2020

Mineral absorption by submerged bone in marine environments as a potential PMSI indicator

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

MINERAL ABSORPTION BY SUBMERGED BONE IN MARINE ENVIRONMENTS AS A POTENTIAL PMSI INDICATOR

by

KRISTINA LYNN MAMMANO

B.S., Bucknell University, 2014

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2020

Approved by

First Reader _____

James Pokines, Ph.D.
Assistant Professor of Anatomy and Neurobiology

Second Reader _____

Tara Moore, Ph.D.
Associate Professor of Anatomy and Neurobiology

ACKNOWLEDGMENTS

I would like to thank every individual and group that helped make this research possible. I specifically would like to thank my thesis advisor, Dr. James Pokines, who helped me cultivate this research idea and guide me throughout the process. I would also like to thank my committee member Dr. Tara Moore for all of her support. My thesis work would not have been completed without the help of Dr. Gregg Harbaugh, who answered any of my questions about statistics. I would like to thank the Boston University Forensic Anthropology program for supplying me with any materials I needed and for funding my research project.

This research project would not have been possible without the help and guidance from the Environmental department at the University of Massachusetts, Boston. I would specifically like to thank Dr. Hannigan for allowing me to use their water facilities for my experimental location for my time trials and for discussing with me the best instrumentation for my bone sample analysis. My bones would not have made it into the water, yet alone been taken out each month, without the assistance of Dock Master Jay Messana! Along with Jay, I would like to thank Jay's assistants Derek Briggs and Aaron Teiveira of the University of Massachusetts, Boston; Derek had the unfortunate luck of helping me scrape off the ascidian encrustations from my cages, while Aaron helped complete the final steps of removing and power washing my empty cages.

I would like to thank the Woods Hole Oceanographic Institution for allowing my advisor to submerge samples off of their marine dock for 18 months. The salinity trial portion of this research would not have been as thorough without these samples.

The elemental analysis of my samples would not have been possible without the approval of Dr. Christian from the University of Massachusetts, Boston and the dedication of Amy Johnston who scheduled all of my time in their laboratories. I would specifically like to thank Joe Brouillet from the University of Massachusetts, Boston, who spent so much time training me on all the instruments and machines I needed to use and giving me advice when things did not go as smoothly as they were planned. Dr. Alan Stebbins was also always willing to give me advice on data collection and analysis.

Finally, I would not be here without the support of my parents and siblings. They guided me through all of the stressful periods with their advice and willingness to listen to me vent. I will be forever grateful to them! I would also like to thank all of my friends for their encouragement to continue on even when I believed this project was impossible.

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ABSTRACT

Human remains enter marine environments in a number of ways ranging from homicides, suicides, accidental drownings, shipwrecks, to burials at sea. Once the remains are discovered, a legal and forensic investigation begins. A key component to this investigation is the postmortem submergence interval (PMSI). Determining this range on skeletonized remains is a complicated process in which there is no accurate test; although barnacle growth data was previously used to determine PMSI, there are still limitations with that method. Therefore, a more reliable component of bone needs to be used as a potential PMSI indicator, such as its elemental composition.

Diagenesis starts affecting bones immediately and continues for thousands of years. Although diagenesis is a slow process, an exchange of elements between bone and the marine environment continually occurs. The purpose of the present study is to determine whether an increase in marine elements is found within the composition of bone after being submerged in a marine environment for up to 20 months. The present study will also determine whether bones submerged in different aquatic environments have significantly different elemental concentrations.

For the time trials, pig femora were submerged in lobster cages off the coast of the University of Massachusetts Boston for 2-20 months. For the salinity trials, pig femora were submerged in a freshwater pond (Holliston, MA), the Inner Boston Harbor,

and an ocean inlet near Woods Hole, MA for 18 months. All bone samples were dried, milled, homogenized, and analyzed by ED-XRF under He purge. The initially produced mass percentages of the identified elements were corrected with certified values of standard reference materials (NIST 1486, 1646a, and 2702). A Pearson's correlation test determined that the concentrations for K, Fe, Zn, Sr, Si, S, Cr, Mn, Cl, Br, Ta, and W were significantly correlated to the amount of time submerged in the water. An ANCOVA analysis was applied to the significant elements noted above. After adjusting for the amount of time submerged, the concentrations of K, Fe, Sr, Si, S, Cl, Br, and Ta were determined to be significantly different between the control samples (never submerged) and the submerged samples (submerged for 2-20 months). K was the only element that had greater concentrations in the control samples than the submerged samples, most likely because of the decrease in mass percent as other environmental elements were incorporated into the bone. S and W were significantly related to the number of months submerged, with S being positively influenced and W being negatively.

A multivariable linear regression was run in order to identify a means of predicting the amount of time submerged from the elemental concentrations of an unknown bone from a marine environment. The regression produced an equation that used the concentrations for K, Sr, Si, S, Cr, Cl, and Br to predict the PMSI in months.

For the salinity trials, a one-way ANOVA was performed on all the elemental concentrations from the different salinity environments. Post hoc tests determined significant differences in elemental concentrations for K, Fe, Si, S, Al, Ti, Cr, Ni, Mn, Cl

and Br among the different submergence locations; elemental concentrations of S, Fe, Mn, Cl, K, and Br were either significantly different between the fresh, brackish, and saltwaters or the freshwater and some form of marine water (brackish and salt). The trends in the other elemental concentrations were less obvious due to the impact of pollution within the surrounding environments.

The linear regression equation created in the present study accounted for the majority of the variance in the outcome (R^2 = 80.2%); however, this equation should not currently be applied in forensic investigations. The study needs to be repeated a number of times with other bone samples from the same and different submergence locations, in order to determine the accuracy and usefulness of the equation. Although not verified, this regression equation may be useful in analyzing samples from brackish and saltwater environments, because the majority of the variables within the equation (K, Sr, S, Cl, Br) were consistent among the fresh, brackish, and saltwater samples.

Time constraints, small sample sizes, and variance among samples were the major limitations of the present study. Even with limitations, significant results were produced by the ED-XRF analysis. Future research should expand upon the methodologies of XRF analyses of bones, especially those from marine environments. Because of their relevance to forensic investigations and PMSI, future research should include longer experimental periods, more salinity locations, more information on the surrounding water components, and more comparisons among instrumentation.

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significant differences among the groups.

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LIST OF ABBREVIATIONS

AAS.....Atomic Absorption Spectrophotometry

ADD..... Accumulated Degree Days

BADS.....Body Aquatic Decompositional Score

BFR.....Brominated Flame Retardant

BU..... Boston University

CV.....Coefficient of Variation

DDT.....Dichlorodiphenyltrichloroethane

DI..... Deionized Water

ED-XRF..... Energy Dispersive X-ray Fluorescence

ENQUAD.....Environmental Quality Department

EPA.....Environmental Protection Agency

FADS.....Facial Aquatic Decompositional Score

LADS..... Limbs Aquatic Decompositional Score

MC..... Measured to Certified

MPRSA.....Marine Protection, Research and Sanctuaries Act

MWRA.....Massachusetts Water Resources Authority

NIST..... National Institute of Standards & Technology

ORF..... Outdoor Research Facility

PBB.....Polybrominated Biphenyls

PBDE.....Polybrominated Diphenyl Ethers

PCA.....Principal Components Analysis

PCB .....Polychlorinated Biphenyl

PMI .....Postmortem Interval

PMSI .....Postmortem Submergence Interval

PSU ..... Practical Salinity Units

SEM/EDS..... Scanning Electron Microscopy/Energy-Dispersive Spectrometry

SRM .....Standard Reference Material

TAD .....Total Aquatic Decomposition

TPI.....Tooth Per Inch

UNESCO.....United Nations Educational, Scientific and Cultural Organization

WHOI.....Woods Hole Oceanographic Institute

XRD .....X-Ray Diffraction

XRF.....X-Ray Fluorescence Spectrometry

INTRODUCTION

Many forensic cases involve human remains that were disposed of in terrestrial environments; therefore, most research on human decomposition and the influence of numerous taphonomic agents has been carried out in various terrestrial environments. The characteristics and timing of each stage of soft tissue decomposition have been studied in a number of terrestrial environments using nonhuman (Matuszewski *et al*. 2008; Payne 1965) and human samples (Galloway *et al*. 1989; Komar 1998; Mann *et al*. 1990; Megyesi *et al*. 2005). For example, Payne (1965) developed stages of decomposition (fresh, bloated, active decay, advanced decay, dry, and remains) when analyzing pig (*Sus scrofa*) remains in the presence of arthropods in South Carolina. The stages and rates of decomposition have also been studied in places like Arizona's arid environment (Galloway *et al*. 1989), Poland's forests (Matuszewski *et al*. 2008), and Canada's colder climates (Komar 1998). The effects of different microenvironments on decomposition rates have been thoroughly studied, such as the effects of shading (Joy *et al*. 2006; Majola *et al*. 2013) and different burial environments (Payne 1968; Rodriguez 1997; Rodriguez and Bass 1985). The scavenging and dispersal of fleshed and skeletal remains by birds (Komar and Beattie 1998; Spradley *et al*. 2012), rodents (Haglund 1992; Hockett 1989; Hoffman and Hays 1987; Pokines *et al*. 2016), and carnivores (Haglund 1997; Haynes 1982; Kent 1981) have also been studied in different terrestrial environments throughout the world, such as the forests of New England (Ricketts 2013) and the Serengeti of Tanzania (Blumenschine 1986). The various taphonomic alterations

to skeletonized remains found in different terrestrial environments also have been previously analyzed, including the characteristics and rates of weathering stages in a number of geographic regions, ranging from the Amboseli Basin in Kenya (Behrensmeyer 1978) to the Ituri Rain Forest in Zaire (Tappen 1994) to Yellowstone National Park in the United States (Miller 2009). As evident from above, terrestrial environments have been the most common setting for studies involving the decomposition and alterations to nonhuman and human remains, because of their prevalence in forensic cases and simpler experimental design; therefore, less is known about the processes affecting remains from other environments, such as aquatic ones.

Despite the fact that terrestrial disposal sites are common, there are many forensic cases in which human remains, fleshed and skeletal, are found in aquatic environments, specifically the ocean. For example, from 1993 to 2015, the Office of the Chief Medical Examiner in Boston, Massachusetts received a minimum of 25 cases in which skeletonized human remains were collected from the ocean or along the shoreline of Massachusetts; this value is excluding all nonhuman bones presented to the authorities and all predominantly fleshed human remains (Pokines and Higgs 2015).

Deposition of Remains into the Ocean

Human remains commonly enter aquatic environments due to drowning. For example, from 2005 to 2014, there was an average of 3536 fatal unintentional drownings each year in the United States, with an additional 332 deaths each year due to drowning in boating related incidents (CDC 2016); specifically in Massachusetts, there were 40

unintentional drowning/submersion deaths that occurred in 2012 alone (Massachusetts Department of Public Health 2015). Other than accidental drownings, suicides, homicides, burials at sea, natural disasters, aircraft crashes, and shipwrecks can all lead to human remains in the ocean (Dumser and Türkay 2008; Ebbesmeyer and Haglund 2002; Haglund and Sorg 2002; Higgs and Pokines 2014; Kahana *et al*. 1999; Lewis *et al*. 2004). For example, a common mode of suicide that results in bodies being introduced to aquatic environments is intentional jumping off of tall bridges. Jumping from bridges was the most common mode of suicide in Marin County, California between 1990 and 2005 (Fussell and Louie 2008); according to Blaustein and Fleming (2009), the Golden Gate Bridge in San Francisco, California is the number one site in the world to commit suicide. Officials stopped counting and publishing the number of Golden Gate Bridge jumpers in 1995 as it increased to 1000, however, as of 2003 the unofficial number of suicides was believed to be greater than 1300 (Fussell and Louie 2008). Blaustein and Fleming (2009) and Abel and Ramsey (2013) noted that bridge suicide counts in general only include cases in which bodies were recovered; therefore, the actual number of suicides from bridge jumping is probably higher, since the number of unwitnessed jumps with unrecovered bodies that were washed out to the ocean is unknown. Suicide by drowning is another mode of death that can introduce human remains to the ocean; if discovered, the remains of these individuals were usually found floating in the sea, washed ashore on a beach, or near jetties (Byard *et al*. 2001; Kaliszan *et al*. 2013).

The ocean is known to be a dumping ground for the bodies of homicide victims who were killed on land. Defendants often try to dispose of their victims' remains by

throwing them into the ocean with hopes that the remains will never be found and with the misguided belief that they cannot be convicted without a recovered body (DiBiase 2015). For example, prominent Delaware Attorney Thomas Capano was convicted of killing Mary Ann Fahey in 1996, even though her remains were never found, because they were weighed down with anchor chains and dropped into the ocean; similarly, Michael Lubahn Clark of California was convicted of killing his wife in 1981 and disposing of her body by weighing it down with cinder blocks and dumping it in the ocean 200 to 500 yards from shore (DiBiase 2015). In 1993, Petty Officer Mark Allen Eby of Washington strangled his wife, stuffed her body into a nylon suitcase, and threw it off the Deception Pass Bridge into tidal waters that led out into the Pacific Ocean; even though the body and suitcase were never found after extensive searching and studying of the currents, Eby was still convicted of first degree murder (Ebbesmeyer and Haglund 2002). The bodies of homicide victims are not always disposed of close to shore; there are some cases in which individuals dump bodies far into the open ocean, as in the case when Lawrence Cowell and Donald Dimascio of California dumped their victim's body into the Pacific Ocean from a plane in hopes of attracting sharks that would eat the remains (DiBiase 2015).

When looking outside the realm of homicides, intact human remains can also be introduced to the ocean due to burials at sea. The Marine Protection, Research and Sanctuaries Act (MPRSA) issued by the United States Environmental Protection Agency (EPA) allows for human remains to be buried at sea, but in accordance with the requirements deemed necessary by the United States Navy, United States Coast Guard or

other civil authority who is performing the burial (EPA 2017). The EPA (2017) suggests wrapping non-cremated remains in natural fibers with additional weights or placing them in a non-plastic casket; the United States Navy (2010) requires intact and casketed remains to be placed in a metal casket weighing at least 300 pounds with twenty holes drilled into it in order to guarantee sinking. Although it is required to weigh down the remains and to dispose of them at least three nautical miles from shore and in ocean waters at least 600 feet deep (EPA 2017), it is possible that these remains are later found. For example, a commercial fishing vessel recovered, from 80 miles south of Block Island, Rhode Island in 700 to 800 feet of water, a skeletonized cranium that was loosely attached to a fleshed torso (London *et al*. 2006). Due to the sewn-closed Y-shaped incision, the stapled-closed surgical incision on the torso, the presence of hardening compound, and intact skin untouched by scavengers, the pathologist and anthropologist determined that this individual underwent surgery and eventually died, and then subsequently was autopsied, embalmed, and buried at sea (London *et al*. 2006). Two other cases involving the recovery of whole body sea burials occurred off of Dade County, Florida, one of which involved an autopsied body being found in the mangroves along Biscayne Bay, Florida. This body was determined to be previously autopsied, because the axillary skin was pink and well-preserved, and there was a hole for a trocar button (London *et al*. 2006). This individual was later identified through the medical examiner's sea burial approval records; it was determined that after the burial at sea about one year earlier, the body was able to float to the surface, travel with the currents, and wash ashore once the wooden casket had disintegrated (London *et al*. 2006).

When airplanes crash into the ocean, the search and recovery of all debris and human remains are very difficult. In some crashes, human bodies are highly fragmented due to the rapid deceleration and impact of the plane, which complicates the recovery, re-association, and identification of all the human remains involved in the crash; this was the case when Swissair Flight 111 crashed into the Atlantic Ocean near Nova Scotia on September 2, 1998 (Transportation Safety Board of Canada 2013). Sometimes the remains of those killed in the crash cannot be found or safely recovered and therefore are left in the ocean. For example, only 154 out of the 228 bodies were recovered from the Atlantic Ocean when Air France Flight 447 crashed in route from Rio de Janeiro, Brazil to Paris, France on June 1, 2009 (Bureau d'Enquêtes et d'Analyses 2012; Stone *et al*. 2011) and only 113 out of 152 bodies were recovered from the Indian Ocean when Yemenia Airlines Flight IY26 crashed in route from Sanaa, Yemen to Moroni, Comoros on June 30, 2009 (Ribéreau-Gayon *et al*. 2017).

Similar to airplane crashes, shipwrecks litter the ocean floor throughout the world. The United Nations Educational, Scientific and Cultural Organization (UNESCO) (2017) estimated that there are about three million shipwrecks throughout the ocean floor from many historical periods; more specifically, there have been more than 3,000 shipwrecks within the 192 mile general coastline of Massachusetts (NOAA 2016; NPS 2012; PHPP 2016). These wrecks involve historical, military, and modern ships, which range from wooden to metal frames and which contain a variety of artifacts. All of the shipwrecks throughout the world are in different states of preservation due to the condition of the ship when it was sunk and the local aquatic environment; in the ocean, some factors that

affect the rate of degradation are the currents, oxygen content, salinity, and sediments (Björdal and Nilsson 2008). Historical wooden ships degrade rapidly in saltwater due to sediment erosion, or scour; mollusks like wood-boring shipworms (family Teredinidae) and crustaceans like gribble worms (family Limnoriidae); and sediment-living fungi and bacteria (Eriksen *et al*. 2015; Gregory *et al*. 2012). Steel-hulled ships also corrode faster in saltwater, because the sodium and chloride ions in the saltwater solution accelerate the electrochemical process that turns strong iron alloys into crumbly hydrated iron oxide known as rust (Dupras and Schultz 2014; Hamilton 1997). The structural collapse of the shipwrecks over time allows for the exposure of the ships inner contents, which potentially includes human remains. Many of these shipwrecks resulted in casualties and unrecovered remains; for example, there are about 1000 individuals still entombed in the USS *Arizona* in Pearl Harbor, since its sinking on December 7, 1941 (Russell *et al*. 2006). Beyond the initial sinking incident, human remains can be released into the ocean as the shipwreck breaks down or is uncovered from the overlying seafloor sediment.

