Evaluation of forensic DNA profiling success on teeth that have been submerged in the ocean

by

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Song: "Ke na le modisa"

Table of contents

List of Figures

List of Tables

List of Equations

List of Abbreviations

%	percentage
<	less than
>	greater than
°C	degrees Celsius
µ	microlitre
bp	base pair
CE	capillary electrophoresis
DI	degradation index
DNA	deoxyribonucleic acid
DO	dissolved oxygen
g	gram
HREC	Human Research Ethics Committee
LCN	low copy number
ml	millilitre
ng	nanogram
ng/µL	nanogram per microlitre
NGS	next generation sequencing
OFPI	Observatory Forensic Pathology Institute
PCR	polymerase chain reaction
pg	picogram
pH	potential of hydrogen
qPCR	quantitative real-time PCR
RFU	relative fluorescence units
RMP	random match probability
STR	short tandem repeats
UCT	University of Cape Town
µg/ml	microgram per millilitres
PMSI	Post-mortem submersion interval

Abstract

Deceased humans that are recovered from marine environments contribute to the burden of unidentified bodies, both globally and in South Africa. Compromised conditions of these bodies make identification difficult. In these instances, DNA analysis becomes an invaluable tool for identification. Nonetheless, there is a notable gap in existing literature regarding the use of DNA from teeth for identifying human remains recovered from marine environments.

This study therefore aimed to evaluate forensic DNA profiling success on human teeth samples (n = 90) that were submerged in two different marine locations along Cape Town's coastline. Thirty adult volunteers each donated three wisdom teeth, where each tooth per individual was subjected to a different condition for 20 days: one was submerged in False Bay, one was submerged in Table Bay and the remaining tooth was kept as an unsubmerged, matched control in the laboratory. DNA was extracted and quantified using quantitative polymerase chain reaction before undergoing DNA profiling.

No significant difference was observed in the likelihood ratio between DNA recovered from samples submerged in Table Bay (median: 1.031×10^{13}; range: 4.221 to 5.633×10^{31}) compared to False Bay (median: 3.501×10^{15} range: 1.557×10^{3} to 2.578×10^{35}). As expected, matched control samples yielded significantly higher DNA concentrations than submerged samples and showed significantly higher Likelihood ratios (median: 3.094×10^{28}; range: 11.11 to 1.614×10^{33}) than submerged samples ($p < 0.001$). Three DNA profiles from False Bay samples showed no allele detection and therefore were uninformative. Further, allele drop-ins were observed in three DNA profiles from False Bay samples but none in Table Bay samples. These were hypothesised to be due to the presence of marine microbial DNA and could potentially confound DNA interpretations. Overall, the results suggested that the protocol used was suitable for DNA profiling of teeth samples recovered from Table Bay and further research is required to obtain insight into aquatic conditions affecting samples submerged in False Bay.

Chapter 1: Introduction

1.1 Background

Determining the identity of deceased individuals is a crucial component of medico-legal investigations and is important for many social, legal, and cultural reasons (Krysztofiak,2016; Suwalowska et al., 2023). Medico-legal death investigations in South Africa are regulated by the Inquest Act, No. 58 of 1959 to determine the identity of the deceased, the cause of death and the circumstances surrounding death.

Some of the most challenging bodies to identify in Cape Town forensic mortuaries are those that are recovered from the ocean or have been washed ashore by wave action (Finaughty et al., 2019). Washed up cases may be due to either natural or unnatural causes, but this depends on the circumstances surrounding death, which will be discussed in the literature review that follows. Along South African coastlines, particularly False Bay and Table Bay where washed-up cases are mostly recovered, identification is challenging due to bodies often being in advanced stages of decomposition (Reid, Martin & Heathfield, 2020). While visual recognition, fingerprinting, forensic odontology and forensic anthropology may be helpful in some cases to establish identity, forensic DNA profiling is sometimes the only method available due to the incomplete and decomposed state of the body (Reid, Martin & Heathfield, 2023).

Hard tissues such as bone and teeth are often the sample type of choice in cases of severe decomposition due the slower rate they decompose, their availability and their ability to preserve DNA when exposed to harsh environmental conditions (Thomas & Weedn, 1997; Higgins & Austin, 2013). Although there have been vast improvements made in human identification methods that use hard tissues as a source of DNA, there is paucity of literature pertaining to successful identification of bodies washed up from the sea using teeth as a source of DNA. Additionally, most research to date has been done on cases from the Mediterranean Sea, which has a considerably warmer temperature (16.6°C–22.1°C) compared to Table Bay (15.2°C–18.95°C) and False Bay (14.4°C–19.2°C) (Sea temperature information, n.d.; World sea temperature, 2024a, 2024b).

The studies conducted in the Mediterranean Sea included investigation into aquatic decomposition and the factors such as temperature on the rate of decomposition (Ellingham, Perich & Tidball-Binz,2017); the recovery, identification process and taphonomic findings on victims of the Kater Radez I shipwreck (Introna & Campobasso, 2013); and a taphonomic study which included the records of water temperature conditions at victim's recovery site (Donno et al., 2014). Due to the influence of temperature on decomposition rates, there is a need for research to be conducted in South African oceans to understand DNA recovery from hard tissues in our context specifically. To situate this research in

context, factors pertaining to forensic taphonomy in the marine environment, including temperature and salinity, need to be understood and thus will be discussed later in this chapter.

Additionally, this chapter will present a literature review that describes the importance of identification with evidence of cases in a migration phenomenon. This will be followed by the global crisis of unidentified bodies and how washed cases contribute to this burden. Further, the theoretical background of forensic DNA profiling will be provided, followed by the anatomy of the teeth, the impact of marine environmental factors on DNA preservation and an overview of the studies that used teeth in a South African marine environment. The chapter will end with the rationale, aim, and objectives of this study.

1.2 Importance of human identification

Identification can be defined as the allocation of a name to an individual, whether living or dead (Blau et al., 2021). In medico-legal death investigations, identification is an essential aspect both in legal and social contexts (Inquest Act, No. 58 of 1959; Krysztofiak, 2016). The necessity to identify the deceased does not only fulfil the obligations of the law or the achievement of justice, but it also acknowledges the rights of an individual to not lose his or her identity even after death (Hoover, 1971; Ellingham, Perich & Tidball-Binz,2017; Blau et al., 2021). Additionally, identification is important for humanitarian reasons because it acknowledges the rights of the family and informs them about the fate of their loved one (Blau et al., 2021).

Identification of the deceased is also required to complete the death certificate. This document holds the key to the discharge of legal claims of property, estate, life insurance, deceased pensions, and other final matters by the spouse or surviving relative (Saukko & Knight, 2015). This is especially important in the context of migration, as described by Franceschetti et al., (2023), where DNA analysis was used to confirm familial linkage and paternal lineage in five different cases. However, in the sixth case, identification was not possible due to the low DNA yield from the human remains of the alleged father, thereby impacting the reunification of the family with their loved one (Franceschetti et al., 2024). This last case highlights the challenge in the identification process especially of highly degraded remains. As such there is a need for improvement of the DNA methods used in these cases to facilitate effective family reunification.

1.3 The burden of unidentified bodies

1.3.1 Unidentified bodies

The burden of identified bodies is both a global and national crisis, with South African mortuaries reporting on averages of 9.2% in Cape Town, 8.1% in Johannesburg, and 7% - 10% in Pretoria of unidentified cases per annum (Evert, 2011; Reid, Martin & Heathfield, 2020; Keyes, Mahon & Gilbert 2022). In most cases, identification is made by visual recognition by the next-of kin or a family member, especially in cases of non-decomposed or non-skeletonised human remains (Reid, Martin, & Heathfield, 2023). However, sometimes this may not be possible due to the condition of the body, which may be in advanced stages of decomposition, dismembered or burnt beyond recognition.

Additionally, if visual recognition is not possible, then fingerprinting analysis is performed. However, this analysis may be limited by the lack of ante-mortem data for reference comparison or not available due to the burnt or decomposition of the body, dismembered, skeletonisation or incomplete bodies (Dahal et al.,2023). Furthermore, visual recognition and fingerprinting may not be possible in cases where the bodies are found in mass disasters such as explosions, tsunamis, and plane crashes (Thakar et al., 2019; Reid, Martin & Heathfield, 2020; Heathfield et al., 2021; Kumar, Aparna & Sharma, 2021). Alternative methods such as DNA analysis may then be utilised. However, although alternative methods are available, efforts to use them to identify unidentified human remains are under-utilised (Reid, Martin & Heathfield, 2020).

Migration has been identified as one of the factors that leads to an increase in the burden of unidentified human remains. South Africa is a hotspot for migrations, and some migrants are undocumented (Keyes, Mahon & Gilbert 2022). As migrants arrive, they may be unemployed, and may be vulnerable to ending up homeless or going missing. In some instances, they may not be reported missing immediately and deceased bodies recovered after a longer period of time may be decomposed or skeletonised (Baliso, Finaughty, & Gibbon, 2019). Identification of undocumented migrants, irrespective of the stage of decomposition, places a strain on the mortuary services as the bodies are often unidentified and unclaimed (Keyes, Mahon & Gilbert 2022).

1.3.2 Washed up bodies

Decedents who wash up from the ocean are a burden both internationally and locally. These deaths have been attributed to a range of causes, including boating and ferry disasters, suicide, the disposal of murdered victims, work-related incidents, shark attack (although rare), recreational accidents and accidental drowning (Heaton et al., 2010; Leggio, Tarzia & Introna, 2021). Interestingly, the majority of these cases are statistically published internationally, while local publication in South Africa is scarce. Nonetheless, these cases contribute to the burden of unidentified bodies due to several reasons which include: 1) limited resources to identify unknown deceased bodies 2) difficulties in obtaining antemortem data from relatives due to problems in contacting and reaching them (Cattaneo et al., 2023),

3) difficulty in visual or fingerprint identification due to aquatic decomposition as described elsewhere (Stuart & Ueland, 2017; Martlin, Anderson & Bell, 2023).

Evidently, in a review by Reid, Martins and Heathfield (2020), of the 2 476 cases of unidentified human remains at Salt River Mortuary in Cape Town, 14.1 % of the bodies were not physically identifiable by the next of kin. Among the 14.1% of bodies that were visually unrecognisable, 12.8% were recovered from aquatic environments such as lakes, oceans, and rivers (Reid, Martin & Heathfield, 2020).

Aquatic decomposition is affected by various factors such as temperature, water flow, water depth, flora and fauna, and water chemistry (Stuart & Ueland, 2017; Zhang et al., 2022). Literature has shown that cooler temperatures reduced bacterial activity by salinity and inhibited insect activity, and overall slowed down the rate of aquatic decomposition (Simmons, Adlam & Moffatt, 2010; Ellingham, Perich & Tidball-Binz, 2017; Franceschetti et al., 2022). However, these factors varied depending on the type of aquatic environment. Hence, it is essential to understand the effect of the water and its components on DNA preservation.

Bodies that wash up from sea differ from bodies recovered from land due to exposure to different environmental factors. Further, bodies that have been in the marine environment for longer period of time become susceptible to greater extends of decomposition and animal scavenging. This may result in the bodies recovered being incomplete and/or skeletonised (Introna & Campobasso, 2013). Consequently, longer post-mortem submersion interval (PMSI), along with other factors, may have an impact on the DNA quantity and quality recovered from human bodies. Studies showed that DNA quantity, DNA degradation and DNA purity decreased as the immersion time increased (Vemuri et al., 2012; Graham & Adamowicz, 2015; Cartozzo et al., 2018, Iroanya & Onuoha, 2022). However, other studies have shown that identification using DNA profiling is still possible even after extensive PMSI (Finaughty et al., 2020).

1.4 Forensic DNA Profiling

Forensic DNA profiling is a process that targets specific regions of DNA, mainly short tandem repeats (STRs) that are spread throughout the twenty-two pairs of autosomal chromosomes (Goodwin, Linacre & Hadi, 2010). These regions have a high degree of variability among different individuals in terms of the number of times that a core repeat unit occurs at each locus, giving STRs a high discriminatory power. The basic process of how DNA profiling is shown in Figure 1.1.

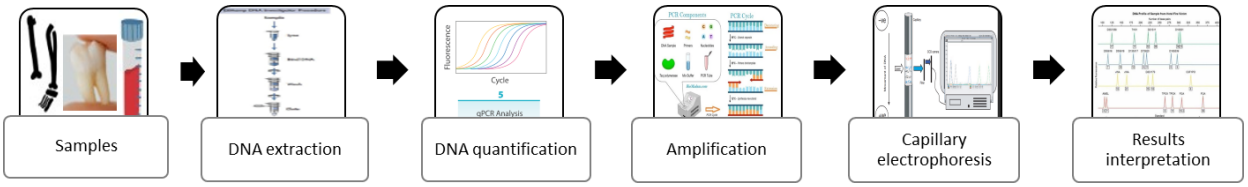

Figure 1.1: Basic flow process of DNA profiling (Adapted from Butler, 2009; Goodwin, Linacre & Hadi, 2010)

The generated DNA profile is compared to the DNA profile(s) generated from known individuals. In casework scenarios, a match may be found against a reference sample, thereby identifying the origin of the unknown DNA profile. However, for unidentified bodies, the reference samples are typically provided by family members and thus kinship analyses are computed. Population genetics principles are applied, based on the population's established allele frequency database, to determine the probability that an individual selected at random from a population could be related to the unidentified deceased individual (Butler, 2009; Goodwin, Linacre & Hadi, 2010).

It is important to note that although the DNA profiling has been highly successful using certain sample types such as blood, saliva and nails (Schlenker et al., 2016; Finaughty et al., 2020; Reid & Heathfield, 2020), these sample types are often unobtainable in cases where bodies are in advanced stages of decomposition (Higgins & Austin, 2013). Unfortunately, there is a lack of data pertaining to DNA recovery from hard tissues such as teeth, especially sampled from human remains recovered from marine environments.

1.5 Teeth as a source of DNA

Teeth have been well-researched as a source of DNA from bodies found in terrestrial environments due to their resistance to environmental factors and long post-mortem intervals (Ohira et al., 2009; Pagan et al., 2012; Higgins & Austin, 2013; Johnston and Stephenson, 2016; Correa et al., 2020; Heathfield et al., 2021; Kumar and Sharma, 2021; Chávez-Briones et al., 2023). To understand how DNA is preserved in teeth and how they withstand harsh conditions, a basic understanding of the tooth structure and its components is required. Structurally, teeth are the hardest organs in the body and are made up of the enamel, which is the outer layer that is highly mineralised. The enamel protects the cells inside the tooth from the external environment such as high temperature, microbial action, and humidity (Higgins & Austin, 2013). The roots are encased within the alveolar bone, which provides a protective barrier that promotes further DNA preservation (Higgins & Austin, 2013). The tooth also consists of a pulp chamber (which comprises DNA-rich blood vessels and nerves), dentine, and cementum as illustrated in Figure 1.2.

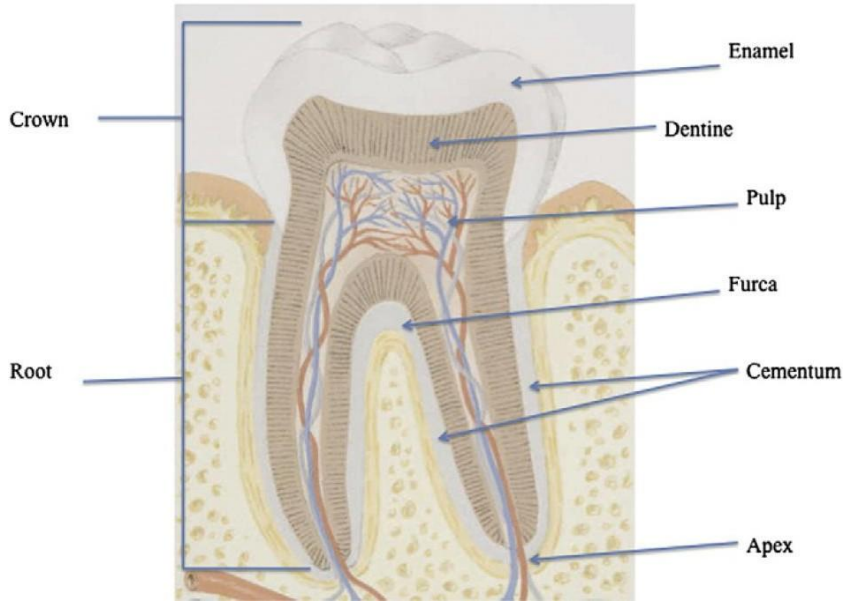

Figure 1.2: The basic composition of a tooth (Higgins & Austin, 2013).

1.6 Impact of marine environmental factors on DNA preservation in teeth

There is limited literature on the effect of marine environmental factors such as temperature, salinity, and microorganism activity on preservation of DNA from human remains in a marine context. The studies that have been carried out have primarily focused on the taphonomy and marine environmental factors that influence marine decomposition (Ellingham, Perich & Tidball-Binz,2017; Stuart & Ueland, 2017).

In an experimental study conducted by de Oliveira Musse et al. (2009) involving immersion of human teeth samples in freshwater and saltwater, DNA was recovered in about 37.5% of the samples, with more from freshwater than saltwater. The authors noted that the exposure of the tooth to water interfered directly with the preservation of DNA. However, the mechanism by which the water affected DNA remains unclear. In addition, greater DNA degradation was observed in DNA from teeth that were submerged in salt water (70%) as compared to freshwater (55%). The authors suggested this may have been due to the differing chemical composition of the aquatic environments (de Oliveira Musse et al., 2009). Contrarily, in another study, low levels of DNA degradation were observed in samples that were submerged in saltwater tank (salinity range: 24 700 to 39 400 ppm) to mimic seawater than in samples submerged in freshwater tank (salinity range: 144 to 787 ppm) (Hughes-Stamm, 2012). These results indicated that salt may be a good preservative of DNA by stabilising the double helix structure of DNA by preventing depurination which causes damage to DNA (Marguet & Forterre, 1998). Overall, the results from these two studies are contradicting each other in terms of DNA degradation in saltwater, this may be due to the use of agarose gel analysis in Oliveira Musse et al. (2009) study, which is a

qualitative measure for DNA degradation as compared to a quantitative measure (qPCR) used in Hughes-Stamm (2012) study.

DNA is destabilised in more acidic and alkaline environments and is less susceptible to damage in neutral environments (Latham & Miller, 2019). In marine context, where the pH of the ocean or seawater or freshwater is neutral (Marion et al., 2011; Collins et al., 2018), DNA degradation may not be due to pH but other factors. Evidently in the study by Hughes-Stamm (2012), pH in both saltwater tank (pH range: 6.7 to 7) and freshwater tank (pH range: 6.8 to 7.2) were relatively stable and could not have impacted DNA degradation as DNA would be stable within those ranges.

