Effects of Different Types of Water on the Degradation Rate of Human DNA in Bone and Tissue

Ema Graham
Forensic Science Department/ Forensic Science and Biology Major
Michael Adamowicz, Ph.D.

Abstract
Human remains that have been subjected to aqueous environments for periods of time are often used for DNA analysis of the tissue and bone for identification purposes. This has posed a problem for investigators in the past due to the degradation and loss of DNA in the aqueous environmental conditions. The purpose of this research was to determine the quantity of viable DNA that can be obtained from human bone and tissue after a 72-hour period of immersion and whether or not a DNA profile can be made. Also, this research studied how different types of water environments such as saltwater, swamp water, or freshwater affect the amount of DNA loss and degradation over the set period of time. In this study human bone and tissue samples were placed in three aqueous environments (saltwater, swamp water, and freshwater) and allowed to incubate for 72-hours. The DNA was extracted, quantified, amplified, and analyzed. The degradation and loss of DNA was studied for each sample of bone and tissue in comparison to a control sample that was not placed in water. It was found that there was significant DNA degradation and loss in both tissue and bone samples that were immersed in water for 72 hours. The bone samples showed on average a ~10,000-fold reduction of detectable DNA. The bone sample that was immersed in saltwater showed such extensive DNA degradation and loss that it was unable to even detect any viable DNA at all. As for the tissue there was significant DNA loss as well. For the control sample (dry sample) there was little to no DNA loss; ~341.8 ng/µL of DNA detected. The tissue samples showed much less detectable DNA than the control sample; ~7.31 ng/µL (freshwater), ~0.77 ng/µL saltwater, and ~3.66 ng/µL swamp water. These findings were consistent with the data collected in a previous study, and support the theory that there is considerable DNA loss and DNA degradation after 24 hours of exposure.

Introduction

In areas along the shore or near larger bodies of water it is not uncommon for forensic investigators to find human remains that have been submerged. When remains are found submerged in water, investigators rely heavily on DNA to help in the identification process. In situations such as national disasters involving water or large accidents, such as a plane crash or a boat sinking, it is vital for the remains of the victims to be identified. On March 8th, 2014 Malaysia Airlines Flight 370 went missing. It has since been theorized that the plane had crashed somewhere in the ocean but the remains of the plane and the victims have yet to be found. When the wreckage is discovered, especially considering the intensity of the crash, the bodies of the victims will be highly decomposed and battered. It will be very difficult to identify the remains of the victims by pure visual identification. Investigators will rely on different methods of identification, such as DNA analysis to try to identify the remains of the victims. Other incidents with mass victims, such as the Tsunami in Indonesia on December 26th 2004, and Hurricane Katrina in August of 2005 required the timely identification of the remains. DNA identification of victims was utilized. The exposure to long periods of immersion made DNA analysis difficult.

When remains are exposed to aqueous conditions for periods of time the soft tissue begins to detach from the bone and is either consumed by organisms living in the environment, taken away by currents, or is decomposed. Since there is such a low chance of there being viable soft tissue on remains that have been submerged in water for long periods of time investigators largely rely on DNA analysis from skeletal remains.

After DNA is extracted a forensic DNA analyst will perform an amplification process on the DNA called *Polymerase Chain Reactions*, also commonly known as PCR. PCR essentially acts as a highly efficient copy machine for DNA to make multiple copies of DNA so that it can undergo further testing and analysis.

If there is not enough viable DNA in a sample then PCR cannot be performed and DNA analysis is not viable. When bodies are subjected to water the amount of DNA in skeletal and soft tissues such as skin that is available for PCR is decreased overtime due to many different factors.

