers, however, for some primers this criterion was not met. Primer set details for *TLR4* primers are presented in Table 2.1. In addition to qPCR, the primers sets were also used for conventional PCR and Sanger sequencing, where appropriate.

The reaction set-up and qPCR conditions followed manufacturer's guidelines using the QuantiNova™ SYBR® Green assay (QIAGEN, Germany) with one deviation: the combined annealing/extension was performed for 30 seconds, instead of 10 seconds, as this was the shortest acceptable time for the 7500 Real-Time PCR system (Applied Biosystems® Thermo Fisher Scientific, USA).

Table 2.1. *TLR4* and *Cytochrome B* (*Cyt B*) primers targeting different regions within the pig genome that were used in the assessment of DNA extracted from teeth.

	Primer Sequence 5' - 3'	Amplicon length	Length	GC content (%)	Tm (°C)
***TLR4* short fragment**					
Forward	CGGGTCACTTCTGTTCACG	96	19	57.9	55.9
Reverse	CTCAGGCAGGAGAGAAAGG		19	57.9	54.8
***TLR4* long fragment**					
Forward	CTGAGGAGAGAAGACGGC	200	18	61.1	54.6
Reverse	CACATACCT CACGCAAG		18	55.6	54.1
Cyt B					
Forward	GAATTTAACCACGACCAATGAC	486	22	40.9	52.2
Reverse	CCTCAGAATGATATTTGTCCTC		22	40.9	50.6

Two separate *TLR4* primers were designed to assess DNA degradation *i.e.* 96 bp and 200 bp. The *Cyt B* primers were designed to amplify a conserved portion within this region of mtDNA (Section 2.3).

2.3 Species Identification

Ancillary to these methods, a sub-set of DNA samples generated from the optimisation stage were selected to confirm that DNA extracted from teeth was indeed of pig origin. DNA barcoding was employed, whereby a conserved region within the *Cytochrome B* (*Cyt B*) gene of mtDNA was targeted. The targeted region within *Cyt B* is conserved within pigs, but varies between species, with sequences flanking the region being conserved between mammals (Hsieh *et al.*, 2001). As such, the primers were designed to bind regions flanking the 'barcode' portion of *Cyt B* for sequencing purposes. Primers were designed to amplify a 486 bp region within the *Cyt B* gene of mtDNA. Bioinformatics checks were performed to ensure that the primers met the same criteria as that outlined for *TLR4* primers (Table 2.1). Depending on the sequence generated, and the variation encountered within this region, the species of the animal could be deduced by comparing it to the reference sequence of known animals.

DNA samples generated from one control tooth and two experimental teeth were sequenced to confirm species in this manner. This step constituted a quality check to assess the origin of DNA extracted from teeth. The sole presence of pig DNA indicated that DNA from other mammal sources, such as humans, was not entering the samples of interest, or was negligible to remain undetected. The methods and results of species identification are detailed below to demonstrate that DNA extracted from teeth referred to in the method development and optimisation stage was that of pig origin.

Each PCR contained 2X GoTaq® Green Master Mix (Promega, USA), 10 µM of each primer at final volumes of 2.5 µl, less than 250 ng of genomic DNA, as quantified by Nanodrop™ Spectrophotometry in a final volume of 25 µl. A no template control was included for each PCR run, which contained molecular biology grade (MBG) water (Lonza, Switzerland) instead of genomic DNA. The PCR conditions were as follows: initial denaturation for three minutes, 30 cycles of denaturation at 98°C, annealing at 62.2°C, and extension at 72°C for 15 seconds with a final extension at 72°C for five minutes. The annealing temperature was based on in-house optimisation where a temperature gradient between T_M = 55°C - 65°C was tested. A temperature of 62.2°C was chosen based on sufficient yield and non-specificity (Appendix C.1).

Agarose gel electrophoresis was performed to visualise PCR products. The gel contained 2% SeaKem® LE Agarose powder (Lonza, Switzerland) dissolved into Tris-Borate-EDTA (TBE) (Sigma Aldrich, Missouri, USA) stained with 7 % GelRed™ (Biotum, USA) for subsequent DNA visualisation (See Appendix D.1 for method). A volume of 5 µl of each PCR product was loaded onto the gel alongside a 50 bp DNA ladder (Quick-Load Purple, New England BioLabs, USA), for amplicon size estimation. Gel electrophoresis took place for one hour at 100 volts and was visualised using GelDoc (Chemi Genius Bio-imaging System, Syngene, India).

Amplified products were subjected to post-PCR clean-up using the Nucleofast 96 well plate (Macherey Nagel, Germany). The manufacturer's protocol was followed and implemented on a Tecan EVO150 robotic workstation (Tecan, Switzerland). Cleaned PCR products underwent sequencing using the forward primer and the BigDye® Terminator v 3.1 sequencing Kit (Applied Biosystems® Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The post-PCR sequencing steps were performed at the Central Analytical Facility at Stellenbosch University.

Electropherograms were viewed using ChromasLite Version 2.4.4. The DNA sequences were aligned with the reference sequence for the *Cyt B* gene of pig (Downloaded from: http://ensembl.org/index.html, date accessed 29rd January 2017) using ClustalW at 1000 bootstraps on BioEdit 7.2.6 Sequencing Alignment Editor.

Figure 2.2 depicts a portion of the *Cyt B* reference sequence aligned with the sequences generated from DNA samples of one control tooth and two experimental teeth. Upon analysis, it was identified that the sequences generated from the three DNA samples displayed consensus with the *Cyt B* reference sequence (Figure 2.2). This confirmed that DNA extracted from teeth samples was indeed of pig origin. In addition, several single nucleotide polymorphisms (SNPs) were observed and are detailed in Appendix B.1. These were attributed to genetic variation of pigs used in this study, and not coming from DNA of other species. While some SNPs have been reported previously, those which had not were observed in multiple DNA samples from different pigs (Chapter 4). Evaluating these SNPs further was outside the scope of the study.

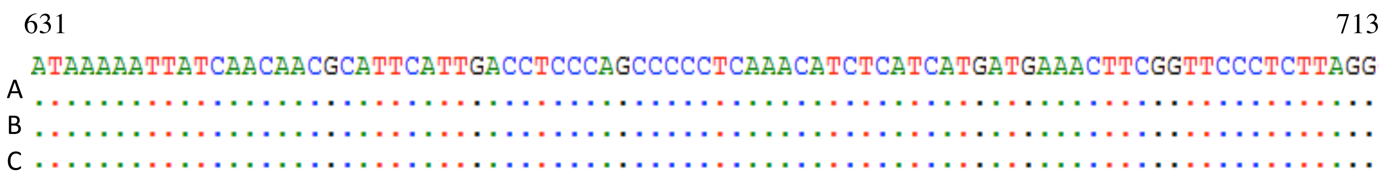

Figure 2.2. The above sequences represent a portion of the 486 bp target region within the *Cyt B* gene. The top sequence represents the reference sequence for pig, below this one are the sequences generated from the control tooth (A) and two experimental teeth (B and C). A dot represents consensus, any deviations from the pig reference sequence will be denoted as A, G, C or T. The alignment was done against the whole *Cyt B* sequence, this image represents a region of the conserved portion used in species identification.

2.4 Materials

Control teeth originating from fresh porcine heads were used in the method development and optimisation stages of the DNA extraction workflow. Pre-molars were preferred, as these represented human teeth most accurately in terms of size, morphology, and function (Herring, 1976). By contrast, pigs' molars are larger compared to humans' and their incisors smaller with a considerably more elongated shape.

The University of Cape Town's Faculty of Health Sciences Animal Ethics Committee confirmed that animal ethics was not required for this aspect of the study, as only waste products from already deceased pigs were used (Appendix A.1). Two fresh porcine heads were obtained from Roelcol Company, Malmesbury (25th January 2017). The mandibular and maxillary bodies that housed the pre-molars were isolated for ease of use and to reduce freeze-

thaw repetitions (Figure 2.3). Details pertaining to the exact methods employed to isolate the bodies of the mandibles and maxilla can be found within Appendix A.2. The summary of the control teeth samples used in optimisation may be found within Table 2.2.

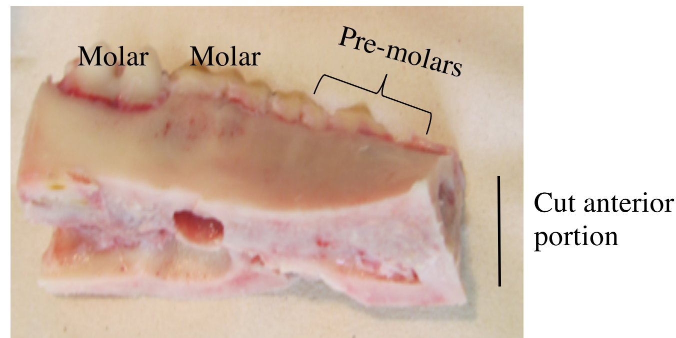

Figure 2.3. Mandibular body containing control teeth from a fresh porcine head. Pre-molars were used in preference to molars and incisors.

Table 2.2. Control teeth sample obtained from fresh porcine heads.

	Head 1				**Head 2**			
Jaw	**Mandible**		**Maxillae**		**Mandible**		**Maxillae**	
Total # teeth	8		7		6		7	
Type tooth	Pm	M	Pm	M	Pm	M	Pm	M
# Teeth	4	4	2	5	3	3	3	4
Pm = Pre-molar; **M** = Molar								

For method development and optimisation purposes there were 12 pre-molars and 16 molars available.

For optimisation, an additional six teeth originating from pig carcasses submerged in a marine environment (False Bay, Cape Town) were obtained via a collaborating project. These teeth were a part of the experimental sample set and will be referred to as experimental teeth within this chapter. Information pertaining to the teeth is detailed within Table 2.3. To control for potential differences between pigs, deployments and sites of submersion, experimental teeth used in optimisation stage were taken from one pig only, during deployment 4. These samples were removed at day 17, 24, and 47 after submersion, which represented different times within the decomposition cycle.

Table 2.3. Information of the six experimental teeth used during the optimisation stages.

	Day 17	Day 24	Day 47
Jaw	Mandible	Mandible	Maxilla
Total # teeth	3	1	2
Type tooth	I	Pm	Pm
Deployment	4		
Pm = Pre-molar; **M** = Molar; **I =** Incisor			

Experimental teeth originated from one pig submerged during deployment 4 within the open marine environment of False Bay, Cape Town.

2.5 Decontamination of Tooth Surfaces

2.5.1 Introduction

Post-mortem samples are prone to external DNA contamination from bacterial, fungal, or other human sources (Alonso *et al.*, 2001; Gilbert *et al.*, 2005; Sampietro *et al.*, 2006; Loreille *et al.*, 2007; Alia-García *et al.*, 2015). Therefore, decontamination is an important first step when extracting DNA from post-mortem teeth samples (Higgins & Austin, 2013). In a forensic context, the increasing sensitivity of newer forensic technologies allows for trace DNA to be detected, which means decontamination procedures need to be efficient at eradicating potential external contamination (Balk, 2015).

One decontamination agent widely used for the purposes of destroying contaminating DNA is sodium hypochlorite (bleach) (Alonso *et al.*, 2001; Adler *et al.*, 2011). Bleach degrades DNA by oxidation and chlorination of bases (Hayatsu, Pan & Ukita, 1971) and is considered a cost-effective method of decontamination (Kemp & Smith, 2005). Champlot *et al.* (2010) reported bleach as having an average of 99.4% success in degrading unwanted PCR products compared to Ultraviolet exposure, which had an average success of 95.6%, indicating bleach to be more effective decontamination agent.

2.5.2 Development of a Method

To evaluate the use of bleach as a decontamination agent, a sample of control teeth (n = 9) underwent a process of wiping the outer surface of the tooth with bleach solution (concentration between 3.5% - 5%) followed by distilled water. The pulp cavity was accessed using a drilling method (Section 2.4) and underwent DNA extraction using the Quick g-DNA™ MiniPrep DNA extraction kit (Zymoresearch, USA) in its factory form and following the manufacturer's protocol (Zymoresearch, USA). The lysis step was performed in the TissueLyser LT (QIAGEN, Germany) for 10 minutes at 50 oscillations per second and DNA was eluted in 50 µl of Zymo Elution buffer.

DNA was quantified spectrophotometrically and the results were compared to control teeth where (i) distilled water was used to wipe the surface (n = 6); (ii) where bleach was used to wipe the tooth surface (n = 3); and (iii) where a toothbrush was used to clean the tooth surface (Table 2.4). A mechanical action has previously been found to aid degrading exogenous contaminating DNA (Champlot *et al*., 2010).

It was found that wiping the outer surface of the tooth with bleach did not reduce the total DNA yield when compared to samples where only distilled water was applied (Table 2.4). In fact, the three samples decontaminated with bleach gave a higher mean DNA yield compared to the seven samples where there was no decontamination (504.3 ng versus 197.5 ng). However, there was one discrepancy where one tooth decontaminated with bleach resulted in a low DNA yield of 6.5 ng. It is possible that the amount of sample collected from within the tooth was not sufficient, therefore, reducing the yield obtained. Despite this, the two other control teeth where bleach was used showed higher DNA yields. Furthermore, later control teeth where bleach was employed as the decontamination agent gave the highest DNA yields (Table 2.5 samples C10 and C11), and were successfully quantified by Qubit® and qPCR. Finally, in samples where the *Cyt B* region was sequenced and bleach was used to clean the outer surface, no other mammal species were detected. Therefore, bleach was employed in the final developed method.

Table 2.4. DNA yields for control teeth that underwent various decontamination steps.

Sample	DNA yield (ng)*	Tooth	Decontamination		
			Wiped with bleach (3.5 % - 5%)	Wiped with water	Mechanical
C1	48.5	Pm		✓	
C2	213.5	Pm		✓	
C3	350.0	Pm		✓	
C4	70.0	Pm		✓	
C5	185.0	M		✓	
C6	318.5	Pm		✓	✓
Mean DNA yield without bleach					**197.5**
C7	6.5	M	✓		
C8	1021.5	M	✓		✓
C9	485.0	M	✓		✓
Mean DNA yield with bleach					**504.3**
C = control tooth; **Pm** = Pre – Molar; **M** = Molar					

Teeth were sampled by the drilling method and DNA extraction was performed with the Quick g-DNA™ MiniPrep kit (Zymoresearch); * the DNA yield was calculated by multiplying the DNA concentration (ng/µl) as measured by a Nanodrop™ 2000 Spectrophotometer with the elution volume of 50 µl.

2.6 Tooth Sampling

2.6.1 Introduction

Several sampling methods to access DNA-rich regions within teeth have been previously suggested (Sweet & Hildebrand, 1998; Sivagami *et al.*, 2000; Shiroma *et al.*, 2004; Pinchi *et al.*, 2011; Hughes-Stamm *et al.*, 2016). Broadly, these methods can be split into two different approaches, one being minimally invasive methods where the inner regions of the tooth are targeted, and others involve a process where the entire tooth undergoes grinding (Smith *et al.*, 1993; Shiroma *et al.*, 2004).

Grinding the whole tooth is currently the method of choice used by the SAPS FSL. This method is also the most common tooth sampling technique (Pinchi *et al.*, 2011). Therefore, it was an objective during method development stage to investigate these two main approaches of tooth sampling (minimally invasive versus whole tooth grinding), and assess DNA results in terms

of quantity and quality. In addition to comparing the two methods, consideration of the cost-effectiveness and reproducibility of the technique was taken into account.

Minimally invasive techniques of tooth sampling are those where the inner regions of the tooth are targeted. Methods to enter the inner region include specialised dental practices, such as endodontic procedures (Pinchi *et al*., 2011; Hughes-Stamm, 2012), or removing the crown at the cemento-enamel junction, with the intention to restore the crown to its original position (Smith *et al*., 1993; Shiroma *et al*., 2004). In addition, these procedures are often designed to preserve the structure of the tooth as far as possible. Endodontic procedures require specialised skills and instrumentation; therefore, a method of tooth sampling whereby easily accessible equipment could be used was rather investigated. The method tested in this study was based on a minimally destructive method (Shiroma *et al*., 2004). Deviations from the original method reported, was the use of a sterile anatomy oscillating saw and hand-held drill. This method will be referred to as the 'drilling method' throughout the chapter and details are presented in Chapter 3. Briefly, the drilling method included using the oscillating saw to cut the crown from the tooth and sampling the pulp cavity and root canal using the hand-held drill, where a powdered sample consisting of pulp, cementum, and dentine was generated.

Techniques where the whole tooth undergoes grinding includes rudimentary methods such as the use of a mallet (Smith *et al*., 1993) or mortal and pester (Sivagami *et al*., 2000), as well as modern techniques of automated grinding (Sweet & Hildebrand, 1998; Tilotta *et al*., 2010; Hughes-Stamm, 2012). In this study, the second method tested involved the use of a Tissue Lyser and Grinding Jars (QIAGEN, Germany), whereby individual teeth were placed into an ice-cold grinding jar and then subjected to oscillation at a frequency of 1/second for 30 seconds. This method is denoted as the 'grinding method', and additional details are presented in Appendix B.2. For each tooth, the weight of the tooth powder samples was recorded using an electronic balance scale (Radwag: model AS 220.R2, Poland). Grinding the whole tooth is arguably more advantageous as it does not require complicated endodontic procedures and a greater sample weight is generated. The larger sample weight also allows for multiple protocol adjustments to be assessed on the same tooth, thus, strengthening conclusions. However, the drawback of this is the possible introduction of PCR inhibitors in the form of minerals from the enamel of the tooth (e.g. calcium) (Higgins & Austin, 2013).

2.6.2 Testing of Tooth Sampling Methods

Five DNA extractions were performed on four control teeth (n = 4) and 15 DNA extractions were performed on six experimental teeth (n =6) to assess the two sampling methods. The full DNA results are summarised in Section 2.5 Table 2.6. DNA yields and purities were found to be better in teeth sampled by the drilling method. However, in some cases additional protocol adjustments incorporated into the DNA extraction method resulted in improved DNA yields from teeth sampled by grinding method (Section 2.7, Table 2.6). Despite this, the overall mean DNA yield was higher, as measured spectrophotometrically, for both control and experimental teeth sampled by the drilling method (Figure 2.4).