Temperature has affected DNA stability through a variety of physical and biochemical mechanisms and this in turn makes DNA susceptible to fragmentation, especially for long autosomal targets (Khorwal et al., 2024). Studies that reported the effect of water temperature on DNA degradation have focused on environmental DNA (Tsuji et al., 2017; Saito & Doi, 2020). In those studies, the rate of DNA degradation was slower in colder temperature as enzymatic activity by microorganism and extracellular nucleases was slowed down. As such, colder temperature can be hypothesised to preserve DNA better than warmer temperature. To date, only one study by Finaughty et al., 2019 reported that more DNA was recovered from porcine teeth during the colder temperatures (in winter season) as compared to other seasons.

Furthermore, DNA recovery was successful from human teeth immersed in saltwater tanks and freshwater tanks (Hughes-Stamm, 2012). However, when experimental studies involving the submersion of teeth in an authentic marine environment were carried out, substantially less DNA was recovered compared to the tank environments (Drake, 2014; Finaughty et al., 2019). The contradicting results between the natural marine environment and the artificial marine environment (i.e. tanks) has been hypothesised to be due the presence of oceanic microbes – a mechanism known to degrade DNA (Finaughty et al., 2019). Hence, the findings of studies utilising artificial marine environments cannot be directly applied to a natural marine environment.

1.7 Studies with the use of teeth in South African marine environment

To understand the unique challenges involving DNA identification of decedents of the marine context in Cape Town, a study was started at the University of Cape Town (UCT) in 2015. This project began with the assessment of DNA recovery from teeth obtained from pigs that were submerged in Table Bay and False Bay for a decomposition study (Finaughty et al., 2019). Overall, poor DNA recovery was observed from the pig teeth, which was insufficient for DNA profiling. DNA recovery was better when the seawater temperature fluctuated less, and when salinity was higher, supporting the protective effects

of colder temperature and salt. Non-specific amplification was also observed in some of the samples, which was found to belong to marine bacterial species. Thus, the authors hypothesised that microbial activity may have negatively influenced DNA preservation (Finaughty et al., 2019). This study prompted a series of optimisation studies to improve the DNA extraction workflow on teeth (Heathfield et al., 2021; Geldenhuys, 2023; Malan, 2023).

These studies were all carried out on human teeth, and subsequently, the protocol was tested on a small number of teeth submerged in the ocean for 20 days. Teeth samples (6 teeth and matched controls) were submerged in Table Bay as well as False Bay oceans in Cape Town, reflecting the sites where most washed-up cases are recovered (Malan, 2023) Malan's pilot study provided the opportunity to optimise the deployment of teeth in the ocean and provided insight into DNA recovery. However, due to the pilot nature of this experiment, the sample size used was small. There is therefore a need to assess the DNA workflow on a larger sample size and build on the results from the pilot study.

1.8 Rationale

As discussed in the literature review, the burden of unidentified bodies is both a global and local crisis (Reid, Martin & Heathfield, 2023; Suwalowska et al., 2023). Among these cases, decedents recovered from the ocean are prominent (Malan, 2023). There is therefore a need to improve the method for identifying these human remains. Teeth may be a valuable sample type for identification in these cases, as they have been reported as valuable sources of DNA due to their resilience against environmental factors. While the use of teeth for identification has been well studied in terrestrial environments, research in a marine context is limited.

To date, there is a notable gap in the existing literature on the use of teeth for identifying human remains in an authentic marine context. Most studies involving hard tissue as a sample type for DNA analysis were in the form of case reports or have used bones (Steinlecher et al., 2006; Byard, Both & Simpson, 2008; Kumar, Chauhan & Sharma, 2019; Finaughty et al., 2020). This is because teeth may not be present due to the skeletal remains being found incomplete or due to teeth being used for odontology.

Due to known factors that affect DNA degradation in a marine environment, including temperature, salinity, and microorganisms, it is important to carry out prospective studies involving human samples in an authentic marine environment. Additionally, it is important that studies are done locally as the environmental conditions vary substantially between oceans. Thus, there is a need for a study using human teeth submerged in the South African ocean to understand these identified factors and their impact on DNA recovery for use in human identification.

As described above, our research group has an ongoing study investigating and optimising DNA recovery from human teeth that have been submerged in the ocean. Additional research is required to build on the internally optimised protocols from previous studies using a larger sample size. The findings of such research will contribute to the development of standard operating procedures for forensic mortuaries and laboratories (e.g. the Observatory Forensic Pathology Institute in Cape Town, South Africa) particularly for the identification of washed-up cases.

1.9 Aim and objectives

Aim

The aim of this study was to evaluate the forensic DNA profiling success on human teeth samples that have been submerged in two different marine locations along Cape Town's coastline.

Objectives

1. Extract DNA from teeth submerged in two different marine locations (False Bay and Table Bay), as well as from matched control teeth.
2. To determine the quality and quantity of DNA using quantitative polymerase chain reaction.
3. To evaluate and compare the forensic DNA profiling success of DNA recovered from the teeth from the different oceans and controls.

Chapter 2: Methodology

2.1 Study design and approvals

This was a prospective, quantitative and experimental study. The quantitative approach included measuring DNA quality (degradation index and PCR inhibition), DNA recovery (concentration) and DNA profiling success (likelihood ratios) from teeth that were subjected to the internally optimised DNA protocol. The experimental approach involved exposure of the teeth samples to three different environmental conditions: room temperature (in the laboratory), Table Bay and False Bay oceans. Ethical approval was obtained for this research project (HREC REF: 093/2024, Appendix A1) from the Faculty of Health Sciences Human Research Ethics Committee (HREC) at the University of Cape Town. Permits (RES 2023-67) for the deployment of the teeth and for the transportation of human specimens (TRA-4-50-0882-4) were obtained (Appendix A2 and Appendix A3).

2.2 Recruitment and teeth samples

During 2023, a total of 90 wisdom teeth were collected as part of the umbrella study (HREC: 222/2019) from 30 adult volunteers (3 teeth per individual) who underwent elective wisdom tooth removal surgery at Kingsbury Hospital in Cape Town, South Africa. The participants' demographic information and sample metadata were not included/considered for statistical analysis (Appendix A, Table A1). Written informed consent was obtained from the participants (Appendix A4). There were no added risks to participants, as the teeth were obtained as a form of donation from patients who underwent surgical tooth removal for medical reasons. There was no compensation or direct benefits for participants. The teeth were stored at -20 °C in an access- controlled freezer at UCT until deployment into the ocean. Each sample was deidentified by allocating a unique participation number to maintain confidentiality.

2.3 Tooth preparation and deployments

One tooth from each participant was assigned for deployment in False Bay, another tooth for deployment in Table Bay, and the remaining tooth as a control (matched control). Prior to deployment, the tips of the roots of all teeth were coated with paraffin wax to simulate teeth embedded in the jawbone and to provide a protective effect that would minimise external contaminants from the ocean environment. For the teeth deployed in marine environments, each tooth was placed into a 50 mL centrifuge tube containing a total of 22 punch-like holes, each approximately 6 mm in diameter. The set of tubes were then placed into mesh bags (Figure 2.1). All the tubes had the same number of holes. During deployment into the ocean from 16 January 2024 to 5 February 2024 (20 days), the mesh bags

were secured to a metal grid on the ocean bed floor. The samples were out of sight from the public and boat traffic. The water temperature was measured daily using the Star-Oddi Starmon mini temperature logger. The average temperature for False Bay was 14.32 ± 2.92 °C, which was significantly warmer than the average temperature for Table Bay (11.68 ± 1.74 °C) ($p<0.0001$) (Appendix B, Table B2). After 20 days of deployment, the teeth were retrieved by trained divers and stored at -20 °C until further analysis.

Figure 2.1: Illustrates a mesh bag containing individual centrifuge tubes, each containing a single tooth. (Photo by Pieter Truter).

2.4 DNA extraction

After submergence, the tooth's surface was cleaned mechanically using a toothbrush as well as chemically using 3.5% bleach, molecular-grade water, followed by 70% ethanol (Finaughty et al., 2019). The outer enamel layer was then removed using a rotary sanding tool (Dremel, Mount Prospect, USA). The teeth were individually placed into pre-cooled grinding jars with liquid nitrogen and ground into a fine powder using a TissueLyser II (Qiagen, Hilden, Germany) for 1 minute at an oscillation frequency of 30 Hz. Approximately 1 ml of Milli-Q water was used as a DNA extraction blank.

The powdered teeth samples were transferred to microcentrifuge tubes using a spatula. Thereafter, 0.05 g of tooth powder and 50 µl of the blank (milli-Q water) samples were subjected to demineralisation following an in-house standard operating procedure, which has previously been optimised. The demineralisation buffer was made up of 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 8.0), buffer

ATL, proteinase K, 1 M dithiothreitol (DTT), and sodium dodecyl sulphate (SDS). A total volume of 1.5 mL of demineralisation buffer was added to the sample and incubated for 20 hours in a Thermomixer C (Eppendorf, Hamburg, Germany) set at 56 °C with a 450 rpm shaking speed. The process was performed to remove the large amount of calcium, which is a PCR inhibitor (Higgins & Austin, 2013). The remaining tooth powder from each sample was stored at -20 °C.

The QIAmp® DNA Investigator Kit (Qiagen, Hilden, Germany) was used to purify DNA from the lysate (i.e. demineralised tooth powder) following the manufacturer's protocol (Qiagen, 2020). DNA was eluted with a final volume of 30 µL of Buffer ATE. The extracted DNA was stored in the 4°C fridge for the duration of the laboratory experiments.

2.5 DNA quantification by qPCR

qPCR was used to assess the quality and quantity of DNA prior to downstream DNA profiling. This entailed the use of the Quantifiler™ Trio DNA Quantification Kit (Thermo Fisher Scientific, Waltham, USA), following the manufacturer's protocol (Thermo Fisher Scientific, 2018), with the exception that half-volume reactions were used, as internally optimised. qPCR amplification was performed on the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific, USA), and HID Real-Time PCR Analysis software (version 1.3) was used to determine DNA concentrations of the large autosomal target (214 bp), small autosomal target (80 bp) and Y-target (75 bp). DNA concentration was calculated by averaging the DNA concentration obtained from the small and large autosomal targets. The HID Real-Time PCR Analysis software uses the ratio of DNA concentrations of the small and large autosomal targets to determine the degradation index. The degradation index was categorised as previously described by Vernarecci et al., (2015) (Table 2.1). Additionally, the presence of PCR inhibition was also assessed, by evaluating if the cycle threshold (C_T) of the internal PCR control (IPC) was greater than 30 (Seo et al., 2010).

Table 2.1: Illustrates the degradation index categories (Vernarecci et al., 2015)

Degradation index categories	**Cutt offs**
Non-degraded	0-1.5
Mildly degraded	1.5–4
Degraded	4–10
Severely degraded	>10

2.6 Forensic DNA profiling

DNA profiling was performed using the GlobalFiler™ PCR Amplification Kit (Thermo Fischer Scientific, Waltham, USA) following the manufacturer's protocol (Thermo Fisher Scientific, 2022) except with half-volume reactions as previously optimised. The average concentrations of the large autosomal target and small autosomal target obtained with qPCR were used to make dilutions of the extracted DNA to 0.5 ng/µL, where necessary. For samples where average concentrations were less than 0.5 ng/µL , the volume of extracted DNA added was adjusted to achieve a total of 0.5 ng for the PCR reaction, where possible. Where concentrations were too low to achieve a final input amount of 0.5 ng, the maximum volume (7.5 µL) of extracted DNA was added to the reaction. PCR amplification was then performed using a T100 thermal cycler (BioRad, Hercules, California) according to the PCR cycling conditions in the manufacturer's protocol (Thermo Fisher Scientific, 2022)

Following PCR amplification, 0.5 µL of allelic ladder, and 0.5 µL of HiDi™ Formamide were added to wells on the 96 well plate that were designated for the allelic ladder. In the remaining wells, 10 µL of a master mix containing DNA size standard (600 Liz™) and HiDi™ Formamide was added. This was then followed by addition of 1 µL of each PCR product to make a final volume of 11 µL.

The plate was sealed with a rubber septa and denatured on a Hybex incubator (SciGene, Sunnyvale, CA, USA) 95 °C for 3 minutes. After denaturation, the plate was immediately snap-frozen using an iso-freeze block for 3 minutes. This was followed by capillary electrophoresis using the Applied Biosystems 3500*xL* Genetic Analyzer (Thermo Fisher Scientific, Massachusetts). A POP-4™ polymer was used, with run conditions of 13kV/1550 sec and injection settings of 1.2kV/15 sec. The data were then analysed using GeneMapper ID-X Software v1.6 (Applied Biosystems, Foster City, USA) with analytical threshold of 50 relative fluorescence unit (RFU) and a stochastic threshold of 100 RFU. Default stutter ratios were used.

2.6.1 Assessment of DNA profiles

All the DNA profiles were manually evaluated to identify presence of artefacts or anomalies. As such artefacts such as stutter peaks (small peaks that are one repeat unit smaller than the true allele), allele drop out (loss of a single allele of heterozygous pair), locus drop out (loss of both alleles at a locus) and allele drop in (appearance of an extraneous allele within a marker) were observed. Further anomalies such as pull-up or bleed-through which occur as a result of spectral overlap, were also noted. Off ladder (OL) peaks are peaks that falls outside of the virtual bin set, and after exclusion of artefacts, allele names were manually calculated using the data points of the OL peak

and the adjacent bins. To determine concordance, three DNA profiles from sample of the same individual that were exposed to different environments were compared to each other.

2.6.2 Likelihood ratio calculation

The DNA profiling success was determined by calculation of the likelihood ratios of each DNA profile. To this end, genotype frequencies were determined using the Hardy-Weinberg equation (Equation 1) and using the South African population allele frequency data published by Ristow et al., (2016). Thereafter the likelihood ratio was determined using Equation 2. The error rate was calculated using Equation 3.

Equation 1: Hardy-Weinberg equation

$$p^2 + 2pq + q^2 = 1$$

Equation 2: Likelihood ratio equation

$$\text{Likelihood ratio} = \frac{1}{\text{Genotype Frequency}}$$

Equation 3: Error rate equation

$$\text{Error rate} = \frac{1}{\text{Likelihood ratio}}$$

2.7 Quality measures

For quality control measures, a fresh wisdom tooth that was stored in the freezer (-20 °C) and not exposed to any of the abovementioned environmental conditions was used as positive tooth control to ensure that the protocol was working efficiently. In all the DNA extraction batches, blank samples were included to check for the presence or absence of exogenous materials (contaminant) during DNA extraction. These positive and negative controls were included throughout the workflow and were assessed using qPCR and DNA profiling as described above. Additionally, no template controls and positive controls were included in qPCR and DNA profiling batches, which all performed as expected. The detailed quality metrics for the qPCR experiments are presented in the results chapter.

Since qPCR and DNA profiling were done after all the DNA extractions were completed, the Qubit dsDNA High Sensitivity (HS) assay kit (Thermo Fisher Scientific, USA) was used to assess DNA extraction blanks using after each batch to verify no contamination prior to DNA extraction of the next batch (Appendix B, Table B3). This was done for the first 15 DNA extraction batches, whereas for the last two batches, DNA extraction blanks underwent assessment using qPCR and DNA profiling only.

Calibration of the Qubit™ 4 fluorometer (Thermo Fisher Scientific, USA) was performed using the standards provided with the kit. Master mix was prepared according to the manufacturer's protocol and added to samples or standards according to Table 2.2 (Thermo Fisher Scientific, 2022). The concentration of each DNA extraction blank was measured in duplicate.

Table 2.2: Illustrates the preparation of standard assays and blank samples preparation.

	Standards assay tubes	**Extraction blank samples tubes**
Volume of the master mix	190 µL	198 µL
Volume of blank samples	-	2 µL
Volume of standards	10 µL	-
To note, "- "represents that no sample was added		

No DNA was detected in any of the DNA extraction blanks using Qubit™ fluorometry. Three of the DNA extraction blank samples showed very low concentrations using qPCR, but the concentrations were below the validated limit of detection of the assay (less than 0.005 ng/µL) and the resultant DNA profiles of these DNA extraction blanks showed no allele amplification. Therefore, DNA samples extracted within these batches were deemed suitable for this study. However, possible DNA contamination was observed in one DNA extraction blank, and thus the samples (n=6) that were extracted in this batch were excluded from all analyses. These samples represented the three teeth from two donors, thus making the sample size for this study 28 donors (with 28 x 3 = 84 teeth samples).

Additionally, the DNA profile data for a further two donors were excluded from the DNA profile analyses specifically, due to discordance and lack of confidence in allele calling, thus making any resultant likelihood ratio unreliable. Thus, the statistics described below and the results that follow in the next chapter are based on these reduced sample sizes, where quality can be assured.

2.8 Statistical analysis

Statistical analysis and data visualisation were performed using IBM SPSS Statistics version 30.0.0.0 (172), Microsoft® Excel® and GraphPad Prism version 10.3.1.509. In all the tests performed, $p < 0.05$ with confidence interval of 95% was set as the level of statistical significance. Shapiro Wilk test was performed to determine the distributions of the ocean temperatures, DNA concentration, DNA degradation and likelihood ratios. The data were not normally distributed and consequently, non-parametric tests were used.

Mann Whitney test was used to determine the significant difference between temperatures of False Bay and Table Bay environments. Kruskal Wallis test was performed to compare the significant difference between the three environments in terms of DNA concentration, DNA degradation and likelihood ratios. This was followed by Bonferreroni correction application for multiple comparison. Descriptive statistics was also used, and the data were represented as mean ± standard deviation or range with median (unless stated otherwise).

Chapter 3: Results

This chapter presents the findings of the study, which aimed to evaluate the success of DNA profiling from samples submerged in Table Bay and False Bay versus matched controls kept at room temperature for the same duration. Internally optimised protocols were used to assess the quality, quantity, and profiling success of the extracted DNA. This was done to evaluate the laboratory's current protocol in terms of its ability to generate DNA profiles, and to inform any further adjustment that may need to be made to the overall DNA workflow. The results are structured according to each objective of the study. For noting upfront, detailed DNA data for each sample (in the form of tables and figures) are available in Appendix B (Tables B1 to B8, Figure B1 and B2).

3.1 DNA quantity and quality assessment for qPCR

3.1.1 Quality assurance for qPCR

For all qPCR batches, quality parameters were met, including the slope metrics of the standard curves (Appendix B, Figure B1 and Table B4), negative controls (NTC) showing no DNA contamination and the IPC successfully amplifying for all reactions. This indicated that the method yielded reliable results. The positive tooth control had a DNA concentration of 5.255 ng/µl and it was not degraded, which was in line with what is usually seen in our laboratory. The results pertaining to the positive controls sample are shown in Figure 3.1 and Figure 3.2 for context.

3.1.2 DNA quantity

The mean DNA concentration was 0.00530 ± 0.01086 ng/µL for samples submerged in Table Bay, which was lower compared to those submerged from False Bay (0.01099 ± 0.02902 ng/µL), although this was not significantly different ($p > 0.05$) (Figure 3.1). Significantly higher DNA concentrations were observed in DNA samples obtained from the unsubmerged matched control (0.3704 ± 1.257 ng/µL), as compared to those from Table Bay ($p < 0.001$) and False Bay ($p < 0.001$) (Figure 3.1).