"DNA degradation results from strand breakage, chemical modifications, and microbial attack. These degradative processes reduce the yield of high molecular mass DNA molecules and increase the chance of subsequent PCR failure" [1]. Of these many factors that lead to DNA degradation, one of the biggest factors in aqueous environments is damage due to hydrolysis, or the breakage of chemical bonds through the addition of water [1]. When hydrolysis occurs it can result in damage to the DNA, which is referred to as *deamination* (when there is a loss of a amine group), *depurination* (when there is a loss of an adenine and guanine group), and or *depyrimidination* (when there is a loss of thymine and cytosine) [1]. Deamination, depurination, and depyrimidination will result in damage to the DNA and inhibit the PCR process. DNA has a high affinity to water and even after death DNA in dead tissues will continue to attract water molecules. When deceased bodies are submerged in large amounts of water for long periods of time, there is a high chance of damage due to hydrolysis. Hydrolysis does not only happen in soft tissues but it also can occur in skeletal material as well. Water can enter bone through a process called *bone dissolution*. As this

occurs the pores of the skeletal material "become larger and allow for hydraulic flow, leading to a greater loss of bone material. The greater the dissolution of the inorganic component of the bone, the greater the chance of DNA loss as the DNA molecules dissociate from the protection of the hydroxyapatite" [1]. Due to hydrolysis that occurs in bone and soft tissues DNA can become damaged and unable to be used for further investigation and analysis.

Other factors besides hydrolysis can affect DNA and cause it to degrade. One of those factors is microbial interaction with DNA. Organisms will eat away at deceased bodies and speed up the decomposition rate. Since organisms and many bacteria thrive in aqueous environments, more so than dry environments, DNA decomposition due to organisms and bacteria is a huge factor in the rate in which DNA decomposes in aqueous environments.

Materials and Methods

Human rib samples were collected from the Yale School of Medicine Department of Pathology (New Haven, CT). Each rib sample was cut to be 1-1½ inches long and placed in a plastic container and transported back to the Forensic DNA Laboratory at the University of New Haven. The tissue samples were then placed in a labeled Ziploc bag and stored at -20°C until needed.

Water samples were collected from the New Haven Sound. The water was then taken back to the lab and the salinity of the water was measured using a portable refactometer. The salinity of the water from the Long Island Sound was approximately 20 parts per thousand (ppt). The average salinity of natural salt water is approximately 35 ppt [2]; therefore, Instant Ocean® Sea salt was added to the Sound water to increase its salinity to 35 ppt. As for natural salt fen water, or swamp water, the average salinity in the wild is 10 ppt. Thus, water from the Long Island Sound water collection was diluted down to 10 ppt, with the use of deionized water, to mimic the salinity of swamp water. Fresh water was collected from a local fresh water lake, whose salinity was 0 ppt. All water samples (fresh water, salt water, and swamp water) salinities and pHs were measured before the start of the experiment. The salinity of the water samples were measured using a portable refractometer and the pH of the water samples was measured using a Corning Pinnacle 530 digital pH meter (Woburn, MA).

A fume hood along with three 2000 mL beakers and a 200 mL beaker were wiped down with 20% bleach and then the detergent Conflikt®. The three 2000 mL beakers were labeled fresh water, swamp water, and salt water. The 200 mL beaker was labeled the control. The beakers (excluding the control beaker) were filled with approximately 1200 – 1400 mL of fresh water, swamp water, and salt water. Each beaker was then aerated using an air stone and a pump system.

Five human tissue samples were removed from the -20°C freezer and allowed to thaw to room temperature. Pictures and weights of each rib sample were noted and recorded. A tissue sample was placed into each of the beakers, including the control beaker. The control beaker was then covered using parafilm paper. The fifth tissue sample was set aside and placed back in the freezer. This tissue sample was then later on used as a reference sample to ascertain the starting quantities of DNA in the tissue and bone samples. The samples were allowed to incubate in the water for an allotted period of time, 72 hours, with water changes every 24 hours.