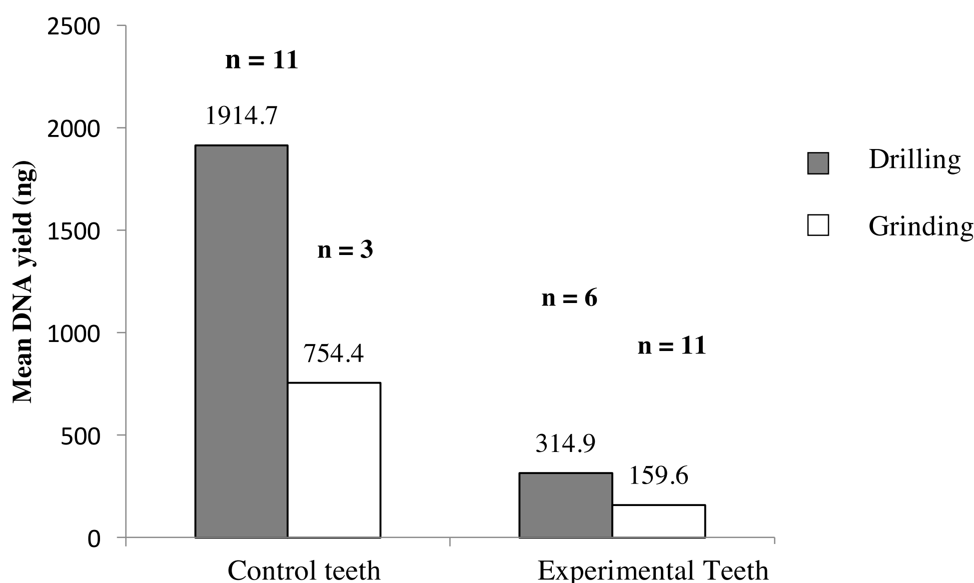

Figure 2.4. Total mean DNA yield, as measured by Nanodrop™ Spectrophotometry, extracted from control teeth and experimental teeth sampled by drilling (dark grey) and grinding method (white).

Purity results as determined spectrophotometrically were variable between DNA samples generated from the two sampling methods. Protein contamination was not a concern as the majority of samples fell within the acceptable range for the A260/280-absorbance ratio (Appendix B.3). In contrast, the A260/230-absorbance ratio, indicating the presence of chaotropic salts or organic molecules did not fall within the acceptable range in majority of samples regardless of the tooth sampling method (Appendix B.3). Particularly, in DNA samples generated from experimental teeth, the A260/230-absorbance ratio was well below the accepted value. The presence of chaotropic salt and organic molecule impurities was

specifically addressed in the DNA extraction section, where multiple protocol adjustments were tested in an attempt to improve the purity, and therefore, will be addressed in section 2.7.

To assess the presence of dsDNA within DNA samples by the two different methods, Qubit® fluorometry was employed. Qubit® was not performed on control teeth sampled by the grinding method, as the yields from fresh control teeth were generally high, whereas the issue of low DNA yields was more prominent in experimental teeth, which required further evaluation. Samples generated from four different DNA extractions from two teeth sampled by the grinding method and four DNA extractions from four teeth sampled by the drilling method underwent dsDNA quantity assessment. The mean DNA yield for control and experimental teeth were lower for those sampled by the grinding method compared to the drilling method (Table 2.6). Furthermore, in one tooth sampled by the grinding method, quantification was not possible due to DNA yields being below the limit of detection for Qubit® (< 0.2 ng).

To further evaluate the forensic usability of DNA samples from teeth subjected to the two different sampling methods, qPCR was performed. It was found that in control teeth sampled by the drilling method, DNA samples met the study's criteria of forensically useable, however, this was not true for control teeth sampled by the grinding method. In an attempt to identify possible PCR inhibitors, qPCR was performed on undiluted and diluted DNA samples. Amplification results demonstrated that PCR inhibition was a possibility in two out of the 20 samples generated from the grinding method, where the undiluted samples did not amplify while the diluted did (Table 2.5). In addition, amplification of DNA samples generated from grinding teeth was more inconsistent compared to those from the drilling method, however, less DNA extractions were performed on teeth sampled by the drilling method, and therefore, a realistic comparison was not possible. It was likely that unsuccessful amplification was mostly due to low DNA copy number in experimental teeth, as a result of the exposure to the marine environment. In summation, considering the lower DNA yields recovered from teeth sampled by the grinding methods, the drilling method was employed in the final developed method to sample teeth.

Table 2.5. Results of qPCR amplification for the 96 bp and 200 bp regions in the *TLR4* gene of pig.

Sample	**Sampling Method**	**EDTA**	**Amplification**			
			96bp	**200bp**	**96bp repeat**	**96bp diluted repeat**
C10	Drilling		✓	✓	✓	✓
C11	Drilling		✓	✓	✓	✓
C12A	Grinding		✓	✓	✓	✓
C12B	Grinding		✓	✓	✓	✓
C13	Drilling	✓	✓	✓	-	-
E1	Drilling		✓	✓	✓	✓
E2A	Grinding		X	X	✓	✓
E2B	Grinding		✓	✓	X	✓
E2C	Grinding		✓	✓	✓	X
E2D	Grinding	✓	X	✓	X	X
E2E	Grinding	✓	X	X	✓	✓
E2F	Grinding	✓	✓	X	-	-
E3	Drilling	✓	X	X	-	-
E4	Drilling		✓	✓	✓	✓
E5A	Grinding		X	✓	X	X
E5B	Grinding		X	✓	✓	X
E5C	Grinding	✓	✓	✓	X	✓
E5D*	Grinding	✓	X	X	X	X
E5E	Grinding	✓	X	✓	-	-
E6	Drilling	✓	✓	✓	-	-

C = control tooth; **E** = experimental tooth; number refers to one tooth; letter represents different extractions done on the same tooth where applicable for the grinding method. Where amplification did not occur once, or more, samples are denoted in bold and underlined; where no amplification occurred, samples are bolded and astrixed (*); ✓ = EDTA added or amplification was successful; X = amplification was unsuccessful.

2.7 DNA Extraction

The DNA extraction protocol was the main focus of the optimisation stage, whereby the results of DNA extraction were used to assess DNA quantity and quality from all steps comprising method development and optimisation. The objective was to incorporate protocol adjustments to obtain forensically useable DNA from both control and experimental teeth.

In recent years, spin-column based DNA extraction methods have gained considerable popularity, considering the reported improved DNA yields and purity of DNA samples,

particularly for bones and teeth (Kemp *et al*., 2006; Rohland & Hofreiter, 2007a; Rothe & Nagy, 2016). As such, this method of DNA extraction was employed.

2.7.1 Optimisation of DNA Extraction

Control teeth were used initially to assess protocol adjustments incorporated into the DNA extraction method. Subsequently, these were implemented on a subset of experimental teeth samples. In total, 13 control teeth and six experimental teeth were used in the optimisation stage, with a total of 31 DNA extractions performed on these samples. The final DNA extraction protocol is detailed in Chapter 3.

Preliminary DNA extractions were performed using the Quick g-DNA™ MiniPrep (ZymoResearch, USA) to investigate initial method development (decontamination) and the ease in extracting DNA from teeth (n = 11 control teeth; n = 2 experimental teeth). Since this kit did not contain Proteinase K, subsequent DNA extractions were performed using the QIAamp® DNA Investigator kit (QIAGEN, Germany). This latter DNA extraction kit, which is also spin-column based, has the added advantage of being validated for forensic use and is used by the SAPS FSL. The manufacturer's protocol included instructions for DNA extraction from bones and teeth, which was used as the basis for the incorporation of protocol adjustments.

Upon assessment of the two DNA extraction protocols on control teeth, irrespective of sampling method utilised, it was found that DNA yields increased when using the QIAamp® DNA Investigator Kit (QIAGEN, Germany) compared to the Quick g-DNA™ MiniPrep kit (Zymoresearch, USA) (Figure 2.5). This was expected, as the ZymoResearch kit did not include an overnight lysis step with Proteinase K, whilst the QIAamp® DNA Investigator Kit did.

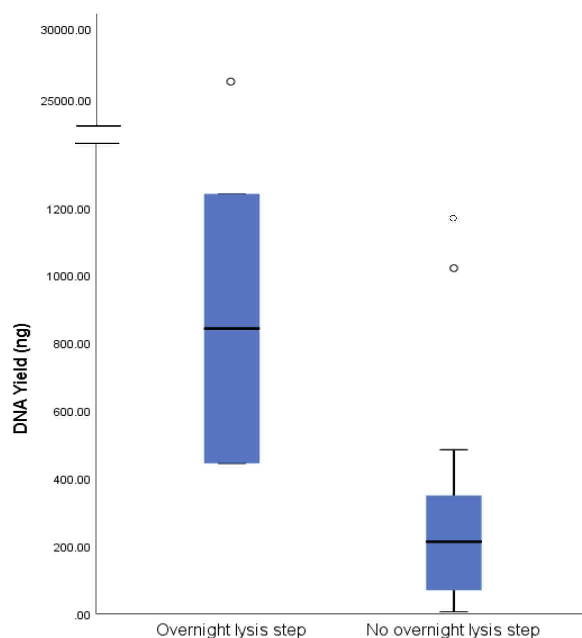

Figure 2.5 DNA yields (Nanodrop™ 2000 Spectrophotometer, Thermo Fisher Scientific, USA) from control teeth where there was no overnight lysis step excluding Proteinase K, and where there was an overnight lysis (between 16 – 20 hours) step with Proteinase K.

Despite improvements in DNA yields from control teeth where the QIAGEN kit was used, DNA recovery from experimental teeth required further optimisation, as yields and quality of samples were poor. An additional problem was that in both control and experimental teeth the existence of impurities absorbed at 230 nm was evident, indicating the presence of chaotropic salts and organic compounds. Protein contamination on the other hand did not pose a problem where mean 260/280-absorbance ratios were above 1.8 (Appendix B.3).

Protocol adjustments incorporated into the DNA extraction method included:

1. Elongated lysis step with Proteinase K
2. Incorporation of an additional ethanol wash step
3. Addition of ethylenediaminetetraacetic acid (EDTA) or EDTA and dithiothreitol (DTT), at different concentrations and volumes to the lysis step
4. Addition of carrier RNA (1 $\mu l/\mu$g)
5. Multiple elutions in lower volumes

DNA yields as measured by Nanodrop™ , Qubit® and qPCR, including all protocol adjustments are presented in Table 2.6.

Table 2.6. DNA quantity results for extractions performed with the QIAamp® DNA Investigator Kit (QIAGEN, Germany) on samples from control and experimental teeth.

Sample	**Day**	**Tooth**	**Sampling Method**	**Sample weight (mg)**	**DNA yield (ng)**		**DNA yield (pg)**	**Protocol adjustment**				
					Nanodrop™	**Qubit®**	**qPCR**	**Overnight with PK**	**Extra ethanol step**	**Carrier RNA**	**EDTA**	**DTT**
C10	N/A	Pm	Drilling	-	7561.5	2322.0	50	✓				
C11	N/A	Pm	Drilling	-	10801.5	3025.0	50	✓				
C12A	N/A	Pm	Grinding	-	577.5	-	0.5	✓				
C12B	N/A	Pm	Grinding	-	445.0	-	0.5	✓	✓			
C13	N/A	Pm	Drilling	63.2	1240.8	1670.0	13.8	✓			✓ 50mM	
E1	17	I	Drilling	27.3	85.0	120.0	<1	✓				
E2A	17	I	Grinding	99.2	215.0	-	<1	✓		✓		
E2B	17	I	Grinding	100	130.0	-	<1	✓	✓			
E2C	17	I	Grinding	60	91.7	-	<1	✓				
E2D	17	I	Grinding	90	201.7	95.0	<1	✓		✓	✓ 50mM	✓
E2E	17	I	Grinding	90	235.0	n/d	<1	✓		✓	✓ 50mM	✓
E2F	17	I	Grinding	27.2	34.6	-	<1	✓			✓ 50mM	
E3	17	I	Drilling	64.7	527.3	93.0	<1	✓			✓ 50mM	
E4	47	Pm	Drilling	Too low	225.0	277.0	<1	✓				
E5A	47	Pm	Grinding	100	143.3	-	<1	✓				
E5B	47	Pm	Grinding	99	128.3	-	<1	✓	✓	✓		
E5C	47	Pm	Grinding	90	230.0	93.0	<1	✓		✓	✓ 0.5M	
E5D	47	Pm	Grinding	90	313.3	63.0	<1	✓		✓	✓ 50mM	
E5E	47	Pm	Grinding	37.9	33.2	-	<1	✓			✓ 50mM	
E6	47	Pm	Drilling	19.3	422.3	155.0	<1	✓			✓ 50mM	

Pm = Pre-molar, **I** = Incisor**;** **C** = Control tooth; **E** = experimental tooth; **n/d** = not detected; number relates to individual teeth; letters represent different protocol adjustments made on the same tooth; samples C10, C11, C12A and C12B were not weighed; the powdered sample generated from tooth E4 was too low to be measured by the electronic scale; (-) represents samples that where not weighed or quantified by Qubit®; bleach solution (3.5%) was used to clean the outer surface of all teeth. DNA concentration of all samples were measured by Nanodrop™ 2000 Spectrophotometer (ThermoFisher Scientiffic, USA); 11 samples the DNA concentration was measured by Qubit® flourometry (ThermoFisher Scientific, USA); the DNA yield was calculated by multiplying the DNA concentration with was elution volume; qPCR results are from amplification of the 96 bp region within *TLR4*.

The incorporation of an extra ethanol wash-step improved the purity ratio for the A260/230-absorbance value, but the improvement was marginal (Appendix B.3) and a decrease in DNA yield was noted (Table 2.6).

Subsequently, the use of EDTA (with and without DTT) was added to the lysis step of the DNA extraction protocol, to try and simultaneously reduce impurities measured at 230 nm and increase DNA yield. This is supported by previous studies where EDTA had been incorporated into extraction procedures and shown an increase in yield and quality of DNA obtained (Loreille *et al*., 2007; Rohland & Hofreiter, 2007a; Jakubowska & Maciejewska, 2012).

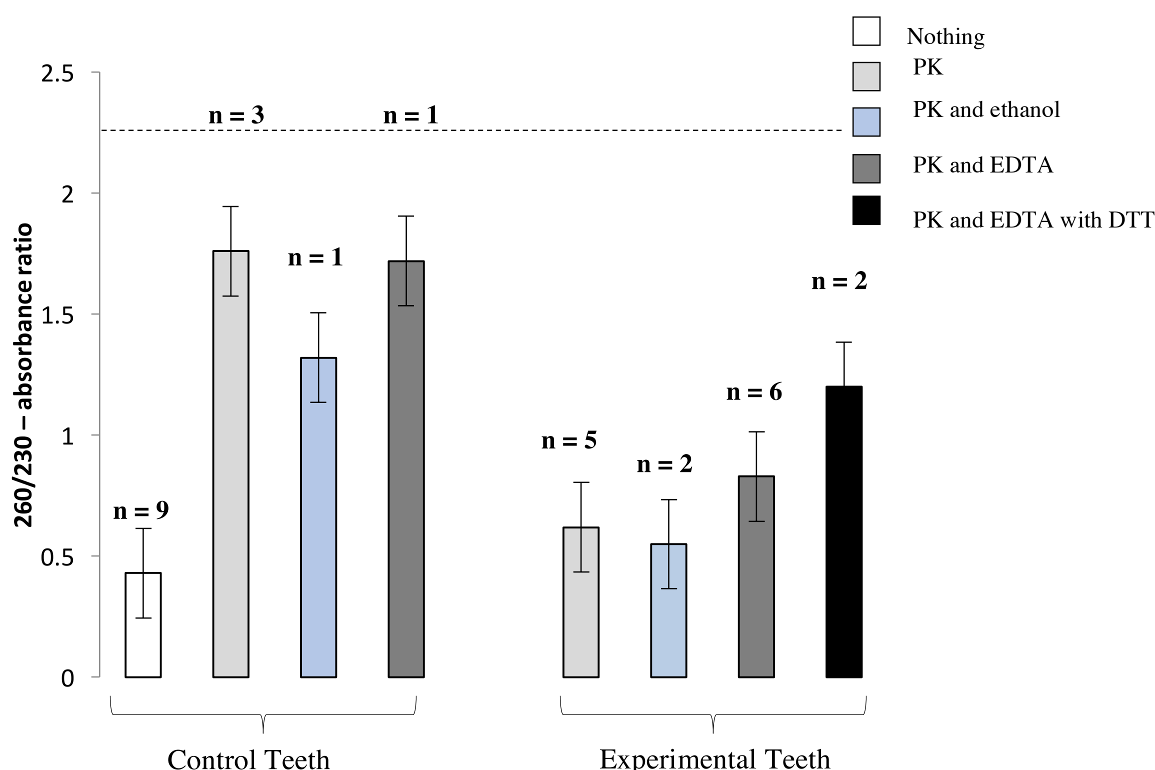

Figure 2.6. 260/230-absorbance ratios of DNA samples from control and experimental teeth, as measured by a Nanodrop™ 2000 Spectrophotometer (Thermo Fisher Scientiffic, USA); using the 260/230-absorbance ratio was used as an indication of chaotropic salt and organic compound contamination in the samples. Ratios of DNA samples from control teeth where PK and EDTA (0.5mM and 0.5M) were incorporated into the DNA extraction protocol and DNA samples from experimental teeth where EDTA and EDTA with DTT were incorporated into the DNA extraction are presented; plotted alongside those where nothing was incorporated in the DNA extraction step (ZymoReserach, USA); a ratio above the black dotted line is deemed acceptable.

The addition of EDTA and DDT was performed as follows: EDTA was added to four samples and two different concentrations were evaluated for effectiveness (0.5 M and 50 mM). A volume of 320 µl of each concentration of EDTA was added to the overnight lysis step. For two samples DTT was also added alongside EDTA, in these cases, two different volumes (10 µl and 20 µl) of a 1M DTT solution were added to the overnight lysis step. For these the volume of EDTA added to the initial lysis step was 310 µl and 300 µl, respectively. These volumes were specifically chosen so that no more than 1 ml of solution was added to the spin-column, as this was the maximum capacity.

The addition EDTA and DTT did not improve DNA yields, however, both acted to improve the purity of the DNA samples (Figure 2.6). The marked improvement in purity of DNA samples motivated the incorporation of EDTA into the final protocol.

The incorporation of carrier RNA was an optional step in the manufacturer's protocol (QIAGEN, 2012) where its addition is thought to increase DNA yields from challenging samples, by reducing the amount of valuable DNA lost during the extraction procedure. Carrier RNA was added to five of the 12 DNA extractions performed on two experimental teeth sampled by the grinding method. These tooth samples were also used in the EDTA experiment above. The addition of carrier RNA did not produce any notable improvements in DNA yield. Any small increase in DNA yield in these samples, as measured by the spectrophotometrically, was rather explained by the increase of carrier RNA present in the sample or due to the addition of EDTA (Table 2.6). Qubit® was done to assess dsDNA within the samples. The concentration of dsDNA concentration for samples where carrier RNA was added were lower compared to those where no carrier RNA was added. As such, carrier RNA was not incorporated into the final developed method.