Other than the natural release of remains into the ocean from shipwrecks, human remains that were once entombed within sunken ships have been recovered by archaeologists who were excavating numerous underwater sites. For example, scattered bones of at least four individuals were found during the 1976 excavations of the Antikythera shipwreck, a 2000 year old merchant ship wreck off the coast of the Greek island of Antikythera (Marchant 2016). Another set of remains consisting of a partial skull with a few teeth, arm bones, femurs, and rib fragments were discovered buried under sand and pottery sherds in 2016 at the same wreckage. In 2008, surface deposited

remains consisting of a complete skull and numerous ribs were discovered at the wreckage site of the HMS *Victory*, which sunk in the western English Channel in 1744 (Cunningham Dobson and Tolson 2010). Over 200 human bones or bone fragments and numerous nonhuman bones were recovered from the stern portion of the HMS *Pandora*, which sank in the Great Barrier Reef in 1791 (Steptoe and Wood 2002). The skeletal remains of two individuals were excavated from the salvaged turret of the ironclad USS *Monitor* after having been submerged in the Atlantic Ocean off the coast of North Carolina since 1862 (Broadwater 2012). During the excavation in Texas's Matagorda Bay of the *La Belle*, one of the ships of French explorer Robert Cavelier de La Salle, the skeletal remains of two individuals as well as 824 animal bones (ship rat, pig, bison, goats/sheep, bird, deer, and turtle) were discovered (Bruseth and Turner 2005). Arguably, the most notable discovery of human skeletal remains from a shipwreck came from that of the *Mary Rose*, which sank in 1545 off the coast of England, resulting in about 385 casualties and producing the remains of at least 179 individuals over 400 years later (Stirland 2013).

Movement of Remains within the Ocean

Once in the ocean, fleshed and skeletal human remains can travel horizontally and vertically throughout it. Fleshed remains can travel horizontally throughout the ocean due to water currents when they are fresh and floating, sinking, bloated and ascending, bloated and resurfaced, or resting on the ocean floor. Even skeletonized remains can travel along the ocean floor due to currents as long as they are not embedded within the

ocean floor. Remains can travel great distances depending upon the strength and direction of the currents. For example, after a bus accident in the Duero River in Portugal, seven bodies floated downriver and out to sea; these bodies traveled 350 to 420 km away over a span of three to eight days (Blanco Pampín and López-Abajo Rodríguez 2001).

In regards to vertical transitions, fleshed remains go through stages of floating and sinking within the ocean environment as they decompose. Some fresh carcasses have been known to float when first introduced to the ocean environment (Anderson and Hobischak 2002, 2004), while others sank immediately (Anderson 2008). Factors such as density of the surrounding water, residual air within the lungs, body fat content, and attached apparel or life preservers affect whether a body initially sinks or floats when introduced to the water (Donoghue and Minnigerode 1977; Haglund and Sorg 2002; Higgs and Pokines 2014). To float, a body's specific gravity, or density relative to freshwater, has to be less than the specific gravity of saltwater (1.026) (Higgs and Pokines 2014). Initially, residual air within the lungs is believed to increase a body's buoyancy enough to float temporarily, until the air is gradually expelled and replaced with water. Donoghue and Minnigerode (1977) have studied the effect of residual air on a body's ability to float by assuming the residual air left in the lungs of a fresh corpse is equal to the residual lung capacity within a living human; residual lung capacity is the amount of air that remains within the lungs after maximum exhalation. Out of the 98 adult male samples studied by Donoghue and Minnigerode (1977), 69% floated in saltwater, while only 7% floated in freshwater. This discrepancy suggested that the residual amount of air within the lungs decreased the body's density enough to be able to

float in the more dense saltwater, but not enough to be able to float in the less dense freshwater. Similar to residual lung capacity, an individual with a higher body fat content will most likely float; this is because fat with the dame mass as muscle occupies more space, which displaces more water and results in a larger buoyant force acting upon a body with a higher fat content (Haglund and Sorg 2002). Life jackets worn on an individual will prolong floating (Higgs and Pokines 2014), but conversely, heavy clothes can weigh down a body and prevent floating.

Once air escapes the lungs and the life jacket deteriorates, then the remains will begin to sink; this rate of sinking is variable (Rodriguez 1997). Later in the decomposition process, remains can rise and potentially resurface as bacterial activity within the abdominal cavity produces decomposition gases, which increases the internal gas pressure causing the central body cavity to expand and potentially overcome the surrounding water pressure. The body can then be lifted off the seafloor and begin to float back to the surface; however, this is sometimes dependent upon water temperature and depth. For example, colder water decreases the activity rate of bacteria within the remains, which then results in the slower production of decomposition gases that would counteract the external water pressure. At a large depth, the water pressure is extremely high, because an additional 1 atm of pressure is exerted on remains for every 10 m they travel deeper in water (Higgs and Pokines 2014); therefore, it would take a large amount of internal pressure within a sunken body to be able to overcome that downward force applied by the water.

Alterations to Remains within the Ocean

Upon immediate entrance into the water and throughout its time traveling throughout the ocean, human remains are altered by numerous taphonomic agents. Natural decomposition, animal scavenging, and interactions with the inorganic forces of the marine environment all aid in the breakdown of soft tissue associated with bones (Haglund 1993; Haglund and Sorg 2002; Higgs and Pokines 2014; Pokines and Higgs 2015; Sorg *et al.* 1997). The first areas that naturally lose soft tissue are the thinly covered portions, such as the head, hands, and shins (Haglund 1993; Haglund and Sorg 2002). As the soft tissue breaks down, the systematic disarticulation of the body occurs. For example, floating bodies are usually oriented with their back-side up and head and appendages hanging down; therefore, disarticulation first occurs in the hands, feet, mandible, and cranium, followed by the rest of the upper limbs and the lower portion of the legs (Haglund 1993; Haglund and Sorg 2002). The rates of decomposition, disarticulation, and skeletonization are extremely variable depending upon the surrounding environment; however, once skeletonized and disarticulated, bones are exposed to the immediate ocean environment.

Just like fleshed human remains, exposed skeletal remains also undergo specific taphonomic alterations when in the ocean. Bones within the marine environment lose their organic components as the fat within the bones leaches out and is washed away. Pokines and Higgs (2015) analyzed 25 skeletal samples originating from the ocean near Massachusetts in order to demonstrate the wide range of effects a marine environment could have on bones. They noted that 44% of the bones still retained an organic sheen,

while 24% of the bones had lost enough organic content to have a chalky outer appearance. There was evidence of fat still leaching from 32% of the bones in the sample and adipocere formation on 20% of the bones, which indicated a presence of fat in those specific areas. Bones within marine environments are battered by sediment and hard substrates when completely or partially exposed to the moving waters; this results in a bumpy texture, rounded edges, increased windowing, eroded cortical bone, and embedded sediment (Higgs and Pokines 2014). In their analysis, Pokines and Higgs (2015) noted abraded surfaces in 24 out of 25 of the cases, as well as evidence of battering on the flat surfaces, rounding of the margins, windowing through thin cortical bone, and expanding of pre-existing holes. The active conditions of the ocean that caused bones to rub against rocks, sand, and suspended sediment can lead to the exposure of cancellous bone and the embedding of sediment. In association with the loss of organic content, the surfaces of the bones usually become bleached when exposed to the saltwater; however, these surfaces can also be stained orange and gray by minerals within the marine environment. For example, the few bones from the *Mary Rose* shipwreck that were directly exposed to the ocean environment were heavily stained with iron oxide (Stirland 2013).

Marine taxa can also affect bones within the ocean by colonizing the bones' surfaces or boring into the bone in order to gain shelter or obtain nutrients (Higgs and Pokines 2014). Algae, bryozoa, barnacles, and mollusks will attach to the surface of bones when in stable water conditions, while marine fungi, bacteria, and cyanobacteria will tunnel into bones (Higgs and Pokines 2014). For example, Pokines and Higgs (2015)

noted that mollusks were attached to two samples and barnacles were attached to nine samples out of a total of 25. They also noted the presence of barnacle cement remnants and mollusk homing scars on the bones' external surfaces. Marine taxa, including marine fungi, bacteria, and cyanobacteria, can also affect bones within the ocean by boring into the bone in order to gain shelter or obtain nutrients (Higgs and Pokines 2014). An unidentified organism produced microscopic tunnels throughout peripheral mandibular and maxillary bone of numerous specimens recovered from the *Mary Rose* shipwreck (Bell and Elkerton 2008).

Discovery and Analysis of Remains from the Ocean

Just like there are a number of ways in which a body can enter the ocean and travel throughout the ocean, there are a number of ways in which human remains from the ocean are discovered. Fleshed and skeletal remains from the ocean can eventually wash ashore due to strong currents and changing tides; they are often discovered along beaches, because of the large number of visitors and the beach's open environment (Higgs and Pokines 2014). Bones can also become trapped in fishing nets and dredges by commercial fishermen as these nets are dragged along the ocean floor (Haglund 1993; Haglund and Sorg 2002; Pokines and Higgs 2015; Sorg *et al.* 1997). As previously mentioned, human bones can even be discovered during underwater archaeological excavations (MBUAR 2006). Once found and delivered to the authorities, a forensic anthropologist analyzes the skeletal remains and constructs a biological profile, which can potentially lead to an identification of the remains depending upon their condition

(Haglund 1993; Sorg *et al.* 1997). In Massachusetts, even before attempting an identification, a forensic anthropologist needs to be able to determine if the remains are less than 100 years old and forensically relevant or greater than 100 years old and require the involvement of the state archaeologist (MBUAR 2006). This determination is easy when the forensic anthropologist knows that the bones were excavated by professional archaeologists from a specific shipwreck. However, when there is no contextual information about the deposition site or any associated material evidence, the accurate determination of how long contemporary and archaeological bones have been submerged in water is complicated. This postmortem submergence interval (PMSI), or the specific amount of time that the remains have been immersed in water, is of particular interest to investigators who are attempting to determine when death occurred, because they assume the remains entered the ocean soon after that event. Researchers have developed a few modern procedures to estimate PMSI from marine samples (see below), yet none of these procedures are universally applicable or irrefutable; therefore, as of the date written, forensic anthropologists do not have validated methodologies that accurately and consistently estimate the PMSI of remains from any aquatic environment (Stuart and Ueland 2017).

In order to calculate the postmortem interval (PMI) in terrestrial environments, forensic anthropologists have thoroughly studied the stages of soft tissue decomposition, the sequence of colonizing insects, the stages of bone weathering, and the radiocarbon dating of bones; however, this is not necessarily the case for remains in marine environments. It was originally accepted that decomposition of a body in aquatic

environments occurred at a slower rate than on land (Anderson and Hobischak 2002, 2004); researchers began to test this theory by performing similar taphonomic experiments as those on land. Although the causes and results of taphonomic changes to carcasses in the ocean were discovered via experiments on animal remains (Anderson 2008; Anderson and Bell 2016; Anderson and Hobischak 2002, 2004; Ayers 2010; Jones *et al.* 1998; Prassack 2011) and reviews of forensic cases (Bytheway and Pustilnik 2013; Dennison *et al*. 2004; Dumser and Türkay 2008; Haglund 1993; Kahana *et al*. 1999; Magni *et al*. 2015; Pokines and Higgs 2015; Sorg *et al*. 1997), there has been little success in establishing distinct time frames for each stage of decomposition in a marine environment. These researchers tried to correlate the condition of recovered remains to observations of human and nonhuman remains made during research experiments and previous forensic investigations with known time frames. Rather than finding constant time frames for each decomposition stage, they discovered that a number of factors in a marine environment, such as marine scavengers and ocean currents, could positively or negatively influence the rate of decomposition, disarticulation, and skeletonization (Anderson 2008; Anderson and Bell 2016; Ayers 2010; Pokines and Higgs 2015; Sorg *et al.* 1997). This makes the rates of decomposition within marine environments less predictable and the associated PMSI estimates less reliable; however, this area of study is still popular. Although many recent studies have focused on decomposition rates of soft tissue in marine environments in the context of developing a PMSI, there has been little research on determining PMSI once the remains have skeletonized (Higgs and Pokines 2014; Sorg *et al.* 1997).

PMSI Determination of Skeletal Remains

Recent research has focused on correlating the colonization and growth of sessile marine plants and animals on remains to minimum PMSI estimates (Bytheway and Pustilnik 2013; Dennison *et al*. 2004; Magni *et al*. 2015; Pirtle 2017; Sorg *et al.* 1997). Although bryozoan and coral colony sizes have been used (Sorg *et al*. 1997), researchers have recently looked into barnacle growth and development as a potential PMSI indicator (Bytheway and Pustilnik 2013; Dennison *et al*. 2004; Magni *et al*. 2015; Pirtle 2017). Barnacles are arthropods of subphylum Crustacea and infraclass Cirripedia that live in marine and brackish water environments (Higgs and Pokines 2014). Although there is a parasitic order of barnacles (order Rhizocephala), the filter-feeding species of orders Pedunculata and Sessilia are the ones that have been studied to aid in forensic investigations and therefore, it is their development that will be discussed below.

The barnacle life-cycle includes two swimming larval phases (naupliar and cyprid) and one adult sessile phase (Anderson 1994; Darwin 1851, 1854). After embryonic development, the first naupliar larval stage is released into the ocean; during the naupliar stage, the larvae swim, feed, and undergo a number of molts (Anderson 1994; Darwin 1851, 1854). A pre-settlement metamorphosis occurs in which the naupliar larvae enter the cyprid larval phase. This bivalve, non-feeding form finds an appropriate substrate to settle upon and uses its antennules to move across the substrate's surface until a suitable area is found; a small amount of polymerized proteinous cement is then secreted in order to loosely attach the cyprid to the substrate in preparation for another

metamorphosis (Dickinson *et al*. 2009; Høeg *et al*. 2012). The sessile barnacles are usually attached to stable, hard substrates, such as rocks, ships' hulls, and even bones and shoes, mainly in shallow and intertidal waters, but also in deeper water on occasion (Higgs and Pokines 2014; Magni *et al*. 2015; Pirtle 2017). The second metamorphosis results in a juvenile barnacle that tightly adheres to the substrate and develops shell plates that progressively calcify (Høeg *et al*. 2012). After numerous molts, the sessile, adult form emerges, continuing to filter feed and incrementally expand its external calcified plates (Anderson 1994); these calcified plates can remain on the substrate even after the barnacle dies.

Researchers have measured basal diameters, remnants of barnacles' adhesion cement, and incremental growth rings on the calcified plates in order to calculate a PMSI (Bytheway and Pustilnik 2013; Dennison *et al*. 2004; Higgs and Pokines 2014; Magni *et al*. 2015; Pirtle 2017). However, there are some obstacles with using barnacle size as a method of estimating PMSI, such as different attachment times, variable growth rates, and increasing likelihood of being scraped off (Bytheway and Pustilnik 2013; Dennison *et al*. 2004; Magni *et al*. 2015; Pirtle 2017; Pokines and Higgs 2015; Sorg *et al.* 1997; Yakovis *et al*. 2013). The duration of the reproduction and brooding cycles and the time of year for hatching are variable among different species of barnacles (Anderson 1994; Hines 1978). Some species have a breeding season that spans the entire year, while others have a single breeding season, which usually follows the trend of a spring larval release and summer growth period. Some barnacle species produce offspring multiple times each year, while others only produce offspring once during the year. For example, *Balanus*

balanoides is known to copulate in late fall, incubate one large brood during the winter, and release all naupliar larvae during the spring when the diatom bloom occurs; on the other hand, *Balanus glandula* produces one major winter brood and then numerous minor broods every month throughout the spring (Hines 1978). Therefore, depending upon the location of the remains, the species of barnacles native to that area, and the time of year that the bones are deposited into the ocean, some bones can be exposed to the ocean environment for a period of time without being colonized by barnacle larvae.

Even when barnacle larvae are in the vicinity of the bones, they may not settle on the bony substrate, because the environment is not providing the appropriate signals; cyprid settlement is dependent upon the amount of light (Hurley 1973; Pirtle 2017), water temperature (Yakovis *et al*. 2013), the water's flow velocity and amount of water disturbance (Qian *et al*. 2000; Smith 1946), surface texture (Hurley 1973), and presence of organisms from its own species and other species (Knight-Jones 1953). Since cyprid settlement and metamorphosis into a juvenile can be delayed, than the durations of each life cycle stage can vary depending upon the immediate conditions of the environment; this affects the accuracy of the estimated PMSI, which was partially derived from the normal durations of each life cycle stage. Once adult barnacles settle on a bony substrate, their growth rates can vary for numerous reasons, which also complicates PMSI calculations and furthers supports the need for a more reliable method of estimating PMSI. For example, growth rates of barnacles vary between species and not all rates are known for each species; the different biology of each species results in various phase transformation times, larval settling times, and sessile growth rates (Sorg *et al.* 1997).

Barnacles of the same species can even have variable growth rates depending upon the local conditions of the water (Sorg *et al*. 1997); for example, growth rates are affected by temperature and current flow (Magni *et al*. 2015; Pirtle 2017). The growth rates of barnacles that delayed metamorphosis are known to be depressed, therefore, resulting in smaller adult barnacles than average for that age (Knight-Jones 1953; Pechenik *et al*. 1993); this would also impact PMSI estimations that were based on basal diameter. Another issue when estimating PMSI from growth rates of basal diameters arises if hummocking of the barnacles has occurred. When large amounts of larvae are recruited to a substrate and competition for space develops, barnacles will stop growing outwards along their bases and start growing upwards, increasing their height, which is referred to as hummocking (Bertness *et al*. 1998). The methods of estimating PMSI from basal diameters do not account for this switch from horizontal growth to vertical growth.