No DNA was detected in a total of three samples, one from each environment (unsubmerged match control, submerged from Table Bay and False Bay), while their corresponding samples that were placed in the other environments showed DNA concentrations that were less than 0.5 ng/µL (n=2) and less than 0.005 ng/µL (n= 4). Each of the samples with no DNA detected were from different participants.

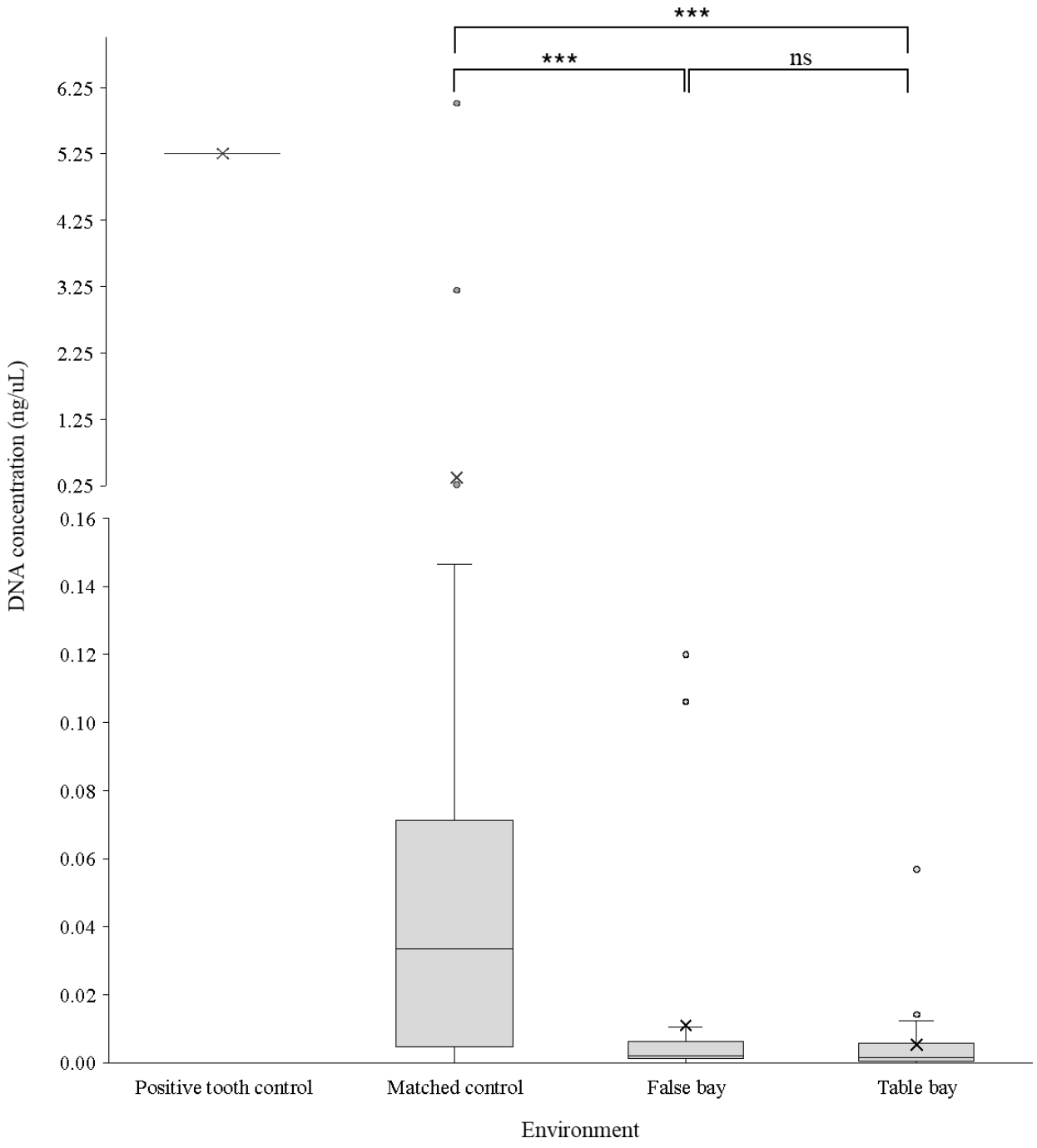

Figure 3.1: A Box and whisker plot of the DNA concentrations (average of the small autosomal and large autosomal targets) of the different environments. Three samples that showed no DNA amplification on qPCR were not depicted in the graph. The significant difference is represented by (***) with $p < 0.001$; and ns representing not significant ($p > 0.05$).

3.1.3 DNA quality (DNA degradation and PCR inhibition)

The DNA degradation of teeth samples is portrayed categorically in Figure 3.2 where most samples from all environments were considered 'non degraded', with the minority of samples falling in the

'severely degraded' category. Two DNA samples from Table Bay were so severely degraded that the large autosomal target did not amplify. Whilst a DNA degradation index could not be determined for these samples, they were represented in the 'severely degraded' category. In a further seven DNA samples (False Bay: n=2, Table Bay: n=4, and unsubmerged matched control: n=1), DNA concentrations of all targets were low – whilst a numerical degradation index could not be calculated, these samples were represented in the non-degraded category (Figure 3.2A).

Severely degraded DNA was not consistently observed within the same set of teeth from an individual across environments. For example, in instances where DNA from teeth submerged in Table Bay were severely degraded, the corresponding samples in the other environments yielded DNA that was mildly degraded and non-degraded (Appendix B, Table B1, row: 31, 65 and 99).

Based on the numerical data, the mean DNA degradation indexes were 0.8960 ± 0.8699 for samples submerged in Table Bay, 2.145 ± 4.313 for those submerged in False Bay and 3.115 ± 4.271 for unsubmerged matched controls (Appendix B, Table B1). A significance difference in the DNA degradation index between samples submerged from Table Bay and the unsubmerged matched control was observed ($p < 0.01$). No significance difference was observed in the DNA degradation index between the unsubmerged matched control and samples submerged in False Bay, as well as between samples submerged in Table Bay and False Bay ($p > 0.05$) (Figure 3.2B).

In terms of PCR inhibition, all samples had IPC C_T values below 30, indicating that no PCR inhibitors were detected by the assay that could have affected the amplification efficiency (Appendix B, Table B1 and Figure B2).

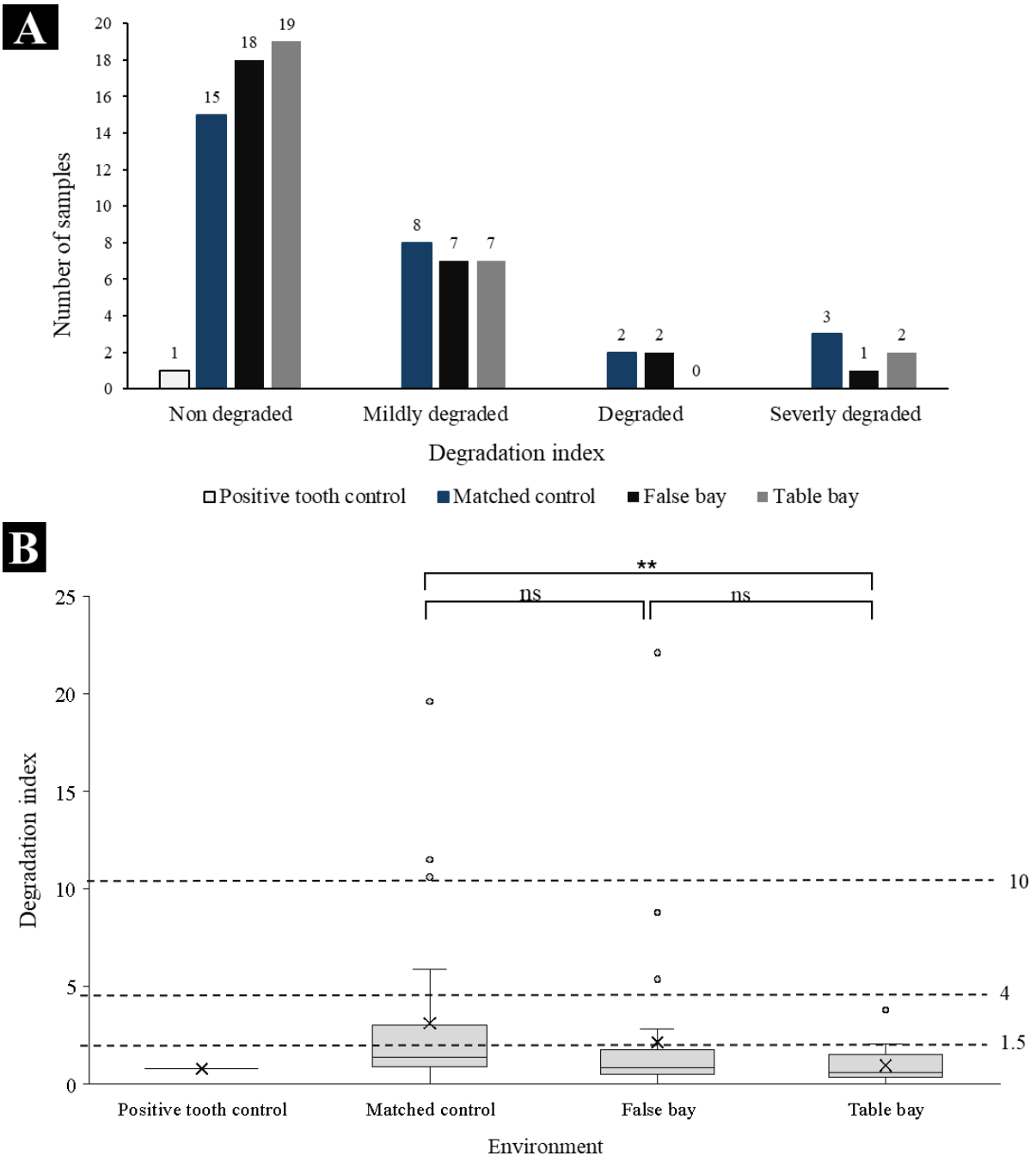

Figure 3.2: Illustrates the quality of extracted DNA from samples exposed to three different types of environments. **A**- represents a graph depicting the degradation index categories for samples of different environments as according to Vernarecci et al., (2015). The nine samples were depicted in this graph, with seven samples represented in the non- degraded category and the remaining two samples were represented in the severely degraded category. **B**-the degradation index for each environment. Nine samples without degradation index were not depicted on the graph as some were severely degraded and others showed no amplification of the small autosomal target or both autosomal targets. P value < 0.05 was regarded as significant difference and p- values represented by asterisk (**) has a $p<0.01$, and (ns) indicates not significant. The dotted lines indicate the degradation index categories according to Vernarecci et al., (2015).

3.2 DNA profiling

3.2.1 Quality assurance for DNA profiles

In all the DNA profiling experiments, DNA extraction blanks and negative controls showed no contamination as no alleles were detected in the resultant electropherograms. Full DNA profiles for the GlobalFiler positive control DNA 007 were observed, which were concordant with the genotypes provided in the manufacturer's manual (Thermo Fisher Scientific, 2019). Additionally, as expected, all alleles in the positive tooth control were detected, which generated a likelihood ratio of 4.207×10^{27} with maximum possible error rate of 2.377×10^{-28}. The results of this sample are given in Appendix B, Table B1 and are portrayed in Figure 3.6 later in this chapter, for context.

3.2.2 Assessment of DNA profiles

After accounting for pull-up, there were three OL peaks: two were novel and one was allele drop in (Appendix B, Table B5). Stutters peaks were observed frequently but did not interfere with interpretation of the electropherograms. The presence of artefacts in electropherograms were inconsistent within an individual, wherein different artefacts were observed in the DNA profiles obtained from the unsubmerged matched control, sample submerged in False Bay and sample submerged in Table Bay for the same individual. The difference in artefact was largely due to allele and locus dropout observed in DNA profiles from samples submerged in False Bay and Table Bay, compared to the unsubmerged matched control (Table 3.1) (Figure 3.3) (Appendix B, Table B6).

Table 3.1: Illustrates the number of samples with allele drop out, per each of the three different environments.

Number of allele drop out instances observed across the 24 loci	**Number of samples with allele drop out (per environment)**		
	Matched control	**False Bay**	**Table Bay**
1	5	1	4
2	2	1	3
3	4	0	1
4	1	5	4
5	2	4	4
6	2	3	2
7	0	2	4
8	1	0	2
9	0	0	0
10	0	2	1
11	0	2	0

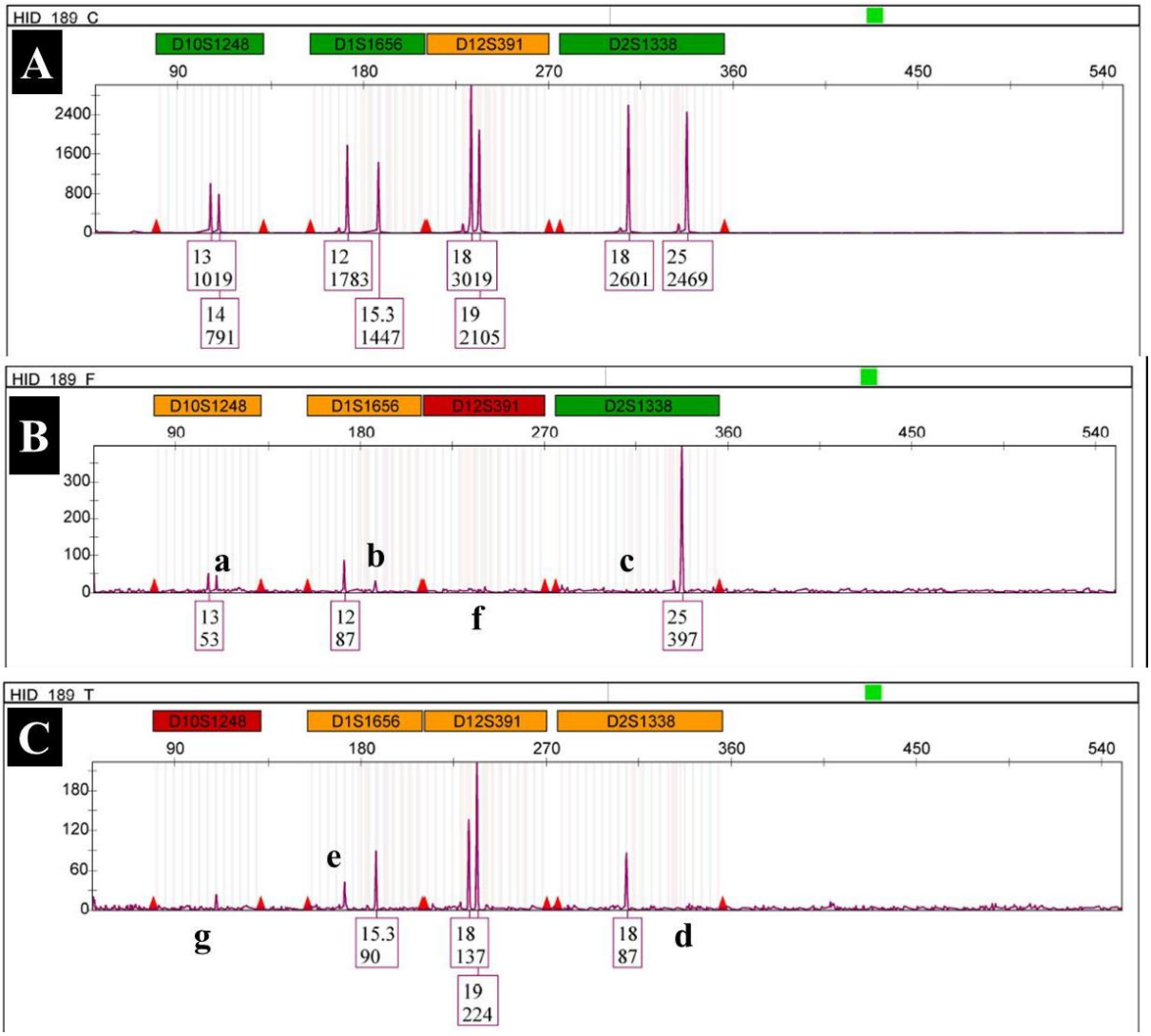

Figure 3.3: Purple channel electropherogram of DNA profiles from **(A)** unsubmerged matched control, **(B)** a sample submerged in False bay and **(C)** a sample submerged in Table bay, showing allele drop out of the following alleles: (a) allele 14 for marker D10S1248; (b) allele 15.3 and (e) allele 12 for marker D1S656; (c) allele 18 and (d) allele 25 for marker D2S1338 in channels B and C. Locus drop out at markers D10S1248 represented by letter (g) and D12S391 represented by letter (f) were also observed in channel B and C, while panel A shows correctly called alleles for D10S1248 and D1S1656.

Allele drop-in was observed in several DNA profiles from False Bay, none from Table Bay and none from matched control samples. Two examples are depicted in Figure 3.4: In the first sample, the marker TPOX is shown: alleles 8 and 12 are observed for the control and Table Bay samples, whereas the DNA profile from False Bay showed drop-in of allele 11 alongside allele 8 and 12. In the second example, D19S433 is shown, where the genotype should be 12, 13. However, the sample from False Bay shows allele drop in of 14.2, giving a genotype of 8, 14.2. The other instances of allele drop-in are detailed in Appendix B, Table B7.

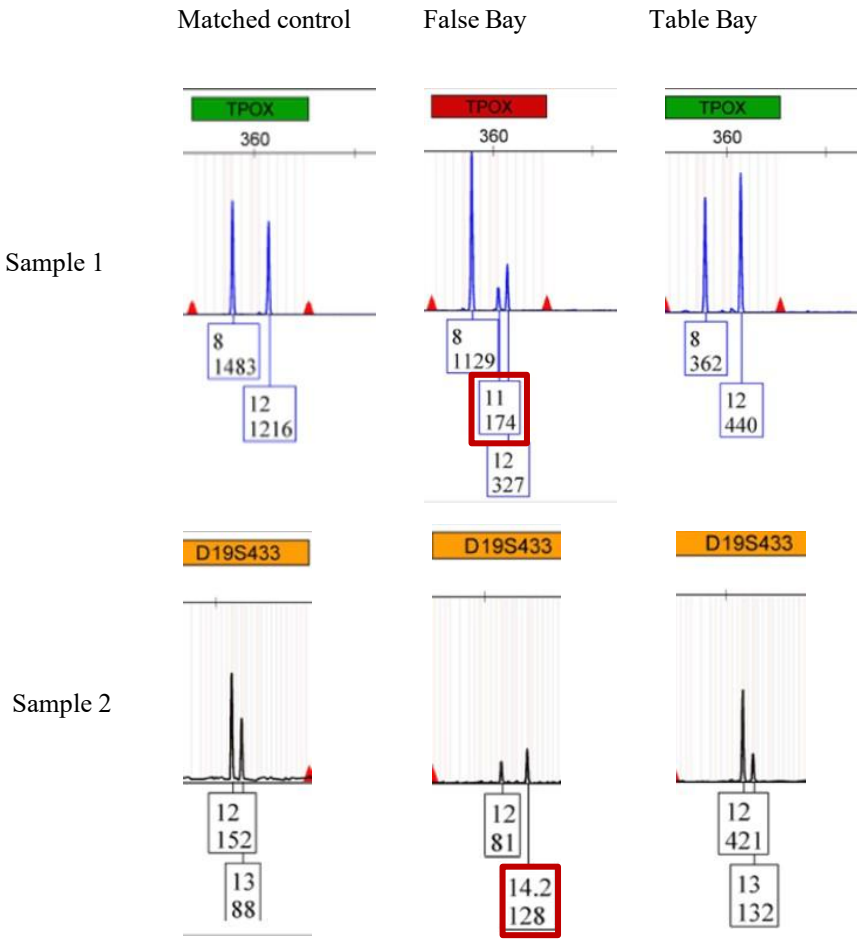

Figure 3.4: Allele drop-in of allele 11 and allele 14.2 observed in the TPOX and D19S433 markers for the DNA extracted from the teeth submerged in False Bay.