The final aspect optimised in the DNA extraction protocol was increasing the concentration of DNA by incorporating multiple elutions in smaller volumes. By doing so there was some improvement in amplification of DNA samples (E2F, E5E, and E6 Table 2.5) and the purity of samples (Appendix B.3). Therefore, this was included in the final DNA extraction protocol.

Overall, the DNA yields of samples generated from the multiple DNA extraction protocol adjustments, for both control teeth and experimental teeth, varied greatly. Comparing the mean

DNA yield, as quantified by Nanodrop™ , Qubit®, and qPCR, it was found that the overall DNA yield from control teeth samples were much higher than that of the overall DNA yield from experimental teeth samples, despite many protocol adjustments (evidenced in Figures 2.4 and Figure 2.7 and Table 2.6). In particular experimental teeth did not meet the criteria of being forensically useable where DNA samples were < 1 pg (qPCR) and amplification of small fragments (96 bp and 200 bp) was unsuccessful in many samples.

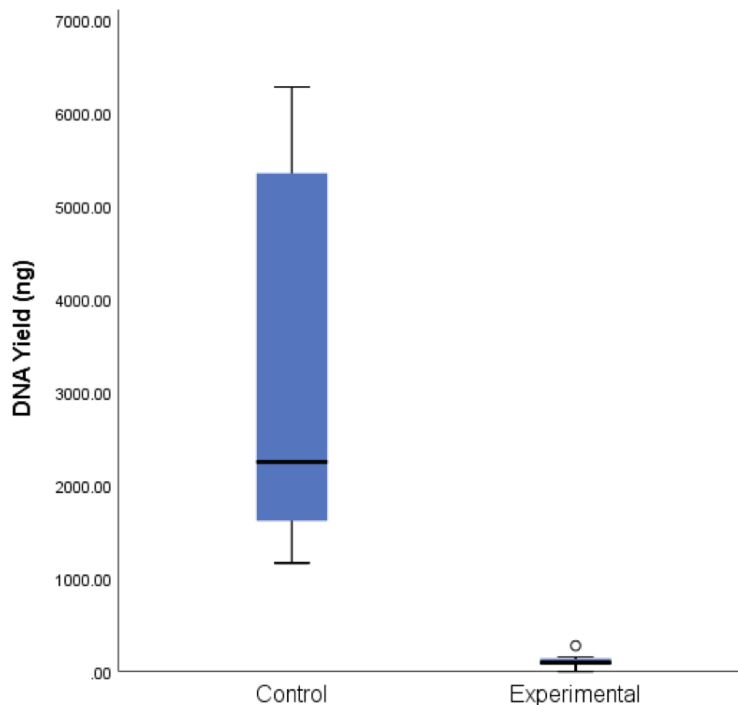

Figure 2.7. Average DNA yields for control and experimental teeth samples, as measured by Qubit® fluorometry. Control teeth displayed much higher DNA yields compared to experimental teeth.

2.8 Summary

In summary, DNA was extracted from both control and experimental teeth in order to test, develop, and optimise a DNA extraction workflow. Bleach was found to be a cost-effective decontamination agent, and the drilling method for tooth sampling gave higher DNA yields from teeth. Protocol adjustments to the DNA extraction stage that resulted in improved DNA results were: an extended lysis step and the addition of EDTA. Particularly, the addition of EDTA reduced chaotropic salt and organic compound impurities, which was high in the majority of DNA samples.

Despite these improvements, qPCR results for DNA samples extracted from experimental teeth were poor, such that, in many samples no amplification of the 96 bp or 200 bp fragments (*TLR4)* occurred, and DNA yields were all < 1 pg (qPCR). Therefore, DNA from the small sample of experimental teeth tested during optimisation stage did not meet the study's criteria of forensically useable. However, the results of species identification on DNA samples extracted from experimental teeth, indicated that mtDNA was sequenceable after 17 and 47 days of submersion in the open marine environment. These promising results motivated the implementation of the optimised method on the remaining larger experimental teeth sample to better represent nDNA survival in seawater and to further investigate mtDNA recovery and preservation.

Chapter 3: Materials and Methods

3.1 Materials

The experimental teeth along with the associated demographics and marine environmental data were provided via a collaborating project, whereby porcine carcasses were submerged in False Bay, Cape Town, to study decomposition in an open marine environment (Speed, personal communication, 2017). Animal ethics for removal of teeth for the purposes of this study was obtained (Faculty Health Sciences Animal Ethics Committee Ref no: 014/004).

The porcine carcasses were deployed 15 meters (m) below sea level at two separate points, as indicated on the maps of False Bay, Cape Town (Figure 3.1). All pigs used in the decomposition study were female, between four and six months old and between 69.5 kilograms (kg) and 70.5 kg. Teeth were removed during data collection visits by divers from the Research Unit (Department of Oceanography, University of Cape Town).

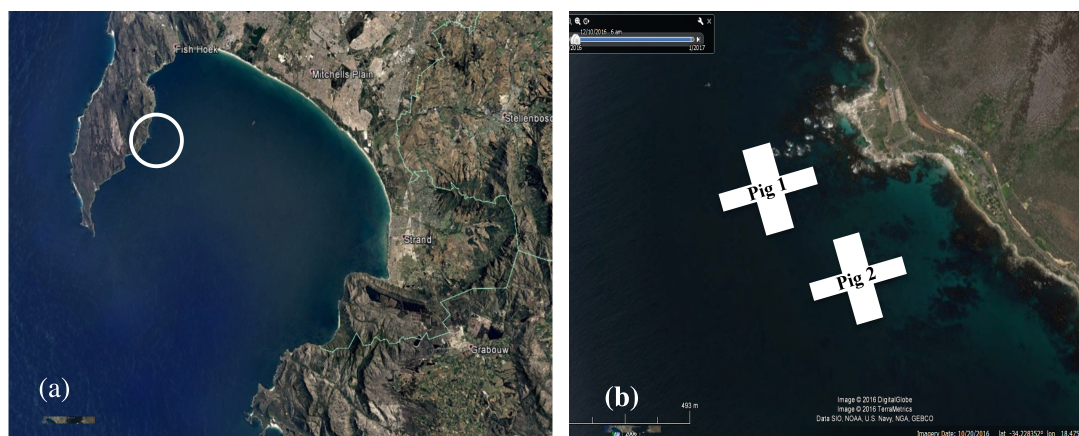

Figure 3.1 **(a)** Aerial view of False Bay, Cape Town, the location of the experimental research site where the decomposition study took place is enclosed within the white circle**;** **(b)** the two separate sites where the pigs were deployed underwater are marked by white crosses. The sites were 500 m apart and were 370 – 450 m from the shoreline. Images are courtesy of Belinda Speed and were downloaded from Google Earth (https://www.google.com/earth/).

The divers used pliers to remove the teeth and were instructed to remove at least one tooth from the mandible and one from the maxilla, and to avoid damaging the teeth. However, limitations such as teeth falling out due to degradation of the periodontal ligament, predators removing the head from the cages, and the difficulty with extracting teeth in early stages of decomposition, meant that this sampling strategy was not consistent for every site visit.

Teeth were removed from carcasses during deployment two, three, four, and five. The type of tooth collected was documented along with what carcass (site) it was collected from and whether it was removed from the mandible or maxilla. In total, 39 teeth were collected from seven pig carcasses used in the decomposition study and six teeth were used in optimisation stage (Chapter 2: Method Development). Five teeth were excluded due to extensive damage. Table 3.1 details the experimental teeth samples originating from the submerged porcine carcasses, and on which the final developed DNA extraction workflow was implemented.

Table 3.1 Summary of the experimental teeth removed from pigs submerged at two different sites in False Bay, Cape Town, and on which the final developed DNA workflow was implemented.

Deployment	Day removed in cycle	Site (1/2)	Jaw	Incisor	Canine	Pre-molar	Total
2	56	-	-	2	2		**4**
3	43	1	Maxilla			2	**2**
3	43	1	Mandible	1		1	**2**
3	43	2	Mandible	1			**1**
4	17	1	Mandible			2	**2**
4	24	1	Maxilla		2		**2**
4	30	2	Mandible	1			**1**
4	30	2	Maxilla			3	**3**
4	42	2	Mandible			1	**1**
4	47	1	Maxilla			1	**1**
5	19	1	Mandible	1			**1**
5	19	2	Mandible		1		**1**
5	35	1	Maxilla		1		**1**
5	35	1	Mandible	1			**1**
5	35	2	Maxilla			1	**1**
5	43	2	Mandible			4	**4**
			Total	**7**	**6**	**15**	**28**

(-) was not recorded; deployments are denoted as: **2** = winter **3** = spring **4** = summer **5** = autumn. Teeth came from one pig in winter deployment; there were 28 experimental teeth in total.

For the associated environmental data, the following methods were employed: minimum and maximum temperatures were measured daily with a *Starmon mini* (Star-Oddi) and at every site visit using a Sea bird conductivity, temperature, and depth (CTD) (SBE 19plus v 2.5.2, Serial number: 7532). Salinity was also measured using the CTD instrument during each site visit. An example of the underwater experimental set-up is depicted in Appendix A.3. For the purposes of the decomposition study, photos were taken every 15 minutes. Environmental data was supplied (Speed, personal communication, 2017) and is summarised within Table 3.2. Other than environmental information the number of visits where teeth were removed is also recorded in the table.

Table 3.2 Environmental information pertaining to each deployment for the two separate sites where two different porcine carcasses were placed.

	Site 1				**Site 2**			
Deployment	**2**	**3**	**4**	**5**	**2**	**3**	**4**	**5**
# Teeth	-	4	5	3	-	1	5	6
# Visits	4	2	4	3	4	2	4	3
Season	Winter	Spring	Summer	Autumn	Winter	Spring	Summer	Autumn
Min Temp (°C)	11.79	12.00	10.53	9.97	12.03	12.01	10.58	9.90
Max Temp (°C)	15.99	17.43	20.58	19.69	15.95	17.49	20.81	19.77
Max – Min (°C)	4.2	5.43	10.05	6.72	3.92	5.48	10.23	9.87
Average Temp (°C)	14.17	15.56	16.78	14.33	14.17	15.65	17.12	13.89
Salinity	35.13	35.29	35.03	34.96	35.14	35.29	35.04	34.95

(-) was not recorded; **Min** = minimum; **Max** = Maximum; deployments are denoted as: **2** = winter **3** = spring **4** = summer **5** = autumn. The seawater temperatures were obtained from a collaborating project (Speed, personal communication, 2017).

3.2 Methods

3.2.1 Tooth Preparation

A sterilisation protocol for the instrumentation and workspace was developed during optimisation, the final protocol is presented in a flow diagram (Figure 3.2). Instruments and workspace surfaces were sterilised between the sampling of individual teeth using this protocol. Personal protective equipment in the form of a lab-coat, gloves, safety glasses, face mask and mop cap was worn to reduce the potential for contamination during sampling. Gloves

were cleaned with 5% bleach and 70% ethanol in between handling instruments and were changed between sampling individual teeth.

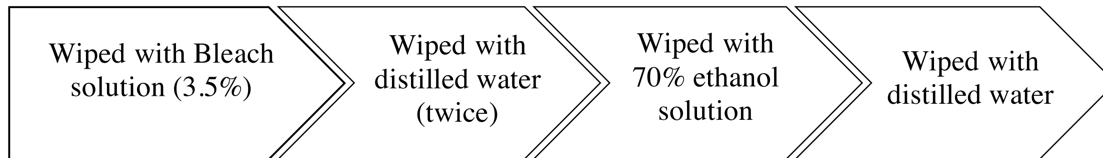

Figure 3.2 Flow diagram of sterilisation protocol for all instrumentation used during the DNA extraction workflow. A clean toothbrush soaked in bleach and then ethanol was used to clean tooth surfaces; clean paper towel was used to wipe the teeth with distilled water.

To decontaminate the outer surfaces, each tooth was brushed using a toothbrush soaked in 3.5% household bleach solution (No Name Brand, Pick-n-Pay, South Africa) followed by wiping with distilled water. Subsequently, the surfaces were again scrubbed with a toothbrush soaked in 70% ethanol, then rinsed with distilled water and wiped dry using clean paper towel. Once each tooth was decontaminated, they were individually secured in a sterile vice grip. The teeth were sampled using a method described by Shiroma *et al.* (2004). Changes from the original method included: scoring the tooth on one side, just above the cemento-enamel junction, and using a sterile oscillating saw (Stryker, USA) to cut across to the opposite side to remove the crown of the tooth (Figure 3.4a). Once the crown was removed, the pulp cavity and root canal was sampled with a sterilised hand-held drill and sterile drill bits (Bosch, GSR 10, 8 – 2 – LT, Professional, Germany) (Figure 3.4b).

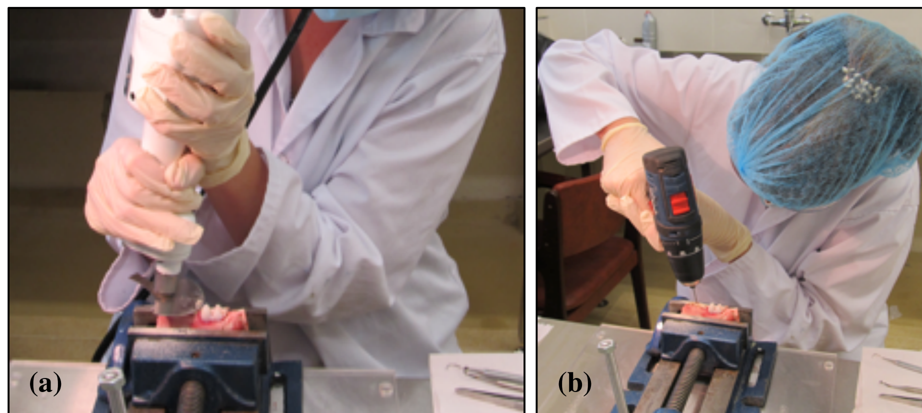

Figure 3.3 **(a)** An example of the sterile oscillating saw being used to remove the crown off a control tooth by cutting just above the cemento-enamal junction; **(b)** the sterilised hand-held drill used to sample the pulp cavities and root canals to generate powdered samples of pulp, cementum, and dentine for each tooth.

The size of the drill bit was chosen based on the size of the tooth and pulp cavity sampled, whereby a small tooth was sampled with a 1 millimetre (mm) drill bit and a larger tooth was sampled with a 2 mm drill bit. A sterile light was used to better visualise the position of the pulp cavity for placement of the hand-held drill. Tooth sampling took place in four batches with seven or eight teeth sampled at a time. In cases where the pulp was still present and "wet" this was noted and collected alongside the powdered samples. Samples were collected in a 1.5 millilitre (ml) sterile microcentrifuge tubes which were placed on ice until DNA extraction was performed later the same day.

3.2.2 DNA Extraction

The QIAamp® DNA Investigator Kit (QIAGEN, Germany) was used to perform all DNA extractions. Buffer preparations are detailed in Appendix D.2. The manufacturer's protocol was followed, and included the following protocol adjustments: 320 µl of 50 mM EDTA was added alongside buffer ATL and Proteinase K, overnight lysis took place for 20 hours, and in the final step DNA was eluted in two-separate 25 µl batches, with a 15-minute incubation time between each elution. The DNA samples from the elution that resulted in a higher DNA concentration were used in further quantification methods and downstream analyses. During the extraction process, DNA samples were designated numbers from 1-28, in the order of sampling. In addition, two negative controls were run alongside the samples *i.e.* every second sampling batch. The negative controls consisted solely of buffers and reagents that were used in DNA extraction.

3.2.3 Assessment of DNA Survival

3.2.3.1 Quantity and Quality

All DNA samples were quantified using Nanodrop™ 2000 Spectrophotometry (Thermo Fisher Scientific, USA), Qubit® Flourometry (ThermoFisher Scientific, USA), and qPCR, the details of these methods are outlined in Chapter 2 Section 2.2 (page 12 – 14).

To further assess quantity and quality of DNA samples, analysis on LabChip® GX Touch HT (Perkin Elmer Inc, USA) was conducted. Twenty-four out of the 28 samples were subjected to

this method, as the Chip could only accommodate this many DNA samples. The LabChip® protocol was prepared according to manufacturer's instructions (DNA high sensitivity Assay User Guide, LabChip® GX Touch/GXII Touch, Perkin Elmer Inc., 2016). The Chip and reagents were equilibrated to room temperature before use and the Chip was prepared for the samples. The active Chip wells were cleaned by rinsing and aspirating twice with MBG water. Gel-dye was added to wells 3, 7, 8, 10, and the DNA HiSens Marker was added to well 4, which generated the DNA ladder. In addition, a 96-well plate was prepared by adding 9 µl of MBG water and 1 µl of DNA to each individual well.

Subsequently both the Chip and plate were loaded onto the LabChip® GX Touch HT, and the assay was run according to manufacturer's instructions (DNA high sensitivity Assay User Guide, LabChip® GX Touch/GXII Touch, Perkin Elmer Inc., 2016). The results were viewed on LabChip® GX Reviewer, Version 5.3.2115.0 (Perkin Elmer Inc., 2016).

3.2.3.2 Sequencing

To further assess the forensic usability of DNA for molecular downstream applications, PCR and Sanger sequencing was done on a subset of DNA samples generated from experimental teeth. Three separate PCR's were performed with primers that were designed to target nDNA (*TLR4*) and mtDNA (*Cyt B)*. The primer sets were the same as those used in method development; therefore, primers are detailed in Chapter 2 Section 2.2. In addition to DNA samples selected specifically for sequencing purposes, amplification of all DNA samples was performed to investigate whether mtDNA was amplifiable, even in very low DNA yield samples, using the same primers from species identification (Chapter 2 Section 2.3, page 13 - 15).

The PCR set-up, cycling conditions and sequencing methodology follow the same as those employed in Chapter 2 (Section 2.3 page 15), except for the sequencing of the regions within the *TLR4* gene, where the annealing temperature was 60°C. Post-PCR, all samples underwent agarose gel electrophoresis (Chapter 2 Section 2.3, page 14) to confirm presence of amplified products. One deviation from the original method of agarose gel preparation described in Chapter 2 was the addition of 10 % SYBR® Safe stain (Life Technologies™ , USA), in place of GelRed™ , for visualisation of amplified products.

Five DNA samples were selected for sequencing of nDNA (*TLR4*) and mtDNA (*Cyt B*) where amplification was successful for the target regions. Only five were chosen due to budget constraints, therefore, samples were chosen to represent the end stages of decomposition within each deployment. Where necessary additional PCR runs and gels were performed on these samples prior to sending them for sequencing.