Once a barnacle dies, the calcified shell can remain adhered to the bone (Higgs and Pokines 2014), but will no longer grow and therefore, no longer represent an accurate PMSI. Earlier barnacle colonies on a substrate are more likely to be scraped off from bones that are exposed in an ocean and interacting with strong taphonomic forces, like abrading sediment, rocks, and waves, for a longer amount of time (Pokines and Higgs 2015). Barnacles can even be displaced by encroaching neighboring barnacles that caused the original barnacle to lose contact with the substrate (Khandeparker and Anil 2007). More barnacles will fall off the bone's surface once the substrate and barnacles dry when exposed to a subaerial environment, sometimes leaving only remnants of the barnacle cement. When the barnacles that originally colonized the bones are removed, only the

minimum PMSI can be estimated. In the end, barnacle size and colonization patterns may not accurately or consistently indicate the amount of time bones have been submerged in the ocean.

In order to avoid the potential error when using less consistent features on bone to develop a PMSI, more universal characteristics should be studied. The elemental composition of living bone is generally consistent. After death, the processes of diagenesis and fossilization can alter that previously stable makeup. Diagenesis is the breakdown of bone in which the bone's organic materials (collagen and other proteins) are replaced with inorganic materials, specifically minerals from the surrounding environment (Pfretzschner 2004). During the early stages, microbial activity and leaching cause the bone's collagen content to slowly decrease. All bones are porous; according to Nielsen-Marsh *et al*. (2000), 12% of the volume of fresh cortical bone consists of pore space (lacunae, Haversian canals, Volkman canals, canaliculae), suggesting that the dense cortical portion of bone as well as the thinner trabecular portion of bone can absorb aqueous solutions. Therefore, as collagen and other elements diffuse out of the bone, elements from the surrounding environment can permeate into the bone. Starting with the outer cortical layers, materials will flow into the bone and gradually diffuse into the deeper cortical layers (Tütken *et al*. 2008). The same gradual process occurs in decaying bones within marine environments; seawater diffuses into the bone, depositing marine minerals within the pore spaces (Hamilton 1999/2001). Upon drying, the salt minerals can crystalize and adhere to the interior and exterior surfaces of the bone (Cronyn 1990; Hamilton 1997). Trujillo-Mederos *et al*. (2012) showed how this exchange process

occurred in bones boiled in freshwater and seawater; there was a decrease in amorphous collagen when boiled in both water samples, but there were elevated concentrations of NaCl in the bones boiled in seawater.

Other minerals can also precipitate within the pores and crevices of a bone; for example, the high pH and low redox environment surrounding the decaying bone causes mobile iron ions from the seawater to reduce and combine with the sulphides from the broken down collagen (Pfretzschner 2004). These newly formed iron sulphides solidify within the bone and later transform into pyrite (FeS_2), a component found in fossils. Fossils from marine environments have been known to contain more pyrite than those found in terrestrial environments, because the additional sulphate ions found within the seawater are also reduced, eventually resulting in more iron sulphides precipitating within the bone (Pfretzschner 2004). This process starts within the outer layers of bone and slowly reaches the inner, central compact layers of bone (Tütken *et al*. 2008).

In addition to the leaching organic content and the surface deposition of marine minerals, diagenesis also involves the recrystallization of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). As the bone structure breaks down hydroxyapatite transforms into a more stable molecule; during this time, substitutions are made with elements from the environment. Common substitutions for calcium are sodium (Na^+), strontium (Sr^{2+}), magnesium (Mg^{2+}), iron (Fe^{2+}), uranium (U^{4+}), zinc (Zn^{2+}), manganese (Mn^{2+}), barium (Ba^{2+}), and a number of Rare Earth Elements (REE) (Pfretzschner 2004; Trueman 1999). The phosphate groups ($PO4^{3-}$) within the bony matrix are often substituted with carbonate ($CO3^{2-}$) and hydrogen phosphate (HPO^{2-}). Finally, the hydroxide groups (OH^-) can be

substituted for fluorine (F^-), chlorine (Cl^-), and carbonate ($CO3^{2-}$). Although it can take hundreds to thousands of years to just complete the early stages of diagenesis (Pfretzschner 2004; Tütken *et al*. 2008), changes within the overall elemental composition of a bone can still be seen within a week to months since deposition, depending upon the environmental conditions (Keenan and Engel 2017). It is this universal feature that should be tested as a more reliable indicator of the PMSI, more specifically with the use of one widely-accepted method of chemical analysis.

The current study investigated whether numerous elements common in marine waters can be detected in bones that were submerged in that environment using X-ray fluorescence spectroscopy (XRF). Analysis of the bone composition of each sample, helped determine whether there was a significant linear relationship between the changes in elemental concentrations and the amount of months submerged. The goal was also to determine if the elemental concentrations are affected by the type of water the samples were submerged in; therefore, the present study investigated whether there is a significant difference between the elemental concentrations of bone submerged in fresh, brackish, and saltwater. Finally, it was discussed how applicable the current study's method is to developing a standard method for calculating a more accurate PMSI for marine forensic cases.

PREVIOUS RESEARCH

Factors Affecting the Rate of Soft Tissue Decomposition in Marine Environments

Before calculating a PMSI for a recovered set of remains, medical examiners and forensic anthropologists have to determine all of the different factors within the environment that affected the decomposing remains. Then they have to determine how these factors affected the remains; for example, did a specific environmental factor increase or decrease the decomposition process. After these determinations are made, a more specific PMSI for the remains can be estimated.

Researchers originally believed that a body would decompose slower underwater than on land, because of the lower temperatures of the aquatic environment, the limited insect activity on the submerged remains, and the decreased bacterial activity within the submerged remains (Higgs and Pokines 2014; Rodriguez 1997). Insects, such as flies from order Diptera and their maggot masses, can only consume the decomposing tissue if they have access to the remains, and submerged remains are only available to terrestrial insects when they are floating (Higgs and Pokines 2014). Flotation of the remains and subsequent access to insects can occur when fresh remains are initially introduced to the water or later during the decomposition process when the remains have bloated enough to resurface (Rodriquez 1997). However, the amount of time that remains float in marine environments is extremely variable (Higgs and Pokines 2014); therefore, unlike in terrestrial environments, insects are usually not the most dominant scavengers of remains that are decomposing in marine environments. The lower temperatures of the water

inhibit bacterial growth, which therefore reduces the rate of decomposition during putrefaction (Higgs and Pokines 2014). Decomposition by bacteria is also hindered in an ocean environment, because the salinity of the water reduces bacterial activity (Rodriguez 1997).

Some recent research has supported the relationship between marine environments and slower decomposition, specifically Anderson and Hobischak's (2002, 2004) studies on submerged pig (*Sus scrofa*) carcasses in the Howe Sound in British Columbia, Canada. However, other studies performed by Ayers (2010), Jones *et al.* (1998), Anderson (2008), and Anderson and Bell (2016) concluded that decomposition occurs more rapidly in aquatic environments than in terrestrial environments. This discrepancy in results suggests that decomposition can occur slowly or more rapidly depending upon the surrounding environment of those remains at that specific time. Some of the major variables that have affected results include floating times, marine scavengers, presence of clothes, currents, and adipocere formation (Haglund and Sorg 2002; Sorg *et al.* 1997).

The amount of time spent floating on the water's surface affects the rate of decomposition, because when a body is at the surface, it can now be scavenged by birds, insects, and insect larvae (Haglund and Sorg 2002). Ayer's (2010) stagnant tub experiment demonstrated how floating carcasses are more susceptible to insect activity; the exposed internal organs of a floating freshwater pig attracted more insect activity, which possibly supported a faster rate of decomposition. This suggests that if a body floating in saltwater had external damage, potentially from a perimortem trauma or

postmortem alteration, insects and insect larvae would be more likely to colonize the exposed tissue and consume the remains at a fast pace.

Marine scavengers are known to affect the rate of disarticulation and skeletonization as the remains travel from the surface to the sea floor. More actively swimming scavengers have an opportunity to feed on the remains as they float and gradually sink after initial or secondary flotation phase (Sorg *et al.* 1997). There are a number of cases in which sharks scavenged human remains, resulting in postmortem trauma and/or loss of tissue. For example, after the recovery of 113 bodies from Yemenia Airlines Flight IY26 crash in the Indian Ocean, areas of missing tissue on 62 bodies were analyzed and described as scooped-out lesions (Ribéreau-Gayon *et al*. 2017). The morphological characteristics of these unique lesions helped identify them as bite marks from cookiecutter sharks (*Isistius* spp.), which were known to have been in the vicinity of the crash site (Ribéreau-Gayon *et al*. 2017). In another case, a set of fleshed remains found in a fisher's trawl net off the coast of Louisiana was autopsied and anthropologically analyzed once macerated (Allaire *et al.* 2012). Irregular gnaw marks in the soft tissue were noted in the autopsy report, and thoroughly described in the anthropological report as punctures without fractures, punctures with fractures, striations with bone shavings, overlapping striations, and incised bone gouges in the skeletal material; these lesions were mostly due to a bull shark (*Carcharhinus leucas*) and one or more smaller requiem sharks of genus *Carcharhinus* (Allaire *et al.* 2012).

Once marine remains are in contact with the sediment, they are rapidly fed on by sea floor fish and numerous types of invertebrates. Anderson and Hobischak (2002, 2004)

demonstrated that pig carcasses that were in contact with the sediment for the majority of their experiment were scavenged and skeletonized more quickly than the remains that remained floating slightly off the sea floor. The increase in disarticulation and skeletonization of carcasses that were on the sea floor also suggests the importance of local fauna that can access the remains. Evidence from numerous studies on submerged animal carcasses and from some forensic cases involving submerged human remains showed that the most common scavengers of soft tissue within a marine environment are invertebrates, such as crustaceans (Anderson 2008; Anderson and Hobischak 2002, 2004; Dumser and Türkay 2008; Jones *et al.* 1998; Sorg *et al.* 1997). Anderson (2008) observed large amounts of squat lobsters (*Munida quadrispina*), Dungeness crabs (*Cancer magister),* and three spot shrimp (*Pandalus platyceros*) scavenging a pig carcass submerged near Vancouver Island in British Columbia, Canada. In this experiment, half of a 26 kg pig was skeletonized within 23 days of submergence. Jones *et al.* (1998) noted that invertebrate scavengers, such as lysianassid amphipods and galathcid crabs, were major contributors to the skeletonization over six days of a sagittally sectioned half of an Atlantic white-sided dolphin (*Lagenorhynchus aculus*) carcass submerged 4000 m to 4800 m on the abyssal sea floor of the northeast Atlantic. Crustaceans and other invertebrates tear at and bore into the skin of carcasses in order to reach the desired soft tissue (Anderson 2008; Anderson and Bell 2016; Sorg *et al.* 1997).

Although local crustaceans and other marine fauna often increase the rate of decomposition by quickly scavenging food-falls, their activity is sometimes hindered by the presence of clothing. Marine scavengers can only eat soft tissue that is accessible to

them and sometimes cannot reach soft tissue underneath certain apparel, depending upon the condition and type of clothing. For example, Dumser and Türkay (2008) described a case in which a body was found still strapped into a seat in a helicopter that crashed into the Mediterranean Sea 34 days earlier. The body was still intact, because it was tightly sealed into its flight suit, while the skull and one ungloved hand, both of which were exposed to the marine environment, were completely skeletonized. Pokines and Higgs (2015) and Sorg *et al.* (1997) have observed in forensic cases where otherwise skeletonized bodies still wearing shoes can have practically intact soft tissue covering the feet.

Adipocere formation on human remains has also been known to slow the rate of decomposition by preserving the remains and preventing excessive marine scavenging. Adipocere is a white, gray, or beige soapy or wax-like substance that is made from hydrolyzed and hydrogenated adipose tissue produced during saponification (Higgs and Pokines 2014; O'Brien and Kuehner 2007). Adipocere usually forms in moist anaerobic environments within an optimal temperature range for the bacterial activity of *Clostridium* species, such as *C. perfringens* (Higgs and Pokines 2014; O'Brien and Kuehner 2007); however, this substance has also formed around remains in marine environments that have less than optimal temperatures, yet have anaerobic conditions with little to no marine scavenging (Higgs and Pokines 2014). In an experiment, O'Brien and Kuehner (2007) tried to initiate saponification on three male cadavers submerged in pits filled with freshwater; they determined that saponification occurred within three

months on two of their three sets of remains when analyzing the macroscopic and chemically changes.

Lewis *et al*. (2004) discussed a case involving the recovery and identification of the missing crew members entombed within the *Ehime Maru*, a Japanese fishing training boat that sunk in 610 m of water after colliding with a *Los Angeles*-class fast attack submarine named the USS *Greeneville*. Due to the wreck's great depths and the necessity to find the missing remains, the wreck was raised off of the seafloor and transported to an area with a depth of 35 m. Upon recovery of eight out of the nine missing individuals, seven remains were found to be intact and six remains at various stages of skeletonization had formed adipocere even at the lower water temperatures of the original wreck site (Lewis *et al*. 2004). Kahana *et al*. (1999) examined the case involving the recovery of the missing crew members entombed within the *Mineral Dampier*, a ship that sunk in the East China Sea at depths ranging from 65 to 85 m. A series of body recoveries were performed over a 433 day period, which allowed for the analysis of changing decompositional features and the formation of adipocere (Kahana *et al*. 1999). Adipocere was first noted to be on recovered remains by the 38th day since submergence; on this individual it was located on the subcutaneous tissue of the face and lower abdomen (Kahana *et al*. 1999). Adipocere continued to cover a larger surface area of the recovered remains as the bodies remained submerged for longer periods of time; by the 433rd day, one of the remains from one of the closed cabins had a thick, hard layer of adipocere on cutaneous and subcutaneous tissue (Kahana *et al*. 1999). The exceptions to this trend were the bodies that were found in an open cabin that contained a strong water current;

these remains were skeletonized and had only remnants of adipocere formation (Kahana *et al*. 1999).

PMSI from Soft Tissue Decomposition in Marine Environments

Although many factors affect the rate of decomposition in marine environments, researchers have attempted to determine the specific amount of time that it would take for a body to go from fresh to skeletonized. Recent research in marine taphonomy has focused on defining the stages of decomposition of remains in the ocean and determining the factors that influence the duration of these stages, since there is a lack of knowledge in these areas when compared to what is known about these processes in terrestrial environments. The stages of marine decomposition that were summarized in Haglund and Sorg (2002) included fresh, early floating, floating decay, bloated deterioration, floating remains, and sunken remains; but, other terms, such as fresh bodies, bloated bodies, active decay, and remains, can be used to separate the stages of decomposition, further complicating the analysis of marine decomposition research.

The determination of a decomposition stage is not always easy, because some features are not clearly defined, while others can occur across multiple stages (Anderson and Hobischak 2002, 2004). The general features of soft tissue decomposition within aquatic environments include: lividity, bloating, marbling, hair shedding, skin sloughing, scavenging, adipocere formation, flesh decaying, internal organ exposure, algae accumulation, silting, disarticulation, skeletonization, and staining of skeletonized remains (Anderson and Bell 2016). Researchers attempted to associate these features with

specific stages of decomposition and tried to identify the starting and ending points of each stage; however, they discovered that the terminal points of each decomposition stages were not well defined. For example, Anderson and Hobischak (2002, 2004) studied submerged pig carcasses in the Howe Sound of British Columbia, Canada. During these studies, pig carcasses were submerged in 7.6 m and 15.2 m of water by a slack rope and weights. Divers would sporadically visit the site to observe and document the stages of decomposition and marine scavenging. They determined that the fresh stage occurred between 0 and 3 days after death, the bloat stage between 3 to 11+ days after death, the active decay and advanced decay stages between 11 to 30+ days after death, and the remains stage 40+ days after death. However, Anderson and Hobischak (2002, 2004) also noted that the rates of decomposition were not constant between trials and were affected by length of time spent floating above the sediment, as well as depth, surface types, and season; this is evident in that during one trial the remains still had not completely skeletonized until 140 days after submergence.

Ayers (2010) observed the stages of decomposition of pigs floating in stagnant tubs of freshwater and saltwater, compared with terrestrial decomposition. The freshwater pigs skeletonized within 11 and 22 days, while the saltwater pigs skeletonized under the surface by the 38th day; the pig on dry land did not skeletonize until the 65th day (Ayers 2010). Although this study did produce a time frame for stages of decomposition in aquatic environments, these results are most applicable to forensic cases involving bodies floating in stagnant waters.

As previously noted, the inconsistent research results and consequently, the more open-ended time frames are due to the fact that many environmental factors affect the rate of decomposition, disarticulation, and skeletonization (Haglund and Sorg 2002; Sorg *et al*. 1997), which complicate the estimation of PMSI during forensic investigations. In an attempt to address this problem, a few researchers recently tried to apply the concept of accumulated degree-days (ADD) to decomposing remains in aquatic environments. An ADD is the sum of the average daily temperatures of the specific environment to which the remains were exposed (Stuart and Ueland 2017); this is used in conjunction with a score of the total aquatic decomposition (TAD). TAD is an overall score of the individual decomposition scores for the face, body, and limbs of a set of remains in an aquatic environment (Stuart and Ueland 2017).

Heaton *et al*. (2010) analyzed forensic cases from rivers and canals in the United Kingdom by noting the PMSI from the forensic case files, then the TAD was calculated by summing up the facial aquatic decompositional score (FADS) with the body aquatic decompositional score (BADS) and the limbs aquatic decompositional score (LADS), which were each derived from a regional-specific description of the decompositional stages. It was determined that duration of time spent in the water and the water temperatures around the body affected the decomposition process. With this information, a single linear regression equation was developed in order to be able to calculate ADD from TAD, which then can be used to estimate PMSI. Although this methodology is relatively simple and easy to calculate, it will not always produce accurate results, because there are other environmental factors that can increase and decrease the rate of

decomposition, which are not accounted for in the linear regression developed by Heaton *et al*. (2010).