The ski-slope effect was observed in nine matched control samples and given the qPCR data, was an indication of DNA degradation in the samples (Figure 3.5 A). Interestingly, a reverse ski-slope with the large loci being preferentially amplified over smaller loci was observed in two matched control samples, four Table Bay samples and one False Bay sample (Figure 3.5 B).

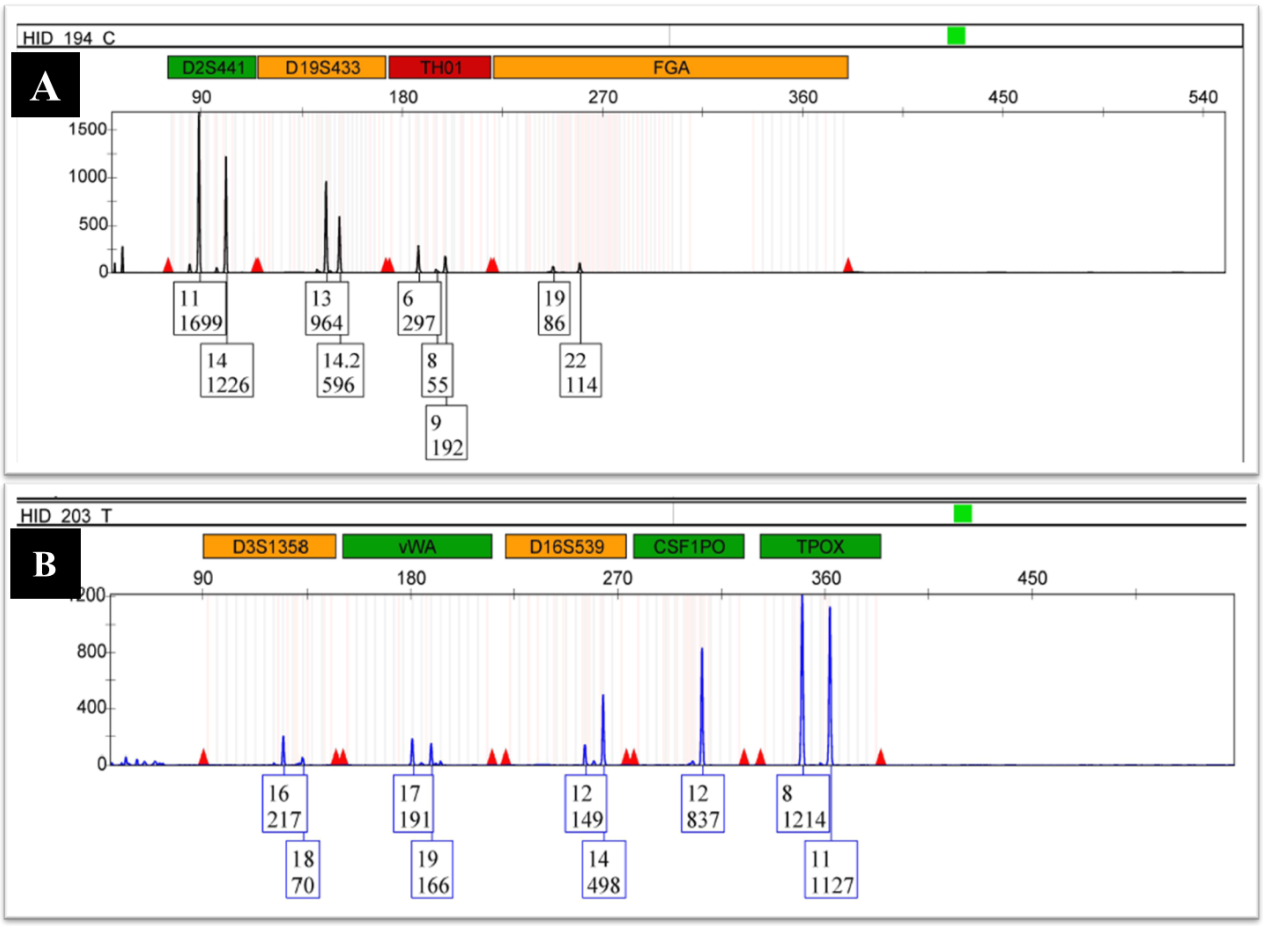

Figure 3.5: Channel A represent an electropherogram obtained from the DNA profile of a matched control sample showing the ski-slope effect, preferential amplification of the small loci and lower amplification of larger loci. Channel B shows a reverse ski-slope effect.

3.2.3 Failed DNA profiles

Three DNA profiles from samples submerged in False Bay showed no amplification and their corresponding DNA profiles from unsubmerged matched controls and one sample submerged in Table Bay showed better amplification, while two remaining samples submerged in Table Bay showed very few loci amplification (see Table 3.2).

Table 3.2: Three failed DNA profiles from samples submerged in False Bay and their corresponding samples indicating observation of amplified loci.

Sample ID	**Matched control**		**False Bay**		**Table Bay**	
	Total number of amplified loci	Concentration (ng/µL)	Total number of amplified loci	Concentration (ng/µL)	Total number of amplified loci	Concentration (ng/µL)
HID_181	22/24	6.016551	0/24	0.001279	22/24	0.005943
HID_190_	24/24	0.068828	0/24	0.006958	7/24	0.000672
HID_195_	22/24	0.067823	0/24	0.000426	7/24	0.001260

3.2.4 Likelihood ratio inferred from DNA profiles

The likelihood ratios from DNA profiles generated from teeth subjected to each environment are tabulated in Table 3.3 and graphically portrayed in Figure 3.6. The likelihood ratios were higher from DNA profiles generated from unsubmerged matched control samples as compared to those generated from teeth submerged in False Bay and Table Bay. False Bay showed slightly higher likelihood ratios than Table Bay and no significant difference was observed in the likelihood ratio between the three environments ($p > 0.05$).

Table 3.3: Descriptive statistics for likelihood ratios as well as their associated error rates calculated using DNA profiles generated from teeth samples submerged in three different environments. Likelihood ratios could not be calculated for three DNA profiles from teeth submerged in False Bay due to no alleles being amplified.

	Unsubmerged matched control samples (n=24)		False Bay (n=21)		Table Bay (n=24)	
	Likelihood ratio	Likelihood ratio error rates	Likelihood ratio	Likelihood ratio error rates	Likelihood ratio	Likelihood ratio error rates
Median	3.094×10^{28}	6.116×10^{-29}	3.501×10^{15}	2.855×10^{-16}	1.031×10^{13}	1.312×10^{-11}
Standard deviation	3.282×10^{32}	Not applicable	5.626×10^{34}	Not applicable	1.146×10^{31}	Not applicable
Range	$11.11 - 1.614 \times 10^{33}$	$6.198 \times 10^{-34} - 8.999 \times 10^{-02}$	$1.557 \times 10^{3} - 2.578 \times 10^{35}$	$3.878 \times 10^{-36} - 6.424 \times 10^{-04}$	$4.221 - 5.633 \times 10^{31}$	$1.775 \times 10^{-32} - 2.369 \times 10^{-01}$

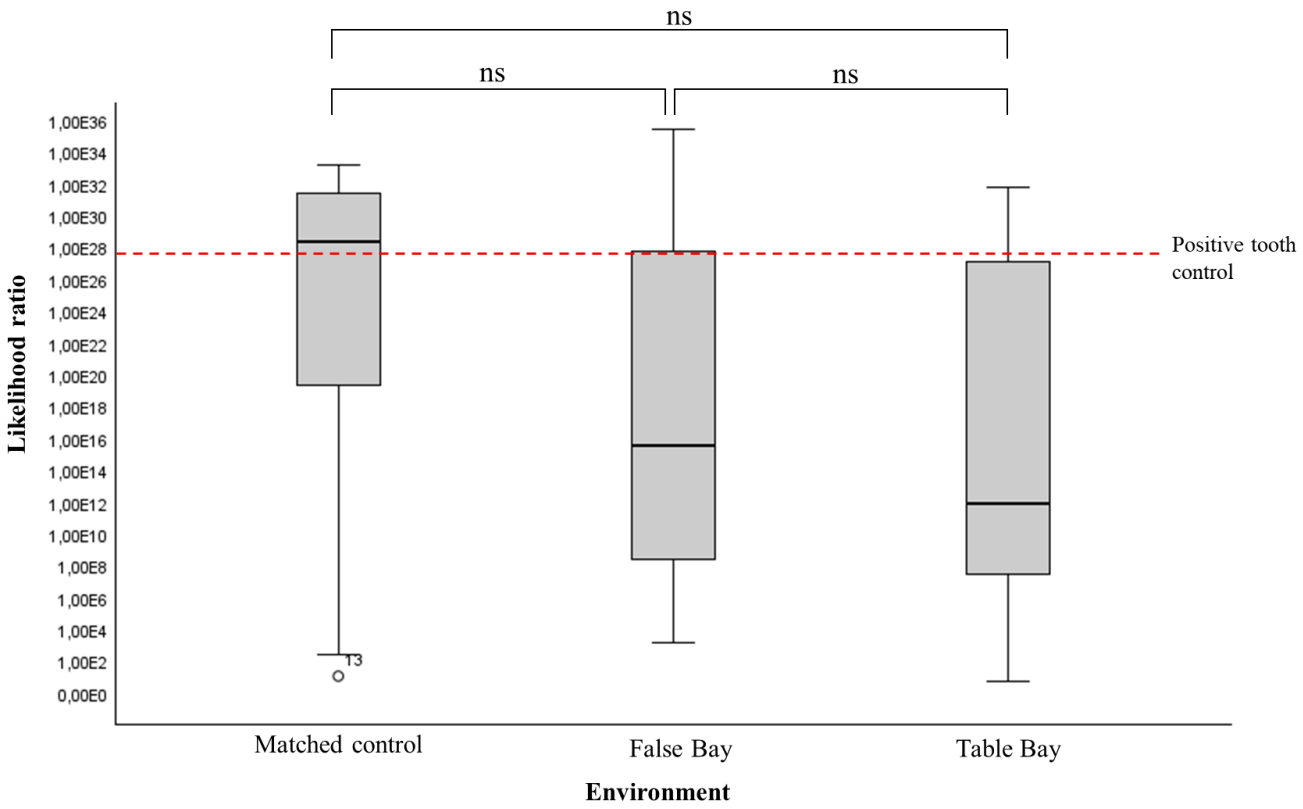

Figure 3.6: Illustrates the likelihood ratio of samples from different environments. The y-axis is represented as a log scale. P value < 0.05 was regarded as significant difference and (ns) indicates not significant. Two set of samples that had discordance were not included in the likelihood ratio calculation. The red dotted line represents the positive tooth control.

Chapter 4: Discussion and Conclusion

The aim of this study was to evaluate the forensic DNA profiling success on human teeth samples that have been submerged in two different marine locations in South Africa. This aim was achieved by following three objectives. This chapter will be structured in terms of each objective, where the results pertaining to each objective will be discussed in the context of the body of literature. Technical aspects relating to each objective will also be discussed.

4.1 Objective 1: Extract DNA from teeth submerged in two different marine locations (False Bay and Table Bay), as well as from teeth kept as controls

This objective was successfully achieved, whereby the teeth samples that were submerged and kept as controls underwent DNA extraction using the previously optimised DNA extraction method. The QIAmp® DNA Investigator Kit was used, which utilised a silica-based membrane for solid-phase extraction. This method has shown to yield varying DNA concentrations from hard tissues (Lee et al., 2010; Kaneko et al., 2015).

In the DNA extraction workflow, a rotary tool was used to sand the tooth surface to remove the highly mineralised enamel, which contains calcium, a known PCR inhibitor. However, this process may generate heat, which may contribute to DNA degradation within the sample. This was mitigated as far as possible by using pre-cooled grinding jars and the addition of liquid nitrogen. However, the presence of severely degraded DNA in six samples (Figure 3.2) suggests that mechanical sanding could have accelerated the pre-existing degradation. While environmental conditions are a primary factor in DNA degradation as discussed later (section 4.4), the potential impact of heat generated during sample preparation should not be overlooked and caution during sample preparation should be taken.

Decontamination and demineralisation processes were necessary in the DNA extraction workflow to avoid exogenous contaminants and the high calcium content from teeth, which may be present in the samples and affect DNA amplification in PCR (Higgins & Austin, 2013; Cafiero et al., 2019). All the experimental samples and controls showed no PCR inhibition (see Appendix B, Table B; Figure B2), indicating the successful removal of inhibitors and external contaminants. This aligns with existing literature, where the incorporation of demineralisation process has shown to successfully remove inhibitors, thus leading to improved amplification and DNA yield (Loreille et al., 2007; Higgins et al., 2015; Hasap et al., 2020; Finaughty et al., 2023).

Notably, literature has reported constant modification of this process in terms of the buffer composition, incubation time and temperature and the volume of EDTA (Loreille et al., 2007; Mckinnon and Higgins., 2021; Nasrollahzadehsabet et al.,2021). This highlights how well established the process is and its necessity for removing as much contamination and inhibitors as possible, especially for hard tissues. Consequently, this will aid in the downstream analysis. Furthermore, demineralisation has also been associated with improved STR profile success according to the meta-analysis by Finaughty et al., (2023), emphasising its importance in forensic workflows.

4.2 Objective 2: To determine the quality and quantity of DNA using quantitative polymerase chain reaction

Determining the DNA concentration is an important step prior to DNA profiling of hard tissues, because different regions of a tooth or bone sample can yield varying concentrations of DNA (Finaughty et al., 2023). It is also important to know how much DNA is available for use, and in case repetition of analysis is required. Since DNA profiling is highly sensitive, precise quantification is necessary to prevent issues arising from excessive or insufficient DNA input. The mean DNA concentrations from teeth submerged in Table Bay and False Bay were 0.00530 ng/µL and 0.01099 ng/µL respectively. Most samples from both sites had concentrations above 0.001 ng/µL (Appendix B, Table B1). This represents an improvement over the findings from the initial pilot project by Longden-Thurgood (2018), where porcine teeth were submerged at two different locations in False Bay, across various deployment days.

As expected, the mean DNA concentrations of samples in this study (Figure 3.1) were lower than those reported in studies where controlled tank environments were used (Hughes-Stamm, 2012). They were also lower than the concentrations reported in studies where Nanodrop spectrophotometry was used to quantify DNA (Vemuri et al., 2012, Iroanya & Onuoha, 2022) – this may be because Nanodrop spectrophotometry quantifies all nucleic acid as opposed to human-specific DNA only. Additionally, the differences in the method of DNA extraction could also explain the amount of DNA recovered. A summary table of DNA concentrations found in other studies is provided in Appendix C, Table C1.

The mean DNA concentration of the submerged teeth were also lower than that of the unsubmerged matched controls (0.370 ng/µL). This suggests the effect of seawater had a negative influence on the DNA concentrations recovered from teeth. Additionally, three samples from each environment showed no detectable DNA (despite having IPC C_T values below 30). This may be due to a myriad of factors, such as the concentration of DNA in the sample being lower than the limit of detection of the quantification method used, inter-individual variation of DNA from donors or the non-homogenous nature of ground tooth powder (Heathfield et al., 2021, Finaughty et al., 2023).

Furthermore, assessing DNA integrity was crucial prior to DNA profiling, as the quality of the extracted DNA may influence the most subsequent DNA profiling approach, based on the degree of degradation or presence or absence of PCR inhibition (Houston et al.,2024). In this study, variation in the levels of DNA degradation was observed across all the three environments (Figure 3.2), with the majority of samples classified as non-degraded (degradation index < 1.5) and the minority of samples as severely degraded (degradation index > 10), as categorised by Vernarecci et al., (2015). Mildly degraded DNA samples of teeth samples exposed to the three environments were also observed which aligned with degradation levels reported by Hughes-Stamm (2012), where samples submerged in saltwater had degradation ratios ranging from 1.5 to 2, while those submerged in freshwater ranged from 2 and 3.5.

Previous studies have reported that damaged or exposed teeth roots accelerated the level of degradation (Leskovar & Pajnic, 2023). In this study, this variable was mitigated by adding paraffin wax to the exposed roots to simulate the natural protection that would have ordinarily have been provided by the gum and jaw of the individual. The variation in DNA degradation observed among some samples submerged in Table Bay were notable. For example, certain teeth exhibited severe degradation while their counterparts submerged in False Bay or unsubmerged matched controls remained mildly degraded or non-degraded. This suggests that particular environmental factors may have played a role in DNA degradation, compared to donor-specific biological factors. These environmental factors will be discussed in more detail in section 4.4.

4.3 Objective 3: To evaluate and compare the forensic DNA profiling success of DNA recovered from the teeth exposed to different oceans and controls

The DNA profiles obtained in this study exhibited various artefacts and anomalies including allele drop out, locus drop out, stutter peaks and allele drop-in (Section 3.2.2). As expected, DNA profiles from samples with low concentrations showed stochastic variation in peak height, with allele drop out and locus drop out being prominent artefacts. Similarly, DNA profiles generated from degraded DNA samples showed features such as ski-slope effects, also including allele drop out and locus drop out. Allele drop out was observed in more DNA profiles from samples submerged in False Bay and Table Bay as compared to unsubmerged matched control (Table 3.1). This observation suggests poorer quality DNA profiles from the teeth that were submerged in the ocean compared to unsubmerged matched controls, which is consistent with previous findings (Corrêa et al., 2017; Lozano-Peral et al., 2021).

DNA degradation and low template DNA have been reported as one of the factors which contributes to failure in DNA profiles (Hughes-Stamm, 2012). In this study, three failed (i.e. completely blank)

profiles were observed in samples submerged from False Bay (Table 3.3). These samples had various degradation indices but all had low concentrations of DNA (Appendix B, Table B1). However, other samples with low concentrations yielded DNA profiles with informative likelihood ratios. This inconsistency is in agreement with previous reports that DNA concentrations and degradation indices generated from qPCR are not accurate indicators of DNA profiling success (Finaughty et al., 2023).