When the allotted time period had elapsed the tissues were removed from the beakers and were photographed and weighed. The control sample and the reference sample (that was placed back in the freezer at the beginning of the experiment) were also photographed, weighed and processed. All five rib samples were then completely defleshed using a scalpel and approximately 1 g of soft tissue was collected and placed in a labeled 1.5 mL microfuge tube. These tubes were then immediately placed in a -20°C freezer until needed. The remaining tissue was discarded in the biohazard waste bags located in the laboratory.

The defleshed bone samples were photographed and weighed. Their mass and description were noted and recorded. The bones were then fragmented using a hammer and chisel. The spongy bone was then removed off of the cortical bone using a scalpel. Approximately 0.5 g – 1.0 g of cortical bone was then pulverized under liquid nitrogen using a SPEX SamplePrep 6770 Freezer/Mill® cryogenic impact grinder (Metuchen, NJ). The SPEX SamplePrep 6700 Freezer/Mill® cryogenic impact grinder cycle settings that were utilized were 10 minutes of pre-cooling, three cycles of alternating grinding at a rate of 10cps for 2 minute intervals and cooling for 2 minute intervals. The bone powder produced by the Freezer/Mill was then placed in labeled 2 mL microfuge tubes and then placed in a -20°C freezer until needed.

To extract the DNA from all of the bone and tissue samples the microfuge tubes containing the soft tissue and bone samples (including the reference and control samples) were removed form the freezer and allowed to thaw to room temperature. 0.05 g of soft tissue was removed and placed in a clean and labeled microfuge tube. The remaining tissue was placed back in the freezer and the 0.05 g tissue samples were used for DNA extraction. DNA was extracted from the tissue samples using the "isolation of total DNA from tissues" protocol from the Qiagen QIAamp® DNA Investigator Handbook [3].

0.3 g – 0.7 g of bone powder was decalcified using 0.5 M EDTA at a pH of 8.0 for 16 – 24 hours with continuous shaking at 25 – 26 °C. Once decalcified, the microfuge tubes containing the bone powder were centrifuged at 8000 rpm for 1 minute. The supernatant was discarded and 1 mL of deionized water was added to each sample. After the addition of water the tubes were inverted and flicked to re-suspend the bone powder. The samples were then centrifuged again for 1 minute at 8000 rpm. The process of discarding the supernatant, adding 1 mL of water, re-suspending the powder, and then centrifuging the samples was repeated two more times. The procedures that were utilized to declassify the bone powder were those that

are employed by the Connecticut Department of Emergency Services and Public Protection in the Division of Scientific Services for the Forensic Laboratory [4]. The DNA from the decalcified bone powder was then extracted using the "isolation of total DNA from tissues" protocol outlined in the Qiagen QIAamp® DNA Investigator Handbook [3].

The DNA extracts from the bone and tissue samples were then quantified using the Quantifilier™ Human DNA Quantification Kit from Applied Biosystems [5]. The following series of dilutions of known human DNA were used to produce a standard sizing curve for quantification: 50 ng/µL, 16.67 ng/µL, 5.56 ng/µL, 1.85 ng/µL, 0.62 ng/µL, 0.21 ng/µL, 0.068 ng/µL, and 0.023 ng/µL. A master mix containing 10.5 µL per reaction of Primer mix and 12.5 µL per reaction of Reaction mix was made. 2 µL of each standard, sample, and negative control (DNAase free H_2O) were pipetted out into individual wells in a 96-well plate. 23 µL of the master mix was then added to each well containing sample, standard or a control. The well plate was then sealed using optically clear plate tape and then centrifuged for a minute to eliminate any bubbles that were present at the bottom of the wells. The plate was then placed in an Applied Biosystems® 7500 Real-time PCR System (Foster City, CA). The samples were then quantified and the results were analyzed using the 7500 System SDS Software (Foster City, CA). The settings that were used for analysis were a 0.2000 Threshold, Manual Ct and Autobase line for all reactions. The slope of the standard curve was checked and had to be close to -3.32 with a R^2 value greater than 0.98 or the run was rejected and the quantification process was preformed again.