Although all of these researchers discussed above were attempting to estimate PMSI, none of them solely studied skeletonized remains. These experiments ended once skeletonization was achieved; thus, the problem of PMSI intervals for the skeletonized elements is still not addressed.

PMSI from Skeletonized Remains in Marine Environments

Forensic investigators have analyzed the size of adhering invertebrates as a way of estimating PMSI. As a general practice, the investigators identify a species of acorn barnacle, research the durations of its life cycle stages, research its average growth rate or its growth rate around the temperatures that preceded the body's discovery, and calculate a potential minimum PMSI. For example, Dennison *et al*. (2004) obtained a partial calvaria off the east coast of New Zealand, which contained one living sub-tidal barnacle *Notobalanus decorus*, three dead barnacles, and one attachment scar. They used scanning electron microscopy (SEM) to visualize and count the growth rings on the barnacle's calcified plates. If the growth rings are laid down twice in a 24 hour period when the barnacle feeds with the tides, than according to the ring counts, the largest barnacles represented at least two years of growth. Dennison *et al*. (2004) believed that the barnacles most likely did not settle immediately upon the bone once exposed to the marine environment, therefore, it probably took 2-12 months before conditions were appropriate for the barnacle to settle. They also needed to take into account that three of

the barnacles had already died before the calvaria was found, which probably occurred after 30-48 months; therefore, they concluded that the calvaria was on the sea floor at depth between 15 and 70 m for at least four years.

Bytheway and Pustilnik (2013) estimated the PMSI from basal diameter measurements of the glycoproteinous adhesion deposits, also referred to as barnacle cement, from acorn barnacles on skeletal and dental remains found in Galveston Bay, Texas. The species of barnacles were determined to be *Balanus improvisus*, which tend to settle at one time each year during March and July. The juveniles usually have a basal diameter less than 5 mm, while the adults have a diameter between 5 mm and 25 mm. The basal diameters of the adhesions ranged from 1.70 to 6.43 mm; statistical analyses concluded that there were two statistically different sized cohorts. Bytheway and Pustilnik (2013) estimated that because there were two settlement cohorts, the remains were in the intertidal zone for 375 to 410 days, a value that took account for the 180 days in the Nauplius stage, 10-45 days in the cypris stage, over 225 days for the development of adult barnacles, and the development of a second cohort of juvenile barnacles.

Magni *et al*. (2015) measured the capitulum length of the largest adhering stalk barnacles (*Lepas anatifera*) from the pants and shoes of a corpse that washed ashore of Italy's Tyrrhenian Sea. The capitulum of a stalk barnacle is the shelled portion of the body that contains the feeding appendages and the pedicle is the stalk section of the body that attaches to the substrate. The maximum capitulum length was 12 mm with a 5 mm pedicle. In an unpublished experiment, Mangi *et al*. (2015) previously estimated growth rates of this species of barnacle from a sample colonizing a mooring in the eastern

tropical Pacific Ocean; they accounted for water temperature changes over the experimental period (26.6-30.2℃), which resulted in estimated growth rates ranging from 0.20-0.74 mm/day. Magni *et al*. (2015) noted that the surface temperature of the Tyrrhenian Sea is usually 12-18℃ in winter and 23-30℃ in summer and that in the months prior to the body's discovery (January to May of 2012) the water temperature ranged from 13-19℃; therefore, they estimated the barnacle age by using the slowest growth rate from their experiment in the Pacific Ocean, which was 0.20 mm/day. Using this growth rate, Magni *et al*. (2015) estimated that the largest barnacle grew 12 mm over the span of 60 days. According to previous research, they assumed that it would take an additional minimum of five days for the cyrpid larvae to attach to a substrate; however, this was only an estimate, since there were no data on larval attachment times at lower temperatures. In addition to the 65 day minimum of floating time, Magni *et al*. (2015) also took into account the effect that cooler water temperatures have on a decomposing body's ability to rise up to the surface, which could take about two to three weeks. Therefore, Magni *et al*. (2015) estimated that the remains' minimum floating time was between 65 and 90 days.

As previously noted, problems arise with each of these methods. For example, Bytheway and Pustilnik (2013) highlight the fact that the remains could have been in the intertidal environment for an unknown amount of time before the barnacles attached or they could have been settled almost immediately upon exposure. In that case, the authors also noted that their reliance on the cement adhesion sizes could lead to inaccuracies, because they did not know the decay rate of the adhesions; therefore, only allowing the

minimum exposure time to be estimated. In addition to an environment with a strong current, barnacles can also not settle on bones that are embedded within the seafloor due to a lack of access for the larvae. In the study by Mangi *et al*. (2015), the barnacle's age was based upon growth rates of barnacles from a completely different ocean and at a significant difference in temperature (13-18℃ versus 26.6-30.2℃). The colder temperature of the water from which the body was found could have caused the adhering barnacles to grow at a slower rate that estimated. Even Magni *et al*. (2015) noted that when estimating their barnacle growth rates other factors other than temperature could have affected shell growth in either direction. Because a number of factors affect barnacle growth rates, only a minimum PMSI can be estimated and even this estimation can be unreliable due to the lack of species, location, and temperature-specific research.

Mineral Content of Seawater

Seawater contains numerous types of organic and inorganic solids, colloids, gases, and solutes (Kennish 2001; Millero 2006; Quinby-Hunt and Turekian 1983); however, the water and solute components of seawater are the portions most often studied when discussing the makeup of saltwater. The solutes are the soluble salts that have dissociated into cations and anions when in water. These components are usually separated into groups of major, minor, and trace elements or ions based on their concentrations in open ocean water, which is considered standard or normal seawater. The major and minor ions are the more abundant solutes with concentrations greater than 1 ppm (1 mg solute in 1 kg of seawater) in standard open ocean water; the six major ions

are chloride (Cl^-), sodium (Na^+), sulfate (SO_4^{2-}), magnesium (Mg^{2+}), calcium (Ca^{2+}), and potassium (K^+), while the minor ions include elements such as carbon (C), bromine (Br), boron (B), strontium (Sr), and fluoride (F) (Table 2.1) (Culkin and Cox 1966; Kennish 2001; Millero 2006; Wilson 1975). The major ions make up over 99% of the dissolved ions in seawater by weight (Kennish 2001; Millero 2006). The trace ions are the solutes with concentrations below 1 ppm, and this group includes nearly all of the remaining elements on the periodic table (Brewer 1975; Millero 2006).

Table 2.1. Composition of 1 kg of seawater with a salinity of 35 PSU and chlorinity of 19.375 ‰ (adapted from Millero 2006).

Ion	Ratio of concentration of solute to concentration of seawater (g/kg)	Ratio of concentration to chlorinity (g/(kg‰))
Cl^-	19.3529	0.998910
Na^+	10.7838	0.556614
SO_4^{2-}	2.7124	0.140000
Mg^{2+}	1.2837	0.066260
Ca^{2+}	0.4121	0.021270
K^+	0.3991	0.020600
HCO^{3-}	0.1070	0.005524
Br^-	0.0672	0.003470
$B(OH)_3$	0.0193	0.000996
CO_3^{2-}	0.0161	0.000830
Sr^{2+}	0.0079	0.000410
$B(OH)_4^-$	0.0079	0.000407
F^-	0.0013	0.000067
OH^-	0.0001	0.000007
Total	35.1710	1.814369

Scientists have studied the composition of water for hundreds of years. Although the first compositional data was collected in the 17th and 18th centuries, the concentrations of seawaters elemental makeup were more thoroughly studied after the 20th century (Culkin and Cox 1966; Millero 2006). More recently, Nozaki (1997) reviewed the previously published data on the distribution of elements in seawater, as well as more recent data collected on modern samples from the North Pacific Ocean. Nozaki (1997) noted that the advancements in methodology and technology allowed for the more frequent and accurate testing of water samples, which led to the discovery that the previously accepted trace metal concentrations were incorrect. Although there were issues with the trace metal concentrations, the concentrations of the other elements, such as the halogens, alkali, and alkaline elements, were well-known because they are easily detectable, largely abundant, conservative in nature, and homogenous through space and time (Nozaki 1997).

All of the major and a number of the minor ions are described as conservative ions, because their relative proportions are nearly constant in the open ocean, meaning that the ratio between any two ions dissolved in open ocean water is fixed over time and space; this concept is called the principle of constant proportion or constancy of composition (Wilson 1975). The more abundant components are homogenous vertically and horizontally in the open ocean due to ocean water circulation (Nozaki 1997). These components are also homogenous over time due to their large residence times, meaning that the ions remain in solution for long periods of time, usually ranging 10^5 to 10^8 years,

which results in their more constant concentrations (Kennish 2001; Nozaki 1997). Unlike the more abundant components, the low concentration, difficult detection, and uneven distribution of the trace elements result in less accurate and less abundant data on the concentrations of these trace elements throughout open ocean waters. In addition, no single laboratory has been able to determine every element in a seawater sample (Nozaki 1997); therefore, the tables describing the overall composition of seawater consists of concentration values that were compiled from the past work of numerous researchers, as seen in the works of Millero (2006), Nozaki (1997), and Quinby-Hunt and Turekian (1983). Although these compiled data are incomplete and changing, the mean concentrations of the more abundant elements remains the same and therefore, the work from the 1960s and 1970s is still being referenced as an accurate data source. A consequence of this collaborative approach to collecting data is that these reference lists of seawater components and concentrations can have slightly different formats providing slightly different information. For example, some provide ion concentrations (Kennish 2001; Millero 2006; Wilson 1975) while others provide overall element concentrations (Brewer 1975; Millero 2006; Nozaki 1997; Quinby-Hunt and Turekian 1983), which results in different values since a single element can appear in a number of different ion complexes. In addition, some researchers expressed their results as a ratio of concentration (mg/kg) to chlorinity (‰) (Culkin and Cox 1966; Morris and Riley 1966), or as concentration of solute (moles or mg) for 1 kg seawater (Table 2.1) (Horibe *et al*. 1974; Millero and Leung 1976). Some researchers who compiled tables of previously

published data converted the results into molar values (M) in order to incorporate volume (Brewer 1975; Millero 2006).

Because the major ions are conservative, homogenous over time and space, and the major solute component of seawater, the open ocean has been calculated to have a relatively constant amount of dissolved salts in solution, meaning it has a relatively constant salinity. The salinity of open ocean water is 35 practical salinity units (PSU), which is equivalent to 35 parts per thousand (‰) (Kennish 2001); this means that 3.5% of the sample of open ocean seawater consists of dissolved salt, while the other 96.5% consists of water molecules, or there is 35 g of dissolved salt in 1 kg of seawater. In order to quantify the overall salinity of the sample, the amount of only one of the single major ions needs to be measured, since there is a consistent proportion to the other major constituents of seawater (Millero 2006). Originally, the concentration of chloride was used to determine salinity, because it was the most abundant solute, but eventually all of the halogens, which included chlorine, were measured to calculate salinity. The use of conductivity meters has made it easier to calculate the salinity of a water sample; these meters measure the electrical charge carried in a solution, which is dependent upon the amount of dissolved ions within the sample (Wilson 1975).

Although the salinity level and the abundancies of the major components of open ocean water are constant, the same is not true for other samples of seawater that are not from the open ocean. Estuaries with more brackish waters, as well as anoxic basins, hydrothermal vents, and evaporated basins all have non-conservative concentrations of ions present in the water, because processes like precipitation, evaporation, dissolution,

and oxidation all affect the distribution of ions horizontally from one area to another and vertically through the water column (Kennish 2001; Millero 2006). Trace element concentrations also fluctuate due to anthropogenic inputs (Kennish 2001; Quinby-Hunt and Turekian 1983). Therefore, the relatively constant concentrations of seawater and salinity values discussed above only refers to open ocean seawater samples with a salinity of 35 PSU, chlorinity of 19.374‰, pH of 8.1, and temperature of 25°C (Millero 2006; Millero and Leung 1976). Therefore, the presence of certain marine elements and their specific concentration levels could help identify what type of water an unknown water sample came from and could potentially determine the type of aquatic environment a solid sample, like a bone, had been exposed to.

Conservation of Bone from Marine Environments

Many archaeologists who are recovering bones from the ocean are initially interested in stabilizing the specimens so that they can be studied further at a later time. All types of archaeological material excavated from shipwrecks within the ocean have to undergo preservation treatments, because they can rapidly deteriorate during an analysis outside of its usual environment. These treatments are necessary, because artifacts and remains from shipwrecks have been in a stable physical, chemical, and biological environment for a long period, and if removed, they will be exposed to oxidizing agents, microbes, chemical changes, and temperature variations. These all can degrade an artifact within 10 days if not properly treated (Steptoe and Wood 2002). Therefore, previous research involving bones in the ocean has been undertaken by underwater archaeologists

interested in the conservation of artifacts and the development of cleaning and stabilizing procedures for materials, such as bone. In order to stabilize these artifacts in a dry atmospheric environment, they have to be slowly rinsed of all soluble salts (Cronyn 1990; Hamilton 1997, 1999/2001; Steptoe and Wood 2002). Bones submerged in seawater are surrounded by dissolved salts, which are continuously in contact with the bones' surface as well as the internal structure via the pore spaces (Dupras and Schultz 2014). When bones from the ocean are exposed to the atmosphere, the water within the bone begins to evaporate, resulting in the formation of salt crystals on the surface of the bone (Dupras and Schultz 2014; Junod and Pokines 2014). Porous materials, such as bone, retain a small amount of water within their capillaries as their surroundings dry up, resulting in the concentration of dissolved salts within these capillaries or pore spaces (Cronyn 1990). The soluble salts recrystallize once the remaining water within the material dries, leading to salt crystals filling up the pores of the bone. The thermal expansion of the crystals could form microcracks within the bone, because this increase in volume of the salt crystals can exert an intense amount of pressure on the internal walls of the artifact or bone (Dupras and Schultz 2014; Cronyn 1990; Junod and Pokines 2014). This pressure can increase even more and further worsen the cracking if the relative humidity of the artifact's environment increases, because the salt crystals can absorb water and expand without dissolving (Cronyn 1990). If all of the salt is not removed, even subsequent submersion in water could result in the dissolution and further penetration of the salt minerals into the microcracks within the bone; upon drying, the salts will recrystallize in this deeper area, which could further enlarge the cracks within the bone (Junod and

Pokines 2014). Any formation and expansion of crystals could impact the internal and external structures of the hydroxyapatite and the remaining collagen within bone and therefore result in the mechanical weakening and eventual destruction of the bone. Prassack (2011) discussed this phenomena when she noted that the submergence of lesser flamingo (*Phoeniconaias minor*) bones in the saline-alkaline lakes of Tanzania did not cause cracking or exfoliation to the bones; rather, it was the continuous cycles of submergence and exposure that caused the bones, especially the longer and denser bones, to crack and fragment. Prassack (2011) believed that the daily heating of the bones by sunlight caused the salt crystals to expand and crack the bone.

There is little research in the field of underwater archaeology on the preservation of human bones from shipwrecks, because large assemblages of bone from a single site are unusual. However, as previously noted, human remains have been discovered at a number of shipwreck sites (Broadwater 2012; Bruseth and Turner 2005; Cunningham Dobson and Tolson 2010; Marchant 2016; Steptoe and Wood 2002; Stirland 2013). The lack of a strict protocol has resulted in variations in preservation procedures among anthropologists who are studying these marine remains. For example, after being excavated from the sunken 18th century Royal Navy vessel, the HMS *Pandora*, the bones were stored in seawater at a cooler temperature in order to prevent biodegradation (Steptoe and Wood 2002). Once at the museum laboratory, the bones were place in tap water that was slightly above freezing temperatures. Steptoe and Wood (2002) noted that the desalination treatment removed the sodium chloride (NaCl) gradually from the bones; however, the process is not explained in detail. The bones were then slowly dehydrated

following an unmentioned method and were slowly introduced to a dry environment in a sealed container where the humidity could slowly be decreased (Steptoe and Wood 2002). The skeletal material from the HMS *Pandora* underwent the desalination process for 11 months and the dehydration process for four months; however, other artifacts could require about four years of treatment. During the subaerial excavation of the U.S. Civil War-era USS *Monitor's* turret, the contents of the internal chamber were continuously rinsed with water to prevent the materials from drying out (Broadwater 2012). After being removed from the turret the human remains were sent to the Central Identification Laboratory, where they underwent for several months a concretion removal process and a desalination process, which was similar to what Hamilton (1999/2001) outlined (Broadwater 2012; Dasbach 2013). The human bones from the Tudor-era *Mary Rose* were placed in netting bags, which were washed for four weeks in a cascade of clean, freshwater that ran through four baths; each group of nets started in the bottom bath and moved up the cascade to the next bath each week (Stirland 2013).

Although detailed methodologies for the preservation of human skeletal remains from the ocean are lacking, there are more detailed procedures for small artifacts that contain bone. According to Hamilton's (1999/2001) conservation manual for material from underwater sites, the surface sediment on an archaeological artifact containing bone first has to be lightly brushed off or quickly rinsed off with soap and water or with alcohol. The soluble salts that were absorbed by the bone have to be removed next before they crystallize and exfoliate the surfaces of the dry bone (Hamilton 1999/2001). Therefore, bone samples are rinsed in successive baths of 100% seawater to a mixture of

75% seawater and 25% freshwater to more diluted mixtures until a bath of 100% freshwater is reached. The bones are next rinsed in a number of freshwater baths until a conductivity meter detects that the soluble salt level has reached that of tap water (Cronyn 1990; Hamilton 1999/2001). After a bath of deionized or distilled water, the bone is dried in a series of alcohol baths in order to prevent any reactions to changing humidity (Hamilton 1997, 1999/2001). Although a conductivity meter is usually used by archaeologists during the soluble salt removal process of bones found in the ocean, it could also potentially be utilized to estimate the total amount of soluble salts that were removed from the bone; however, this has not been researched. Other chemical analyses would be more successful at analyzing what specific marine elements penetrated into the bone.