3.3 Data Analysis

Graphs were generated in Excel, Version 14.4.1 (Microsoft® Excel® Mac, 2011) and Prism Version 7.0c (GraphPad Software Inc, 2017). No statistical analysis was performed on the data due to limited and uneven sample sizes. Sequencing data was viewed on ChromasLite Version 2.4.4. DNA sequences were aligned to the reference sequence using using ClustalW at 1000 bootstraps on BioEdit 7.2.6 Sequencing Alignment Editor. Reference sequences were obtained from Ensembl Genome browser (available at: http://www.ensembl.org). To investigate the origin of non-specific amplification, where applicable, FASTA sequences were entered into BLAST® (available at: https://blast.ncbi.nlm.nih.gov/Blast.cgi) and performed against the Nucleotide collection (nr/nt) database.

Chapter 4: Results

To assess DNA survival in the cold-water marine environment of False Bay, a process of optimisation was employed to develop methods of DNA extraction on porcine teeth. The objective in the method development stage was to ensure that forensically useable DNA could be extracted from fresh control teeth and experimental teeth exposed to the marine environment of False Bay. Forensically useable DNA was successfully extracted from control teeth, using the methods developed in the optimisation stage (Chapter 2). Despite the successes in extracting DNA from control teeth, optimisation methods tested on the small sample of experimental teeth, from a single deployment, did not yield forensically useable DNA. Considering this, it was deemed necessary to assess any patterns of molecular degradation associated with environmental parameters, such as the effect of seasonality, duration of submersion, and mtDNA preservation. Therefore, the optimised DNA extraction method was tested on a larger sample of experimental teeth (n = 28).

The dental morphology, including tooth type and root closure was noted in the experimental sample. Pre-molars accounted for 53% (15/28), whilst incisors and canines accounted for 25% (7/28) and 22% (6/28), respectively (Appendix A.4). In 68% (19/28) of teeth the root apex was open and in 43% (12/28) pulp was present (Appendix A.4).

4.1 DNA Survival

To assess DNA recovery, DNA samples were subjected to four quantification techniques. In this section quantification results are outlined first, followed by results of DNA quality. Sanger sequencing was performed on a subset of DNA samples as an additional quality assessment, results of this are presented separately.

4.1.1 DNA Quantity

DNA yields generated from the different quantification methods produced varying results for individual DNA samples (Table 4.1). This was expected as each method differed in its sensitivity and specificity, but were valuable in holistically assessing DNA recovery.

Quantification results were variable not only depending on the method used, but between DNA samples from the different deployments and days of submersion (Table 4.1). No DNA was detected in any of the negative controls, indicating no exogenous contaminating DNA was present in the buffers or reagents, and that the decontamination procedure was sufficient. In addition, it demonstrated that no other sources of DNA were introduced during the process of DNA extraction. A noteworthy finding was that DNA samples originating from teeth removed during deployment 2, compared with later deployments were of better quality as demonstrated by the quantification results. Deployment 2 occurred in winter, whilst deployments 3, 4, and 5 were in spring, summer, and autumn, respectively (Speed, personal communication, 2017).

Longitudinal survival of nDNA could not be assessed in teeth removed from deployments 2 and 3, as these were removed on the same day at the end stage of decomposition, within each deployment (day 56 for deployment 2 and day 43 for deployment 3). For deployments 4 and 5, teeth were removed from both early and late stages within the decomposition cycle. DNA recovery from teeth samples removed from pigs after the shortest time of submersion investigated (17 days, deployment 4) was poor (Table 4.2). None of the DNA samples, in any deployment or duration of submersion, met the study's criteria of forensically useable, as determined by qPCR. DNA yields were < 1 pg and intact DNA was smaller than 300 – 600 bp, where in most cases a 200 bp fragment could not be amplified.

Table 4.1. DNA yields of experimental samples as determined by four different quantification methods.

Dep.	Day	Tooth type	Sample	Sample weight (mg)	Nanodrop™	Qubit®	Labchip® GX	qPCR	
					DNA yield (ng)			96 bp (pg)	200 bp (pg)
2		I	1	86.4	150.75	102.5	122.5	<0.00025	<0.0025
		I	2	43.0	380.00	165	502.5	<0.0025	<0.025
		C	3	37.4	122.50	40.5	27.5	<0.0025	<0.00025
	56	C	4	0.3	296.75	8.25	-	<0.0025	-
3		PM	5	15.1	302.50	42.5	15	<0.0025	<0.0025
		PM	6	43.5	122.50	-	-	-	-
		I	7	69.7	130.00	-	-	-	<0.000025
		PM	8	33.3	539.25	107.5	75	<0.00025	<0.0025
	43	I	9	37.1	114.25	-	-	-	<0.00025
4		PM	10	35.5	145.00	185.75	137.5	<0.00025	-
	17	PM	11	48.8	89.25	105	67.5	<0.0025	<0.025
		C	12	73.7	87.50	-	-	<0.00025	-
	24	C	13	64.9	87.50	-	-	-	-
		PM	14	85.5	88.25	-	-	<0.0025	-
		PM	15	61.6	209.25	-	-	<0.00025	<0.000025
		PM	16	59.7	206.75	-	-	-	-
	30	I	17	48.5	270.00	-	-	-	-
	42	PM	18	66.1	47.50	-	12.5	<0.00025	<0.000025
	47	PM	19	63.5	124.25	-	10	-	-
5		C	20	75.3	80.00	-	-	-	-
	19	I	21	65.2	80.75	-	2.5	-	<0.000025
		PM	22	41.4	67.50	-	2.5	<0.00025	-
		C	23	63.0	170.25	-	-	-	<0.000025
		PM	24	55.5	137.50	-	-	<0.0025	-
		PM	25	32.2	123.25	-	•	<0.00025	<0.000025
		PM	26	19.6	140.75	-	•	-	-
		PM	27	38.6	85.75	-	•	<0.0025	-
	35	I	28	108.1	123.25	-	•	<0.0025	-
			Mean	**52.6**	**161.53**	**94.63**	**88.64**	**(-)**	**(-)**
			Median	**52.2**	**123.75**	**103.75**	**27.5**	**(-)**	**(-)**
			Min.	**0.3**	**47.50**	**8.25**	**2.5**	**<0.00025**	**<0.000025**
			Max.	**108.1**	**539.25**	**185.75**	**502.5**	**<0.0025**	**<0.025**

Dep. = Deployment; **Min**. = Minimum; **Max**. = Maximum; **ng** = nanograms; **pg** = picograms; Deployments are denoted as: **2** = winter, **3** = spring, **4** = summer, **5** = autumn. The DNA yield (ng or pg) is calculated by multiplying the DNA concentration (ng/µl) by the elution volume of 25 µl; the dash [-] represents where the DNA concentration for the samples were out of range for Qubit® (< 0.5 ng/µl), LabChip® (< 0.2 ng/µl), or where no amplification occurred in qPCR analysis; minimum values were not recorded for Qubit® and LabChip® as samples fell out of range for quantification by these methods; (-) represents where the mean DNA yield was not calculated for qPCR, as DNA concentration was too low to accurately determine; all concentrations were less than 0.001 ng/µl as quantified by qPCR; DNA yields for qPCR are represented with "<" this is translates to a concentration between the number in front of the sign the next decimal point *i.e.* <0.0025 is equivalent to concentration between 0.00025 < pg < 0.0025.

4.1.2 DNA Quality

The results of qPCR and LabChip GX were used in this study to assess the quality of DNA in samples. No amplification was observed in the no-template controls, which indicated that no contaminating pig DNA was within the buffers and reagents used in qPCR. Quantification of DNA samples by qPCR was possible in 60% (17/28) for the 96 bp (Figure 4.1) and 46% for the 200 bp fragment (13/28) (Figure 4.2), as evidenced by the qPCR software. To assess if amplification during qPCR was specific to the *TLR4* region of interest, gel electrophoresis was performed. Non-specific amplification occurred in 14% (4/28) of samples for 96 bp fragment and in 10% (3/28) for the 200 bp fragment (Appendix C.1).

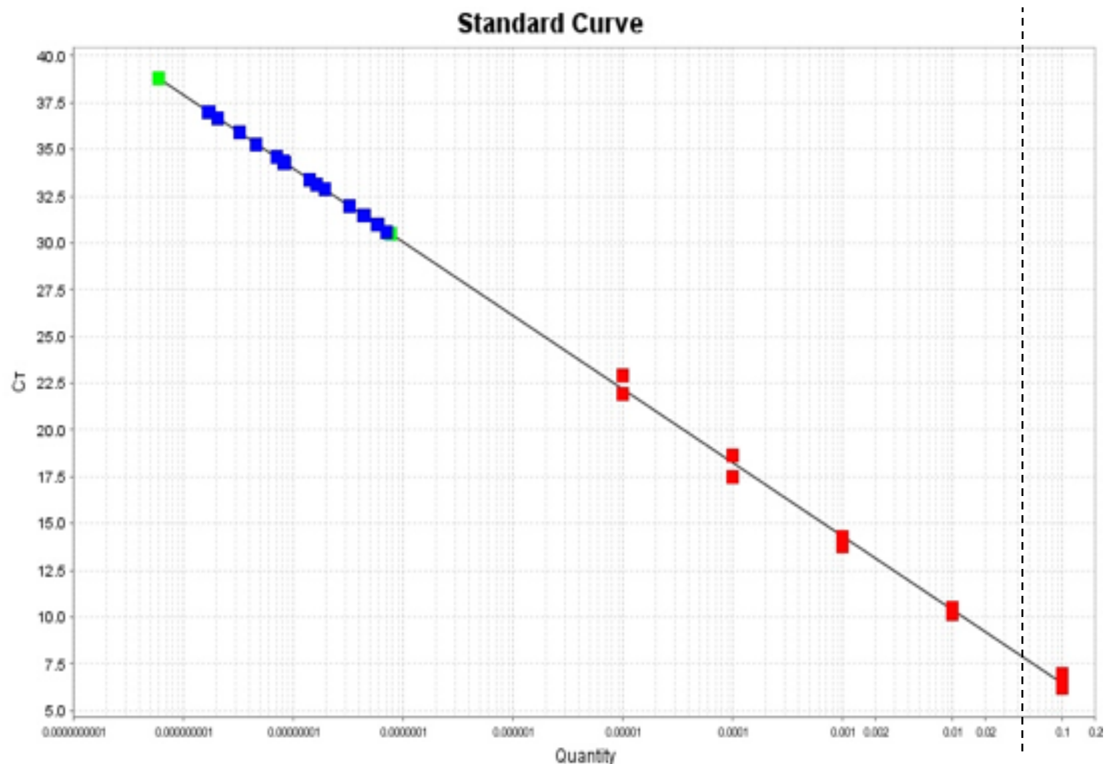

Figure 4.1 Standard curve from performing qPCR using primers targeting a 96 bp region within the *TLR4* gene. The red squares represent the concentrations of the DNA standards, blue squares represent the concentration of the DNA samples, green squares green represent samples that were flagged as either having multiple melting points or those where amplification was unsuccessful; $R^2 = 0.994$; Efficiency = 79.5%; the dotted line represents the cut-off of forensically useable, anything left of this was quantified as < 1 pg.

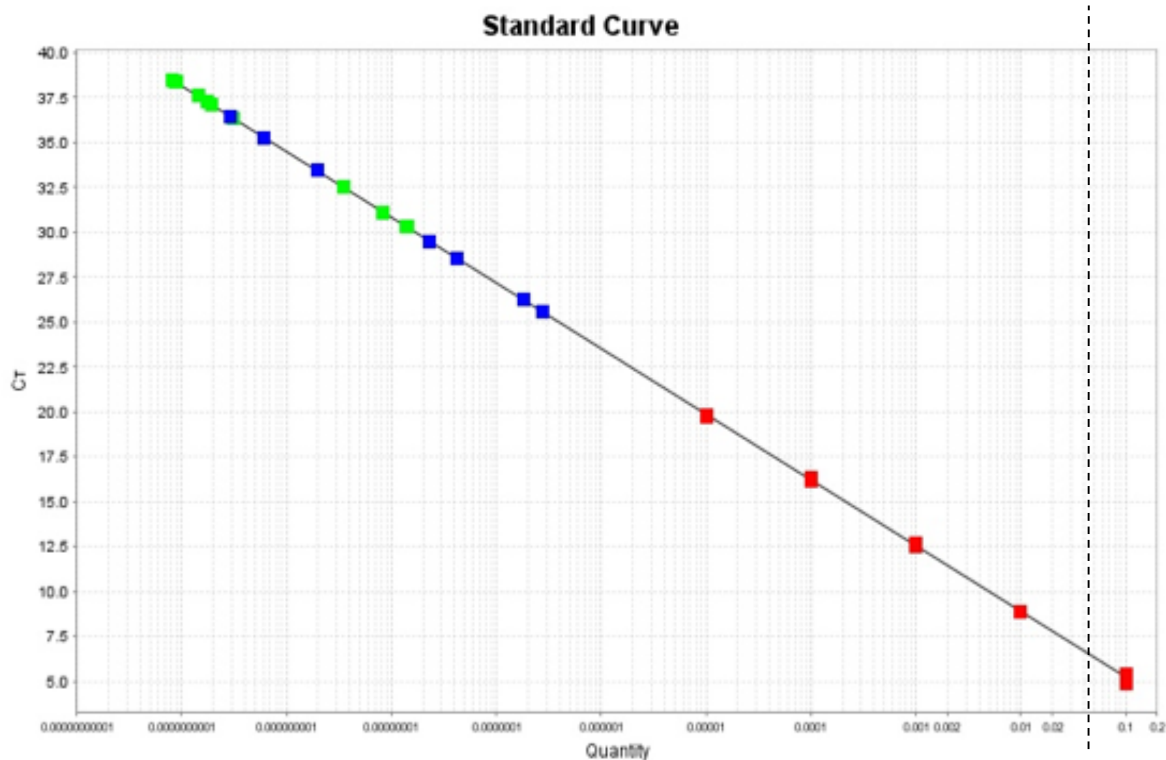

Figure 4.2 Standard curve from performing qPCR using primers targeting a 200 bp region within the *TLR4* gene. The red squares represent the concentrations of the DNA standards, blue squares represent the concentration of the DNA samples, green squares green represent samples that were flagged as either having multiple melting points or those where amplification was unsuccessful; R^2 = 0.999; Efficiency = 87.5%; the dotted line represents the cut-off of forensically useable, anything left of this was quantified as < 1 pg.

The LabChip GX instrument was used to assess total genomic DNA (gDNA) in 24 DNA samples. A virtual gel was generated and the presence of gDNA was detected in 29% (7/24) of samples analysed (Figure 4.3). However, of these, several failed the gDNA quality score (GQS), which indicated DNA concentration was < 0.2 ng/µl. Samples where this occurred are highlighted by a yellow circle exclamation mark in the virtual gel image (Figure 4.3).

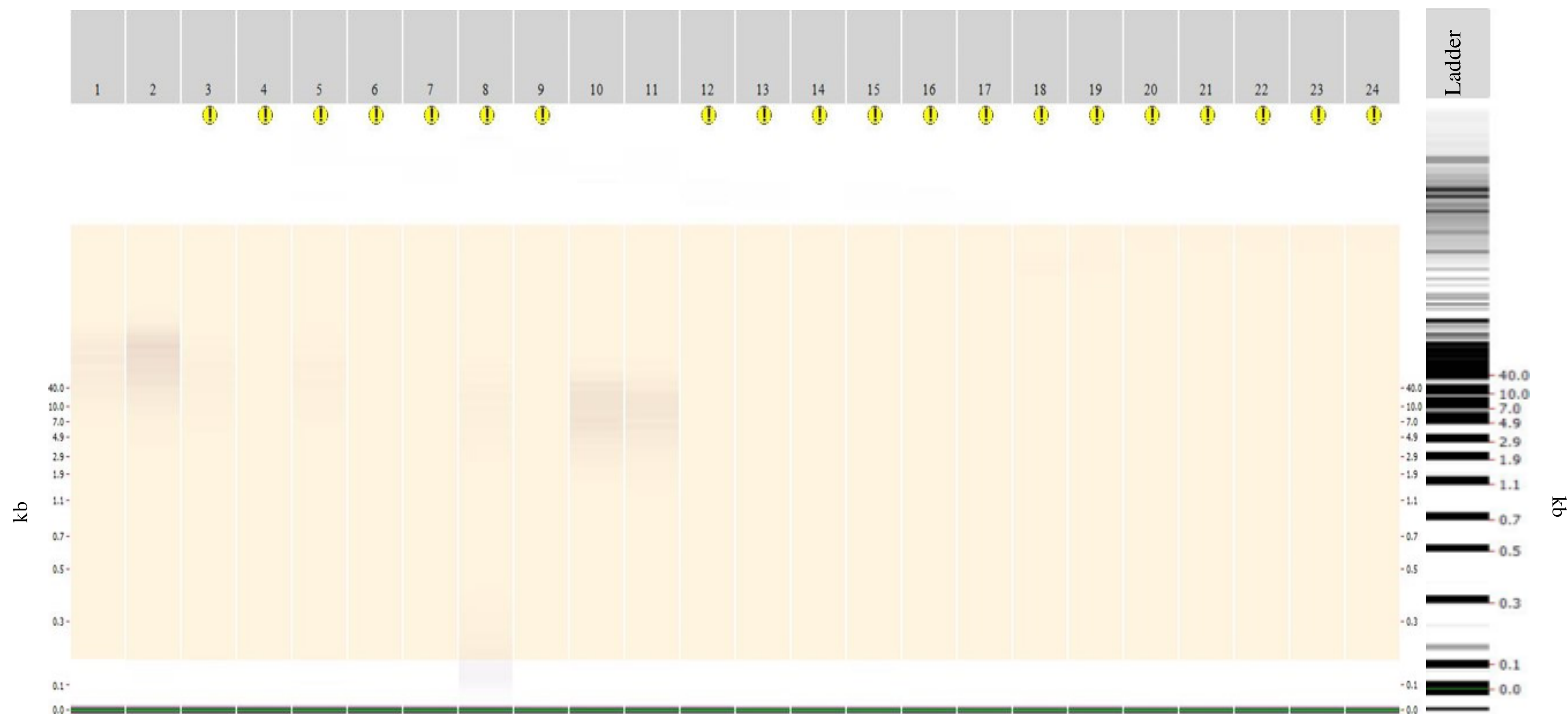

Figure 4.3 Results of the LabChip® GX Touch HT (Perkin Elmer Inc, USA) analyses, a highly sensitive microfluidic gel system performed on 24 samples (1 – 24); total genomic DNA was quantified using 1 µl of sample; the contrast was set to maximum for visualisation purposes, this image can be found in Appendix C.1; lanes are numbered 1-24 to represent the DNA samples analysed; yellow circled exclamation marks indicate failed gDNA GQS *i.e* where DNA concentration was < 0.2 ng/µl.