From the results obtained from quantification, dilutions for the DNA extracts were calculated so that the mass of DNA was 1 ng. The appropriate dilutions for each sample were pipetted out, including the negative control. Each sample was then amplified using the Promega PowerPlex® 16HS Kit (Madison, WI). A master mix was made that contained 5 µL per reaction of 5X master mix and 2.5 µL per reaction of primer pair mix. PCR tubes were obtained and 7.5 µL of master mix and the appropriate volume of sample and water were pipetted into the PCR tube to achieve a total volume of 25 µL. The samples were then amplified in the Applied Biosystems® GeneAmp PCR System 9700 thermal cycler (Foster City, CA). All samples, including the negative control, were run for 30 cycles and the recommendations for amplification from the manufacturer of the PowerPlex® 16HS Kit were used [6].

To prepare the amplified samples for injection, 9.5 µL of Hi-Di™ formamide and 0.5 µL of internal lane standard (ILS600) were pipetted into individual wells of a clean 96-well plate. For each module, 1 µL of allelic ladder was placed into two of the wells. Then in the remaining wells 0.5 µL to 1 µL of amplified sample products was added. Any of the remaining wells that were not being used (did not contain allelic ladder nor amplified sample) were filled with 10 µL of Hi-Di™ formamide. A rubber septum was placed on the well plate in order to seal it, and then the well plate was centrifuged to bring all samples to the bottom of each well. The 96-well plate was then placed in the Applied Biosystems® GeneAmp PCR System 9700 thermal cycler (Foster City, CA) for 6 minutes to denature the samples. After denaturation the 96-well plate was then placed in the Applied Biosystems® Prism 3130xl Genetic Analyzer (Foster City, CA) to separate and detect each sample. Each injection was run for five seconds at 3kV following the manufacturer's recommended settings.

The data from the samples that were separated using the Applied Biosystems® GeneAmp PCR System 9700 thermal cycler (Foster City, CA) was analyzed and edited using the Applied Biosystems® Genemapper ID v.3.2.1. software (Foster City, CA). All the electropharagrams were assessed and edited to eliminate allelic drop out, allelic drop in, and artifacts. The parameters for analysis were set at a minimum peak height of 50 relative fluorescent units (RFU) for the blue, green, yellow, and red channels. The sizing algorithm used was the Local Southern method. "The data from the electropharagrams was assessed based on the number of correct alleles present, the number of loci that has a 70% or more peak height balance and how the average peak height between the smallest locus (D3S1358) and the largest locus (FGA) differed."[7].

Results

Significant DNA loss was observed in the bone samples treated in all three water environments. The starting quantity of DNA in the bone (at time zero) was ~36.02 ng/µL. ~0.003 ng/µL of DNA was detected for bone samples that were incubated in freshwater for 72-hours. This was a significant loss of DNA; ~10,000 fold. ~0.02 ng/µL of DNA was detected for bone samples that were incubated in saltwater for 72-hours; ~10,000 fold. No detectable DNA was found for bone samples incubated in swamp water. The time control bone sample (incubated dry) exhibited some DNA loss, but it was not as significant as the values of the bone samples that were placed in water; ~1.12 ng/µL of DNA (~36 fold).

The tissue from the rib samples closely resembled the findings from that of the bones. The control tissue sample (dry) yielded ~341.8 ng/µL of DNA. ~7.31 ng/µL of DNA was detected for tissue samples that were incubated in freshwater for 72-hours; ~50 fold. ~3.66 ng/µL of DNA was detected for tissue samples that were incubated in swamp water for 72-hours; ~70 fold. ~0.77 ng/µL of DNA was detected for tissue samples that were incubated in saltwater for 72-hours; ~350 fold.