Elemental Analysis of Bone

The organic portion of bone consists mainly of collagen, but also includes proteoglycans, glycosaminoglycans, and proteins such as osteonectin, osteocalcin, and osteopontin (Schultz 2006). The inorganic portion of bone consists of phosphate, calcium, carbonate, citrate, nitrate, sodium, magnesium, fluoride, and strontium; some of these combine to form the hydroxyapatite crystals. Other than the major components, researchers have also identified a number of minor and trace elements found within bone.

Forensic anthropologists and archaeologists have used the known elemental composition of bone in order to differentiate between osseous and nonosseous samples. By visually and statistically comparing calcium to phosphorous ratios obtained from

elemental analyses, osseous materials, such as human bones, animal bones, and teeth, have been differentiated from nonosseous materials, such as shells, ceramics, and some coral, even in altered or damaged states (Christensen *et al.* 2012; Kuzel *et al.* 2016; Meizel-Lambert *et al.* 2015; Zimmerman *et al.* 2015). This area of study was later expanded to include differentiating between human and nonhuman bone fragments (Buddhachat *et al*. 2016). Gonzalez-Rodriguez and Fowler (2013) even researched whether differences in the elemental concentrations within bone samples could be used to separate individuals in commingled archaeological assemblages.

In bioarchaeology, anthropologists analyze the chemical composition of bone, because they wanted to identify any geochemical markers that identified what past populations ate and drank, where they migrated from, and what was the status of their health (Zapata *et al*. 2006). The ability to identify and analyze these geochemical markers is complicated by the interference of postmortem environmental contamination. When researchers have studied how taphonomic processes affect bone composition, it is usually because they are concerned about the preservation of the samples and its ability to help answer the previously stated bioarchaeological questions, rather than any forensic ones. Some studies have specifically compared different preparation techniques and analytical methodologies in order to find the best way of removing the environmental contamination so that antemortem geochemical signals can be detected. For example, Lambert *et al*. (1991) analyzed the elemental composition of bones from Illinois sites dating around 175 ± 80 AD and 1000 ± 70 AD in order to study the transition from hunting and gathering to farming of maize; however the specific goal of their research

was to determine whether the removal of the outer layer of bone via abrasion would reduce the elemental concentrations of environmental contaminants. Other researchers, like János *et al*. (2011), have embraced the fact that environmental contamination occurs due to diagenesis and use that to determine whether assemblages from different sites were exposed postmortem to similar or different environmental conditions.

Many archaeological studies have focused on the later stages of diagenesis and fossilization rather than the earliest stages which occur within the first few years; many forensic-based studies have worked with samples that are in the early diagenesis stage, but their research questions do not involve looking at the chemical changes that occur over time. López-Costas *et al*. (2016) compared elemental differences between three types of bones (thoracic, long, and cranial) from 30 archaeological assemblages of two different time periods (Roman and post-Roman) that were recovered from two different soil environments (acidic and alkaline) at the same site. After obtaining the compositional data, they determined what chemical signals were different and what factors caused these differences. The analysis showed that postmortem changes in elemental concentrations did occur and that soil environment and bone type significantly influenced these changes. Krajcarz (2019) questioned how quickly after deposition in a soil environment does the chemical signature of bone change due to environmental conditions; therefore, to study early diagenesis, elemental analysis of bones placed in different sediments for 2.5 years were analyzed; Krajcarz (2019) concluded that 2.5 years was early enough to see chemical composition changes from that of fresh unaltered bone, under certain environmental conditions (i.e., moist, rich organic sediment).

McElreath (2018) performed a study in which the effects of early diagenesis were monitored in remains from a marine environment. In order to help the field of wildlife forensics and wildlife criminal investigations, McElreath (2018) monitored elemental concentrations in bones of a harbor porpoise (*Phocoena phocoena*), Kemp's Ridley sea turtle (*Lepidochelys kempii*), harbor seal (*Phoca vitulina*), gray seal (*Halichoerus grypus*), Harp seal (*Pagophilus groenlandicus*), and a domestic cow (*Bos taurus*) that were submerged off the coast of Maine for 0, 62, 335, and 427 days. The trends in elemental changes over time were compared among the different species and to samples that were placed in a terrestrial environment. For example, iodine and zirconium concentrations in the cow bones decreased over time when submerged in the ocean, but increased over time when on land. Although this study did examine the elemental concentration of bone during early diagenesis in a marine environment, it only included four time intervals, which did not provide enough data to explain some inconsistent trends. This study also only discussed the trends in concentration changes for certain elements rather than using this information to produce a method of calculating a more specific PMSI.

Methodologies for Elemental Analysis

Similar to how there are many different reasons to analyze the chemical composition of bone, there are many methodologies and instruments available to do so, such as powder X-ray Diffraction (XRD), Scanning Electron Microscopy paired with

Energy-Dispersive X-ray Spectrometry (SEM/EDS), and Atomic Absorption Spectrophotometry (AAS).

XRD is qualitative method of analysis that identifies crystalline compounds within a powdered sample by analyzing a unique diffraction pattern created when X-rays hit the different alignments of the mineral's lattice structure and diffract back in specific directions (Herrera and Videla 2009). In order to identify these minerals within a sample like bone, the experimental peak patterns from the XRD readout are compared to known compound patterns listed in the Joint Committee on Powder Diffraction Standards (Trujillo-Mederos *et al.* 2012). XRD peaks are only seen if the crystals are larger than 3 nm and if they make up more than 3% of the sample (Trujillo-Mederos *et al.* 2012); these peaks also do not indicate the concentrations of the crystalline compounds within the samples, making this a qualitative form of chemical analysis rather than a quantitative. In preparation for powder XRD, the sample has to be ground up into a homogenous, fine powder; therefore, the original structural integrity of the sample is destroyed, but the powder sample will not be destroyed during the analysis process. Trujillo-Mederos *et al.* (2012) used XRD to detect the presence of hydroxyapatite and sodium chloride in human bone samples that had been boiled in fresh and saltwater.

Another method of analyzing the elements of bone is SEM/EDS. SEM is used to identify the morphology of different components found within a scanned areas of the sample. This instrument captures a high resolution, magnified image of that specific area's surface topography by using electron emissions (Herrera and Videla 2009). When coupled with EDS, the elements within the scanned area can be identified (McElreath

2018; Trujillo-Mederos *et al.* 2012). This method is semi-quantitative, meaning it can calculate the mass percentages of the present elements; a mass percent represents the percentage of a component in a mixture relative to all the other components within that mixture, rather than the actual element's concentration that is present in the sample. SEM/EDS does require samples to be prepped, sometimes in a mildly destructive way. SEM/EDS requires a small sample of bone to be removed in order to be analyzed within a vacuum as a solid fragment or as a homogenous, fine powder. If the sample is a homogenous powder, than the EDS analysis is representative of the entire sample; however, if only a fragment's surface is analyzed, than the sample is not homogenous and an EDS analysis of each area of that fragment has to be performed in order to get results representing the bulk of the sample (Trujillo-Mederos *et al.* 2012). In order to obtain better images, the sample's conductivity may need to be improved by applying a thin layer of gold or carbon on the sample's surface (Herrera and Videla 2009). SEM/EDS is often utilized in the archaeology and biological anthropology fields as a way of differentiating between osseous and nonosseous materials (Meizel-Lambert *et al.* 2015) and a way to study morphological changes due to taphonomy (Lambert *et al*. 1991; Trujillo-Mederos *et al.* 2012).

AAS is a quantitative method that vaporizes liquid samples, breaking them down to their atoms; the concentrations of these atoms are measured by absorption of a characteristic wavelength of light (Harris 2010). This method is highly sensitive and can differentiate between elements within a complex sample. Over 70 different elements can be identified, with their real concentrations rather than percent mass. Although this

method is extremely useful, the sample preparation needed for analysis is quite complex, and the process is destructive. The sample has to be completely digested and in a liquid form in order to be analyzed; to achieve this, the samples are broken down with a number of acids. For example, the procedure used by Cáceres-Saez *et al.* (2016) included the pulverization and homogenization of bone as well as the digestion of the dried samples in a microwave with nitric and perchloric acids. Digestion procedures have to be developed through trial and error in order to guarantee that all of the sample was digested properly. For example, Lambert *et al*. (1991) followed a previous protocol, but noted that they had to make a number of modifications; elements of intermediate and high sensitivity underwent a dilution ranging from 2-fold to 200-fold depending upon the particular element in question. Some elements had to be analyzed in a different mode or depressants were needed to reduce ionization of certain elements.

X-ray Fluorescence

All of the above elemental analyses produce different results and therefore are only used when a specific goal is in mind. The current study examines elemental concentrations of bone samples without losing any evidence of marine contamination and without causing major alterations to the bone, such as the complete destruction with acids or the addition of metal coatings. Therefore, the present study used X-ray fluorescence (XRF) as a means of elemental analysis.

XRF is a common, relatively non-destructive type of chemical analyses that has often been used in geological and archaeological studies. Energy dispersive XRF (ED-

XRF) is used to identify the elemental concentrations of a sample in mass percents. In order to do this, high energy protons from an X-ray beam hit a sample, which usually causes an excitation of a K or L shell electron within the sample's atoms (Byrnes and Bush 2016; Daar *et al*. 2015). The excited electron is ejected from the atom, forcing an outer orbital electron to drop down into the empty space; this shift in orbital electrons results in the emission of a secondary X-ray from the atom that has a quantified amount of energy. In ED-XRF, the detected emissions are sorted through by energy (not wavelength) and displayed in an energy spectrum as numerous peaks; these peaks correspond to a specific elemental signature and the height or intensity of a peak corresponds to the amount of that specific element within the sample. It should be emphasized that the mass percents obtained via ED-XRF are calculated by dividing the area under each specific peak by the total area, therefore, the value is more of a proportional amount rather than the actual concentration of each element in the sample (Buddhachat *et al*. 2016). Although the produced values are relative concentrations, the data are still extremely useful, especially when statistical tests are applied.

There are two main types of XRF spectrometers: portable and benchtop. Portable XRF spectrometers are handheld units that can be brought into the field and perform in situ elemental analysis on samples. They are a common tool for archaeologists who want to analyze the superficial layers of objects, such as surface coatings or paints on ceramics and adhering deposits (Čechák *et al*. 2007); they also use these portable units to differentiate between osseous and nonosseous fragments (Christensen *et al.* 2012; Kuzel *et al.* 2016; Zimmerman *et al.* 2015). These units are easy to use, especially with their

point-and shoot capabilities. Although they are easy to use and convenient, an important limitation is that lighter elements, such as phosphorus or magnesium cannot be detected, because lower energy X-rays are absorbed in the air; therefore, some elements of a bone sample cannot be detected.

Benchtop spectrometers, like the one used in the present study, are stationary units kept in laboratories that are associated with a vacuum or gas purge system (SPECTRO Analytical Instruments GmbH 2011). These vacuum or purge systems limit the amount of X-rays that are absorbed by the surrounding air and promote the detection of lighter elements (Smith 2007). Benchtop XRF spectrometers have the capabilities of analyzing solid, powdered, pressed pellets, or liquid samples, depending upon the specific unit; pressed pellets are a powdered sample, occasionally mixed with a binding agent, that are compressed into a solid disk by use of a manual hydraulic press (Smith 2007; SPECTRO Analytical Instruments GmbH 2011). In the present study powdered samples were analyzed under helium purge; however, if a pressed pellet was analyzed, it would need to be in a vacuum.

Like any form of chemical analysis, a number of issues arise when using XRF as a means of detecting marine elemental contamination in bone. For portable XRF spectrometers, the surfaces of the fragments need to be flat and smooth in order to obtain accurate readings (Christensen *et al.* 2012; Kuzel *et al.* 2016; Zimmerman *et al.* 2015). This might require scraping the top layer of bone, which can subsequently remove salt crystals as well as other elements from the bone sample (Lambert *et al*. 1991). The handheld XRF spectrometer cannot detect low mass elements below magnesium

(Zimmerman *et al.* 2015), which suggests that sodium (22.990 g/mol) will not be detected; therefore, other elements that make up marine salts, other than sodium chloride, need to be identified. Theoretically, table top units will be able to detect lighter elements than the handheld ones; however, use of these units requires more sample preparation. For example, samples may have to be milled to form a homogenous powder and then either placed within an XRF capsule of pressed into a pellet. Depending upon which form of spectrometer is used and which comparative standards are available, a vacuum or helium atmosphere will be required. Because no elemental analyses are perfect, standard reference materials (SRMs) are required for instrument calibration, accuracy and precision tests, as well as data correction.

METHODS

Experiment 1: Time Trials (TT)

The first part of the present study, the time trials (TT), analyzed the change in certain elemental concentrations within submerged bone over time. This experiment included a control group of five, never submerged bones (Month 0: Samples A-E) and ten submerged experimental groups, each consisting of five bones (Months 2-20: Samples A-E); an experimental group of five bones was removed from the ocean every two months for a 20 month period. The five bones for each submergence time interval (0-20 months submerged) were then analyzed with an ED-XRF in order to identify any changes in elemental concentrations.

The experimental osseous remains consisted of 55 commercially purchased, previously frozen pig femora, which were largely defleshed with adhering unfused epiphyses (Figure 3.1, a). Scissors and scalpels were used to remove any large chunks of meat that were still adhering to the epiphyses and diaphyses (Figure 3.1, b). The defleshed pig remains were used in the present study as a proxy for human remains that had already decomposed in an aquatic environment to the point of skeletonization and are now able to absorb soluble salts and other elements directly from the surrounding seawater. Five defleshed femora were not included in the submerged experimental group, because they represented the control group of dry, unaltered bone at time zero; these control bones were frozen until processing.

The remaining fifty pig femora were submerged in two commercial-grade metal lobster cages (approximately 0.76 m x 0.47 m x 0.31 m) in order to control the location of the bone samples within the marine environment. These cages had a waterproof coating that prevented the oxidation of the metal; this feature prevented the cages from chemically influencing the bones while they were submerged for the duration of the experiment. The metal cages were modified so that they no longer contained the interior trapping mechanism, therefore allowing small and medium marine taxa access to the bones while excluding large taxa that could remove the bones from the cages. The fifty pig femora were separated between the two cages in order to minimize the chances of all experimental materials being lost due to any extreme weather or other circumstances during the experimental time period.

The femora were individually attached to the interior of the lobster cages. A heavy duty plastic cable tie was secured to the interior side of each cage's lid and linked to a second cable tie, which was fastened around the midshaft of each femur; this allowed the femora to hang down into the interior cavity of the cages (Figure 3.2). The hanging femora were evenly distributed throughout the cages and were free to move with the water currents.

Within the center of each cage for the time trial experiment, an Onset HOBO Pendent Temperature Data Logger was attached with a cable tie in order to monitor the temperature of the water surrounding the samples for the duration of the experiment. The temperature data loggers recorded the temperature of the surrounding water once every hour during the duration of the study. The first set of loggers were replaced after the first

four months of submergence; during this time frame these two data loggers malfunctioned after only recording from the launch date to 13 December 2016 at 3:00pm and 30 December 2016 at 8:00am. After this incident, the two data loggers were replaced every two months when the next batch of bones were removed from the cages. A portion of the missing temperature data was supplemented with temperatures collected by the Environmental Quality Department (ENQUAD) of the Massachusetts Water Resources Authority (MWRA) (2017) at harbor monitoring stations 038 and 084, both of which surround the test site (Figure 3.3); approval to use this temperature data, as well as other physical harbor measurements like salinity, was given by Wendy Leo and Kristin MacDougall of MWRA. The available temperature data were used to monitor the average daily water temperature throughout the experimental period (Figure 3.4).

The lobster cages containing the pig femora were secured to the John T. Fallon State Pier (N 42^{o} 18’ 55.60” W 71^{o} 01’ 57.31”), located on the campus of the University of Massachusetts in Boston and adjacent to the John F. Kennedy Presidential Library (Figure 3.5); approval for the use of the pier was given by Dr. Robyn Hannigan, Dean of the School for the Environment at the University of Massachusetts, Boston. This location was chosen due to its distance from the Inner Boston Harbor where there is a mixing of saltwater from the ocean and freshwater from the Mystic and Charles Rivers; throughout the experimental period for the time trials, the chosen experimental site had a slightly higher average salinity level (29.73 PSU, Monitoring Station 084) than other locations within the Inner Boston Harbor (26.46 PSU, Monitoring Station 014), because it contains

a mixture of saltwater and freshwater from only one river, the Neponset River (MWRA 2017).

With the assistance of Mr. Jay Messana, Dock Master of the University of Massachusetts Boston, the lobster cages containing the pig femora were submerged in the saltwater located in the deepest portion of the inner section of the pier in order to avoid boat traffic (Figures 3.6 and 3.7). The cages were dropped into the water, allowed to hit the sea floor, then raised up off the floor; once suspended off the seafloor, the cages were tied to the pier. The ropes were tied to the pier in a locked enclosure to avoid unwanted tampering by visitors. The initial launch of the cages into the water took place on 12 December 2016, and the final collection of the last bone samples and the removal of the cages took place on 17 August 2018. Throughout the experimental period, the cages were submerged approximately 1.5 to 6 m below the surface, depending upon the regular tides which rise overall approximately 3.13 m daily in this location (NOAA 2018).