4.2 mtDNA Survival

To investigate the preservation of mtDNA, the 486 bp region within the *Cyt B* gene, previously targeted in the optimisation stage (Chapter 2– species identification), was amplified. The mtDNA fragment was detected in 57% (15/28) of samples (Figures 4.4 and 4.5) and in seven samples, mtDNA was amplified where the nDNA fragments were not.

Non-specific amplification was observed in three samples, where there was a second band present below the main band (Figure 4.4). This required investigation by Sanger sequencing given that the primers designed to target this region can detect other species. Results are presented in section 4.4.

The trend of mtDNA survival followed a similar trend to nDNA, wherein earlier deployments (*e.g.* deployment 2) the quantity was higher and mtDNA was more amplifiable, compared with later deployments (Figure 4.4 white arrow). Sanger sequencing was also performed on a sub-set of DNA samples to evaluate the sequencing quality of mtDNA; results are presented below alongside those of nDNA.

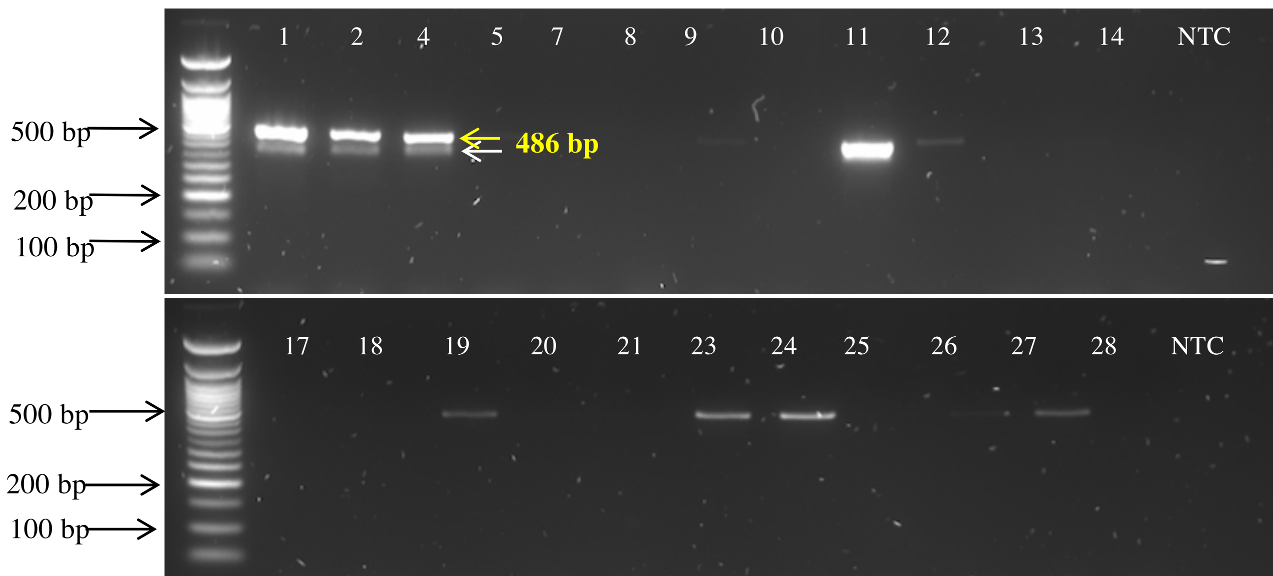

Figure 4.4 Agarose gel electrophoresis (2%) of PCR amplified products using a primer set to target a conserved region within the pig *Cyt B* gene, as indicated by the yellow arrow (486 bp); electrophoresis was performed at 100 V for 1 hour. The far-left lane is the 50 bp DNA ladder; numbers 1 – 28 represent the 28 DNA samples from experimental teeth; two no template controls (NTC) were run alongside samples. Non-specific amplification was observed in samples 1, 2, and 4, as indicated by the white arrow; sample 1 and 3 were sent for sequencing.

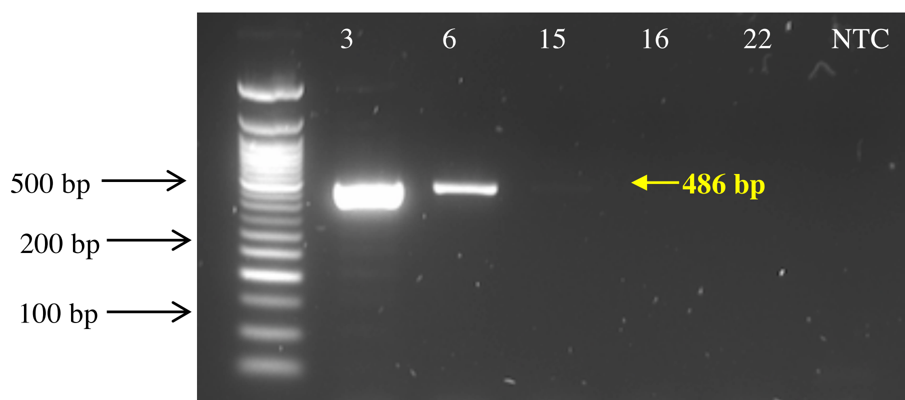

Figure 4.5. Agarose gel electrophoresis (2%) of PCR amplified products using a primer set to target a conserved region within the pig *Cyt B* gene, as indicated by the yellow arrow (486 bp); electrophoresis was performed at 100 V for 1 hour. The far-left lane is the 50 bp DNA ladder; numbers 3, 6, 15, 16 and 22 represent the DNA samples chosen for sequencing; a no template control (NTC) was run alongside samples. A very faint band is present for sample 15.

4.3 Sequencing - nDNA and mtDNA

To evaluate the integrity of nDNA and mtDNA, five DNA samples were chosen for Sanger sequencing. The five samples were chosen to represent those that were quantified consistently (Nanodrop, Qubit, LabChip and qPCR) and those that were not (3, 6, 15, 16, and 22). Samples were also chosen to represent the different deployments. Only samples that showed positive amplification as represented on an agarose gel were sequenced for mtDNA and nDNA targets.

Initially, three of these five samples were amplifiable for mtDNA, whereas no amplification of nDNA was observed (Figure 4.5). PCR optimisation was performed to improve the amplification of samples chosen for sequencing. While increasing the DNA input and cycle number had no positive effect on the mtDNA samples (Appendix C1 Figure, C.5 a), increasing the cycle number to 40 cycles resulted in successful amplification in two nDNA samples (96 bp) (Appendix C1, Figure C.5 b). However, one of these samples (sample 16) represented non-specific amplification, sequencing results for this sample are presented in section 4.4.

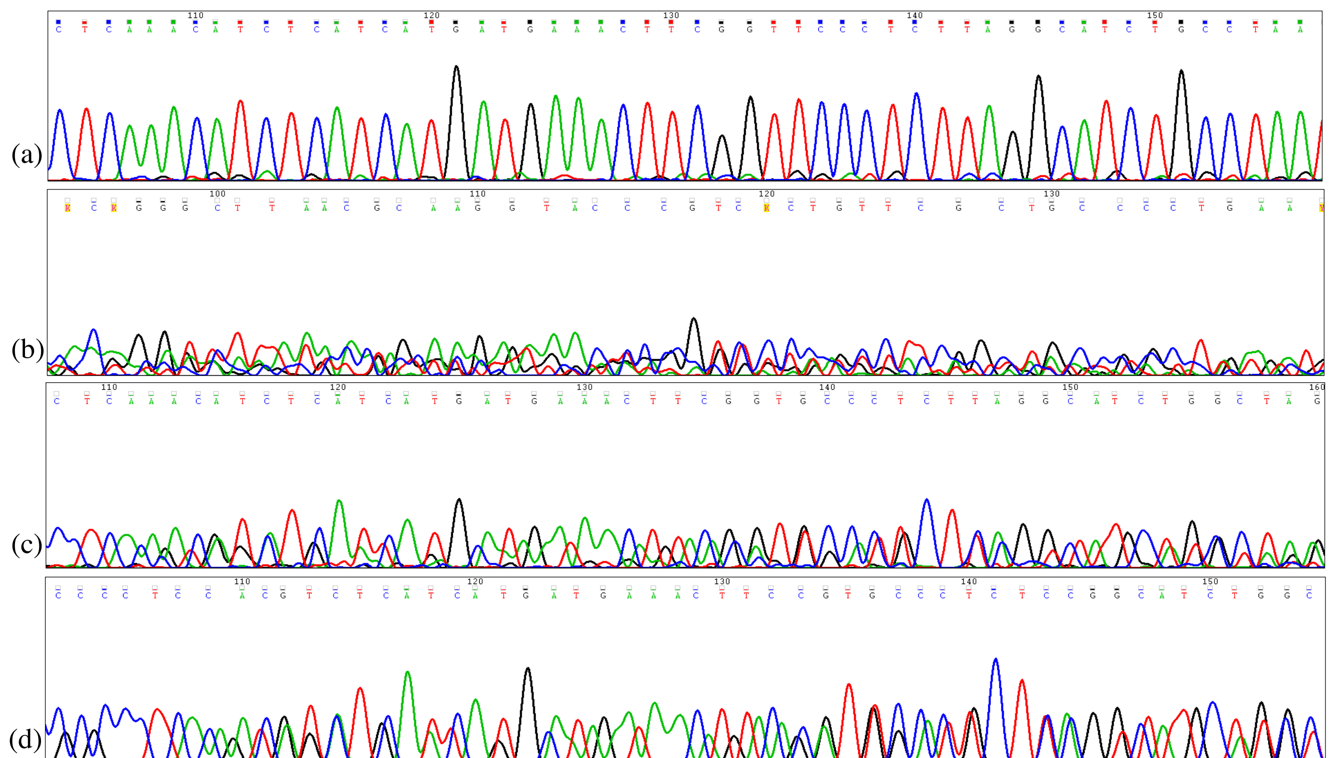

Figure 4.6 The above electropherograms represent portions of the 486 bp target region on the *Cyt B* gene of mtDNA; (a) successful sequencing (sample 3); (b) and (c) poor and incomplete sequencing (6 and 15, respectively); (d) possible additional species or interference where multiple peaks are visible (sample 22).

Based on the results of PCR optimisation, sequencing was performed on four samples for mtDNA, and one sample for nDNA. In addition, the nDNA sample that displayed non-specific amplification was also sequenced (see section 4.4). Sequencing of specific nDNA and mtDNA targets were successful in one sample for each (Figure 4.6 a and Figure 4.7). In the other three samples for mtDNA, poor and incomplete sequencing occurred in two (Figure 4.6 b and c) and interference was observed in the last (sample 16), where two peaks were visible in the same electropherogram (Figure 4.6 d).

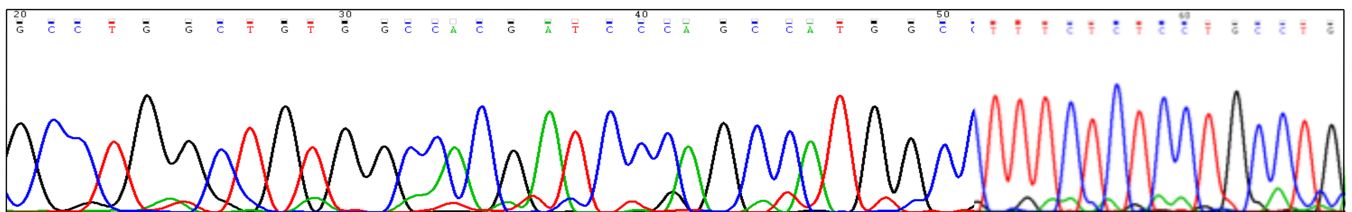

Figure 4.7 The above electropherogram represents the 96 bp target region of the *TLR4* gene within nDNA that was sequenced for sample 3.

4.4 Non-Specific Amplification

To investigate the origin of non-specific amplification, Sanger sequencing was done on the three samples where this occurred (Figure 4.4 and 4.5 – sample 1, 3 and sample 16). ClustalW Alignment (BioEdit 7.2.6 Sequencing Alignment Editor alignment) was chosen, where each sample was aligned to their respective reference sequences, *i.e.* *Cyt B* for mtDNA and *TLR4* for nDNA (Figures 4.8 and 4.9). For mtDNA, sequences generated from samples during the species identification stage of method development were also included to represent those of known pig origin.

Alignment results confirmed that one sample was of pig origin (Figure 4.8), as it displayed consensus with the *Cyt B* reference sequence, and the sequences generated from samples of known pig origin, *i.e.* the control sample and experimental sample from species identification (Chapter 2 Section 2.3). The additional secondary bands observed in samples 1 and 3, may have been as a result of nuclear mitochondrial DNA. Furthermore, deviations from the reference sequence were identified as SNPs, and were the same as those discussed in chapter 2 (Figure 4.8 b and c). The SNPs observed were attributed to genetic variation between subspecies of pig. Three of the SNPs encountered were novel but interpretation of these were

outside the scope of the study (Appendix B.1). Alignment of the final sample with the *TLR4* reference sequence and the nDNA sample of known pig origin displayed no consensus (Figure 4.9).

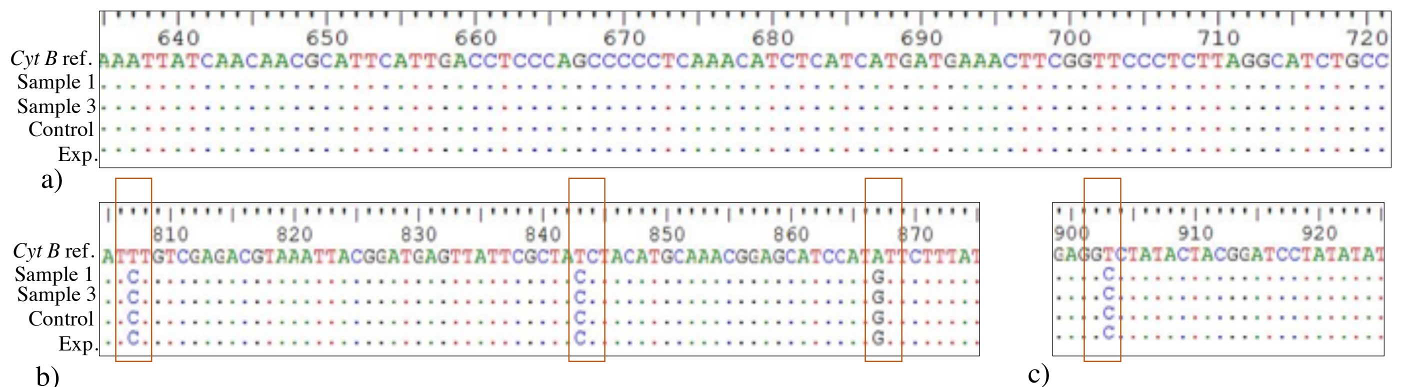

Figure 4.8 The above sequences represent portions of the 486 bp region within the *Cyt B* gene of mtDNA; a dot represents consensus, any deviations from the *Sus scrofa* reference sequence will be denoted as A, G, C, or T; the orange boxes indicate were SNPs were observed; **ref.** = reference sequence; **Control** = control tooth; **Exp**. = experimental tooth. The alignment was done against the whole *Cyt B* sequence therefore, the image represents the conserved portion used in species identification.

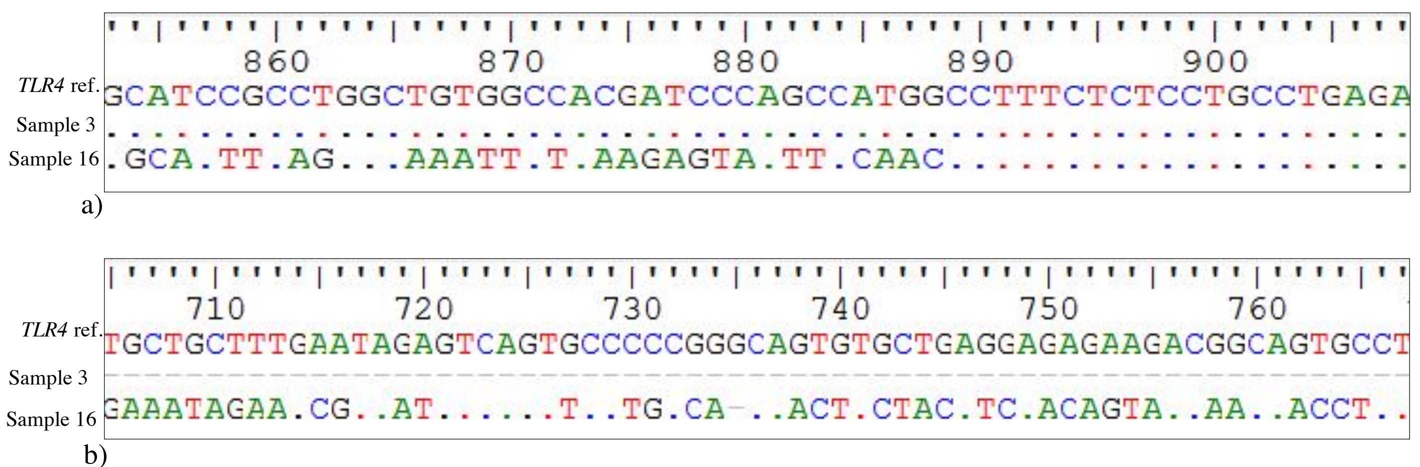

Figure 4.9 The above sequences represent portions of the TLR4 gene aligned with the 96 bp region from sample 3 and the unknown sequence from sample 16; a dot represents consensus, any deviations from the Sus scrofa reference sequence will be denoted as A, G, C, or T; ref. = reference sequence. The alignment was done against the whole *Cyt B* sequence therefore, the image represents the conserved portion used in species identification.

The sequence of unknown origin (query sequence) was analysed in BLASTn (Version 2.7.0) and two programs were selected. The first program was for highly similar sequences (megablast), and the second was for somewhat similar sequences (blastn). The results from performing megablast against pig and human genomes revealed that no genome sequences

were similar. The results of megablast performed against all genomes, with no restrictions, revealed that one species, *Oceanisphaera*, had the highest similarity (Figure 4.10). Other Microbe species that shared similarities in sequence with ~~to~~ the unknown sequence included: *Halodesulfovibrio marinisediminis, Magneto-ovoid bacterium,* and *Magnetococcus marinus* (Appendix C.2).