It was found that there were large amounts of DNA loss in both in bone and tissue from samples that were incubated in all three water environments for 72-hours. The bone samples showed much more extensive DNA loss than that of the tissue samples. There is less DNA in bone samples to begin with, resulting in proportionally larger DNA loss. The saltwater environment showed the most amount of DNA loss out of all three. This was consistent in both the bone samples and the tissue samples. From these results it is conclusive that there is a large loss of DNA in human remains that have been immersed for 72 hours.

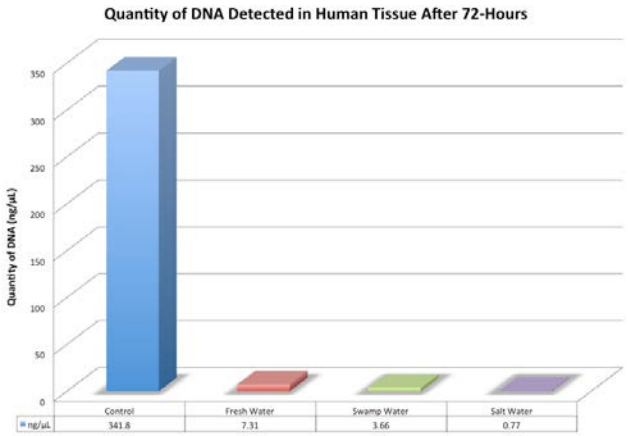

Figure 1. DNA quantification results from the human tissue samples reported in ng/µL. Freshwater, swamp water, and saltwater all showed a large loss of DNA over the 72-hour period. This data shows that aqueous environments had a large affect on the DNA degradation in this specific time period.

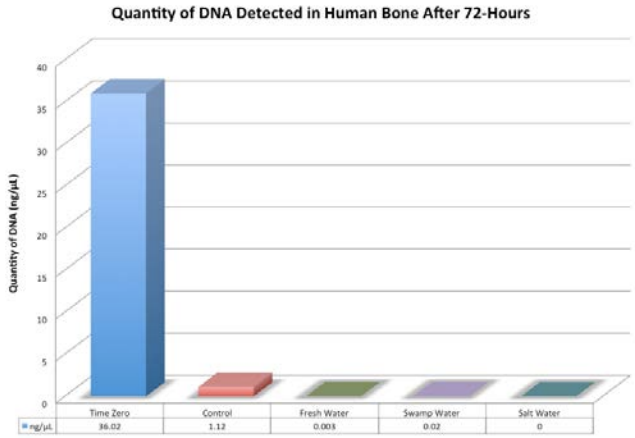

Figure 2. DNA quantification results from the human bone samples reported in ng/µL. Freshwater, swamp water, and saltwater all showed a large loss of DNA over the 72-hour period. This data shows that aqueous environments had a large affect on the DNA degradation in this specific time period.