Throughout the time trial experiment, five femora were removed for analysis every two months for a 20-month period; these samples were labeled "0, 2, 4…, 20 Months: Samples A-E." The femora were transported back to a laboratory at Boston University's Medical Campus in gallon-sized plastic bags where the associated cable ties were removed and the femora were left to air-dry on trays for a week within a fume hood. After the drying period, the femora were placed into labeled plastic bags and frozen until ready for analysis. No rinsing of any kind occurred as it would alter the elemental concentrations of the samples by removing precipitated salts (Cronyn 1990; Hamilton

1997, 1999/2001; Steptoe and Wood 2002) and other elements, such as K, S, Cu, Ni, Zn, P, and Ba (Zwolsman and van Eck 1999).

During the experimental period, the cages became enveloped with ascidians, specifically between the June 2017 and August 2017 collections and the June 2018 and August 2018 collections (Figure 3.8). Ascidians (Phylum Chordata, Subphylum Tunicata, Class Ascidiacea), also called sea squirts or tunicate, are sessile, fouling marine invertebrates that inhabit areas from the shore to the deep sea; although there are native species to different regions of the world, non-native species have invaded most harbors around the world (Shenkar and Swalla 2011). Twenty-six species of ascidians inhabit the Massachusetts coastline, including *Ascidiella aspersa*, *Botrylloides violaceus*, and *Styela clava*, which are commonly found within the Massachusetts Bay and the Boston Harbor (Bell *et al*. 2005; Pederson *et al*. 2003; Shenkar and Swalla 2011). This was an issue for the 2017 collection dates, because the cages became too heavy to pull up manually to the pier during collection times; if these remained on the cage, the author and Mr. Messana were concerned that this would complicate the fall and winter sample collections and potentially cause the ropes securing the cages to break from too much tension. Therefore, the ascidian encrustation was scraped off of the cages during the August 2017 collection date with putty knifes and a long-handled sidewalk ice scraper. At the end of the experimental period (August 2018) the second ascidian encrustation was removed manually and with a pressure washer, once the last bone samples were collected. Any ascidians that were attached to the collected bones were gently removed by hand in order to not dislodge any adhering barnacles on the bones.

Experiment 2: Salinity Trials (ST)

In order to test the impact of water salinity on elemental concentrations within bone, three collections of pig femora that were previously submerged for 18 months in three different aquatic environments were also tested; these femora were initially submerged in September 2013 and removed from the ocean and harbor environments on 18 March 2015 and from the freshwater environment on 11 April 2015.

Forty pig femora were submerged in a costal ocean environment at 10 m and 18 m (sea floor) in the harbor facility of the Woods Hole Oceanographic Institute (WHOI) in Woods Hole, Massachusetts (N 41^{o} 31’ 24.40” W 70^{o} 40’ 19.00”). The femora were equally split among four submerged cages, two cages for each depth. The closest salinity monitoring station was Menauhant Station in the Eel Pond Inlet of Waquoit Bay (N 41^{o} 33.156’ W 70^{o} 32.912’), approximately nine miles northeast from Woods Hole at the junction of estuarine and marine waters; the average salinity during the experimental period was 31.24 PSU (NOAA NERRS 2012). The experimental area at Woods Hole was situated in a harbor with no significant freshwater inflow, so therefore the average salinity of the surrounding was estimated to be closer to that of the open ocean, 35 PSU (Brewer 1975; Wilson 1975), than the salinity at the monitoring station in Waquoit Bay, which is fed by the Quashnet/Moonakis and Child’s Rivers as well as the Atlantic Ocean.

Forty pig femora were submerged in the Boston Inner Harbor, from the Massachusetts State Police Marine Unit Dock in Boston, Massachusetts (approximately N 42^{o} 22’ 05.02” W 71^{o} 03’ 38.97”). The femora were equally split among four cages

that were resting along the sea floor; two cages were submerged at a depth of 3 m and two cages were submerged at a depth of 6 m, when measured from the low tide mark. The Boston Inner Harbor consists of brackish waters; freshwater flows out from the Charles and Mystic Rivers, and saltwater flows in from Atlantic Ocean. Due to the estuarine nature of this location, the salinity is lower, with an average value of 27.76 PSU, which is based on both surface (0.1 m) and bottom (~10 m) salinity measurements taken at Monitoring Station 014 between September 2013 and October 2014 (MWRA 2017).

Ten pig femora were submerged in a freshwater pond at the Boston University Outdoor Research Facility (ORF) in Holliston, Massachusetts (N 42^{o} 12’ 20.90” W 71^{o} 25’ 06.90”; site at 48 m a.m.s.l.). The femora were separated into two cages that were tied to land via a rope and were completely submerged in water that fluctuated from approximately 1.0 to 1.5 m deep. The pond is a remnant of a commercial cranberry bog from the late 1800s. The pH of the water is around neutral (7.0).

Upon removal from the different experimental locations, the bones were dried in a fume hood within a laboratory located at the Boston University School of Medicine; none of the bones were rinsed at any point during this experiment. Once the femora were completely dried, they were stored in large plastic bags organized by submergence location. After three years of storage, five bones from each of the different experimental locations were randomly selected to be analyzed for the salinity trials (ST) of the current study; these bones were labeled Holliston pond, Boston Harbor (3 m), Boston Harbor (6 m), Woods Hole (10 m), Woods Hole (18 m): Samples A-E.

Preparation for Analysis

The control samples for the TT experiment (0 Months: Samples A-E) were thawed for 5 hours within their plastic bags, then dried on trays at ambient temperatures within a fume hood for one day. After this time, more of the remaining adherent flesh was removed using scalpels. These remains were then placed with a dermestid beetle colony (Dermestidae) in the Department of Anatomy and Neurobiology at Boston University School of Medicine. After four weeks, the control samples had minimal to no adhering soft tissue; at this point the bones were removed from the colony, stored in plastic bags and frozen until ready for processing.

In preparation for ED-XRF analysis, all bone samples from both the TT and ST experiments would need to be stripped of adhering barnacles, cored, and milled. Prior to this processing, the frozen bones from the TT experiment (0-20 Months: Samples A-E) were thawed in a fume hood within their bags for 24 hours, then segregated onto different trays based on collection time and dried at ambient temperatures within a fume hood for a day before being photographed (Figures 3.9-3.19). Since the bones from the ST experiment were already dried, the five dry bones (Samples A-E) from each of the five salinity environments (Holliston pond, Boston Harbor [3 m] and [6 m], and Woods Hole [10 m] and [18 m]) were just photographed prior to processing (Figures 3.20-3.24).

The majority of the TT bone samples and the Boston Harbor and Woods Hole samples from the ST experiment contained adhering barnacles; these eventually had to be removed prior to coring. Prior to removal, data were also collected on the adhering

barnacles for potential future research, although it was not further analyzed in the present study, because it goes beyond the scope of the current research project. The minimum number of barnacles adhering to each bone from the TT and ST experiments were recorded. As explained by Pirtle (2017), the exact number of adhering barnacles could not be determined, because barnacles less than 0.5 mm were difficult to see without magnification and others were obscured by dense barnacle colonies that also developed on the bones. The maximum basal diameter of the smallest barnacle and the largest barnacle were measured using digital calipers. The barnacles were then removed from the bones and analyzed for species identification. Any other organisms found adhering to the bones or the cages during the experiment were noted as well. When collecting the barnacle data from the bones of the ST experiment, it was noted that many barnacles that were once adhering to the bones from the Woods Hole (10 m and 18 m) and Boston Harbor (3 m and 6 m) locations had fallen off and were collected at the bottom of the original storage bags; therefore, the minimum number of adhering barnacles was not an accurate representation of the number of barnacles that were originally adhering to the bones when they were first removed from the water three years prior. The diameters of the largest and smallest adhering barnacles would also most likely not represent the size of smallest and largest barnacles that were originally adhering to the bones when they were first removed from the water. Due to this issue, the detached barnacles at the bottom of the original storage bags were measured in order to determine the diameters and, although noted, the minimum number of adhering barnacles was not analyzed.

After all adhering barnacles were removed from the affected TT and ST bone samples, the location of each bone's midshaft was calculated from the length of the anterior surface of the bone's diaphysis. This process was complicated by the fact that the epiphyses of the majority of the bones had fallen off when submerged during the experimental period; therefore a consistent method for measuring the bones with and without epiphyses was developed. To calculate the anterior surface's diaphyseal length, one arm of a digital caliper was placed along the proximal epiphyseal-diaphyseal junction and the other along the distal epiphyseal-diaphyseal junction; this method was not affected by the presence or absence of epiphyses. The bones were then sawn in half using a Delta band saw fixed with a 14 tooth per inch (TPI) carbon band saw blade and set to a speed of 80 feet per minute (fpm). The marrow was then removed using a metal spatula in order to reduce the amount of time spent cleaning the coring drill bit used in the next processing steps (see below). The saw blade and spatula were cleaned with soap and water between uses on each sample from both the TT and ST experiments.

Each bone was cored in order to make sure that the samples to be milled were approximately the same size and from the same location for each bone in the TT and ST experiments. The steps for using a drill press to collect bone cores were discussed by Owen (2002) and Stein and Sander (2009). A Delta table-top drill press set to a speed of 1100 rpm was used in conjunction with a 16 mm, diamond-coated tip, coring drill bit to drill 8 cores of cortical bone from each diaphysis; no cores were collected from the few remaining epiphyses of the bones. The halves of bone were clamped to the drill press stand using a one-handed bar clamp and leveled using wooden shims, in order to make

the diaphyseal surface perpendicular to the drill bit. On each half of the bone, one core was drilled from each of the four sides of the diaphysis (anterior, posterior, medial, lateral) along the cut midline; therefore, the cores represented the approximate makeup of the 32 mm section at mid diaphysis (Figure 3.25). Each core was slowly drilled with only light pressure being applied from the drill press and frequent pauses in order to avoid any overheating of the drill bit and thermal alteration of the bone. Upon drilling through to the medullary cavity, the core was removed from the drill bit and then the bone was rotated in preparation for the next core to be drilled. Any remnants of marrow on the internal surface of each core were wiped away with a paper towel. The cores of each bone were combined and stored together in labeled plastic bags, until they could be milled. The drill bit was washed with soap and water and dried before coring a new bone in order to avoid contamination.

Although not necessary for this research project's data analysis, the total mass of all the cores from one bone was measured with a Cen-Tech® 1000 gram digital scale, which has an accuracy of ± 0.1 g. The diameter and minimum depth of each core was also measured using digital calipers, which have an accuracy of 0.02 mm; the approximate volume of each core was mathematically calculated, as well as the overall volume and density of all the cores from each bone sample.

To improve grinding and homogenization, the cores were dried in an oven at 35°C for 48-96 hours; the oven temperature was kept at a physiological level in order to guarantee the bone samples were not thermally altered (Keenan and Engel 2017). Similar to Bell *et al*. (2009) and Krajcarz (2017), a ball mill was used to expedite the grinding

process rather than a mortar and pestle, which other researchers, such as Keenan and Engel (2017), have used. The cores from each bone were ground in an 8004 Tungsten Carbide Vial Set using an 8000M SPEX CertiPrep Mixer/Mill located in the analytical geochemistry laboratory at the University of Massachusetts Boston. The resulting homogenized powder would now more accurately represent the overall elemental composition of each bone, since there is naturally a differential accumulation of elements within bones (Pemmer *et al*. 2013; Wobrauschek *et al*. 2002), that is also intensified by the effects of diagenesis (Tütken *et al*. 2008). Following the standard operating procedure of Atwood (2015), the SPEX mill was cleaned before and after each bone sample was processed in order to reduce cross contamination. Cleaning consisted of first rinsing all components with deionized (DI) water. The metal components were then rinsed with 5% HCl in order to dissolve any soluble materials. All parts were thoroughly rinsed with DI water followed by ethanol, in order to remove any excess HCl and water. All components were wiped with a KIM wipe and allowed to fully dry before the next sample was transferred to the container.

Approximately 5 g of powder from each bone sample was weighed out on a Mettler PM 4600 Delta Range® microbalance and placed into 32 mm diameter XRF sample cups (Chemplex® Industries Series 1300 XRF sample cups, re-sealable/ventable caps, snap-on rings) with a 2.5 µm SpectroMembrane® Mylar® thin-film. Following the protocols of Atwood (2015), the remaining space within the container was filled with polyester pillow stuffing, which slightly compressed the powder onto the film side of the container in order to decrease pore space and prevent the powder from shifting. Using the

same capsules and XRF instrument, Smith (2007) determined that the backing material caused no noticeable signal during sample analysis, since the depth of the sample in the XRF cup was larger than the penetration depth of the X-rays.

XRF Analysis

The bulk elemental composition was determined by Energy Dispersive X-ray Fluorescence (ED-XRF) analysis. Following the protocols outlined in Stebbins (2015), the 5 g powdered samples were measured in triplicate under helium purge using a SPECTRO XEPOS Benchtop ED-XRF Spectrometer located at the Environmental Analytical facility of the University of Massachusetts Boston. A total of nine runs were performed, during which 12 capsules (3 standard reference materials [SRM] and 9 experimental samples) were analyzed per run. The factory-installed global calibration method Tqk-7058g for He purge and MCA re-calibration were performed before analysis. The spectrometer was equipped with a Turboquant data-processing program for powdered samples that automatically transforms the measured radiation intensities into elemental concentrations and absolute errors (expressed as mass %) for 48 elements with atomic masses ranging from Mg to U. Unfortunately, Na is below the detection capabilities of this specific instrument, however, other elements associated with bone composition and the marine environment can be detected. The following elements found within the analyzed bone samples were either below or near the lower detection limit and, therefore, excluded from statistical analysis: V, Co, Cu, Ga, Ge, As, Se, Rb, Y, Zr, Nb, Mo, Ag, Cd, In, Sn, Sb, Te, I, Cs, Ba, La, Ce, Hf, Hg, Ti, Bi, U; however, two bone

samples submerged in the Holliston pond produced values for Cl and K that were below the detection limit, yet overall statistical analyses still included Cl and K. The elements of interest for the present study were those that represented natural bone composition (Ca, P), the local marine environment and diagenetic processes (i.e., Cl, Mg, Sr, Si, Fe, Zn, Mn), as well as other elements that the XRF detected; it should be noted that some of these elements that potentially represent the marine environment and diagenetic processes are naturally found within bone (i.e., Zn, Fe, or Mn).

Three National Institute of Standards & Technology (NIST) SRMs were included in each sample run in order to monitor the spectrometer's performance and for sample data correction. These SRMs were NIST 1486 (Bone Meal), NIST 1646a (Estuarine Sediment), and NIST 2702 (Inorganics in Marine Sediment). Because of its similar matrix with the present study's samples, NIST 1486 was used to correct the measured values for Mg, P, K, Ca, Fe, Zn, Sr, and Pb. For the elements that do not have certified values in NIST 1486, NIST 1646a (Si, S, Al, Cr, Mn, Ti) and NIST 2702 (Ni, Th) were used for data correction. Table 3.1 compares the SRMs' measured and certified values for the elements analyzed in the present study. The methods described in Smith (2007) were used for data correction. First, a measured-to-certified (MC) ratio was calculated by dividing the SRM's mean measured value by its certified value for each element of concern. The mean measured elemental concentrations for each experimental sample was divided by the MC ratio for the appropriate sample run number. The measured results for elements Cl, Br, Ta, and W were not corrected, because none of the studied SRMs had

certified values for these elements; therefore, when these concentrations are reported, they are always labeled as uncorrected.

Table 3.1. Certified concentrations and ED-XRF measured values obtained in the present study for SRM NIST 1486, 1646a, and 2702.

Derived from SRM:	Element	Certified Value	Measured XRF Value (Mean SD)
NIST 1486	**Mg** (g/kg)	4.66 ± 0.17	141.32 ± 0.73
	P (g/kg)	123 ± 1.9	127.7 ± 1.2
	K (mg/kg)	412 ± 4	532 ± 21
	Ca (g/kg)	265.8 ± 2.4	199.6 ± 1.4
	Fe (mg/kg)	99 ± 8	159 ± 4
	Zn (mg/kg)	147 ± 16	138 ± 1
	Sr (mg/kg)	264 ± 7	254 ± 1
	Pb (mg/kg)	1.335 ± 0.014	4.078 ± 0.348
NIST 1646a	**Si** (g/kg)	400 ± 1.6	369.1 ± 0.9
	S (g/kg)	3.52 ± 0.04	2.08 ± 0.01
	Al (g/kg)	22.97 ± 0.18	27.83 ± 0.26
	Ti (g/kg)	4.56 ± 0.21	5.08 ± 0.01
	Cr (mg/kg)	40.9 ± 1.9	68.7 ± 6.3
	Mn (mg/kg)	234.5 ± 2.8	248.1 ± 3.9
NIST 2702	**Ni** (mg/kg)	75.4 ± 1.5	103.6 ± 2.6
	Th (mg/kg)	20.51 ± 0.96	27.75 ± 0.90

Statistical Analysis

All statistical analyses were performed using the software package SPSS.25. The skewness, kurtosis, and homoscedasticity of the data were assessed to determine the normality of the data distribution and the homogeneity of variance for both the time trials and salinity trials. The Kolmogorov-Smirnov test and Shapiro-Wilk test were also

performed to determine how well the data fit a normal distribution. A Levene's test was performed to assess the variance of outcomes for each group. There were several instances of non-normal distributions and heteroscedasticity within the time and salinity trial data; therefore, all data were transformed by taking the natural logarithm of the elemental concentrations (Buikstra *et al*. 1989; Lambert *et al*. 1991). All statistical analyses for both the time and salinity trials were all performed on the transformed data.