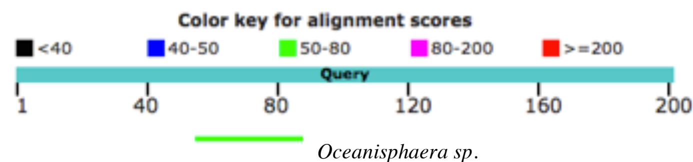

Figure 4.10 The above image represents the graphic summary from BLAST (http://blast.ncbi.nlm.nih.gov, accessed 4th October 2017) showing *Oceanisphaera sp*; Query = sequence under investigation; black indicates an alignment score of lower than 40, blue a score between 40 – 50; green a score between 50 – 80; pink a score between 80 – 200; and red a score greater than 200.

In summation, these results demonstrate that nDNA preservation was poor in teeth removed from pigs submerged in the marine environment of False Bay, Cape Town. Seasonality had an apparent effect on both nDNA and mtDNA survival, where teeth removed during the winter deployment displayed improved DNA recovery over those from the other three seasons. This was not tested statistically due to the limited and uneven sample size, where tooth types, sites of submersion, removal from jaw, and days of submersion were not controlled due to the sampling of teeth by a third party.

Qualitative preservation of mtDNA was better than nDNA, as evidenced by the improved amplification of the 486 bp fragment of *Cyt B*, and in seven samples mtDNA was detected where nDNA was not. Non-specific amplification was encountered in multiple nDNA and mtDNA samples, and in the former instance, was most likely attributable to a microorganism originating from the marine environment. Negative and no template control results indicated that this was not due to operator contamination. Non-specific amplification in the mtDNA sample was not linked to exogenous DNA contamination.

Chapter 5: Discussion and Conclusions

The aim of this study was to optimise a DNA extraction workflow and then assess the DNA quantity and quality from porcine teeth exposed to a marine environment. This was conducted through a process of method development, optimisation, and implementation.

5.1 Method Development and Optimisation

5.1.1 Decontamination of Tooth Surfaces

Incorporation of a decontamination step in the DNA extraction workflow, was necessary to prevent exogenous contaminating DNA originating from human handlers, and or microorganisms entering the samples (Balk, 2015). Bleach was used based on its extensive use in DNA extraction methods on teeth (Gaytmenn & Sweet, 2003; Kemp & Smith, 2005; Rohland & Hofreiter, 2007b; Adler *et al*., 2011; Pinchi *et al*., 2011; Hughes-Stamm *et al*., 2016) and results from method development supported this. However, concerns have been raised regarding the use of bleach, as it has the potential to degrade precious endogenous DNA (Higgins & Austin, 2013). In this study, to assess the potential negative effect of bleach, a sample of control teeth where the surfaces were decontaminated with and without bleach was evaluated. Bleach did not reduce the amount of extractable DNA, contradicting the results of a previous study where Higgins *et al*. (2013) found bleach reduced the presence of distinguishable cementoblasts, and resulted in reduction of DNA yields from fresh human teeth. However, the bleach treatment was more extensive compared to what was implemented in our study, where the outer surfaces were simply wiped with bleach rather than soaked. The effect of bleach on DNA yields from teeth may require more research, however, in this study bleach was deemed an appropriate decontamination agent.

5.1.2 Tooth Sampling

Two methods of tooth sampling were tested on control teeth in the optimisation stage, these being drilling and grinding. Amplification was more consistent in teeth sampled by the drilling method and yields were found to be higher, which is supported by other studies (Smith *et al.*, 1993; Tilotta *et al.*, 2010; Hughes-Stamm *et al.*, 2016). In a forensic context, a further advantage of using the drilling method was that the tooth structure could be maintained, therefore, allowing the possibility of additional analyses (Alakoç & Aka, 2009; Pinchi *et al.*, 2011).

Regardless of the improved DNA yields, it was found that DNA quantities from control pig teeth, sampled by the drilling method, were lower compared to what has been reported in human teeth samples (Tilotta *et al.*, 2010). This could be due to the anatomical and morphological differences between the two species; where pigs have shorter crowns, with much longer and thinner roots compared to humans (Li, 1993). As a result, the size and surface area of the pulp cavity and root canal is smaller, posing difficulties in sampling these elements, which may lead to a reduction in the amount of DNA-containing dentine tissue collected. This suggests that future work should be conducted on human samples to fully investigate sampling teeth via the drilling method.

5.1.3 DNA Extraction

In the optimisation of the DNA extraction method, multiple protocol adjustments were tested on control and experimental teeth. A 10-fold improvement in DNA yields was noted in control teeth where there was a 20-hour overnight lysis step with Proteinase K. These findings are supported by other studies who showed improved DNA yields from degraded aDNA bone and teeth samples (Rohland & Hofreiter, 2007b; Gibbon, 2008). Importantly, the DNA yields from incorporating these changes met the study's criteria of forensically useable. Therefore, the protocol was applied to a sub-sample of experimental teeth for testing.

Application of the method on experimental teeth resulted in DNA yields, which did not meet the standard of forensically useable. In addition, chaotropic salts and organic compound impurities were present in the samples (Loughrey & Matlock, 2016). The presence of

impurities were suspected to be as a result of immersion in the seawater environment. However, sea-salt (NaCl) is known to absorb at higher wavelengths (> 400 nm) (Chai *et al.*, 2008), thus, should not affect this ratio. This would need further investigation to identify the chemical affecting the absorbance at 230 nm.

Despite numerous optimisation efforts, DNA extracted from experimental teeth did not meet the criteria of being forensically useable. These results corroborated with a previous study where teeth exposed to a similar environment also displayed poor DNA recovery (Drake, 2014). Irrespective, these results only represented DNA recovery from six experimental teeth. Therefore, the implementation of the DNA extraction workflow on a larger sample size was required to assess the patterns of molecular degradation associated with exposure to a marine environment, such as seasonality, duration of submersion, and salinity. Furthermore, in the optimisation stage only nDNA was assessed for forensic use. Considering the results of species identification, which demonstrated that mtDNA was sequenceable in two experimental teeth after prolonged submersion, it was necessary to investigate mtDNA recovery in teeth exposed to the open marine environment of False Bay.

5.2 DNA Survival

The developed DNA extraction workflow was implemented on a sample of 28 teeth retrieved from the open marine environment after different lengths of submersion, and from four different seasons. The aim of this was to assess DNA recovery from teeth by using multiple DNA quantity and quality assessments to evaluate the survivability of both nDNA and mtDNA in teeth, with a comparison made between the two molecular targets. This section of the discussion is separated into nDNA, mtDNA, and molecular preservation in the open marine environment of False Bay.

5.2.1 nDNA

In the optimisation stage it was demonstrated that DNA extracted from a small sample of experimental teeth did not meet the study's criteria of being forensically useable. A larger sample of experimental teeth confirmed that forensically useable DNA was unobtainable, where DNA was quantified by qPCR at < 0.025 pg, in all samples. Amplification by qPCR was

possible in 60% (17/28) for the 96 bp fragment, and in 46% (13/28) for the 200 bp fragment. Despite positive amplification results, this indicated that DNA recovery within teeth exposed to the open marine environment of False Bay was poor. Sequencing results also confirmed this where only one sample was sequenceable for the 96 bp fragment of *TLR4*, and none for the 200 bp. Considering that at least 600 bp of intact DNA is required to generate a full traditional STR profile (QIAGEN, 2016), it is unlikely that this would be successful for the times of submersion investigated in this study. However, more sensitive techniques, such as mini-STR's or SNP-based technology by next generation sequencing may have resulted in successes. This was not investigated in this study, as such alternatives were not available for porcine tissue (Caratti *et al*., 2010; Lin *et al*., 2014).

Studies conducted in artificial marine environments have demonstrated superior nDNA recovery. Schwartz *et al*. (1991) demonstrated human teeth placed in jars containing 25°C seawater for one month, high molecular weight DNA was present in 33% of samples. A study by Hughes-Stamm (2012) observed that after six months of submersion in water containing the salt concentration of seawater (3.5% NaCl), full STR profiles were generated in 100% of human teeth samples. These highly contradicting reports on DNA survival compared to what has been reported in this study, re-affirms Drake's (2014) conclusion that nDNA survival is overestimated in studies conducted in artificial marine environments.

The rapid degradation of nDNA encountered in this sample of teeth exposed to the waters of False Bay, validates the results of a study conducted in a cold-water, semi-open marine environment (Drake, 2004). Drake (2004) found that the detection of the *GADPH* gene was greatly reduced in DNA samples from human and pig teeth exposed to a marine harbor environment after just one week. The earliest day of removal from the False Bay environment for this study was 17 days, taking Drakes's results into account, a shorter time of submersion may not have resulted in the extraction of better quantity and quality nDNA from pig teeth. However, Drake (2004) does not provide information on the size of the gene amplification, and, with no published environmental conditions from this study, a useful comparison could not be made. Therefore, further investigation into shorter times of submersion is required for the False Bay marine environment.

The failure to report environmental conditions is also encountered in another study. Alvarez García *et al*. (1996) found DNA extracted from human teeth exposed to a marine environment after a period of six months, that in 16% of their sample a 600 bp fragment was amplifiable. These results for nDNA are in contradiction with this study, but as previously stated, without a description of the environmental parameters, comparative inferences were restricted (Alvarez García *et al*., 1996). In addition, these results were derived from human teeth samples obtained from dental extractions, thus the superior DNA recovery could be explained by the difference in species studied, where human teeth may provide better protection from environmental insult. However, Drake (2014), demonstrated that pig teeth housed in the mandible had improved amplification of the *GADPH* compared to human teeth derived from dental extractions. Therefore, the difference in species does not fully explain the variable results reported with regard to nDNA recovery from studies investigating its recovery from teeth exposed to marine environments. In fact, it is more likely that these disparities could be attributed to the difference in environments investigated. This demonstrates that it is imperative for studies to report the environmental parameters when investigating DNA preservation and recovery from post-mortem samples.

5.2.2 mtDNA

Considering the poor preservation of nDNA in teeth exposed to an open marine environment, it was necessary to investigate other molecular targets of forensic interest. In the optimisation stage, species identification demonstrated that mtDNA was successfully sequenced in two experimental teeth exposed to the marine environment for 17 and 47 days, respectively. This demonstrated that even in lengthy periods of submersion, mtDNA was present and sequenceable. This was not unexpected, given that mtDNA has multiple protective properties, such as higher copy number (Robin & Wong, 1988) and a circular structure, affording it better protection from environmental degradation (Foran, 2006). Importantly, in forensic identification, mtDNA can be used in familial testing (Bär *et al*., 2000; Alvarez-Cubero *et al*., 2012), and recently whole mtDNA sequencing has been used to distinguish between individuals, using next generation sequencing technologies (Templeton *et al*., 2013; Zhang *et al*., 2016; Amer *et al*., 2017). Therefore, mtDNA is viewed as a valuable alternative molecular target, supporting its investigation in this study.

Amplification of a 486 bp region within the *Cyt B* gene was observed in 57% (16/28) of samples, despite its larger fragment size compared to nDNA (96 bp and 200bp). Sequencing of this region was successful in 33% (2/6) of samples. Recovery of mtDNA from teeth exposed to seawater has been reported previously (Corte-Real et al., 2006). Corte-Real *et al*. (2006) demonstrated that in 100% of human teeth samples, mtDNA was amplifiable and sequenceable. The results of Corte-Real *et al*. (2006) indicates greater success in obtaining mtDNA from human teeth, however, the environmental conditions were not disclosed. Therefore, as the case with nDNA, it is possible an overestimation of mtDNA preservation was reported. In another study investigating forensic human teeth samples exposed to realistic immersed environments, mtDNA recovery was poor (Graw *et al*., 2000). Amplification was unsuccessful in all samples investigated, but remains were exposed to the environments for much longer periods, and thus, further investigation into mtDNA preservation in a marine environment is required.

5.2.3 Molecular Preservation in a Marine Environment

In the overall evaluation of nDNA and mtDNA preservation in teeth, it was found that mtDNA demonstrated improved survival. However, both were still susceptible to degradation or PCR inhibition due to exposure to the marine environment of False Bay. Assessing the recovery of these molecular elements qualitatively, certain environmental conditions had similar effects on the recovery of both molecular elements. Therefore, this section is dedicated to discussing reasons behind this.

It was observed that certain environmental conditions pertaining to the unique environment of False Bay, acted to improve recovery of nDNA and mtDNA. This was noteworthy for teeth removed during the winter deployment (Deployment 2), where DNA samples displayed improved DNA quality compared to other seasons. The average seawater temperature between seasonal deployments did not differ greatly, however, differences between maximum seawater temperatures were noted. In winter, the maximum temperature of seawater was 2 - 4.5°C lower compared to other seasons, and there was less fluctuation in seawater temperature. Porcine carcasses also decomposed slower in deployment 2, where time to skeletonisation was 2 - 3 weeks longer compared to other seasons (Speed, personal communication, 2017).

Summer (deployment 4) and autumn (deployment 5) deployments yielded the poorest nDNA and mtDNA results. Fluctuations in seawater temperature were greatest in summer, where there was a 10.14°C difference between the maximum and minimum temperature. This was followed by autumn, where the difference was 8.30°C. High and fluctuating temperatures have previously been associated with poor DNA preservation in post-mortem samples (Perry *et al.*, 1988; Burger *et al.*, 1999). However, this study is the first to suggest that seawater temperature is associated with nDNA survival in an open environment, and was the first to investigate mtDNA preservation under these conditions.

Other than seawater temperature, the effect of salinity was also considered. Salinity did not vary greatly between the four deployments, where the average was between 34.71 and 35.29 practical salinity units (Speed, personal communication, 2017). This was not unexpected, since salinity is self-regulated within marine environments. Therefore, it was challenging to associate fluctuating salinity with molecular preservation in the environment of False Bay. However, the results of a study conducted by Hughes-Stamm (2012) illuminated the possible effects salt could have on DNA preservation. In Hughes-Stamm's (2012) study, human teeth were exposed to an enclosed salt-water environment containing 3.5% sodium chloride, equivalent to natural seawater. It was observed that after six months of immersion, reasonable quality DNA could be extracted from human teeth, thus, suggesting that salt-water in the study conducted by Hughes-Stamm (2012) may have acted to preserve DNA within teeth. Contrastingly, teeth exposed to freshwater displayed poorer preservation after just six weeks (Hughes-Stamm, 2012). The result indicates that sodium chloride does not negatively affect DNA, which is not unexpected considering this compound is used in traditional salting-out DNA extraction procedures (Nasiri *et al.*, 2005). However, the results of our study indicated that sea-salt did not act to preserve DNA, as DNA degraded rapidly evidenced by the poor DNA recovery from teeth samples investigated. Therefore, other factors are most likely contributing to DNA degradation in this environment, such as other chemicals or microorganims occurring naturally within seawater. This reinforces the need to conduct research in realistic marine environments.

Burger *et al.* (1999) demonstrated that large numbers of bacterial colonies increased DNA degradation within teeth exposed to a terrestrial environment. In post-mortem contexts, the decomposition process leads to the release of numerous nutrients, which encourages the growth of multiple microorganisms (Cymerman *et al.*, 2008; Handke *et al.*, 2017). Importantly, many

microorganisms possess natural nucleases, which act to degrade DNA (Antheunisse, 1971). For this reason, microorganisms are seen as one of the prominent drivers of DNA degradation in post-mortem samples (Burger *et al.*, 1999; Rollo *et al.*, 2002). In DNA preservation studies conducted in marine environments, studies have not considered marine microbes driving DNA degradation. Furthermore, studies to date have not reported the presence of marine microbes within the internal environment of teeth exposed to an open marine environment.

Decontamination protocols in the DNA extraction workflow were employed to ensure exogenous DNA from external sources did not enter the samples of interest. However, these decontamination techniques focused on the exterior surface of teeth, not taking into account the possibility of internal contamination, which has been reported in terrestrial post-mortem teeth samples (Gilbert *et al.*, 2005; Brundin *et al.*, 2013). The detection of non-specific amplification in certain experimental DNA samples made it necessary to investigate possible internal sources of DNA from marine organisms. It was found that in one sample, this could be attributed to DNA from marine bacterial species, candidates included: *Halodesulfovibrio marinisediminis* (Shivani *et al.*, 2017), *Oceanisphaera* (Romanenko *et al.*, 2003; Xu *et al.*, 2014), *Magneto-ovoid bacterium* (Ji *et al.*, 2017), and *Magnetococcus marinus* (Bazylinski *et al.*, 2013).

Certain marine bacteria have been reported to possess high DNAase activity (Maeda & Taga, 1976), which poses a distinct possibility that the presence of these species may have resulted in increased DNA degradation. The existence of marine bacterial DNA within teeth raises the question of the mode of bacterial entry into the tooth's internal environment. Despite the protective properties teeth afford DNA, they are particularly porous in their roots, potentially offering a route into the interior of the tooth (Gilbert *et al.*, 2005). Furthermore, the porcine teeth used in this study were mostly deciduous, and due to their lower mineral content and open nature of their root apexes (Wilson & Beynon, 1989), they could be more susceptible to contamination from external sources.

Even more concerning is that experimental teeth were removed *in-situ*, and it has been reported that the bony casing provides additional protection against the marine environment (Drake, 2014). This raises the question of how impermeable teeth truly are, which would require further investigation. Finally, the detection of bacteria within samples also poses an additional

challenge with regard to decontamination of teeth, where it would be difficult to rid the inner environment from contaminating sources without potentially further degrading endogenous DNA (Gilbert *et al.*, 2005).

In addition to the observations of this study, increased DNA hydrolysis has been suggested as a factor for rapid DNA degradation in marine environments (Alvarez García *et al.*, 1996). Hydrolysis can affect both nDNA and mtDNA, where the glycosidic-base sugar undergoes hydrolytic cleavage, resulting in base losses and a loss of genetic information (Lindahl, 1993; Alaeddini *et al.*, 2010). The effects of hydrolysis were not explicitly explored in this study. However, considering the immersed state of the experimental teeth, it is likely that DNA was subject to this.

5.3 Study Limitations and Recommendations

In this pilot study investigating DNA extraction from teeth exposed to an open marine environment, there were several limitations that required addressing. These will be presented separately, focusing first on the method development and optimisation stage, and then on the investigation into DNA preservation within teeth in a marine environment.

5.3.1 Method Development and Optimisation

In optimisation, commercial DNA kits were tested, and any protocol adjustments were incorporated as part of the manufacturer's instructions. The number of protocol adjustments that could be tested was restricted since there were finite resources for this study on which multiple variables could be tested. In future, fewer variables should be tested on a large sample to confer significance, which can be achieved by testing one variable in duplicate from the same and different individuals, and by conducting experiments in batches.