An unexpected finding was allele drop in, which was observed in samples that were submerged in False Bay. Allele drop in was noted upon comparison of DNA profiles from False Bay to those from the same donor that were subjected to the other two environments. Some studies have attributed allele drop in being due to minute dust particles introduced during sample preparation for profiling which result in spurious amplification (Gill et al., 2000; Buckleton & Gill, 2004). However, we hypothesise that allele drop in observed in this study was due to non-specific amplification from aquatic microbes. The hypothesis is drawn from two studies: Chierto et al., (2024) also found non-specific PCR product in the DNA profiles of bone and teeth samples from buried bodies. They subjected the DNA samples to next generation sequencing and analysis of taxa, which indicated that the samples contained DNA from humans as well as microorganisms. They attributed the allele drop in to the non-specific binding of STR primers to the DNA of the microorganisms that were present in the samples. The second study was the original pilot study in our research group on submerged porcine teeth, where non-specific amplification was also observed, and Sanger sequencing showed alignment to an aquatic microbial species (Finaughty et al., 2019). Thus, further research could use next generation sequencing to investigate this hypothesis and understand the underlying cause of the allele drop in.

The likelihood ratio is one of the approaches used in forensic science for evaluation of DNA evidence given two competing hypotheses (Butler, 2009; Goodwin, Linacre & Hadi, 2010). The likelihood ratio was chosen to portray DNA profiling success in this study as it facilitates comparison of DNA profiling results between studies that have used kits with a different number of loci (Mameli, Piras & Delogu, 2014; Finaughty et al., 2018; Olivieri et al., 2018). For example, a ‘partial’ DNA profile comprising 10 out of 15 genotyped STRs may be more statistically informative than a ‘full’ profile of 9 out of 9 genotyped STRs. Additionally, the definitions of full, partial, or failed DNA profiles varies between studies. A likelihood ratio is also easily compared with random match probabilities (RMP), which are often reported in case studies.

A wide range of likelihood ratios within each environment was observed (Table 3.3), with likelihood ratio values of samples submerged in False Bay and Table Bay being lower than those of unsubmerged matched controls. Slightly higher likelihood ratio values were obtained from samples submerged in False Bay than those in Table Bay, however, this was not statistically significant. The likelihood ratios obtained in this study were comparable to those reported in two case reports involving DNA analysis

of bone samples recovered from the ocean: In the first case, bone samples were recovered from a beach in Table Bay, Cape Town, which yielded a likelihood ratio of 3.88×10^{12} (Finaughty and Reid et al., 2020). The mean likelihood ratio obtained for teeth samples submerged in Table Bay in this study was 1.031×10^{13}, a factor of 10 higher than the likelihood ratio obtained in the case report. In the second case report, human bone samples were recovered from the sea near the northeast coast of Sardinia and yielded an likelihood ratio of 18.3×10^{16} (Mameli, Piras & Delogu, 2014), which was slightly higher than the mean likelihood ratios obtained in this study (False Bay: mean likelihood ratio = 3.501×10^{15}; Table Bay: mean likelihood ratio = 1.031×10^{13}).

4.4 Environmental factors

When assessing the results holistically, there were no statistically significant differences in DNA concentrations, degradation or DNA profile likelihood ratios between the teeth submerged in Table Bay and False Bay. Despite the DNA concentration being slightly lower from teeth submerged in Table Bay, the degradation indices were slightly higher compared to teeth submerged in False Bay. While DNA profiles from teeth submerged in Table Bay exhibited allele drop out, at least some genotypes could be established from every sample, resulting in likelihood ratios ranging from 4.221 - 5.633×10^{31}. Contrastingly, three teeth submerged in False Bay yielded blank DNA profiles, and the others yielded DNA profiles with likelihood ratios ranging from 1.557×10^{3} - 2.578×10^{35}.

Perhaps the single factor that was completely different between the DNA profiles from the two oceans was the presence of allele drop in seen in four DNA profiles from False Bay only, which has implications in human identification in casework. Understanding the effect of aquatic environmental factors on DNA preservation and DNA profiling success is challenging, especially given the limited literature surrounding this. Nonetheless, environmental factors may not affect DNA preservation in isolation, but may interact with one another in affecting DNA preservation. Environmental factors and tooth integrity are discussed below and may possibly explain the results obtained in this study.

Significant differences in the water temperature was observed between Table Bay and False Bay with mean temperatures of 11.64°C ± 1.457°C and 14.37°C ± 2.699°C, respectively (Appendix B, Table B2). These temperatures were lower than the mean water temperatures of 16.78°C and 17.12°C for the two sites of False Bay in the equivalent summer period as reported in Longden-Thurgood (2018) in the original pilot study. Longden-Thurgood (2018) reported that DNA recovery was negatively affected during the summer deployment and the DNA quantities (of < 100 pg). Most samples from the summer deployment failed to amplify, but those recovered from the winter months (with colder seawater temperatures) showed some amplification success (Longden-Thurgood, 2018; Finaughty et al., 2019).

This study shows an improvement over the original study, where DNA recovery from majority of the teeth samples submerged from the two ocean environments yielded DNA that was sufficient for successful DNA amplification (Appendix B, Table B1). Additionally, the majority of DNA samples submerged in False Bay and Table Bay environments remained intact. Given the cooler seawater temperatures in this study (despite the summer deployment), it again suggests that the cooler temperatures may have positively affected DNA recovery and DNA profiling success. Meanwhile the few samples which did not yield DNA profiles with high likelihood ratios may have been affected by different environmental factors.

As discussed in chapter 1 section 1.6, it is unlikely that pH could have influenced DNA preservation as the pH typically ranges around neutral in both the ocean study sites. Whilst dissolved oxygen (DO) decreases with increasing ocean depth, which may slow the rate of decomposition (and possibly DNA degradation) (Latham & Miller, 2019), this factor was not varied in this study.

Salinity has been shown to protect DNA from degradation by disrupting the enzymatic activity of microorganisms, as reported in Hughes-Stamm (2012), and as discussed in chapter 1. Furthermore, a recent study by Birne et al., (2024) demonstrated that DNA in bone samples stored in salt was preserved well and high quality STR profiles were also obtained. This salinity present in the seawater could have thus contributed to the overall preservation of DNA in the current study.

Microbial activity is one of the primary contributors to DNA degradation in both terrestrial and oceanic environments, although the effect of these activities in a marine environment is not well understood (Finaughty et al., 2019). The mechanism by which it induces degradation includes the release of nucleases that break down the DNA molecule leading to rapid fragmentation. The activity of microorganisms has been reported to be influenced by other factors such as temperature, salinity and oxygen (O_2) (Latham & Miller, 2019). Given the significantly colder seawater temperatures in Table Bay compared to False Bay, the temperature in Table Bay may have reduced microbial activity and thereby slowed down DNA degradation and prevented the presence of allele drop in.

4.5 Limitations and recommendations

In this study, DNA profiling was performed once for most of the samples across all the environments, and this is a limitation, as artefacts and anomalies were observed in some of the DNA profiles. This included allele drop out, locus dropout and allele drop in, which made interpretation challenging. This was complicated by these samples being low in concentration. In this instance a consensus approach should be applied, which involves generating a composite profile by combining data from three DNA

profile replicates of the same sample in each environment, which may alleviate challenges with profile interpretation. Additionally, buccal swabs should be collected from the donors in the future to serve as a reference sample.

Low DNA concentrations were observed in some samples submerged in Table Bay and False Bay, with only a few successfully amplified loci. This could be improved by exploring the use of a different DNA profiling kit, such as the Promega PowerPlex systems which allows for the addition of 5X AmpSolution® (Promega Corporation, WI, USA) to improve amplification efficiency. The use of next generation sequencing could also be considered in future work. The observation of allele drop in certainly warrants further exploration, such as undertaking shotgun sequencing to identify other species that may be present. This would help researchers understand the cause of the allele drop in and to possibly adapt primer design to avoid non-specific amplification.

4.6 Conclusion

This study aimed to evaluate forensic DNA profiling success from teeth submerged in different ocean locations in South Africa. The results herein showed that the DNA workflow was successfully applied to teeth samples submerged in the ocean and to those what were kept as controls. DNA concentrations and degradation indices were obtained from the majority of the samples, and DNA profiles were obtained from all but three teeth samples from False Bay. These results were an improvement from the few similar published studies, including the research group's former studies. This suggests that the optimisation of the workflow was effective and after validation, it can be applied to relevant forensic casework where bodies wash up from the ocean and where teeth are available.

Overall, the study showed the complexity one may encounter when identifying human remains that may be recovered from the sea, especially with using teeth as a source of DNA. Additionally, the consistent pattern of no significant difference between samples submerged in False Bay and those in Table Bay across all the investigated parameters (such as DNA concentration, DNA degradation and likelihood ratios), suggests that, despite being distinct oceanic environments, both environments had a slightly similar impact on DNA preservation. This further implies that the utilised protocol would be effective and could be reliable when used for forensic casework involving human bodies recovered from either of the two oceans.

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Appendix A

Appendix A1

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

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12 February 2024

HREC REF: 093/2024

A/Prof L Heathfield
Division of Forensic Medicine & Toxicology
Department of Pathology
Email: Laura.heathfield@uct.ac.za
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Dear A/Prof Heathfield

PROJECT TITLE: EVALUATION OF FORENSIC DNA PROFILING SUCCESS ON TEETH THAT HAVE BEEN SUBMERGED IN THE OCEAN (SUB-STUDY - 222/2019)
(MASTER OF PHILOSOPHY IN BIOMEDICAL FORENSIC SCIENCES CANDIDATE : MISS FRANCINA DIMPHO MPHAKA)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review and approval.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is only granted for one year until the 28 February 2025.

Please submit a progress report, using the standardised Annual Progress Report Forms (FHS016) **or** (FHS 017) if the study continues beyond the approval period. Please submit a Standard Closure form (FHS 010) when the study has been completed, this includes after publication or thesis submission and final completion.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

The HREC acknowledge that the student: Miss Francina Mphaka will also be involved in this study.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the HREC REF 093/2024 in all your correspondence.

Yours sincerely

PROFESSOR MARC BLOCKMAN
CHAIRPERSON, FACULTY OF HEALTH SCIENCES HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637. Institutional Review Board (IRB) number: IRB00001938 NHREC-registration number: REC-210208-007

HREC REF 093.2024

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2020), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI), and Declaration of Helsinki (2013) guidelines. The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

HREC REF 093.2024

UNIVERSITY OF CAPE TOWN
IYUNIVESITHI YASEKAPA • UNIVERSITEIT VAN KAAPSTAD

FACULTY OF HEALTH SCIENCES
Human Research Ethics Committee

FHS016: Annual Progress Report / Renewal

HREC office use only (FWA00001637; IRB00001938) This serves as notification of annual approval, including any documentation described below.			
☐ Approved	Annual progress report	Approved until/next renewal date	28.02.2026
☐ Not approved	See attached comments		
Signature Chairperson of the HREC/ Designee		Date Signed	10/2/2025

Note: Please email this form and supporting documents (if applicable) in a combined pdf-file to hrec-enquiries@uct.ac.za.

Please use the latest form found on our website:
http://www.health.uct.ac.za/fhs/research/humanethics/forms

Comments to PI from the HREC

Principal Investigator to complete the following:

1. Protocol information

Date (when submitting this form)	7 February 2025		
HREC REF Number	093/2024	Current Ethics Approval was granted until	28 Feb 2025
Protocol title	Evaluation of Forensic DNA profiling success on teeth that have been submerged in the ocean (MPhil candidate: Dimpho Mphaka)		
Protocol number (if applicable)			
Are there any sub-studies linked to this study?	☐ Yes	☑ No	
If yes, could you please provide the HREC Reference number for all sub-studies? **Note**: A separate FHS016 must be submitted for each sub-study.			
Principal Investigator	A/Prof Laura Heathfield		
Department and email address	Pathology | laura.heathfield@uct.ac.za		

HUMAN RESEARCH ETHICS COMMITTEE
10 FEB 2025
HEALTH SCIENCES FACULTY UNIVERSITY OF CAPE TOWN

1.1 Does this protocol receive US Federal funding?	☐ Yes	☑ No

1.2 If the study receives US Federal Funding, does the annual report require full committee approval? **Note:** Any annual approvals for **Full Committee** review MUST be submitted on the monthly HREC submission dates. (Please send electronic combined copy if for full committee review to hrec-submission@uct.ac.za)	☐ Yes	☑ No

If yes in 1.2 please complete section 1.3 below for invoicing purposes

1.3 Ethics Renewal Fee

Please **(tick ✓)**appropriate box for billing purposes:

Submission Type	*Description*	*New fee (Vat Incl.)*	*tick ✓*
Research funded solely from UCT departmental/ divisional/group budget/self-initiated research	Annual evaluation of research progress report for re-certification	**R0,00**	☐
Non-sponsored student research for degree purposes at UCT/Other Universities & Colleges	Annual evaluation of research progress report for re-certification	**R0,00**	☑
Annual re-certification / Progress report (FHS016 Form)	Clinical Trial & International Grant Funded Research - Annual evaluation of research progress report for re-certification for Full Committee Approval	**R7700,00**	☐
Annual re-certification / Progress report (FHS016 Form)	Clinical Trial & International Grant Funded Research - Annual evaluation of research progress report for re-certification for Expedited review	**R3800.00**	☐
Annual re-certification / Progress report (FHS016 Form)	National grant funded research - Annual evaluation of research progress report for re-certification for Full Committee Approval	**R5000.00**	☐
Annual re-certification / Progress report (FHS016 Form)	National Grant funded research for Annual evaluation of research progress report for re-certification for Expedited review	**R1650,00**	☐

NB: Protocols funded by UCT (e.g. departmental funding / student research) and by certain grant funding organizations (e.g. MRC, NRF, CANSA,) are exempt from these charges.

Please provide details for Invoicing, either complete section 1 or 2 :

1. Invoice billing – Directly to Sponsor

Sponsor's name	
Billing Address of Sponsor:	
Vat Number:	
Contact person	
Telephone number	
Email Address	

2. **Internal Journal Billing:**	
Fund Number:	
Cost Centre Number:	
Account Holder Name:	
Division of Account Holder:	

2. List of documentation included to support this approval where applicable

None

3. Protocol status (tick ✓)

☐	Open Enrolment
☐	Closed to enrolment (tick ✓)
☐	Research-related activities are ongoing
☐	Research-related activities are complete, long-term follow-up only
☑	Research-related activities are complete, data analysis only
☐	Main study is complete but sub-study research-related activities are ongoing
☐	Publication or thesis submitted and final completion?
☐	Study is closed → Please submit a Study Closure Form (FHS010)

4. Enrolment

Number of participants enrolled to date	-
Number of participants enrolled, since last HREC Progress report (continuing review)	-
Additional number of participants still required	-

5. Refusals

Total number of refusals (participants invited to join the study, but refused to take part)	-

6. Cumulative summary of participants

Total number of participants who provided consent	-

Number of participants determined to be ineligible (i.e. after screening)	-
Number of participants currently active on the study	-
Number of participants completed study (without events leading to withdrawal)	-
Number of participants withdrawn at participants' request (i.e. changed their mind)	-
Number of participants withdrawn by PI due to toxicity or adverse events	-
Number of participants withdrawn by PI for other reasons (e.g. pregnancy, poor compliance)	-
Number of participants lost to follow-up. Please comment below on reasons for loss of follow-up.	-
Number of participants no longer taking part for reasons not listed above. Please provide reasons below:	-

7. Progress of study

Please provide a brief summary of the research to date including the overall progress and the progress since the last annual report as well as any relevant comments/issues you would like to report to the HREC:
This study is a laboratory study that assessed forensic DNA recovery from teeth that were deployed in the ocean. The teeth were previously collected from volunteers and deployed in the ocean as part of the umbrella study (HREC: 222/2019). This study only involved analysis of the teeth by the Masters student. Data collection and laboratory component has been completed. Data analyses are underway. There are no issues to report to the HREC.

8. Protocol violations and exceptions (tick ✓ all that apply)

☑	No prior violations or exceptions have occurred since the original approval
☐	Prior violations or exceptions have been reported since the original approval and have already been acknowledged or approved If so, did these occur in the last review period
☐	Unreported minor violations that have occurred since the last review, as well as significant deviations not yet reported, are attached for review

9. Amendments (tick ✓ all that apply)

☑	No Prior amendments have been made since the original approval
☐	Prior amendments have been reported since the last review and have already been approved
☐	New protocol changes/ amendments are requested as part of this continuing review (See note below)

Note: If new protocol changes are being requested in this review, please complete an amendment form (FHS006).
Specific changes in the amended protocol and consent/assent forms must be **bolded**, *italicised* or tracked and all changes must include a rationale.

10. Adverse events

10.1 Please provide below or attach a narrative summary of serious adverse events and/ or unanticipated problems since the last progress report. Please indicate changes made to the protocol and informed consent document(s) as a result (if not already reported to the HREC). Please comment on whether causality to any study procedure or intervention could be established.
None

10.2 Have participants received appropriate treatment/ follow-up/ referral when indicated (e.g. in the case of abnormal or incidental clinical findings, distress or anxiety)?		
☐ Yes	☐ No	☑ Not applicable
If yes, please describe:		

11. Summary of Monitoring and Audit Activities (tick ✓)

11.1 Was this study monitored or audited by an external agency (e.g. SAHPRA, FDA)?		
☐ Yes	☐ No	☑ Not applicable

11.2 Did a Data and Safety Monitoring Board publish a report?		
☐ Yes	☐ No	☑ Not applicable

11.3 If yes, please identify the agency and attach a summary of the findings.					
Agency Name		Report attached	☐ Yes	☐ No	☑ Not applicable
		DSMB report attached	☐ Yes	☐ No	☑ Not applicable

11.4 Has there been any agency, institutional or other inquiry into non-compliance in this study, or any finding of non-compliance concerning a member of the research team?	
☐ Yes	☑ No
If yes, please explain:	

12. Level of risk (tick ✓)

12.1 In light of your experience of this research, please indicate whether the level of risk to participants has:	
☐	Increased
☐	Decreased
☑	Shown no change
If there has been a change, please explain:	

12.2 Please provide a narrative summary of recent relevant literature that may have a bearing on the level of risk.
N/A

13. Insurance

Please confirm that valid no fault insurance is still in place? **(tick ✓)**			
☐ Yes		☑ No	
If yes, please complete the following:			
Insurer's name:			
Policy no.		*Coverage Period:	
For UCT sponsored studies please liaise the Insurance office via fhs.sponsorship@uct.ac.za regarding the required documentation and information required obtain a renewed UCT No-fault Insurance Certificate.			

14. Statement of conflict of interest

Has there been any change in the conflict of interest status of this protocol since the original approval? (tick ✓)	
☐ Yes	☑ No
If yes, please explain and if necessary, attach a revised conflict of interest statement (Section #7 in the New Protocol Application Form FHS013):	

15. Signature

My required signature certifies that the above is complete and correct.			
Signature of PI		Date	7 February 2025

Appendix A2

forestry, fisheries & the environment
Department:
Forestry, Fisheries and the Environment
REPUBLIC OF SOUTH AFRICA
Foretrust Building, Martin Hammerschlag Way, Foreshore, Cape Town, 8001

Reference:
Enquiries: M Attwood & L Swart (permit administrators)
E-mail :researchpermits@dffe.gov.za

OCEANS AND COASTAL RESEARCH AND FISHERIES RESEARCH AND DEVELOPMENT PERMIT TO ENGAGE IN SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT ISSUED IN TERMS OF SECTION 83 OF THE MARINE LIVING RESOURCES ACT, 1998 (ACT NO. 18 OF 1998).