The transformed elemental concentration data from the TT experiment were graphed overtime. General trends in the data were noted prior to statistical analysis. All of the transformed elemental concentrations were analyzed by a Pearson's correlation test, in order to identify any correlations between an elemental concentration and time submerged. The Pearson's correlation test could not specify if correlation between the elemental concentrations and time were due to a significant difference between the control samples (never submerged) and the experimental samples as a whole (submerged anywhere from 2-20 months) or due to a significant difference that is dependent upon the amount of time submerged. Therefore, the transformed elemental concentrations that were significantly correlated with time according to the Pearson's test were further analyzed by a one-way ANCOVA, which would determine if there was any significant difference in the elemental concentrations between the two groups when their means were adjusted for the amount of time submerged in the water (covariate). A post hoc analysis with a Bonferroni adjustment was performed to determine the type of relationship between the control and experimental groups that had significantly different means when adjusted for the amount of time submerged.

Only the elements that were significantly correlated with the amount of time submerged according to the Pearson's correlation test were used in a multivariable linear regression in order to determine a way to predict the amount of time submerged from a combination of elemental concentrations. After creating the initial model, the elements with significant ($p < 0.05$) unstandardized coefficients and those with relatively large coefficients that were close to being significant ($p < 0.1$) were used to redefine the model. The significant elements from this redefined model were used to create the final linear regression equation.

For the ST experiment, a one-way ANOVA was performed on all the transformed elemental concentrations for each submergence location (Holliston pond, Boston Harbor [3 m], Boston Harbor [6 m], Woods Hole [10 m], Woods Hole [18 m]). Another Levene's test was performed in order to assess the variance of the newly transformed outcomes. When equal variances were assumed, a post hoc Tukey test was applied; when equal variances were not assumed, a Welch's ANOVA was run and the Games-Howell post hoc test was used.

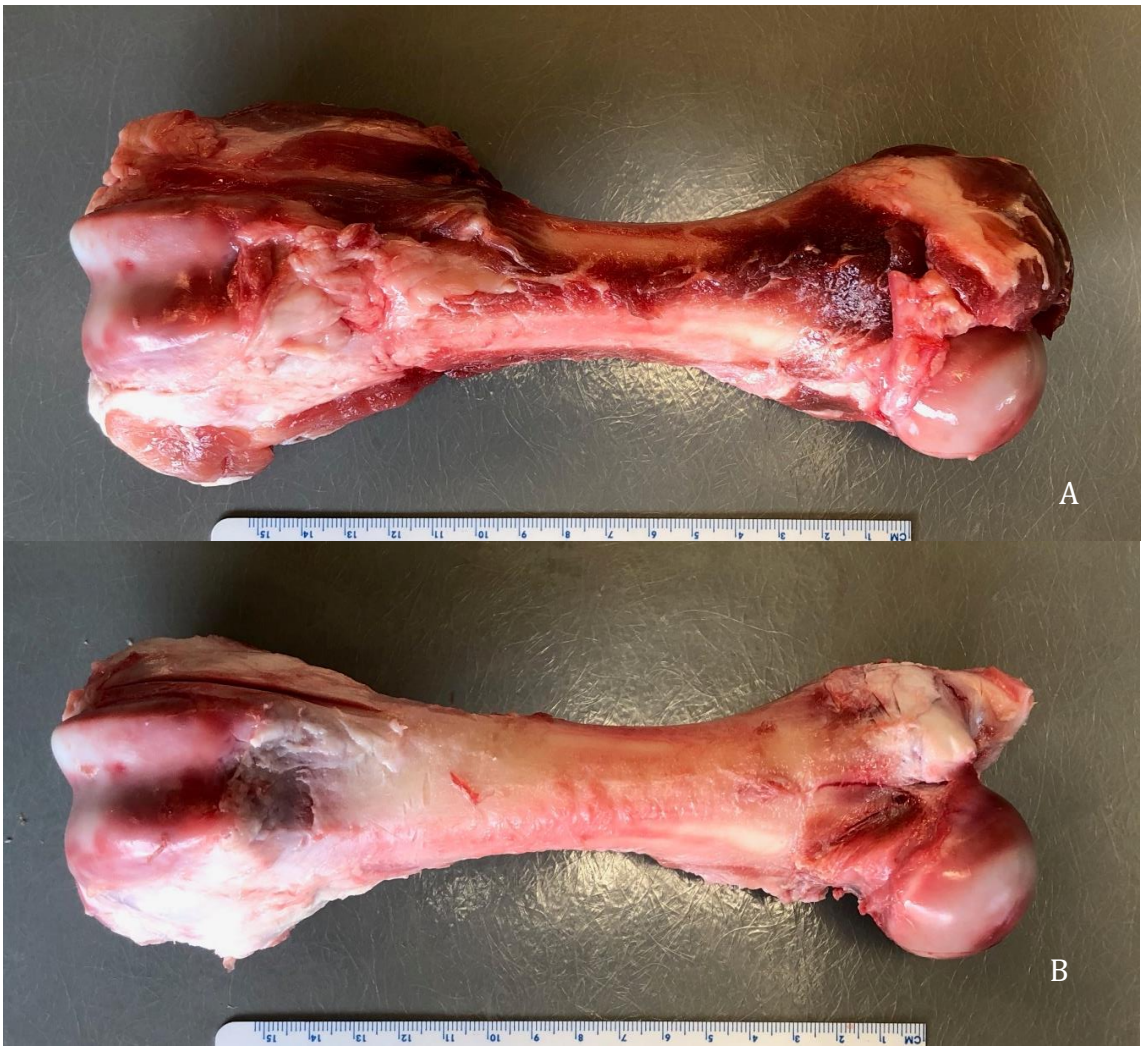

Figure 3.1: Fresh pig femora (a) before and (b) after the majority of the adhering flesh was removed with scissors and scalpels.

Figure 3.2: Pig femora suspended within cage prior to initial launch of the time trials at the John T. Fallon Pier.

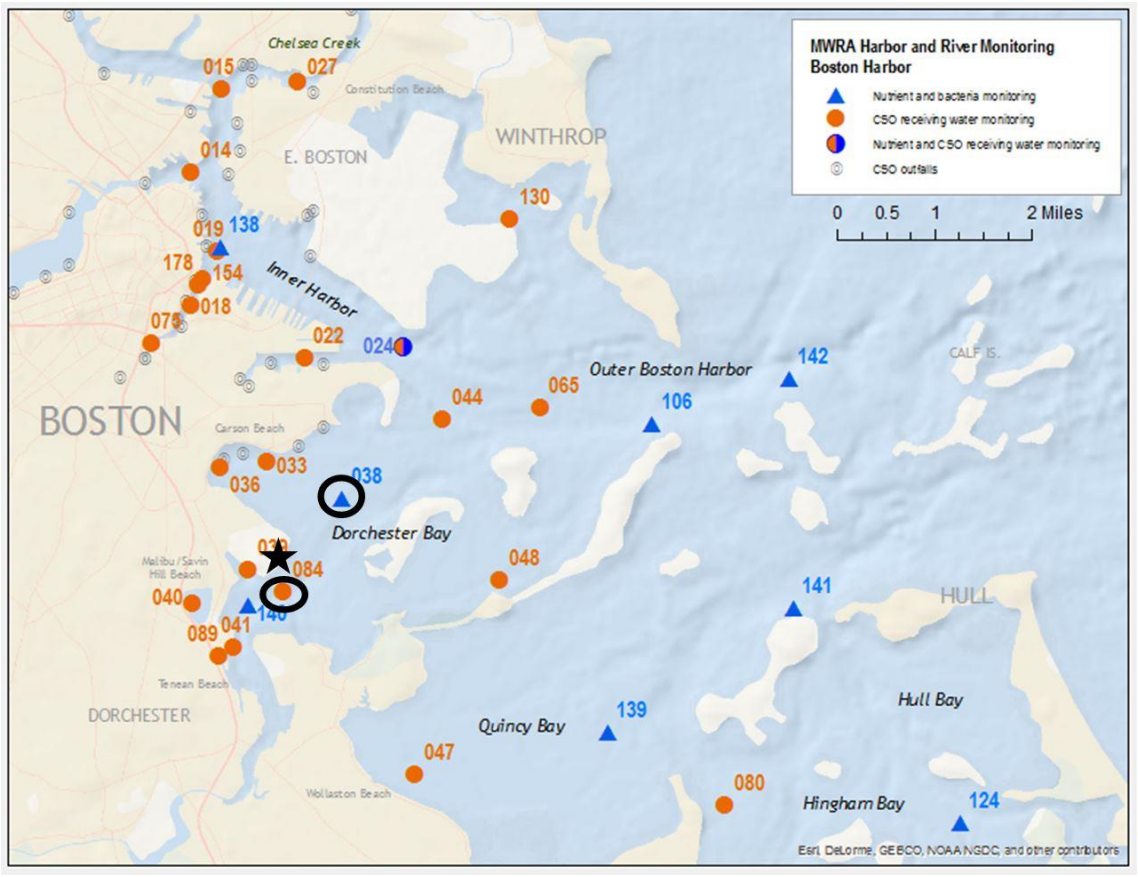

Figure 3.3: Map of the MWRA monitoring stations within the greater Boston Harbor area. A star marks the experimental location. Missing temperature data was obtained from stations 038 and 084 (encircled).

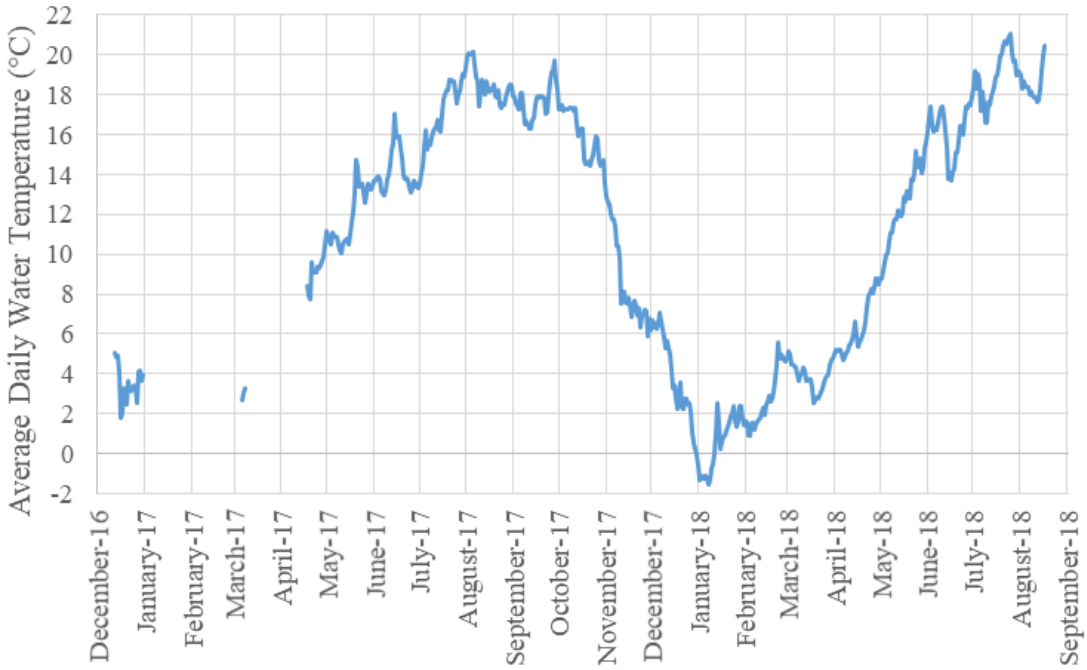

Figure 3.4: Average daily water temperatures at the John T. Falon Pier during the experimental period for the time trials. Due to malfunctioning data loggers, the temperature data from 12/13/16 to 12/30/16 and 12/14/17 to 2/8/18 were collected by one logger, and no data were collected from 12/30/16 to 4/21/17; some of the missing temperature data were supplemented eith data collected by ENQUAD of MWRA (2017).

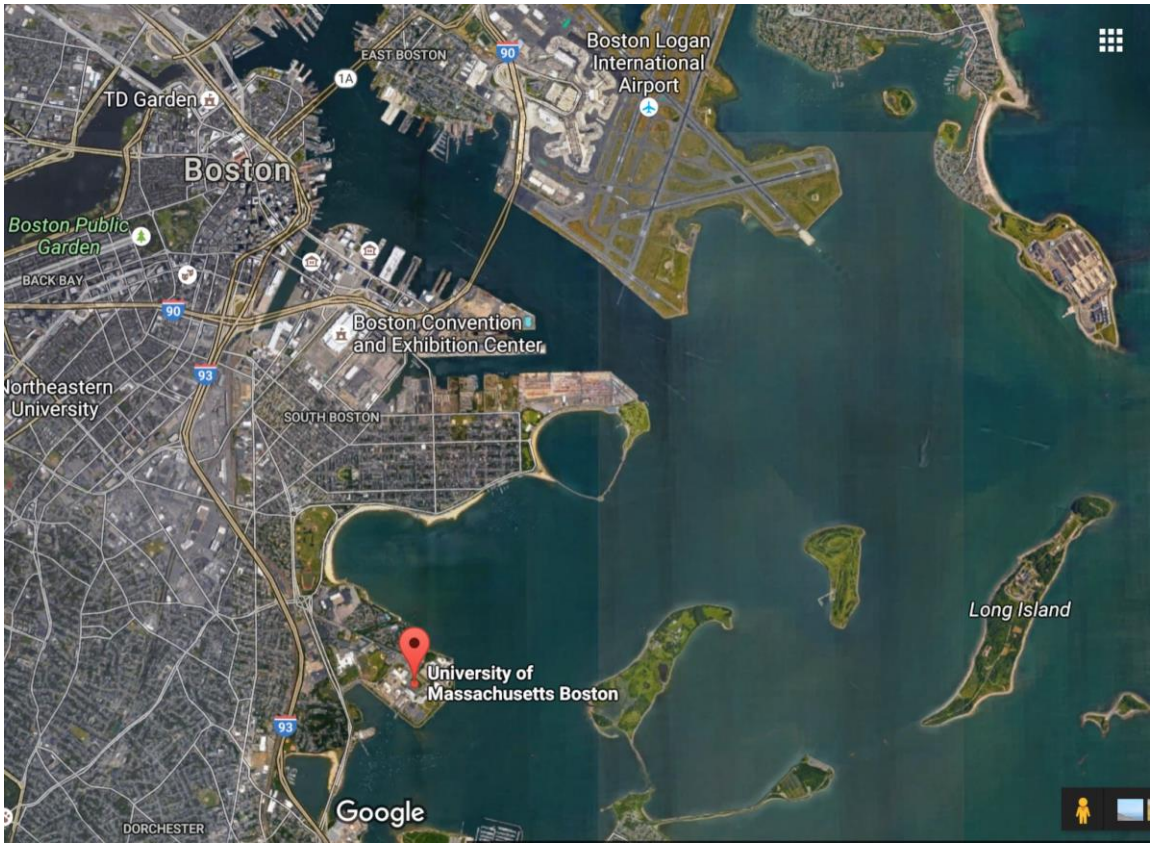

Figure 3.5: University of Massachusetts Boston campus in relation to the Boston Harbor (Google Earth Pro 2018).

Figure 3.6: Satellite view of the John T. Fallon Pier at the University of Massachusetts Boston (Google Earth Pro 2018). The star marks the location of the submerged cages during the experimental period for the time trials.

Figure 3.7: The John T. Fallon Pier at the University of Massachusetts Boston. The arrow marks the location of the submerged bones during the experimental period of the time trials.

Figure 3.8: Ascidian colonization of a cage at the August 2017 collection.

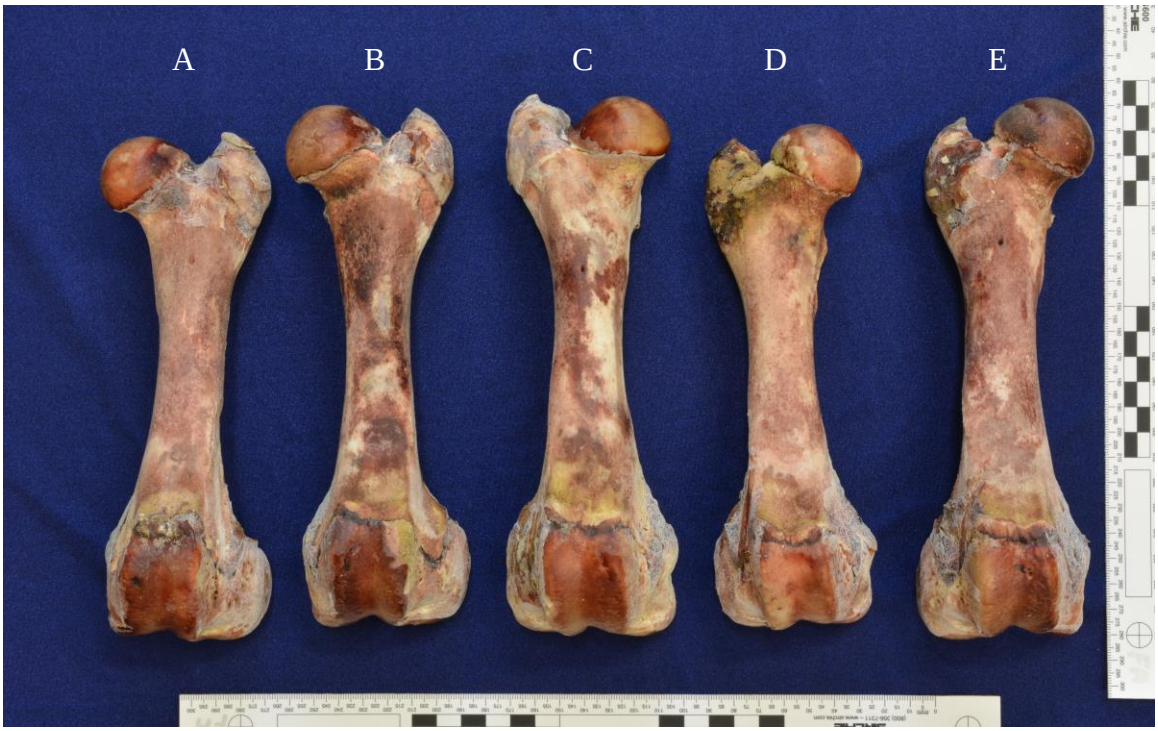

Figure 3.9: Group of control pig bones for the time trials representing zero months submerged in the water; Samples A-E.