5.3.2 DNA Preservation in a Marine Environment

This study was the first to investigate DNA preservation within teeth exposed to the open marine environment of False Bay, Cape Town. Without collaboration with a marine decomposition study, this research would not have been. Despite the advantages, there were

multiple restrictions that were challenging to overcome related to data collection, the use of pig teeth, environmental data available, and the extraction of teeth at regular intervals to assess effects of seasonality.

Data collection was performed by a third party that was not well-versed in the anatomy of pig teeth. It is recommended for future studies that the investigator attends and aids in collecting the samples directly. Teeth were also difficult to extract in early stages of decomposition when the periondontal ligament was still intact. Consultation with a dentist could be explored to improve methods of extracting teeth *in-situ*. Time of submersion was also a limitation, as the earliest time teeth could be extracted was 17 days post-deployment. Therefore, in future studies it will be necessary to investigate earlier times of submersion, *i.e,* less than a week. This would be valuable to investigate at what point forensically useable DNA is unobtainable from teeth exposed to a marine environment. Environmental data, although highly informative, did not provide a full picture since pH could not be recorded by the instruments used. Therefore, routine pH measurement could be done in future studies to assess this measure across the different seasons.

Loss of tooth samples due to predators and teeth falling out of the tooth sockets of pig mandibles and maxillae was a problem. To negate this in future studies, extra protective measures could be employed to the cranial area, and covering it with a mesh-like material to "catch" lose teeth, could prevent this from occurring. Furthermore, the use of pig teeth was a limitation. Even-though pigs are regarded as suitable models, and were acceptable for use in this pilot study, it was limiting in that a forensic DNA profile could not be generated. Forensic kits are designed to be human specific and while there are pig-specific kits, they are fewer and require higher quantities of DNA (Caratti *et al*., 2010; Lin *et al*., 2014), which may not have been suitable for degraded samples. Therefore, our analysis was prohibited from completing this final step in the forensic DNA workflow due to resource restrictions. To overcome this human samples should be used in future studies.

In the future, ideally a larger sample of uniform human teeth should undergo a similar investigation in the open marine environment of False Bay, Cape Town. Particularly, human molar teeth still intact within the mandible will be valuable, since previous studies have found that in a marine environment the bone provides protection (Drake, 2014). This would allow for

a more accurate representation of DNA recovery from teeth exposed to an open marine environment and the ability to test variables statistically. The move to human teeth would also be an advantage as there are many commercial assays available to assess DNA quantity and quality, which are also more sensitive to low copy number and fragmented DNA. In addition, more sensitive methods should be investigated, such as next generation sequencing.

5.4 Conclusions

In this pilot study, DNA survival in the open marine environment of False Bay, Cape Town was investigated. Methods were developed and optimised to extract forensically useable DNA from porcine teeth. The developed methods for decontamination, tooth sampling, and the optimised DNA extraction protocol were successfully performed on control pig teeth, with forensically useable DNA obtained. To assess DNA preservation in teeth exposed to an open marine environment, these methods were implemented on a larger sample of experimental teeth.

Forensically useable nDNA could not be extracted from teeth submerged in the False Bay marine environment. However, the earliest stage of submersion was 17 days, and therefore, it may be possible to extract nDNA from shorter submersion times, which could not be assessed in this study. The relatively poor preservation of nDNA within the experimental teeth motivates for further research on other sample types. However, environmental parameters such as colder and stable seawater temperatures appeared to act positively to preserve molecular elements, where improved preservation of nDNA and mtDNA, even after 60 days of submersion, was observed. Therefore, the availability of environmental data was valuable in understanding the recovery of DNA from teeth exposed to a marine environment. Further statistical analysis on a larger sample size would be required to fully investigate this relationship.

Poor molecular preservation may have also been due to DNAase activity of marine microbes, with this study being the first of its kind to comment on this. Terrestrial studies have demonstrated that DNA degradation increases in the presence of high microbial activity, thus, supporting this finding. DNA hydrolysis has previously been theorised to increase DNA degradation in the marine environment. However, this would need further investigation in future studies, as it was not assessed.

The positive results obtained from mtDNA in this study, motivates for further investigation into the possible routine use of mtDNA for forensic human identification in SAPS FSL. In addition, mtDNA recovery and preservation within teeth exposed to an open marine environment of False Bay has not been previously evaluated, thus, this study provided preliminary results supporting the use of mtDNA in identifying human remains that have washed-up.

The study emphasises that recovering DNA from teeth submerged in an open marine environment is complex and requires further investigation on human samples. It also re-affirms that DNA preservation is overestimated in artificial marine environments. Current techniques are not sufficient in retrieving DNA from samples exposed to seawater environments. Therefore, the importance of conducting research in realistic settings to improve the identification process for individuals who have died at sea, is demonstrated. With constant technological advancements in the field of genetics, such as mini-STRs and SNP-based technologies, continuous improvements in success rates for highly degraded samples are being made.

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Appendix A: Sample

A.1 Ethics Approval

☆ **Nosi Tsama**
To: Chandra Thurgood
RE: Animal ethics application

17 January 2017 at 3:27 PM
Hide Details
Inbox - Windows Live

Dear Chandra

Thank you for submitting your application. This application does not require ethics clearance. A letter to that effect will be sent shortly.

Kind regards
Nosi

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Animal Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 404 7682 • **Facsimile** [021] 406 6411
Email: nosi.tsama@uct.ac.za
Website: www.health.uct.ac.za/research/animalethics/forms

31 January 2017

Mr C Longden-Thurgood
Forensic Medicine & Toxicology
Pathology Department
Falmouth Building

Dear Mr Longden-Thurgood

Ethics Approval for the project entitled: *Optimisation of DNA Extraction from Teeth Submerged in Sea Water in False Bay, South Africa*

It is noted that the above project will only extract DNA from teeth, and to test the DNA extraction process from a fresh head of a pig carcass. This will be sourced this from a butchery and therefore the animal would not be killed for the purposes of the project. Since no animals will be used in the above project, no ethics approval is required. Thus, all institutional ethics requirements have been satisfied.

Yours sincerely

PROFESSOR PATRICK COMMERFORD
CHAIRPERSON, FHS ANIMAL ETHICS COMMITTEE

A.2 Isolation of Mandibular Bodies

Fresh porcine heads were donated from Roelcol Company (Malmesbury). The heads were packaged and transported to the Department of Human Biology, University of Cape Town. Upon arrival, the soft tissue of the crania was removed and a Stryker autopsy saw model M810 (Stryker, Portage, Michigan, USA) was used to cut the mandibles and maxillae from each of the crania. In order to isolate the mandibular bodies, the mandibular rami were removed from the mandibles and the anterior portion of both the mandibles and maxillae were cut using the saw. Leftover tissue was cleaned from the bone and teeth, using sterilised scalpels, scissors and a sterilised toothbrush. The mandibles and maxillae were then placed in a fume hood to dry for four hours, or until there was no evidence of the bone being wet (Loreille et al., 2007). Subsequently these were packaged individually and stored at -20°C, until they were required for the optimisation stages. The periodontal ligament made it impossible to remove the teeth from the boney matrix of the mandibles and maxillae without damaging them. Therefore, the teeth were left intact in the bodies of the mandibles and maxillae and were sampled in this way.

A.3 Marine Decomposition Set-Up

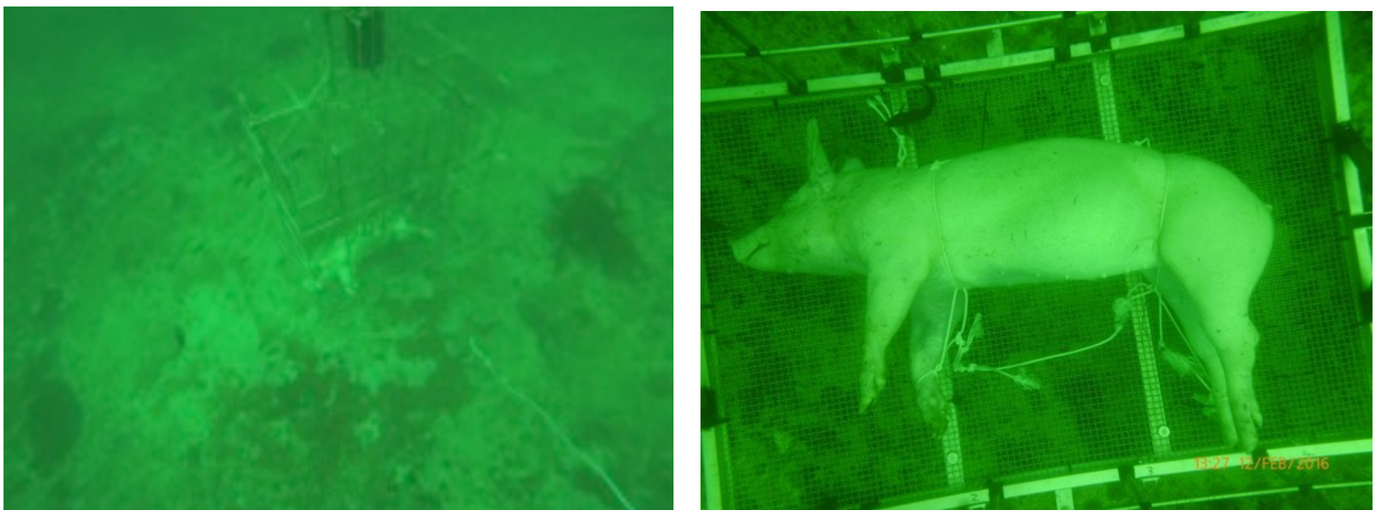

Figure A.1 Example of the experimental cage set-up at one underwater site in False Bay, Cape Town. The pigs were secured in the cages with 5 mm paracord and cameras were attached to each cage; a) overview; b) close-up of the experimental marine decomposition set-up. Images courtesy of Belinda Speed.

A.4 Experimental Sample

Table A.1 Experimental sample information

Dep.	Sample	Tooth root (open/closed)	Pulp present (yes/no)	Open	Yes
2	1	Open	Yes	2/4	4/4
	2	Closed	Yes		
	3	Closed	Yes		
	4	Open	Yes		
3	5	Open	Yes	5/5	3/5
	6	Open	Yes		
	7	Open	No		
	8	Open	Yes		
	9	Open	No		
4	10	Open	No	9/10	2/10
	11	Open	No		
	12	Open	Yes		
	13	Open	Yes		
	14	Open	No		
	15	Open	No		
	16	Open	No		
	17	Open	No		
	18	Open	No		
	19	Closed	No		
5	20	Open	Yes	3/9	3/9
	21	Closed	No		
	22	Open	No		
	23	Closed	Yes		
	24	Closed	No		
	25	Closed	No		
	26	Closed	No		
	27	Closed	No		
	28	Open	Yes		

Out of the 28 experimental teeth, 19 had root apexes that were distinctly open and 12 had distinguishable pulp.

Appendix B: Additional results from the optimisation stage

B.1 Species Identification – Confirming *Sus scrofa species*

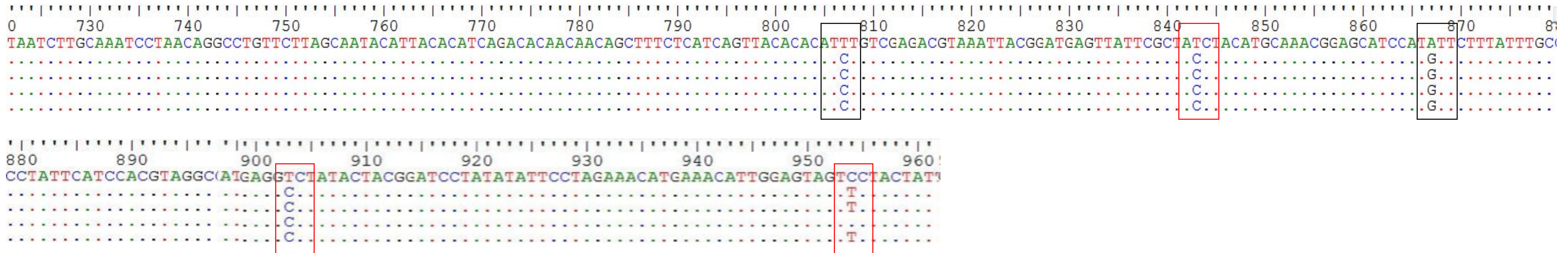

Figure B.1 Alignment of the sequence generated from mtDNA samples showing the position of the SNPs; black = reported SNPs, 807 T>C (rs55619209) and 867 A>G (rs81317944); red = unreported SNPs: 843 T>C, 903 T>C, and 954 C>T. The alignment was done against the whole Cyt B sequence therefore, the image represents the conserved portion used in species identification.

The above SNPs outlined were reported in subsequent sequencing on the larger experimental sample when exploring the origin of non-specific amplification. The fact they were encountered in multiple samples coming from different individual pigs and two being reported, these deviations from the reference sequence were attributed to normal variation within the local South African pig population.

B.2 Grinding Method

For the sampling of teeth by the grinding method, Grinding Jars (QIAGEN, Germany) were used to generate a powdered sample of the whole tooth. The control tooth was removed from the mandibular bone using a Dremmel tool (Dremmel: Racine, WI, USA) with a 22 mm x 0.6 mm circular attachment (pg® mini). This was not necessary for experimental teeth, which had been removed from the boney matrix during data collection visits.

It was necessary to remove as much of the enamel as possible to reduce the mineral content in the powdered sample, which may inhibit downstream processes. To prepare the tooth for grinding, the Dremmel tool with a 22 mm x 0.6 mm rounded attachment (pg® mini) was used. The teeth were cut into three or four individual pieces (depending on size of tooth) and placed into sterilised stainless-steel grinding jars (QIAGEN), which had been stored at -20°C for a minimum of 24 hours prior to use. The grinding jars were then attached to the TissueLyser 85220 (QIAGEN, Retsch) instrument and shaken at a frequency of 30 oscillations per second for 30 seconds. A sterilised metallic spatula was used to scoop the entire powdered sample into a sterile 1.5 ml microcentrifuge tube. Samples were weighed (within the tubes) on an electronic scale (RADWAG, model AS 220-R2) and stored at -20°C, if DNA extraction was not performed immediately.

B.3 Optimisation Raw Data

Table B.1 DNA quantity and quality results for all DNA samples generated in the method development and optimisation stage

	Nanodrop™ Spectrophotometry				**Protein contamination**				**Chaotropic Salt contamination**				**Qubit® Fluorometry**		
Sample	**(ng/µl)**			**Mean (ng/µl)**	**A260/280**			**Mean ratio**	**A260/230**			**Mean ratio**	**(ng/µl)**		**Mean (ng/µl)**
C1	1.30	0.80	0.80	0.97	1.21	-	1.34	1.28	0.04	0.08	0.08	0.07	Not done	Not done	Not done
C2	4.00	4.40	4.40	4.27	1.82	2.37	1.67	1.95	0.54	0.59	0.57	0.57	3.36	3.34	3.35
C3	7.30	6.60	7.10	7.00	1.91	2.11	1.98	2.00	0.32	0.30	0.32	0.31	3.16	3.08	3.12
C4	2.00	1.90	0.30	1.40	8.74	2.65	3.07	4.82	2.27	1.43	1.50	1.73	Not done	Not done	Not done
C5	3.60	3.60	3.90	3.70	2.25	1.87	1.77	1.96	0.31	0.29	0.31	0.30	2.36	2.30	2.33
C6	6.40	6.10	6.60	6.37	2.18	1.97	1.79	1.98	0.26	0.25	0.26	0.26	4.06	4.04	4.05
C7	0.10	0.30	0.00	0.13	-0.54	-0.15	-	-0.35	0.01	0.03	-	0.02	Not done	Not done	Not done
C8	20.10	20.70	20.50	20.43	1.95	1.98	1.97	1.97	0.43	0.44	0.43	0.43	12.70	12.40	12.55
C9	9.40	9.80	9.90	9.70	2.15	2.09	1.92	2.05	0.20	0.21	0.21	0.21	4.94	4.94	4.94
C10	144.80	177.40	131.50	151.23	1.94	1.83	1.92	1.90	2.00	1.37	1.92	1.76	9.40	9.18	9.29
C11	211.50	223.30	213.30	216.03	1.92	1.92	1.92	1.92	2.05	2.05	2.06	2.05	12.30	11.90	12.10
C12A	6.90	8.00	8.20	7.70	2.03	1.82	1.75	1.87	1.64	1.27	1.48	1.46	Not done	Not done	Not done
C12B	6.10	6.50	5.20	5.93	1.82	1.79	1.85	1.82	1.17	1.2	1.78	1.32	Not done	Not done	Not done
C13	(-)	(-)	(-)	49.63	(-)	(-)	(-)	1.89	(-)	(-)	(-)	1.72	68.40	65.20	66.80
E1	1.60	1.90	1.60	1.70	4.70	1.71	2.87	3.09	1.41	0.83	0.46	0.90	0.262	0.218	0.240
E2A	3.70	4.90	-	4.30	1.53	1.38	-	1.46	0.69	0.60	-	0.65	Not done	Not done	Not done
E2B	1.90	2.80	3.10	2.60	1.53	1.53	1.54	1.53	0.47	0.45	0.52	0.48	Not done	Not done	Not done
E2C	2.10	1.90	1.50	1.83	1.58	1.21	1.91	1.57	1.01	0.75	0.62	0.79	Not done	Not done -	Not done
E2D	3.20	4.40	4.50	4.03	2.83	2.04	2.01	2.29	1.22	1.18	1.09	1.16	0.19	-	0.19
E2E	4.70	4.40	5.00	4.70	2.11	1.63	1.89	1.88	1.25	1.22	1.23	1.23	Not done	Not done	Not done
E2F	(*)	(*)	(*)	3.46	(*)	(*)	(*)	2.05	(*)	(*)	(*)	0.31	Not done	Not done	Not done
E3	(*)	(*)	(*)	7.03	(*)	(*)	(*)	1.69	(*)	(*)	(*)	0.45	0.37	1.85	1.11
E4	4.80	4.50	4.20	4.50	2.07	1.84	2.03	1.98	0.22	0.21	0.20	0.21	0.592	0.514	0.553
E5A	4.50	2.20	1.90	2.87	1.35	1.66	1.84	1.62	0.56	0.52	0.54	0.54	Not done	Not done	Not done
E5B	3.20	2.10	2.40	2.57	1.51	1.47	1.47	1.48	0.49	0.71	0.65	0.62	Not done	Not done	Not done
E5C	5.60	4.20	4.00	4.60	1.75	2.43	2.62	2.27	1.05	1.83	1.11	1.33	0.194	0.178	1.86
E5D	5.40	7.40	6.00	6.27	2.97	2.30	3.59	2.95	2.32	1.31	2.20	1.94	0.142	0.11	0.13
E5E	(*)	(*)	(*)	3.32	(*)	(*)	(*)	2.00	(*)	(*)	(*)	0.31	Not done	Not done	Not done
E6	(*)	(*)	(*)	5.63	(*)	(*)	(*)	1.71	(*)	(*)	(*)	0.62	0.62	0.618	0.62

C = Control tooth; **E** = experimental tooth; number relates to individual teeth; letters represent different protocol adjustments made on the same tooth; DNA concentration was measured by Nanodrop™ 2000 Spectrophotometer (ThermoFisher Scientiffic, USA) for 11 samples DNA concentration was measured by Qubit™ flourometry (ThermoFisher Scientific, USA); - = DNA yield was too low to be measured; (-) = not recorded; (*) as multiple elutions were performed and an average of this was calculated.