SECTION A

Permit Reference Number: **RES 2023-67**

Permit Holder: Dr Laura Heathfield

Institution: UCT

Validity Period: 1 February 2023 until 31 January 2024

Scientific Investigation or Practical Experiment Authorised: To optimised DNA extraction workflow for human teeth submerged in an open marine environment

Local of activities: Fasle Bay and Table Bay, Western Cape

Authorised vessels: MARE 1. DTC 8057 B

This permit is issued subject to the general conditions in Section B and the special conditions in Section C.

NAME: Dr GJ Cilliers
DIRECTOR: BIODIVERSITY AND COASTAL RESEARCH
DEPARTMENT OF FORESTRY, FISHERIES AND THE ENVIRONMENT
DATE: 03/02/2023

SECTION B: SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT PERMIT CONDITIONS

1. . GENERAL

1.1. This Permit is issued subject to, among others, the following legislation:

1.1.1. Marine Living Resources Act, 1998 (Act No. 18 of 1998) (the Act);
1.1.2. National Environmental Management Act, 1998 (Act No. 107 of 1998), and in particular, the Control of Use of Vehicles in the Coastal Area Regulations (GNR.496 of 27 June 2014);
1.1.3. National Environmental Management Biodiversity Act, 2004 (Act No. 10 of 2004);
1.1.4. National Environmental Management Protected Areas Act, 2003 (Act No. 57 of 2003);
1.1.5. Sea Birds and Seals Protection Act, 1973 (Act No. 46 of 1973);
1.1.6. Prevention of Pollution from Ships Act (Act No. 2 of 1986);
1.1.7. National Environmental Management Integrated Coastal Management Act, 2008 (Act No. 24 of 2008); and
1.1.8. any other relevant law.

1.2. This permit is intended to authorise *bona fide* research: the primary purpose of the use of the permit may not be commercial gain.

1.3. This permit may not be used in marketing materials of the Permit Holders, or in any way which seeks to solicit business for the Permit Holder

1.4. The relevant Delegated Authority shall be entitled to amend these permit conditions when deemed necessary.

1.5. Any reference to the Permit Holder in these permit conditions is a reference to the entity or person that holds the permit; and shall in the case of an entity include the directors; and in the case of either an entity or a person include his/her/its employees (whether permanent, full-time or part-time), his/her/its contractors, agents or advisers, - acting in course and scope of their contractual relationship and in the case of a university shall include authorised students.

2. DOCUMENTATION

2.1. This permit shall only be utilised by the Permit Holder.

2.2. If the permit is in the name of an institution/company/close corporation the individual utilising the permit shall, in addition to a certified copy of this permit, be in possession of identification and a document which letter identifies the individual as being authorised to participate in the authorised activities. [If this does not happen it will be a breach of a permit condition.

2.3. The Permit Holder shall store at their/its registered place of business/residence the original permit issued.

2.4. The Permit Holder shall, at all times, have available a certified copy of the permit, inclusive of the permit conditions, which must be produced on demand by any law enforcement official.

2.5. Where an additional exemption or permit is required under the Act in order to utilse the permit, the Permit Holder must, at all time when acting in terms of the permit, be in possession of a certified copy of the additional exemption and/ or permit issued under the Act.

2.6. The Permit Holder must submit a progress report to researchpermits@dffe.gov.za

3. VALIDITY OF PERMIT

3.1. This permit is valid for the period indicated in section A after which it shall lapse and be of no force or effect.

3.2. This permit shall automatically expire and become invalid should it be cancelled or revoked in terms of section 28 of the Act.

4. COMPLIANCE

4.1. A breach of the provisions of the Act, its regulations and/or these permit conditions by the Permit Holder may result in the initiation of legal proceedings, which may include proceedings in terms of section 28 of the Act and/or criminal proceedings).

4.2. An application for a future permit may be refused if the conditions of this permit are not adhered to and/ or if the applicant is under criminal investigation for an offence in terms of the Act or any other environmental legislation.

4.3. In terms of section 57 of the Act, the Permit Holder is obliged to report to the Minister any contravention of the provisions of the Act by any other person.

5. STORAGE OF WASTE AND REMOVAL OF INSTALLATIONS

5.1. The Permit Holder must safely store all inorganic waste material, garbage and pollutants on board the vessel or at the site of research activities if on land and safety dispose of it according to any relevant laws.

5.2. Any installations/structures must be removed and any adverse impact must be rehabilitated upon termination of the project(s) or expiry of the permit, whichever comes first

6. TREATEMENT OF SPECIMENS

6.1. Specimens collected in terms of this permit shall not be sold or offered for sale.

6.2. No harmful chemicals may be used when collecting marine species.

6.3. Limited use of fish anaesthetics (including rotenone) is permitted if no other suitable technique is available to collect fish, which should be kept to a minimum. Local authorities should be advised when rotenone is to be used to collect fish.

6.4. No specimens collected in terms of this permit may be used for bioprospecting.

7. USE OF VEHICLES

7.1. No vehicle may be used in the coastal zone unless the permit holder is in possession of a valid permit to use a vehicle in the coastal area in terms of the Regulations for the Control of Use of Vehicles in the Coastal Area (GNR 496 of 27 June 2014).

8. SUBMISSION OF INFORMATION

8.1. Reports required in terms of these permit conditions must be submitted to the Directorate: Biodiversity and Coastal Research for the attention of L Swart by email to researchpermits@dffe.gov.za.

8.2. The reports referred to in condition 8.1 must be submitted within the timeframes provided and shall provide the dates, locations, species and quantities collected where applicable.

8.3. Reporting templates provided by the Department must be utilised.

8.4. No renewal of this permit or application for any other permit will be considered unless and until the information required in terms of these permit conditions has been received.

SECTION C: SPECIFIC CONDITIONS

9. SPECIFIC CONDITIONS FOR MARINE PROTECTED AREAS/ HABITATS AND PROTECTED SPECIES

9.1. The human teeth used in the experiment must have the necessary legislative approval and sourced from the Department of Human Biology (University of Cape Town).

9.2. The research may only occur off Miller's Point in False Bay, but outside the Castle Rock restricted area zone of the Table Mountain National Park Marine Protected Area.

9.3. The metal cages used must be securely locked and anchored to the sea floor. The depth of the cages must be at least 15m depth.

9.4. An information sign explaining the reason for the metal cages and providing contact details of the researcher must be attached to each cage.

9.5. The cages must be removed at the end of the project.

9.6. The permit holder must submit a progress report to DEA by end October 2023.

9.7. This permit may not be utilised within a marine protected area as defined in the National Environmental Management Protected Areas Act, 2003. unless specifically authorised in this permit and only in the areas specifically described.

9.8. Collecting of species within Marine Protected Areas is conditional upon prior written permission of the relevant Management Authority.

9.9. The holder must provide the relevant Management Authority with a certified copy of this permit.

9.10. The holder shall inform the relevant Management Authority at least one (1) week prior to undertaking a trip within a Marine Protected Area

forestry, fisheries
& the environment

Department:
Forestry, Fisheries and the Environment
REPUBLIC OF SOUTH AFRICA

Foretrust Building, Martin Hammerschlag Way, Foreshore, Cape Town, 8001

Permit Reference Number:Res2024-10
Enquiries: L. Swart (permit administrator)
E-mail :researchpermits@dffe.gov.za

FISHERIES RESEARCH AND DEVELOPMENT PERMIT TO ENGAGE IN SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT ISSUED IN TERMS OF SECTION 83 OF THE MARINE LIVING RESOURCES ACT, 1998 (ACT NO. 18 OF 1998).

SECTION A

Permit Reference Number: Res2024-10

Permit Holder: Dr Laura Heathfield

Institution: UCT

Validity Period: Date of issue until 28 February 2025

Scientific Investigation or Practical Experiment Authorised: Decomposition studies

Location of activities: False Bay.

This permit is issued subject to the general conditions in Section B and the special conditions in Section C.

NAME: DR GJ CILLIERS
DIRECTOR: BIODIVERSITY AND COASTAL RESEARCH
DEPARTMENT OF FORESTRY, FISHERIES AND THE ENVIRONMENT
DATE: 27/03/2024

SECTION B: SCIENTIFIC INVESTIGATION OR PRACTICAL EXPERIMENT PERMIT CONDITIONS

1. GENERAL

1.1. This Permit is issued subject to, among others, the following legislation:

1.1.1. Marine Living Resources Act, 1998 (Act No. 18 of 1998) (the Act);
1.1.2. National Environmental Management Act, 1998 (Act No. 107 of 1998), and in particular, the Control of Use of Vehicles in the Coastal Area Regulations (GNR.496 of 27 June 2014);
1.1.3. National Environmental Management Biodiversity Act, 2004 (Act No. 10 of 2004);
1.1.4. National Environmental Management Protected Areas Act, 2003 (Act No. 57 of 2003);
1.1.5. Sea Birds and Seals Protection Act, 1973 (Act No. 46 of 1973);
1.1.6. Prevention of Pollution from Ships Act (Act No. 2 of 1986);
1.1.7. National Environmental Management Integrated Coastal Management Act, 2008 (Act No. 24 of 2008); and
1.1.8. any other relevant law.

1.2. This permit is intended to authorise *bona fide* research: the primary purpose of the use of the permit may not be commercial gain.

1.3. This permit may not be used in marketing materials of the Permit Holders, or in any way which seeks to solicit business for the Permit Holder

1.4. The relevant Delegated Authority shall be entitled to amend these permit conditions when deemed necessary.

1.5. Any reference to the Permit Holder in these permit conditions is a reference to the entity or person that holds the permit; and shall in the case of an entity include the directors; and in the case of either an entity or a person include his/her/its employees (whether permanent, full-time or part-time), his/her/its contractors, agents or advisers, - acting in course and scope of their contractual relationship and in the case of a university shall include authorised students.

2. DOCUMENTATION

2.1. This permit shall only be utilised by the Permit Holder.

2.2. If the permit is in the name of an institution/company/close corporation the individual utilising the permit shall, in addition to a certified copy of this permit, be in possession of identification and a document which letter identifies the individual as being authorised to participate in the authorised activities. [If this does not happen it will be a breach of a permit condition.

2.3. The Permit Holder shall store at their/its registered place of business/residence the original permit issued.

2.4. The Permit Holder shall, at all times, have available a certified copy of the permit, inclusive of the permit conditions, which must be produced on demand by any law enforcement official.

2.5. Where an additional exemption or permit is required under the Act in order to utilse the permit, the Permit Holder must, at all time when acting in terms of the permit, be in possession of a certified copy of the additional exemption and/ or permit issued under the Act.

2.6. The Permit Holder must submit a progress report to researchpermits@dffe.gov.za

3. VALIDITY OF PERMIT

3.1. This permit is valid for the period indicated in section A after which it shall lapse and be of no force or effect.

3.2. This permit shall automatically expire and become invalid should it be cancelled or revoked in terms of section 28 of the Act.

4. COMPLIANCE

4.1. A breach of the provisions of the Act, its regulations and/or these permit conditions by the Permit Holder may result in the initiation of legal proceedings, which may include proceedings in terms of section 28 of the Act and/or criminal proceedings).

4.2. An application for a future permit may be refused if the conditions of this permit are not adhered to and/ or if the applicant is under criminal investigation for an offence in terms of the Act or any other environmental legislation.

4.3. In terms of section 57 of the Act, the Permit Holder is obliged to report to the Minister any contravention of the provisions of the Act by any other person.

5. STORAGE OF WASTE AND REMOVAL OF INSTALLATIONS

5.1. The Permit Holder must safely store all inorganic waste material, garbage and pollutants on board the vessel or at the site of research activities if on land and safety dispose of it according to any relevant laws.

5.2. Any installations/structures must be removed and any adverse impact must be rehabilitated upon termination of the project(s) or expiry of the permit, whichever comes first.

6. TREATEMENT OF SPECIMENS

6.1. Specimens collected in terms of this permit shall not be sold or offered for sale.

6.2. No harmful chemicals may be used when collecting marine species.

6.3. Limited use of fish anaesthetics (including rotenone) is permitted if no other suitable technique is available to collect fish, which should be kept to a minimum. Local authorities should be advised when rotenone is to be used to collect fish.

6.4. No specimens collected in terms of this permit may be used for bioprospecting.

7. USE OF VEHICLES

7.1. No vehicle may be used in the coastal zone unless the permit holder is in possession of a valid permit to use a vehicle in the coastal area in terms of the Regulations for the Control of Use of Vehicles in the Coastal Area (GNR 496 of 27 June 2014).

8. SUBMISSION OF INFORMATION

8.1. Reports required in terms of these permit conditions must be submitted to the Directorate: Biodiversity and Coastal Research for the attention of L Swart by email to researchpermits@dffe.gov.za.

8.2. The reports referred to in condition 8.1 must be submitted within the timeframes provided and shall provide the dates, locations, species and quantities collected where applicable.

8.3. Reporting templates provided by the Department must be utilised.

8.4. No renewal of this permit or application for any other permit will be considered unless and until the information required in terms of these permit conditions has been received.

SECTION C: SPECIFIC CONDITIONS

9. SPECIFIC CONDITIONS FOR MARINE PROTECTED AREAS/ HABITATS AND PROTECTED SPECIES

10.1 The human teeth used in the experiment must have the necessary legislative approval and sourced from the Department of Human Biology (University of Cape Town).

10.2 The metal cages used must be securely locked and anchored to the sea floor. The depth of the cages must be at least 15m depth.

10.3 An information sign explaining the reason for the metal cages and providing contact details of the researcher must be attached to each cage.

10.4 The cages must be removed at the end of the project.

10.5 This permit does not allow any sampling in a marine protected area

10.6 No mammals, turtles, marine birds or any TOPS protected marine species may be Collected or disturbed.

10.7 This permit does not allow for any specimens to be used in any way in a bioprospecting context.

10.8 The Permit Holder must submit a progress report to researchpermits@dffe.gov.za By end October 2024.

Appendix A3

Western Cape Government

Inspectorate of Anatomy
Protea Court, Francie van Zyl Drive, Tygerberg, 7505
IOA@westerncape.gov.za | Tel: 021 826 5730

REFERENCE: TRA | 4 | 50 | 0822 | 4
ENQUIRIES: Ms. L. Korasie

DIVISION OF FORENSIC MEDICINE AND TOXICOLOGY
FACULTY OF HEALTH SCIENCES
UNIVERSITY OF CAPE TOWN

Attention: DR L HEATHFIELD

Dear Dr Heathfield

RE: PERMISSION FOR THE TRANSPORT AND USE OF HUMAN TEETH SAMPLES

Permission is granted for the transportation and use of the human teeth samples for a research project, Investigating Forensic DNA Profiling Methods on human teeth exposed to marine environments to improve human identification, HREC REC 434/2022, for the year of 2022 - 2023.

Permission is granted to the 3 researchers to transport the samples to the places as indicated below:

Pieter Johan Truter

- Department of Oceanography, RW James Building, University Avenue, UCT Upper Campus, Woolsack Drive, Rondebosch, Cape Town, South Africa
- Millers Point
- Table Bay

Andrea Plos

- Department of Biology, John Day Building, University Ave, UCT Upper Campus, Woolsack Drive, Rondebosch, Cape Town, South Africa
- Millers Point
- Table Bay

Lisa Malan

- Claremont Life Kingsbury Hospital, 23 Wilderness Rd, Claremont, Cape Town, 7708
- Biomedical Forensic Science Laboratory, Falmouth Building Level 5, Entrance 2, Anzio Road, Observatory, 7925
- Department of Biology, John Day Building, University Ave, UCT Upper Campus, Woolsack Drive, Rondebosch, Cape Town, South Africa
- Department of Oceanography, RW James Building, University Avenue, UCT Upper Campus, Woolsack Drive, Rondebosch, Cape Town, South Africa
- Millers Point
- Table Bay

Yours faithfully

MR RYAN CLAYTON
Health Officer
Provincial Department of Health
Date: 18th August 2022

Appendix A4

Optimisation and investigation of DNA recovery from different biological sample types for use in forensic human identification

Principal Investigator: Dr Laura Heathfield

Information form

We are inviting you to participate in a research study.

What is the research about?

In forensic science, DNA evidence is often used to help identify people. DNA is a molecule found in the cells of all living organisms, including human beings. It contains the genetic information which is inherited from both parents and contains instructions for the body to work. Most of the DNA between people is the same, but there is a small portion that varies between people. In forensics, we are interested in these variable regions because statistically, there is an extremely low chance that two people will share the same combination of variations in their DNA. Identification is achieved by a matching principle, where the 'DNA profile' from a biological sample is a match that of a known individual.

Therefore, if a skeleton is found, we can analyse the DNA from the teeth and bones to help us assess if the skeleton might belong to a missing person. This method of identification relies on creating a 'DNA profile', which is a set of numbers that represent variations in the DNA. By law, the DNA profile itself cannot reveal medical or behavioural information about you.

In forensic science, we are often faced with only a few types of biological samples for DNA analyses. In the context of human remains, these are often the harder tissue types, such as fingernails, toenails, bone and teeth. These samples can be tricky for DNA analyses. Research is needed to improve the methods that we use to recover high amounts and good quality DNA. To do this research, we are in search of teeth from willing volunteers who have undergone tooth removal surgery for sounds medical reasons, so that we can improve our methods.

What are we are asking from you?

To participate in our study, we ask that you provide the following:

- Informed consent
- Fill in a short questionnaire
- Teeth which will be removed in surgery

The teeth that you donate for our research will undergo a process to isolate the DNA from the tooth. The DNA will be analysed in the laboratory using molecular techniques, to generate a DNA profile. During the process of generating DNA profiles, the sample itself will be used up, but the DNA will be stored for the period of the research. You also have a choice if you would like the DNA samples to be kept in the laboratory for other similar research where DNA is needed for forensic genetics research. In this case, the DNA sample will be stored in an access-controlled freezer at UCT for 20 years. Alternatively, your DNA sample will be discarded after this project is finished.

Are there any risks?

There are no risks to participate in this study. The surgical removal of your teeth will not be done for the purposes of this study, but rather for medical reasons. The operation will be explained to you by your medical practitioner. This study will entail the *donation* of the removed teeth for research. There will be no risk of any incidental findings regarding your genetics, as the parts of the DNA which are analysed in this project are not linked to physical information about you, except for your sex. Lastly, participation in this study cannot link you to any crime, whatsoever. This project is completely separate from the South African Police Service.

Are there any benefits?

There are no direct benefits for participating in the study and you will not be compensated for participation. The community at large however, will benefit as these results will allow better DNA evidence to be recovered from this sample type in the future. This will assist with the overall identification of human remains and their reuniting with their families.

Will information be confidential?

Yes, your DNA profile will be kept confidential. Your samples will be allocated a unique participation number, which will be used to identify your samples during processing in the laboratory. No individual name will be linked to specific samples. The results of the study at large will be anonymised when published, and there is no way you could be linked to the data that will be published.