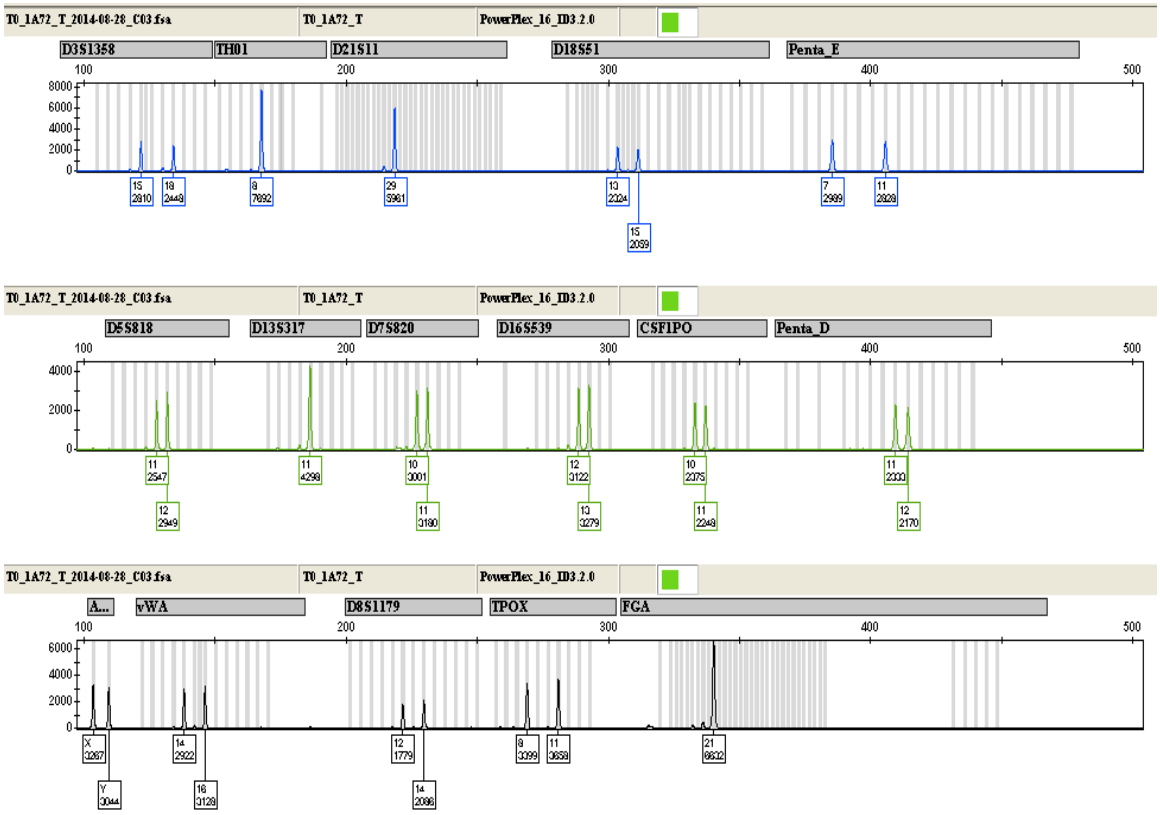

Figure 3. Electropherogram of time-zero tissue sample. This shows a DNA profile of human tissue is not degraded.