Appendix C: Results

C.1 Results Gels

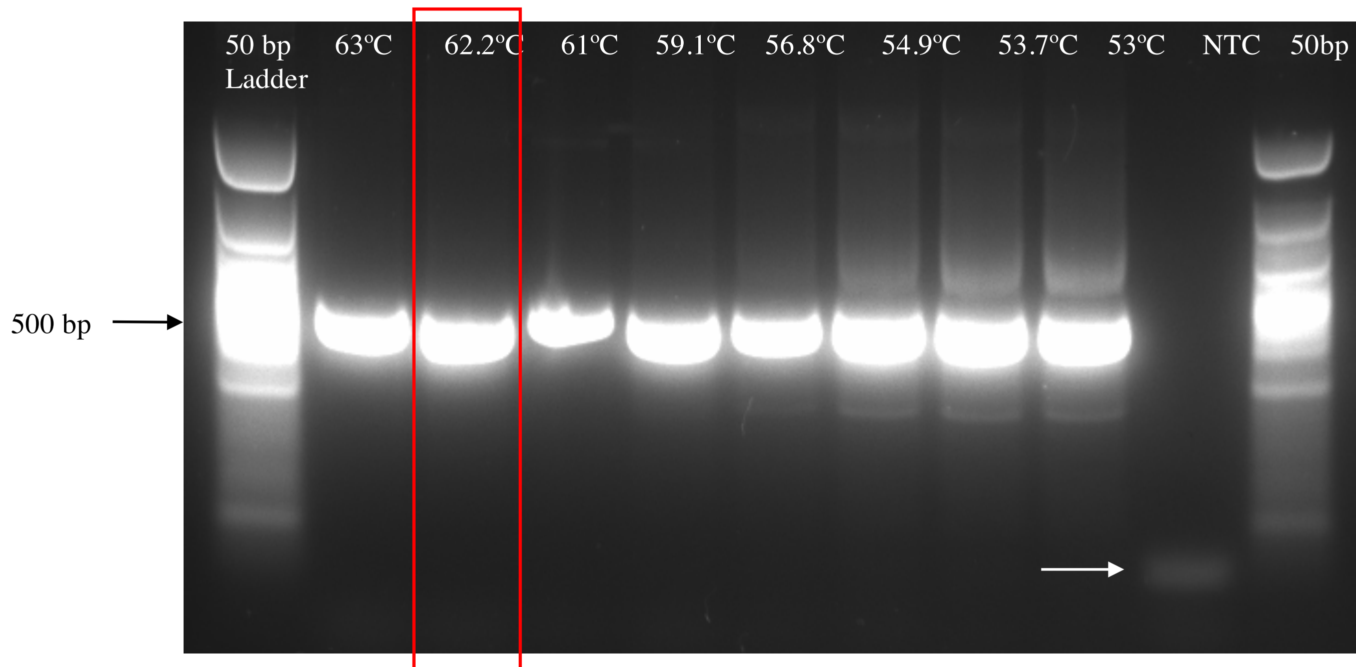

Figure C.1 Agarose gel electrophoresis (2%) of annealing temperature gradient for the *Cyt B* primers; electrophoresis was performed at 100 V for 1 hour. The far-left and far-right lane is the 50 bp DNA ladder; a no template control (NTC) was run alongside samples; white arrow indicated primer dimers; red rectangular box indicated the optimal primer annealing temperature.

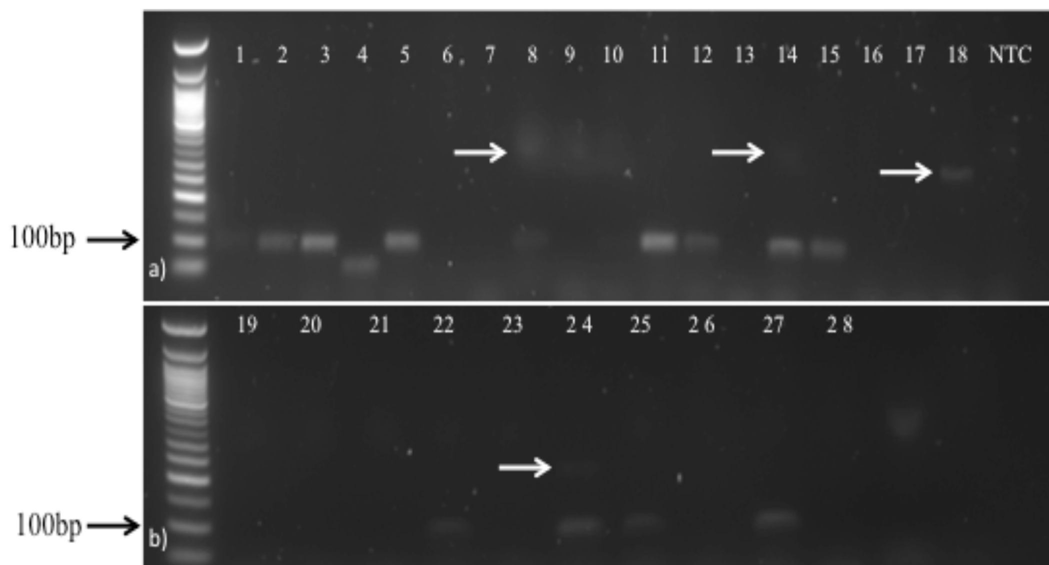

Figure C.2 a), b) Agarose gel electrophoresis (2%) of qPCR amplified products using *Sus scrofa* (pig) specific primer set (*TLR4,* 96 bp); electrophoresis was performed at 100 V for 1 hour. The far-left lane is the 50 bp DNA ladder; numbers 1 – 28 represent the 28 DNA samples from experimental teeth; a no template control (NTC) was run alongside samples; non-specific amplification was observed in samples 8, 9, 10, 14, 18, and 24, as indicated by the white arrows; band at around 50 bp for sample 4 are primer dimers.

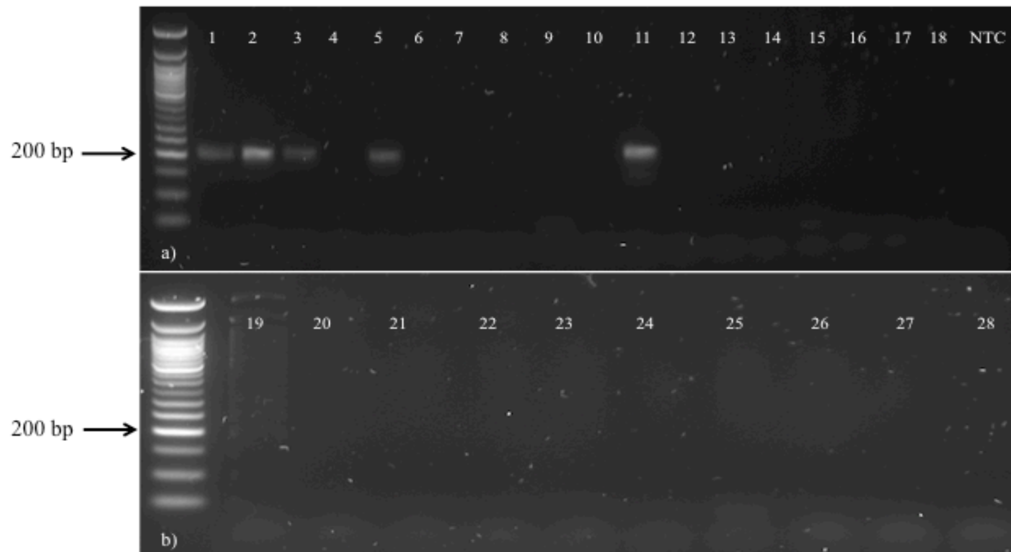

Figure C.3 a), b) Agarose gel electrophoresis (2%) of qPCR amplified products using *Sus scrofa* (pig) specific primer set (*TLR4,* 200 bp); electrophoresis was performed at 100 V for 1 hour. The far-left lane is the 50 bp DNA ladder; numbers 1 – 28 represent the 28 DNA samples from experimental teeth; a no template control (NTC) was run alongside samples.

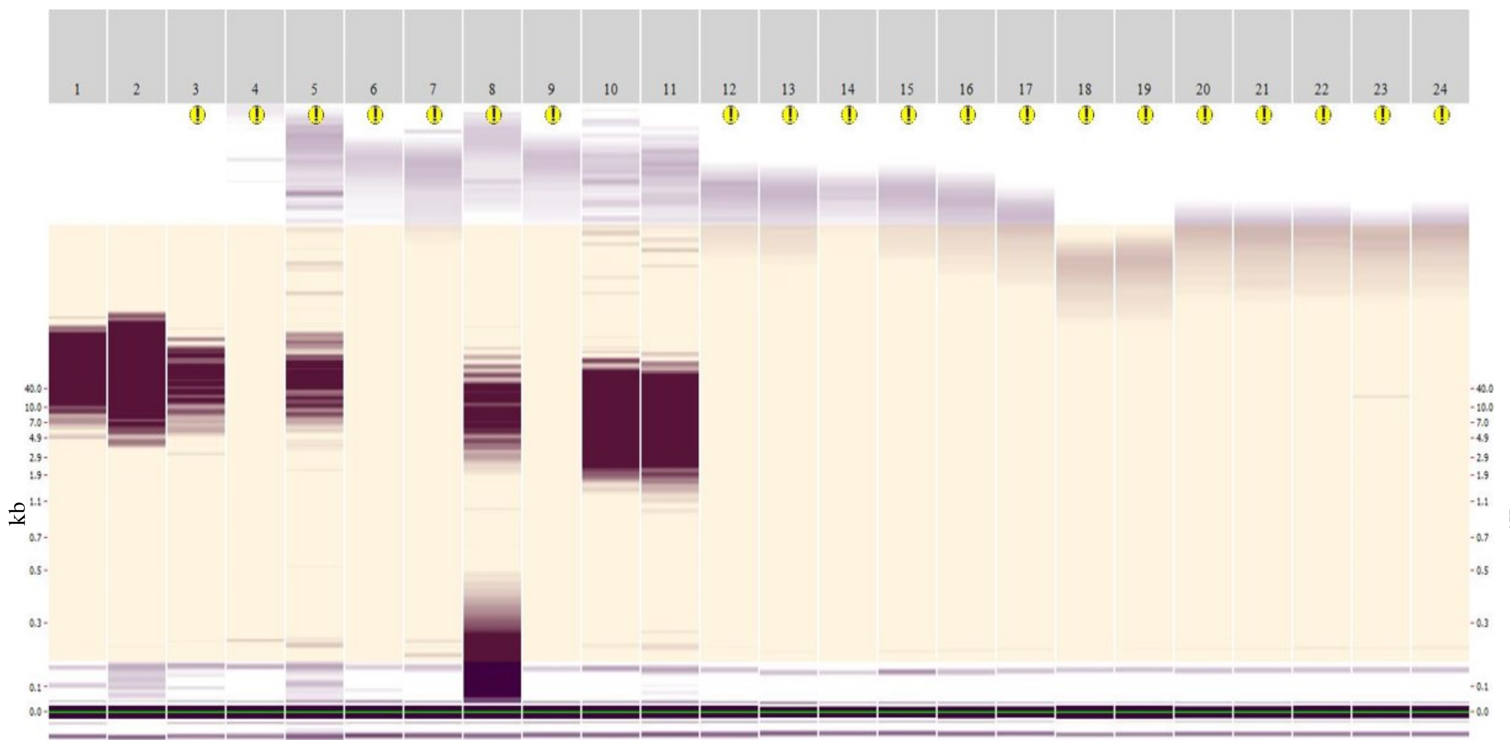

Figure C.4 Maximum contast image from analysis on LabChip® GX Reviewer, Version 5.3.2115.0 (Perkin Elmer Inc., 2016); yellow circled exclamation mark indicates a failed gDNA quality score (GQS) and where DNA concentration was below 0.2 ng/µl.

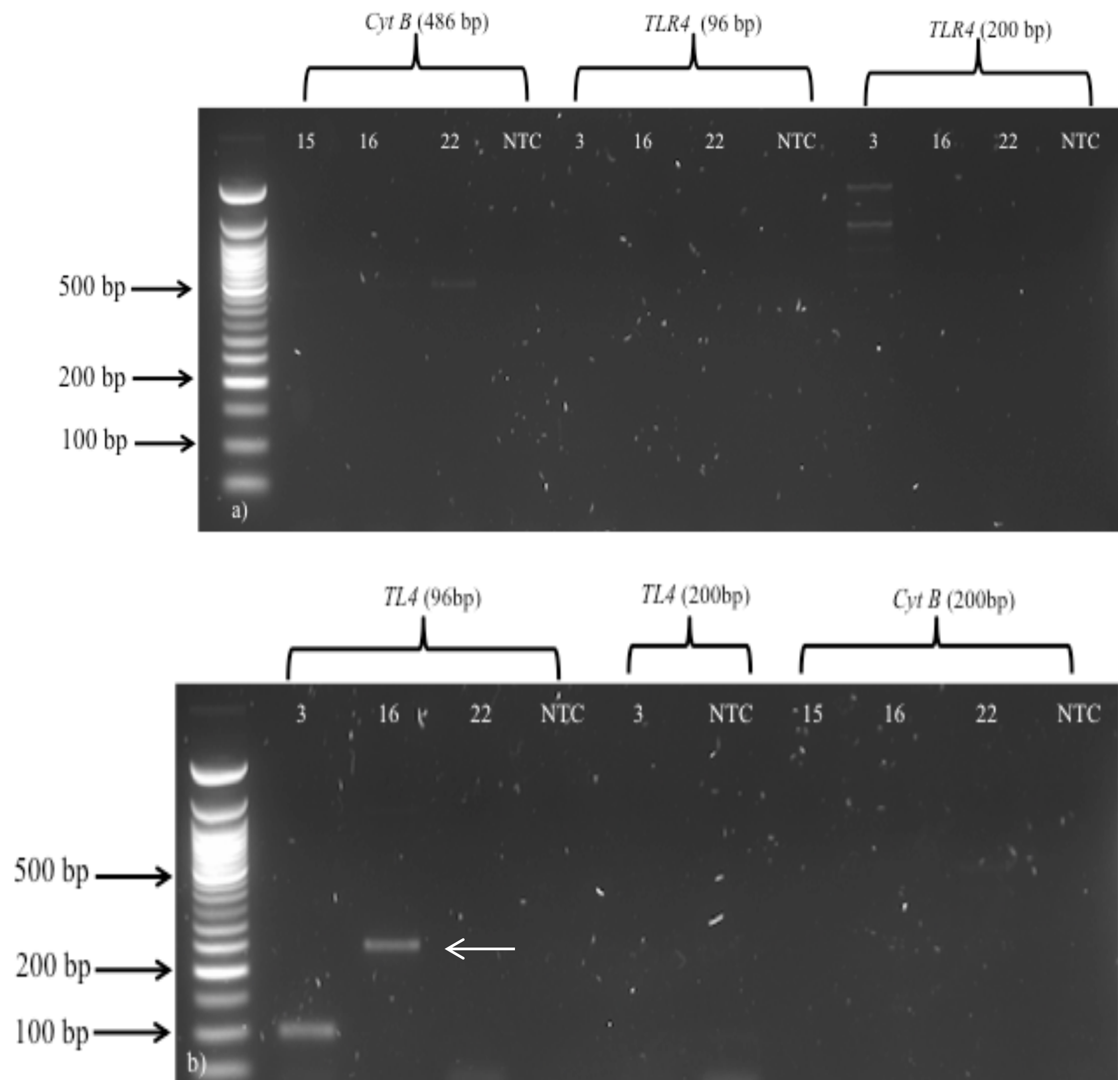

Figure C.5 (a), (**b)** Agarose gel electrophoresis (2%) of PCR products using pig specific primers (*TLR4,* 96 bp and 200 bp) and primers targeting *Cyt B* gene within pig genome (486 bp); electrophoresis was performed at 100 V for 1 hour. The far-left lane is the 50 bp DNA ladder; numbers 3, 15, 16 and 22 represent DNA samples from experimental teeth; a no template control (NTC) was run alongside the samples; (**a)** increased volume of DNA sample was added to the PCR reaction; (**b)** increasing cycle number to 40 in the PCR reaction; non-specific band was observed indicated by the white arrow for sample 16.

C.2 Results of Sequencing

Table C.1 Summaries from BLAST of the query sequence (http://blast.ncbi.nlm.nih.gov, accessed 4th October 2017).

Species	**Alignment score**	**Query cover (%)**	**Expected value**	**Identical value (%)**
Halodesulfovibrio marinisediminis	113	80	4×10^{-22}	75
Oceanisphaera sp.	56.5	16	3×10^{-4}	97
Magneto-ovoid bacterium	86	81	3×10^{-13}	80
Magnetococcus marinus	64.4	67	1×10^{-6}	72

An alignment score of more than 80, query score closer to 100%, expected value closer to 0, and identical value closer to 100 indicates high similarity of the query sequence against the known species.

Appendix D: Recipes

D.1 2% Agarose:

2 g SeaKem® LE Agarose powder (Lonza, Switzerland)

100 ml Tris-borate (TBE) ready mix (Sigma-Aldrich, USA)

Heat and mix until powder is dissolved

Aliquot the appropriate visualisation stain into the liquid agarose

D.2 Buffer AW1 and AW2 (QIAamp® DNA Investigator kit (QIAGEN, Germany)

AW1: Add 25 ml 99.9% absolute ethanol (Kimix, South Africa) to the bottle containing buffer AW1 concentrate.

AW2: Add 30 ml 99.9% absolute ethanol (Kimix, South Africa) to the bottle containing 13 ml Buffer AW2 concentrate.

D.3 0.5 M ethylenediaminetetraacetic acid (EDTA) 250 ml

46.53 g EDTA Disodium Salt Dihydrate ($C_{16}H_{14}N_2Na_2O_8.2H_2O$) (Merck, South Africa)

250 ml Distilled water

Add Sodium Hydroxide (NaOH) pellets (Merck, Germany) until pH reaches 8.0