Voluntary participation

Participation is completely voluntary, and your allocated participation number will ensure traceability of your sample. We will not be collecting names, so we will not be able to know whose teeth belong to who. Therefore, if you donate your teeth, it will not be possible to withdraw your sample from the overall results.

Any questions?

If you may have any questions with regards to the rights and welfare of a research subject in the study, please contact the Chairperson of the University Of Cape Town Faculty Of Health Science Human Research Ethics Committee, **Professor Marc Blockman** on (021) 406 6496. If you require any further information about this study please contact **Dr Laura Heathfield** at (021) 406 6569 or email at laura.heathfield@uct.ac.za.

Participant number: _____

Consent form

If you agree to participate, indicate your understanding and consent by marking a tick or cross in each block:

☐ I confirm that I am 18 years or older.

☐ I confirm have read and understand the research information form for the above study and have had the opportunity to ask questions.

☐ I agree to participate in this study and understand that my participation is voluntary.

I consent to:
(Tick the appropriate box)

☐ The use of my DNA and information to be used ONLY for this study and to be destroyed after conclusion of this project.

☐ The use of my DNA and information to be used for this study AND stored for the ONLY purpose of possible future continuation of this specific research, if approved by the human research ethics committee.

☐ The use of my DNA and information to be used for this study AS WELL AS my samples to be stored and used for future forensic genetics research that is approved by the human research ethics committee.

My current age is _____ years old.

I am:

☐ Male

☐ Female

_____ _____

Signature of Witness Date

Table A1: The information for all the teeth samples.

HID	Sample	Sex (F/M)	Age (yrs)	Tooth Collection	Tooth pathology			Tooth types
					Roots exposed	Caveties/crevicese	Pulp visible	
F17	HID_F17				N	N	N	
203	Control	M	21	2019	N	N	N	Mandible
203	False Bay	M	21	2019	N	N	N	Maxilla
203	Table Bay	M	21	2019	Y	N	N	Mandible
179	Control	M	39	2022	N	N	N	Mandible
179	False Bay	M	39	2022	N	N	N	Maxilla
179	Table Bay	M	39	2022	N	N	N	Maxilla
190	Control	M	19	2019	Y	N	N	Maxilla
190	False Bay	M	19	2019	Y	N	N	Mandible
190	Table Bay	M	19	2019	N	N	N	Maxilla
191	Control	F	19	2019	Y	N	Y	Mandible
191	False Bay	F	19	2019	N	N	N	Maxilla
191	Table Bay	F	19	2019	Y	N	N	Mandible
184	Control	F	19	2022	Y	N	Y	Maxilla
184	False Bay	F	19	2022	Y	N	N	Mandible
184	Table Bay	F	19	2022	N	N	N	Maxilla
192	Control	M	20	2019	N	N	N	Maxilla
192	False Bay	M	20	2019	N	N	N	Maxilla
192	Table Bay	M	20	2019	N	N	N	Mandible
193	Control	F	19	2019	Y	N	Y	Mandible
193	False Bay	F	19	2019	N	N	N	Maxilla
193	Table Bay	F	19	2019	Y	N	Y	Mandible
200	Control	F	20	2019	Y	N	N	Mandible

HID	Sample	Sex (F/M)	Age (yrs)	Tooth Collection	Tooth pathology			Tooth types
					Roots exposed	Caveties/crevicese	Pulp visible	
200	False Bay	F	20	2019	Y	N	N	Maxilla
200	Table Bay	F	20	2019	Y	N	N	Mandible
181	Control	F	25	2022	N	N	N	Mandible
181	False Bay	F	25	2022	N	N	N	Maxilla
181	Table Bay	F	25	2022	N	N	N	Maxilla
183	Control	F	35	2019	N	N	N	Mandible
183	False Bay	F	35	2019	N	Y	Y	Mandible
183	Table Bay	F	35	2019	N	N	N	Maxilla
176	Control	?		2022	N	N	N	Maxilla
176	False Bay	?		2022	Y	N	N	Mandible
176	Table Bay	?		2022	N	N	N	Maxilla
185	Control	F	22	2022	N	N	N	Mandible
185	False Bay	F	22	2022	N	N	N	Mandible
185	Table Bay	F	22	2022	N	N	N	Maxilla
178	Control	F	20	2022	Y	N	Y	Maxilla
178	False Bay	F	20	2022	Y	N	Y	Mandible
178	Table Bay	F	20	2022	Y	N	Y	Mandible
187	Control	F	23	2022	N	N	N	Maxilla
187	False Bay	F	23	2022	N	N	N	Mandible
187	Table Bay	F	23	2022	N	N	N	Maxilla
182	Control	M	22	2019	Y	Y	Y	Mandible
182	False Bay	M	22	2019	N	N	N	Maxilla
182	Table Bay	M	22	2019	N	N	N	Maxilla
188	Control	F	23	2022	N	N	N	Maxilla
188	False Bay	F	23	2022	N	N	N	Mandible

HID	Sample	Sex (F/M)	Age (yrs)	Tooth Collection	Tooth pathology			Tooth types
					Roots exposed	Caveties/crevicese	Pulp visible	
188	Table Bay	F	23	2022	N	N	N	Mandible
189	Control	F	19	2022	N	N	N	Mandible
189	False Bay	F	19	2022	N	N	N	Mandible
189	Table Bay	F	19	2022	N	N	N	Maxilla
194	Control	F	30	2019	Y	N	Y	Maxilla
194	False Bay	F	30	2019	Y	N	N	Mandible
194	Table Bay	F	30	2019	Y	N	N	Maxilla
196	Control	F	20	2019	Y	N	Y	Mandible
196	False Bay	F	20	2019	Y	Y	Y	Maxilla
196	Table Bay	F	20	2019	Y	N	N	Mandible
204	Control	M	25	2019	N	N	N	Mandible
204	False Bay	M	25	2019	N	N	N	Mandible
204	Table Bay	M	25	2019	N	N	N	Maxilla
177	Control	F	32	2022	Y	N	N	Maxilla
177	False Bay	F	32	2022	N	N	N	Mandible
177	Table Bay	F	32	2022	N	Y	N	Maxilla
180	Control	F	30	2022	N	N	N	Mandible
180	False Bay	F	30	2022	N	Y	N	Maxilla
180	Table Bay	F	30	2022	N	Y	N	Mandible
201	Control	M	19	2019	N	N	N	Mandible
201	False Bay	M	19	2019	N	N	N	Mandible
201	Table Bay	M	19	2019	Y	Y	Y	Maxilla
197	Control	F	21	2022	N	N	N	Maxilla
197	False Bay	F	21	2022	N	N	N	Maxilla
197	Table Bay	F	21	2022	N	N	N	Mandible

HID	Sample	Sex (F/M)	Age (yrs)	Tooth Collection	Tooth pathology			Tooth types
					Roots exposed	Caveties/crevicese	Pulp visible	
198	Control	F	20	2019	N	N	N	Mandible
198	False Bay	F	20	2019	N	N	N	Maxilla
198	Table Bay	F	20	2019	N	N	N	Maxilla
199	Control	M	21	2019	Y	N	N	Mandible
199	False Bay	M	21	2019	N	N	N	Mandible
199	Table Bay	M	21	2019	N	N	N	Maxilla
202	Control	F	30	2022	N	N	N	Mandible
202	False Bay	F	30	2022	N	N	N	Maxilla
202	Table Bay	F	30	2022	N	N	N	Mandible
175	Control	M	18	2022	N	N	N	Maxilla
175	False Bay	M	18	2022	Y	N	N	Mandible
175	Table Bay	M	18	2022	N	N	N	Maxilla
195	Control	F	19	2022	Y	N	Y	Mandible
195	False Bay	F	19	2022	Y	N	Y	Maxilla
195	Table Bay	F	19	2022	N	N	N	Maxilla
186	Control	M	26	2019	N	Y	Y	Mandible
186	False Bay	M	26	2019	N	N	N	Mandible
186	Table Bay	M	26	2019	N	N	N	Maxilla

Appendix B

Table B1: shows the qPCR results (DNA quantity and quality) and DNA profiling results.

	HID	Sample Name	T.Large Autosomal	T-Small Autosomal	T.Y	Average concentration (ng/µL)	IPC CT	DI	Sex (F/M)	Profiles	
										Likelihood ratio	Error rates
	A. Positive control										
1	F17	Blank_F17					27.64				
2	F17	HID_F17	5.867	4.643		5.255	29.36	0.7914		4.207E+27	2.377E-28
	B. Blank samples (GB= Griding batch; EB= extraction batch) n= 14										
3	203	Blank_GB1_EB1					27.72				
4	190-191 (179)	Blank_GB5_EB2					27.82				
5	200	Blank_GB7_EB3					27.87				
6	181-183	Blank_GB8_EB3					27.58				
7	192-193 (184)	Blank_GB6_EB4	0.000617		0.000603		27.84				
8	176-185	Blank_GB9_EB5			0.000612		27.65				
9	177-180-201	Blank_EB9					27.50				
10	Blank (186)	Blank_EB11					28.22				
11	178-187	Blank_GB10_EB6	0.00029299				27.97				
12	189-194	Blank_GB12_EB7					28.44				
13	196-204	Blank_GB13_EB7					27.97				

14	197-198	Blank_GB14_EB8					27.67				
15	199-202	Blank_GB15_EB8					27.78				
16	Blank (175-195)	Blank_EB10					28.10				
17				Mean			27.87				
18				Median			27.83				
19				Standard deviation			0.26				
20				Standard error			0.07				
21				Minimum			27.50				
22				Maximum			28.44				
	C. Matched control n = 28										
	HID	**Sample Name**	**T.Large Autosomal**	**T-Small Autosomal**	**T.Y**	**Average concentration (ng/µL)**	**IPC CT**	**DI**	**Sex (F/M)**	**Profiles**	
										Likelihood ratio	Error rate
23	175	HID_175_C	0.005383	0.0128	0.018141	0.009104	27.73	2.383	M	1.2985E+26	7.701E-27
24	176	HID_176_C	0.005662	0.0027		0.004188	28.15	0.479	?	##	
25	177	HID_177_C	0.003740	0.0034		0.003567	27.46	0.907	F	2.801E+12	3.570E-13
26	178	HID_178_C	0.013342	0.0395		0.026431	28.17	2.962	F	1.116E+31	8.964E-32
27	179	HID_179_C	0.095692	0.0481		0.071895	28.41	0.503	M	2.842E+31	3.519E-32
28	180	HID_180_C	0.170929	0.1222	0.178706	0.146558	27.74	0.715	F	9.271E+31	1.079E-32
29	181	HID_181_C	6.358552	5.6745		6.016551	28.79	0.892	F	2.056E+31	4.864E-32
30	183	HID_183_C	0.000508	0.0059		0.003180	27.79	11.520	F	2.942E+02	3.399E-03
31	184	HID_184_C	0.004209	0.0091		0.006671	27.84	2.170	F	2.163E+22	4.624E-23
32	185	HID_185_C	0.001361	0.0011		0.001240	27.85	0.822	F	1.120E+04	8.928E-05
33	186	HID_186_C	0.001942	0.0206	0.044144	0.011284	27.67	10.621	M	##	
34	187	HID_187_C	0.054354	0.0969		0.075614	28.07	1.782	F	1.952E+32	5.123E-33

35	189	HID_189_C	0.038599	0.0478		0.043219	27.60	1.239	F	1.614E+33	6.198E-34
36	190	HID_190_C	0.043642	0.0940	0.16355	0.068828	27.56	2.154	M	2.735E+29	3.657E-30
37	191	HID_191_C	0	0		0.000000	28.12	0.000	F	1.111E+01	8.999E-02
38	192	HID_192_C	0.023704	0.0080	0.01572	0.015856	28.34	0.338	M	5.220E+28	1.916E-29
39	193	HID_193_C	0.001670	0.0062		0.003919	27.80	3.693	F	2.764E+16	3.618E-17
40	194	HID_194_C	0.004111	0.0242		0.014159	27.72	5.888	F	6.077E+25	1.645E-26
41	195	HID_195_C	0.034849	0.1008		0.067823	29.57	2.892	F	1.504E+30	6.647E-31
42	196	HID_196_C	0.108307	0.1348		0.121542	27.72	1.244	F	**	
43	197	HID_197_C	0.040692	0.0479		0.044278	28.06	1.176	F	2.334E+30	4.285E-31
44	198	HID_198_C	0.035422	0.0454		0.040405	27.77	1.281	F	4.840E+31	2.066E-32
45	199	HID_199_C	0.254351	0.2767	0.278664	0.265533	28.25	1.088	M	**	
46	200	HID_200_C	0.000983	0.0051		0.003039	27.63	5.182	F	1.408E+03	7.100E-04
47	201	HID_201_C	0.050456	0.0617	0.136923	0.056073	27.76	1.223	M	9.507E+31	1.052E-32
48	202	HID_202_C	0.004637	0.0140		0.009341	28.01	3.029	F	3.489E+26	2.866E-27
49	203	HID_203_C	0.309671	6.0765	0.423771	3.193105	27.89	19.623	M	9.693E+27	1.032E-28
50	204	HID_204_C	0.040105	0.0575	0.098492	0.048807	27.75	1.434	M	4.723E+27	2.117E-28
51				Mean		0.370436	27.97	3.116		8.788E+31	
52				Median		0.033418	27.82	1.358		3.094E+28	
53				Standard deviation		1.257453	0.43	4.272		3.28204E+32	
54				Standard error		0.237636	0.08	0.807		8.772E+31	
55				Minimum		0.000000	27.46	0.000		11.112944556	
56				Maximum		6.016551	29.57	19.623		1.614E+33	
	**** represent samples that were excluded from DNA profiling section entirely; ## represent DNA profiles that showed discordance**										

	D. False Bay n =28										
	HID	Sample Name	T.Large Autosomal	T-Small Autosomal	T.Y	Average concentration (ng/µL)	IPC CT	DI	Sex (F/M)	Profiles	
										Likelihood ratio	Error rate
57	175	HID_175_F	0.004975	0.0062846	0.001375	0.005630	27.50	1.2634	M	5.652E+24	1.769E-25
58	176	HID_176_F	0.003410	0.00069	0.001156	0.002050	28.29	0.2023	?	##	
59	177	HID_177_F	0.011035	0.003262		0.007149	28.00	0.2956	F	4.270E+27	2.342E-28
60	178	HID_178_F	0.000122	0.0026889		0.001405	27.61	22.1133	F	2.542E+08	3.935E-09
61	179	HID_179_F	0.003747	0.006594		0.005171	27.60	1.7598	M	2.425E+28	4.123E-29
62	180	HID_180_F	0.002176	0.001071	0.002324	0.001624	27.79	0.4922	F	2.843E+05	3.517E-06
63	181	HID_181_F	0.000261	0.002296		0.001279	27.58	8.7969	F		
64	183	HID_183_F	0.002922	0.001667		0.002295	27.56	0.5705	F	1.557E+03	6.424E-04
65	184	HID_184_F	0.001394	0.000808		0.001101	28.18	0.5796	F	2.657E+15	3.764E-16
66	185	HID_185_F	0	0		0.000000	27.88	0.0000	F	8.947E+06	1.118E-07
67	186	HID_186_F	0.000886	0.001352	0.000162	0.001119	27.54	1.5260	M	##	
68	187	HID_187_F	0.006883	0.0059042		0.006394	27.85	0.8578	F	5.812E+30	1.721E-31
69	189	HID_189_F	0.000201	0.0005737		0.000387	27.90	2.8528	F	3.502E+15	2.855E-16
70	190	HID_190_F	0.007549	0.006367		0.006958	27.84	0.8434	M		
71	191	HID_191_F	0.076067	0.136267		0.106167	27.86	1.7914	F	2.578E+35	3.878E-36
72	192	HID_192_F	0.150093	0.089909	0.134783	0.120001	27.57	0.5990	M	5.220E+28	1.916E-29
73	193	HID_193_F	0.002461	0.001368		0.001915	27.45	0.5559	F	3.015E+06	3.317E-07
74	194	HID_194_F	0.000863	0.0020217		0.001442	27.91	2.3435	F	2.488E+09	4.019E-10
75	195	HID_195_F	0.000296	0.000555		0.000426	27.84	1.8735	F		
76	196	HID_196_F	0.000577	0.0003748		0.000476	28.01	0.6500	F	**	
77	197	HID_197_F	0.000638	0		0.000638	28.19	0.0000	F	2.612E+05	3.828E-06
78	198	HID_198_F	0.000492	0.0026463		0.001569	27.69	5.3793	F	5.137E+08	1.947E-09

79	199	HID_199_F	0.007062	0.005971	0.008223	0.006517	27.85	0.8455	M	**	
80	200	HID_200_F	0.004137	0.006377		0.005257	27.58	1.5415	F	1.363E+14	7.335E-15
81	201	HID_201_F	0.008802	0.00342	0.011674	0.006111	27.94	0.3885	M	4.429E+31	2.258E-32
82	202	HID_202_F	0.001813	0.0013147		0.001564	27.93	0.7252	F	2.919E+22	3.426E-23
83	203	HID_203_F	0.003195	0.002933	0.002768	0.003064	27.50	0.9180	M	9.232E+16	1.083E-17
84	204	HID_204_F	0.016283	0.0045509	0.012923	0.010417	27.93	0.2795	M	5.920E+27	1.689E-28
85				Mean		0.011004	27.80	2.1444		1.22805E+34	
86				Median		0.001982	27.84	0.8445		3.502E+15	
87				Standard deviation		0.029018	0.23	4.3127		1.50374E+34	
88				Standard error		0.005484	0.04	0.8150		1.063E+34	
89				Minimum		0.000000	27.45	0.0000		1.557E+03	
90				Maximum		0.120001	28.29	22.1133		2.57841E+35	
	** represent samples that were excluded from DNA profiling section entirely; ## represent DNA profiles that showed discordance										
	E. Table Bay n= 28										
	HID	**Sample Name**	**T.Large Autosomal**	**T-Small Autosomal**	**T.Y**	**Average concentration (ng/µL)**	**IPC CT**	**DI**	**Sex (F/M)**	**Profiles**	
										Likelihood ratio	Error rate
91	175	HID_175_T	0.0045784	0.006214	0.003054	0.005396	27.68	1.3573	M	3.818E+10	2.619E-11
92	176	HID_176_T	0.0007700	0.001089		0.000930	27.66	1.4143	?	##	
93	177	HID_177_T	0.0070120	0.008599		0.007806	27.62	1.2263	F	2.086E+24	4.793E-25
94	178	HID_178_T	0.0004364	0.000655		0.000546	27.99	1.5014	F	1.640E+10	6.097E-11
95	179	HID_179_T	0.0028470	0.002971		0.002909	27.66	1.0436	M	2.364E+22	4.231E-23
96	180	HID_180_T	0.0044560	0.001992	0.003483	0.003224	27.66	0.4470	F	1.774E+07	5.637E-08