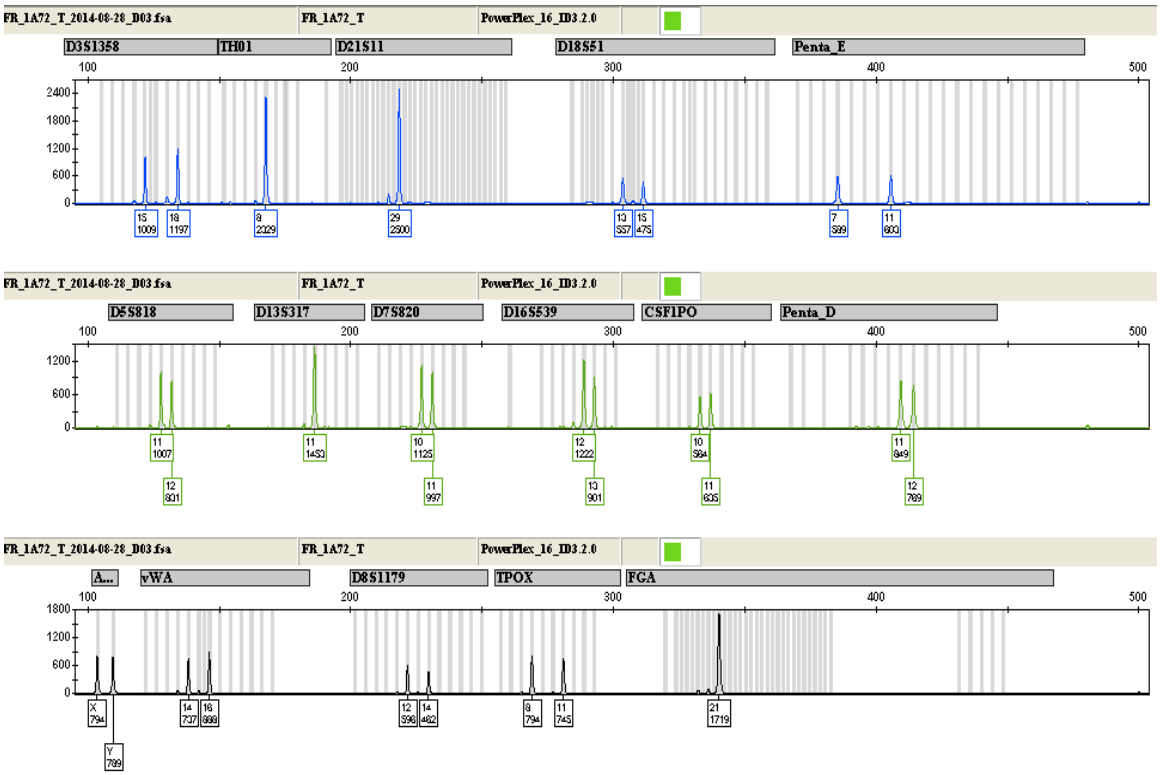

Figure 4. Electropherogram of tissue sample after being incubated in freshwater environment for 72 hours. Even though the sample showed a large loss in DNA quantity, a profile is obtainable.

Conclusion

The 72-hour time period is very important in the timeline of DNA loss of human tissue and bone in aqueous environments. In the research previously done by Shanae Armstrong [8], it was found that there was a critical loss of DNA in between the time periods of 24 hours and 1 week. The results of the 72-hour experiment were consistent with this previous data. It was found that there was not as extensive DNA degradation but more DNA loss, especially in the saltwater samples. When compared to the control and time zero samples it is indicative that there is much more substantial DNA loss and decomposition due to the aqueous environment, proving that the types of water do in fact have an affect on the human DNA.

References

[1] Lathamand, K. E., & Madonna, M. E. (2013). DNA Survivability in Skeletal Remains. In J. Pokines, & S. A. Symes, *Manual of Forensic Taphonomy* (pp. 403-426). CRC Press.

[2] Ocean Water: Salinity. (n.d.). *Office of Naval Research*. Retreived August 15, 2013 from http://www.onr.navy.mil/focus/ocean/water/salinity1.htm

[3] QIAGEN® (2010). *QIAamp® DNA Investigator Handbook*. Isolation of Total DNA from Tissues, 39-41. http://www.qiagen.com/

[4] Conneticut Department of Emergency Services and Public Protection, Division of Scientific Services, Forensic Laboratory. (Revision #004). (2013). *Bone/Tooth Extraction*. Document ID: mtDNA WI-05.

[5] Applied Biosystems (2004). *Quantifiler™ Human DNA Quantification Kits PCR Amplification*. Life Technologies™. http://www.lifetechnologies.com/

[6] Promega (2012). *PowerPlex® 16 HS System Technical Manual TMD022*. Madison, WI: Promega Corporation. http://www.promega.com/

[7] Armstrong S. J. (2014). The Effects of Water Exposure on Typeable Human DNA from Tissue Samples, UNH Master's Thesis. March, 2014.

Acknowledgements

I would like to thank Dr. Michael Adamowicz, whom mentored and assisted me throughout the duration of the project. I also would like to thank the Henry C. Lee College of Criminal Justice and Forensic Sciences for the provision of materials and instruments, as well as the SURF program at UNH and the Board of Governors for the funding received for this research. We would also like to thank the Yale School of Medicine, Department of Pathology for their collaboration in collection samples for this study.

Biography

Ema Graham is currently a junior at the University of New Haven double majoring in Forensic Science with a concentration in Biology and Biology with a concentration in Pre-Medicine, along with a minor in Chemistry. Ema plans on continuing her research on this topic for the rest of her time at the University of New Haven. After college she aspires to pursue a Ph.D. in Forensic Biology or Molecular Biology.