97	181	HID_181_T	0.0079320	0.003954		0.005943	27.91	0.4985	F	3.848E+30	2.599E-31
98	183	HID_183_T	0.0012650	0.001959		0.001612	27.90	1.5486	F	5.536E+06	1.807E-07
99	184	HID_184_T	0	0.000542	0.001211	0.000542	27.55	0.0000	F	2.385E+01	4.193E-02
100	185	HID_185_T	0.0005860		0.000457	0.000586	27.86	0.0000	F	1.239E+06	8.068E-07
101	186	HID_186_T	0.0150420	0.009468	0.016747	0.012255	27.69	0.6294	M	##	
102	187	HID_187_T	0.0013226	0.002038		0.001680	27.54	1.5407	F	2.057E+13	4.861E-14
103	189	HID_189_T	0.0039959	0.001339		0.002667	27.76	0.3350	F	1.552E+30	6.442E-31
104	190	HID_190_T	0.0009150	0.000429		0.000672	27.83	0.4689	M	3.777E+07	2.648E-08
105	191	HID_191_T	0.0002550	0.000975		0.000615	28.21	3.8235	F	3.866E+07	2.586E-08
106	192	HID_192_T	0.0097980	0.001845	0.003989	0.005822	28.31	0.1883	M	3.713E+25	2.693E-26
107	193	HID_193_T	0.0002540	0		0.000254	27.77	0.0000	F	4.222E+00	2.369E-01
108	194	HID_194_T	0.0011716	0.001519		0.001345	27.83	1.2963	F	4.907E+18	2.038E-19
109	195	HID_195_T	0.0012603	0		0.001260	27.98	0.0000	F	6.288E+08	1.590E-09
110	196	HID_196_T	0	0		0.000000	27.97	0.0000	F	**	
111	197	HID_197_T	0.0847556	0.028992		0.056874	28.06	0.3421	F	2.334E+30	4.285E-31
112	198	HID_198_T	0.0001209	0.000249		0.000185	28.16	2.0583	F	2.384E+07	4.194E-08
113	199	HID_199_T	0.0009964	0.001815	0.000916	0.001406	27.44	1.8214	M	**	
114	200	HID_200_T	0	0.000723		0.000723	27.81	0.0000	F	8.600E+07	1.163E-08
115	201	HID_201_T	0.0100180	0.018220	0.008549	0.014119	27.50	1.8187	M	5.224E+30	1.914E-31
116	202	HID_202_T	0.0039395	0.002300		0.003120	27.82	0.5838	F	6.237E+26	1.603E-27
117	203	HID_203_T	0.0156250	0.008866	0.011892	0.012246	27.75	0.5674	M	5.633E+31	1.775E-32
118	204	HID_204_T	0.0067636	0.003933	0.007458	0.005349	28.10	0.5816	M	3.250E+27	3.077E-28
119				Mean		0.005360	27.82	0.8962		2.887E+30	
120				Median		0.001646	27.81	0.5827		1.031E+13	

121				Standard deviation	0.010831	0.22	0.8706		1.146E+31	
122				Standard error	0.002047	0.04	0.1645		3.064E+30	
123				Minimum	0.000000	27.44	0.0000		4.2216	
124				Maximum	0.056874	28.31	3.8235		5.633E+31	
	**** represent samples that were excluded from DNA profiling section entirely; ## DNA profiles that showed discordance**									

Table B2: Shows the average water temperature between False Bay and Table Bay.

Days	Time (False Bay)	False Bay temperature (°C)	Time (Table Bay)	Table Bay temperature (°C)
1	11:00 to 23:00	15.91	14:00 to 23:00	10.59
2	00:00 to 23:00	18.77	00:00 to 23:00	9.8
3	00:00 to 23:00	18.47	00:00 to 23:00	9.81
4	00:00 to 23:00	14.69	00:00 to 23:00	10.39
5	00:00 to 23:00	10.68	00:00 to 23:00	10.76
6	00:00 to 23:00	11.42	00:00 to 23:00	11.03
7	00:00 to 23:00	18.86	00:00 to 23:00	10.38
8	00:00 to 23:00	17.66	00:00 to 23:00	10.31
9	00:00 to 23:00	14.39	00:00 to 23:00	11.34
10	00:00 to 23:00	12.45	00:00 to 23:00	10.8
11	00:00 to 23:00	11.4	00:00 to 23:00	10.94
12	00:00 to 23:00	12.04	00:00 to 23:00	11.4
13	00:00 to 23:00	11.01	00:00 to 23:00	12.02
14	00:00 to 23:00	11.44	00:00 to 23:00	13.64
15	00:00 to 23:00	11.35	00:00 to 23:00	14.19
16	00:00 to 23:00	14.32	00:00 to 23:00	14.82
17	00:00 to 23:00	16.11	00:00 to 23:00	13.09
18	00:00 to 23:00	15.93	00:00 to 23:00	13.23
19	00:00 to 23:00	15.18	00:00 to 23:00	12.97
20	00:00 to 23:00	14.74	00:00 to 23:00	11.5
21	00:00 to 09:00	15.09	00:00 to 11:00	11.43
Mean		14.38		11.64
Median		14.69		11.34
Standard deviation		2.699		1.457
Minimum temperature (°C)		10.68		9.8
Maximum temperature (°C)		18.86		14.82

Table B3: Qubit quantification results of all the blanks

	HID	Sample Name	Reading 1	Reading 2	Reading 3
1	F17	Blank_F17	-	-	-
2	203	Blank_GB1_EB1	-	-	-
3	190-191 (179)	Blank_GB5_EB2	-	-	-
4	186-200	Blank_GB7_EB3	-	-	-
5	181-183-186	Blank_GB8_EB3	-	-	-
6	192-193 (184)	Blank_GB6_EB4	-	-	-
7	176-185-186	Blank_GB9_EB5	-	-	-
8	Demin 177-180-201	Blank_Demin_EB9	-	-	-
9	177-180-201	Blank_EB9	-	-	-
10	175-178-187	Blank_GB10_EB6	-	-	-
11	175-182-188	Blank_GB11_EB6	-	-	-
12	175-189-194	Blank_GB12_EB7	-	-	-
13	195-196-204	Blank_GB13_EB7	-	-	-
14	195-197-198	Blank_GB14_EB8	-	-	-
15	195-199-202	Blank_GB15_EB8	-	-	-
16	Blank (175,195)	Blank_EB10	*		
17	Blank(186)	Blank_EB11	*		

- re**present out of range (too low); *** the samples did not undergo qubit quantification.

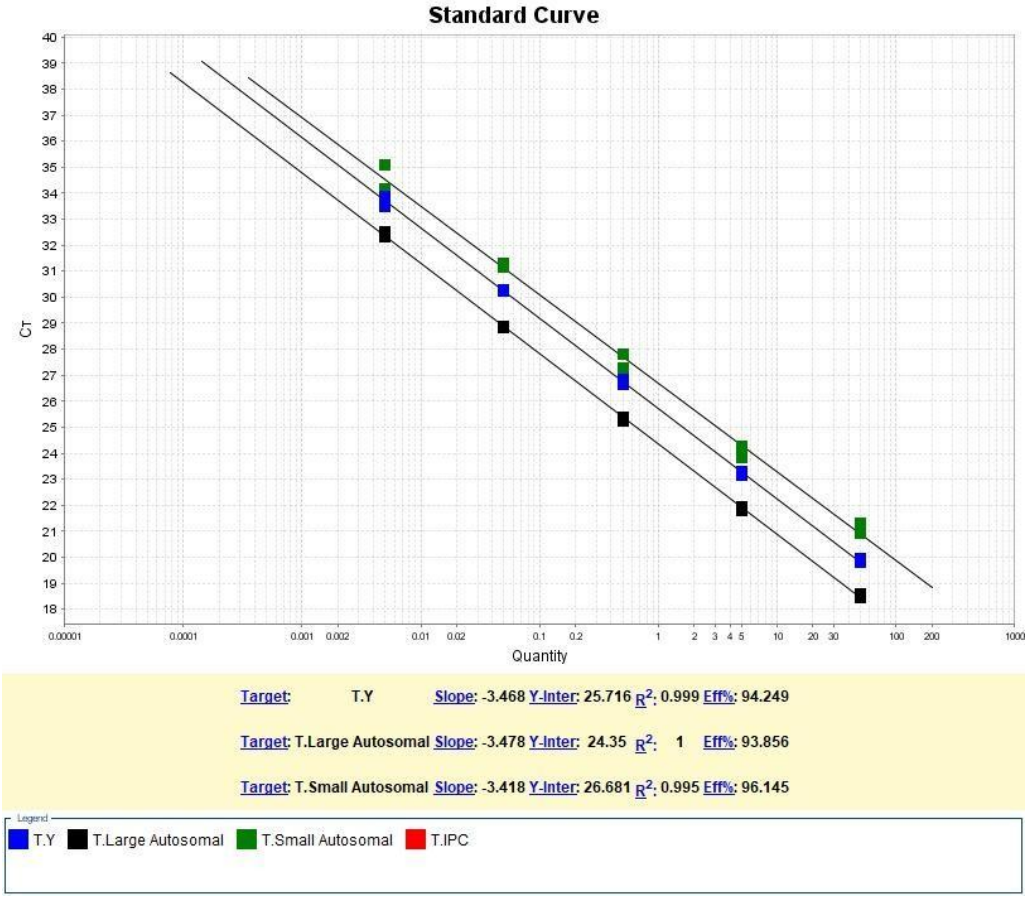

Figure B1: Standard curve showing serially diluted DNA standards concentrations plotted against Ct values.

Table B4: Illustrates a summary of the qPCR quality parameters.

	Large autosomal target			Small autosomal target			Y-target		
Experiment run	**Slope**	**R2**	**Efficiency (%)**	**Slope**	**R2**	**Efficiency (%)**	**Slope**	**R2**	**Efficiency (%)**
Run 1	-3.557	1	91.031	-3.437	0.989	95.42%	-3.484	0.997	93.661
Run 2	-3.594	0.877	89.779	-3.36	0.82	98.439	-3.581	0.904	90.204
Run 3	-3.478	1	93.856	-3.418	0.995	96.145	-3.468	0.999	94.249

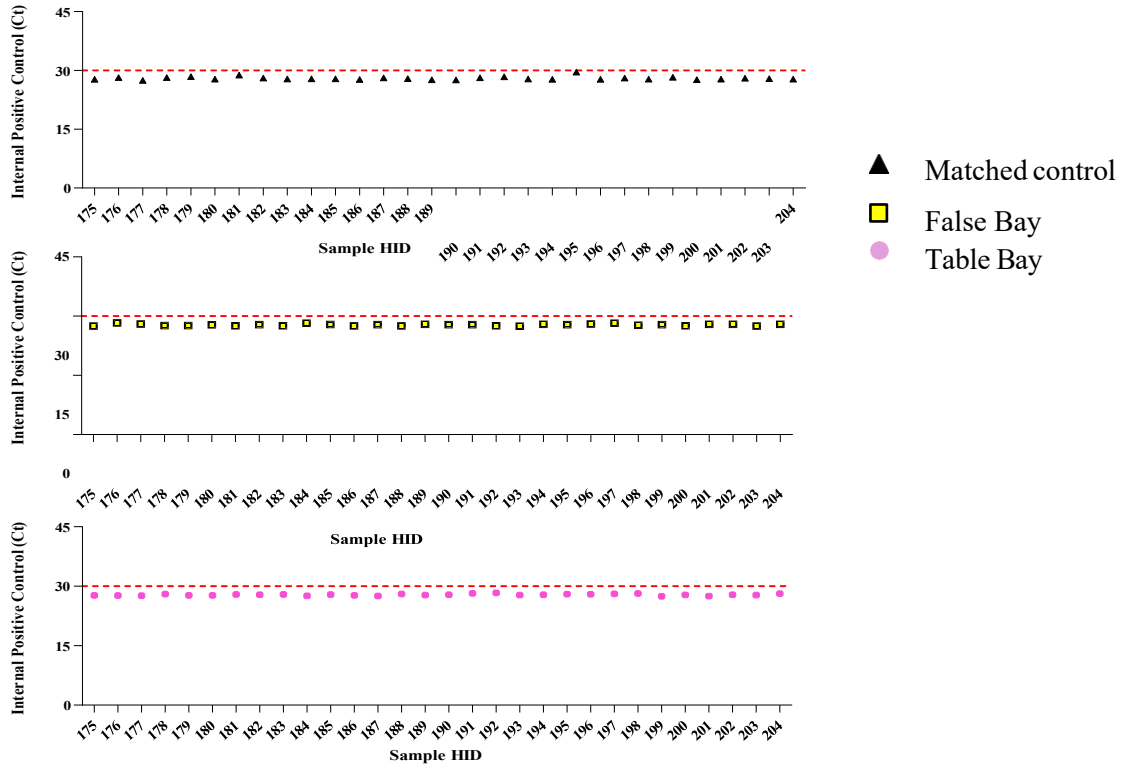

Figure B2: Scatter plots representing the IPC Ct cycles between different environments. The red dotted line represents the cutoff Ct value of approximately 30 cycles for the IPC.

Table B5: List of off-ladders observed on DNA profiles from samples exposed to different environments.

Sample ID	Loci	Allele	Matched control	False Bay	Table Bay	Artefact
175	D10S1248	10	1			True peak
176	TPOX	8.8	1			Novel peak
	D12S391	19.3	1			True peak
178	D8S1179	6	1			Pull up
		8	1			Pull up
	D2S441	11.2	1			Pull up
		13.3	1			Pull up
	D19S433	14.2	1			Pull up
	D22S1045	9.3	1			Pull up
	D10S1248	16.2	1			Pull up
	Yindel	1.1	1			Pull up
179	D21S11	25	1			Pull up
	THO1	3	1			Pull up
	D22S1045	19.2	1			Pull up
	D2S1338	11.2	1			Pull up
181	D1S1656	10.3	1			Pull up
	D12S391	15.3	1			Pull up
186	D16S539	5.1	1			Novel peak
187	D8S1179	7	1			Pull up
		8	1			Pull up
191	D5S818	17.2		1		Pull up
197	D8S1179	8.3	1			Pull up
	D5S818	15.1	1			Pull up
	D13S317	15.1	1			Pull up
	SE33	8	1			Pull up
198	D8S1179	9	1			Pull up
	D22S1045	13.3	1			Pull up
	Amelogen	OL	1			Pull up
200	D2S441	11		1		True peak
	D10S1248	12		1		Allele drop in
201	D5S818	8.2	1			Pull up
		17.3		1		Pull up
202	D2S441	OL		1		Pull up
		OL			1	Pull up
		8			1	Pull up
		9.2			1	Pull up
		10.2			1	Pull up
204	D8S1179	7	1			Pull up
		8	1			Pull up

Table B6: Allele drop out and locus drop-out observations from DNA profiles of samples exposed to different environments.

	Allele drop out observations across 24 loci			Locus drop out observations across 24 loci		
Sample ID	**Matched control**	**False Bay**	**Table Bay**	**Matched control**	**False Bay**	**Table Bay**
175	4/24	11/24	2/24	5/24	5/24	16/24
177	6/24	6/24	7/24	14/24	4/24	5/24
176	8/24	10/24	1/24	4/24	8/24	22/24
178	0/24	5/24	7/24	2/24	16/24	15/24
179	0/24	8/24	6/24	2/24	3/24	6/24
180	2/24	4/24	4/24	0/24	19/24	16/24
181	0/24	0/24	4/24	2/24	24/24	2/24
183	1/24	7/24	4/24	21/24	15/24	15/24
184	5/24	6/24	1/24	7/24	13/24	22/24
185	3/24	2/24	5/24	20/24	18/24	17/24
186	5/24	11/24	1/24	7/24	16/24	1/24
187	0/24	5/24	8/24	2/24	2/24	13/24
189	0/24	7/24	10/24	2/24	12/24	3/24
190	1/24	0/24	4/24	0/24	24/24	17/24
191	1/24	0/24	5/24	23/24	2/24	18/24
192	1/24	1/24	8/24	1/24	1/24	2/24
193	2/24	5/24	1/24	11/24	17/24	23/24
194	3/24	4/24	5/24	3/24	17/24	11/24
195	0/24	0/24	7/24	2/24	24/24	17/24
197	0/24	4/24	0/24	2/24	18/24	2/24
198	0/24	5/24	5/24	2/24	16/24	16/24
200	3/24	4/24	3/24	20/24	10/24	18/24
201	0/24	4/24	6/24	0/24	0/24	1/24
202	3/24	6/24	7/24	3/24	7/24	3/24
203	6/24	10/24	2/24	5/24	9/24	1/24
204	1/24	0/24	2/24	0/24	0/24	0/24
Total number of samples with artefact observation	17	21	25	22	24	25

Table B7: Allele drop-in observed in three DNA profiles of samples submerged in False Bay. Bold alleles in brackets represent allele drop in.

Sample ID	**Locus**	**Genotype**		
		Matched control	**False Bay**	**Table Bay**
HID_176	D2S441	10, ?	10, 14 (**11**)	10, ?
HID_200	D10S1248	locus drop out	13, 16 (**12**)	13, ?
HID_201	TPOX	8, 12	8, 12 (**11**)	8, 12
	SE33	19, 29.2	19, 29.2 (**28.2**)	19, 29.2

Table B8: Illustrates loci that showed discordance in two set of samples. Bold allele represents the discordant allele, "?" represents allele drop out and "*" represents locus drop out. Replicated samples are represented by "i " and "ii".

	Loci											
Samples ID	D13S317 Allele 1	D13S317 Allele 2	D18S51 Allele 1	D18S51 Allele 2	D19S433 Allele 1	D19S433 Allele 2	vWA Allele 1	vWA Allele 2	TH01 Allele 1	TH01 Allele 2	D7S820 Allele 1	D7S820 Allele 2
HID_176_C	11	14			14	15	?	19			?	10
HID_176_F_i	11	?			*		18	?			9	?
HID_176_F_ii	11	**12**			**13**	?	**17**	?			?	**14**
HID_176_T	*				*		*				*	
HID_186_C	8	11	?	14	12	13			6	9.3		
HID_186_F_i	?	**12**	*		?	**14.2**			*			
HID_186_F_ii	?	**12**	**10.2**	?	12	**14.2**			?	**7**		
HID_186_T	8	11	12	14	12	13			6	9.3		

Appendix C

Table C1: List of studies showing DNA concentrations from teeth samples that were submerged and recovered in different environments.

No.	Author and year of publication	Type of quantification	Sample type	DNA concentration (units)	Type of environment
1	Kumar, Chauhan & Sharma, 2019.	qPCR	Human teeth	1.22 ng/µLto 2.45 ng/µL	River bed
2	Hughes-Stamm, 2012	qPCR	Human teeth	0.10 ng and 0.09 ng 0.48 ng and 0.64 ng	Saltwater tank Freshwater tank
3	Vemuri et al., 2012	Nanodrop Spectrophotometer	Human teeth	23 ug/ml to 34 µg/ml	Sea water
4	Iroanya & Onuoha, 2022	Nanodrop spectrophotometer	Human teeth	152.83 ng/µL and 255.4 ng/µL 40 ng/µLand 203.17 ng/µL 2.2 ng/µL and 51 ng/µL	Lagoon water