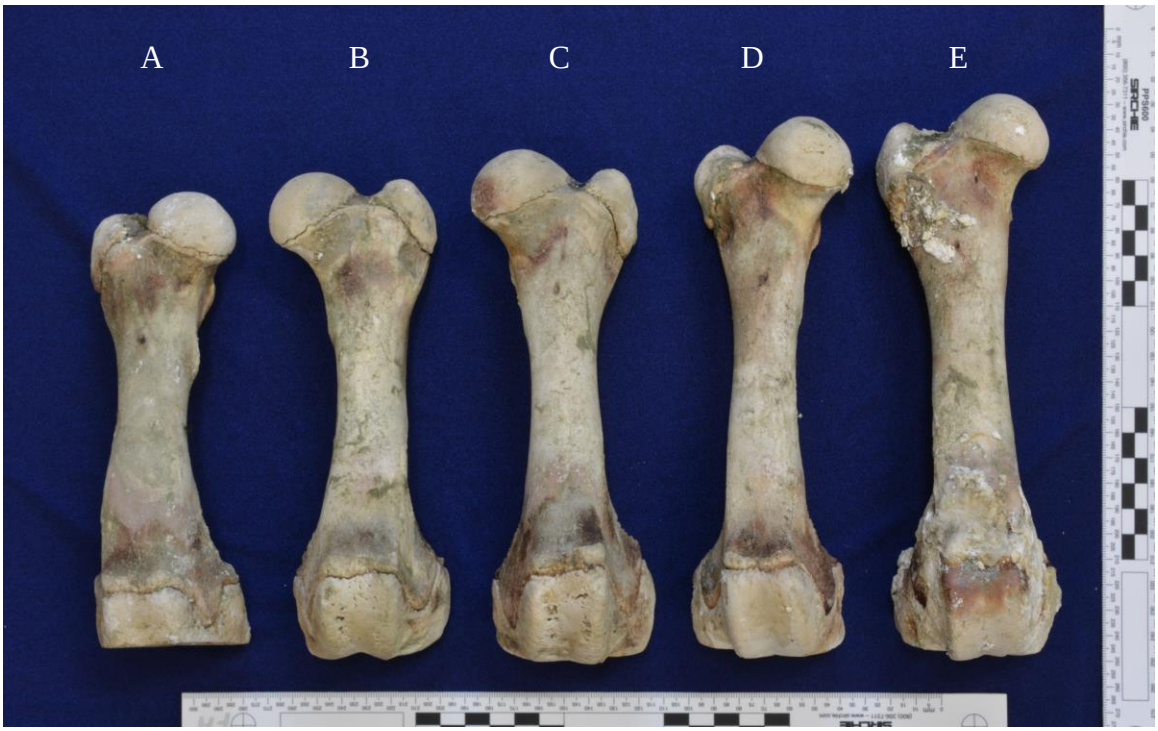

Figure 3.10: First experimental group for the time trials that were submerged for two months (removed February 2017); Samples A-E.

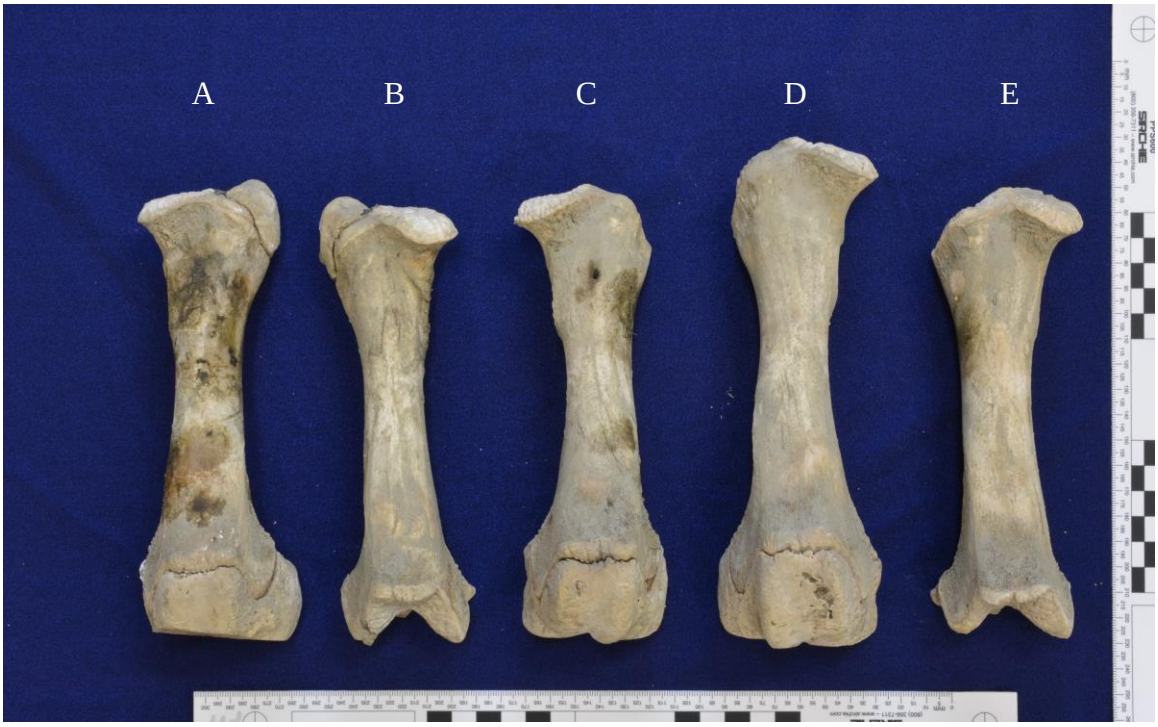

Figure 3.11: Second experimental group for the time trials that were submerged for four months (removed April 2017); Samples A-E.

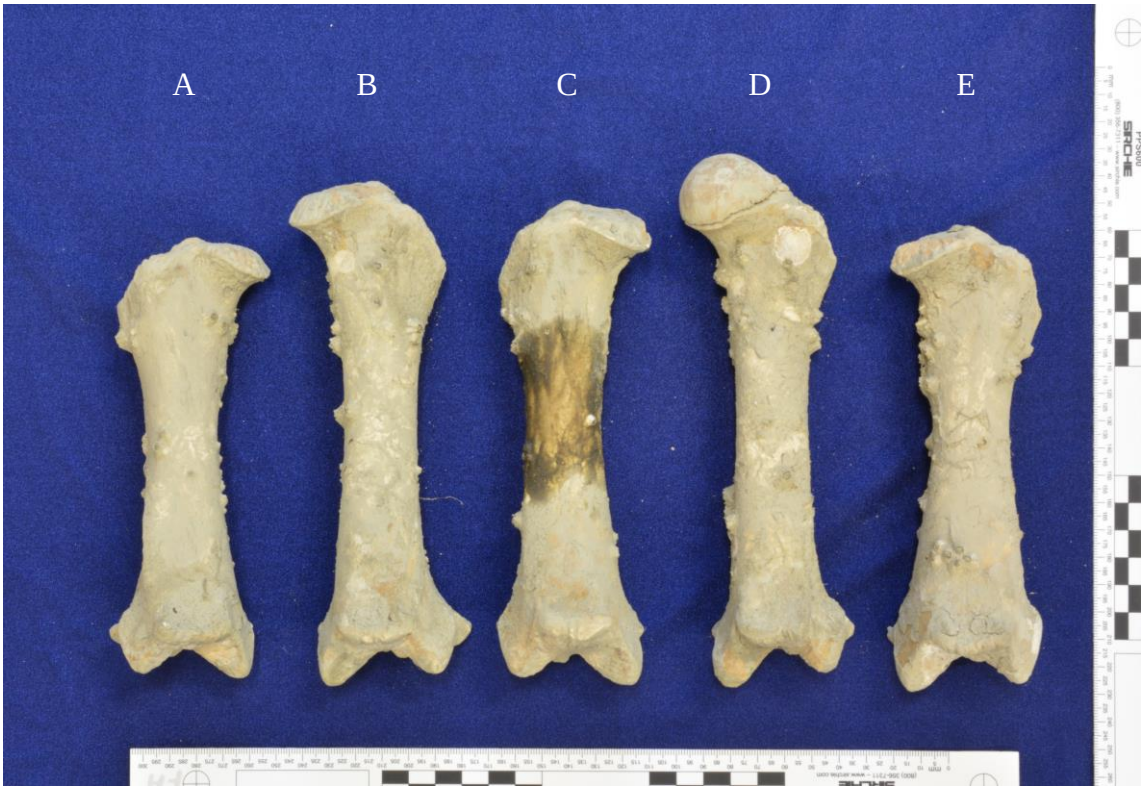

Figure 3.12: Third experimental group for the time trials that were submerged for six months (removed June 2017); Samples A-E.

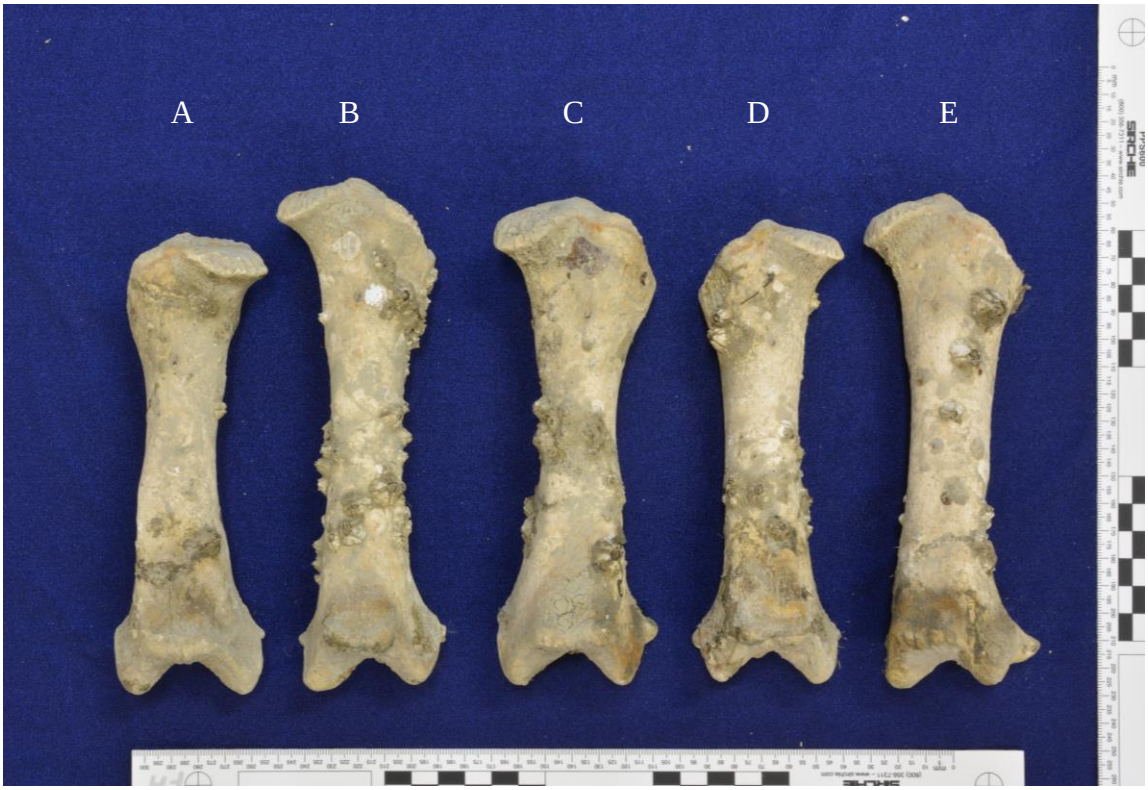

Figure 3.13: Fourth experimental group for the time trials that were submerged for eight months (removed August 2017); Samples A-E.

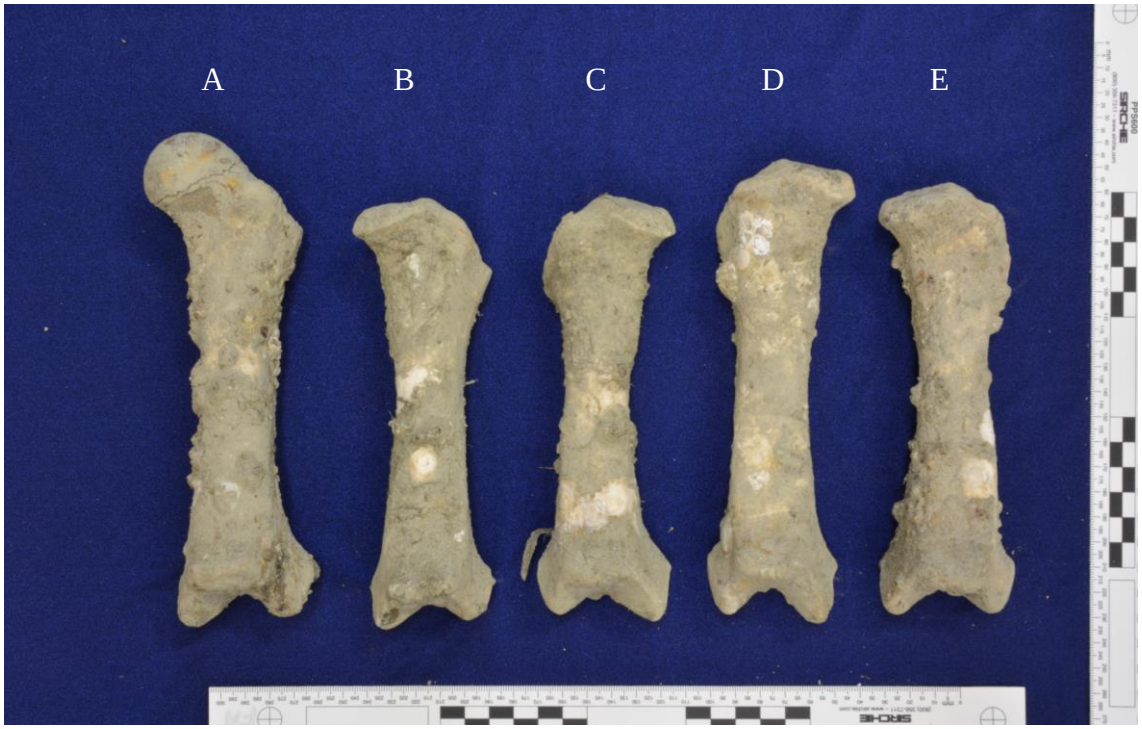

Figure 3.14: Fifth experimental group for the time trials that were submerged for ten months (removed October 2017); Samples A-E.

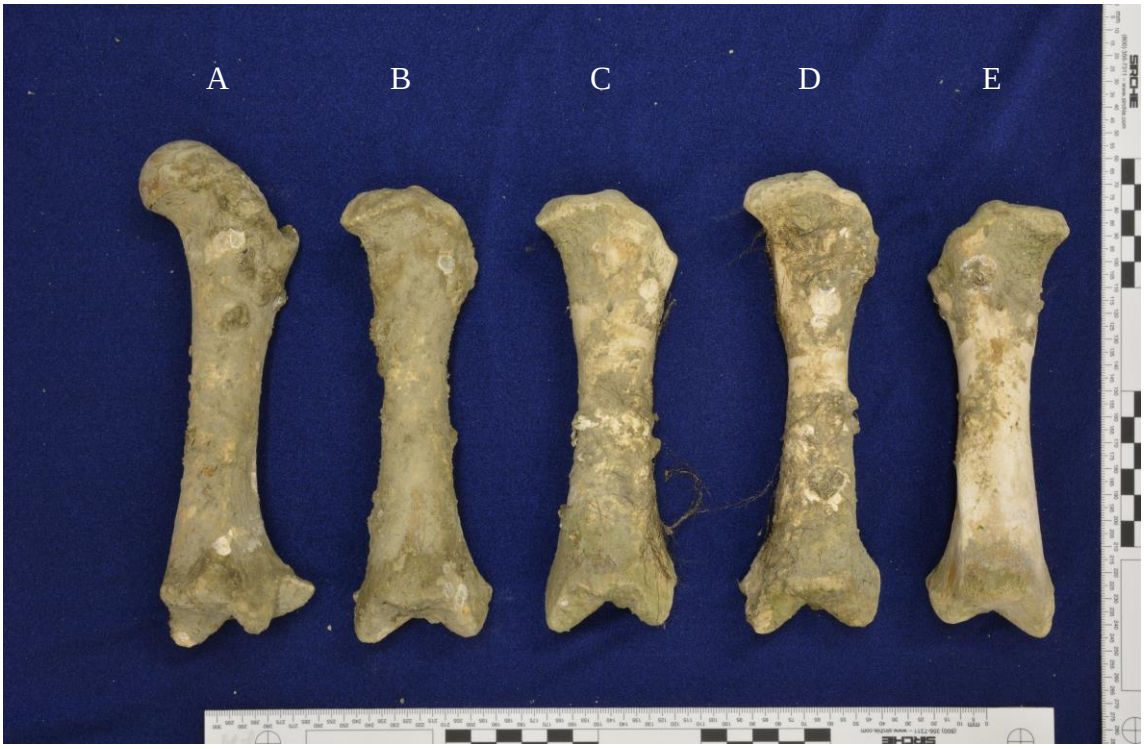

Figure 3.15: Sixth experimental group for the time trials that were submerged for 12 months (removed December 2017); Samples A-E.

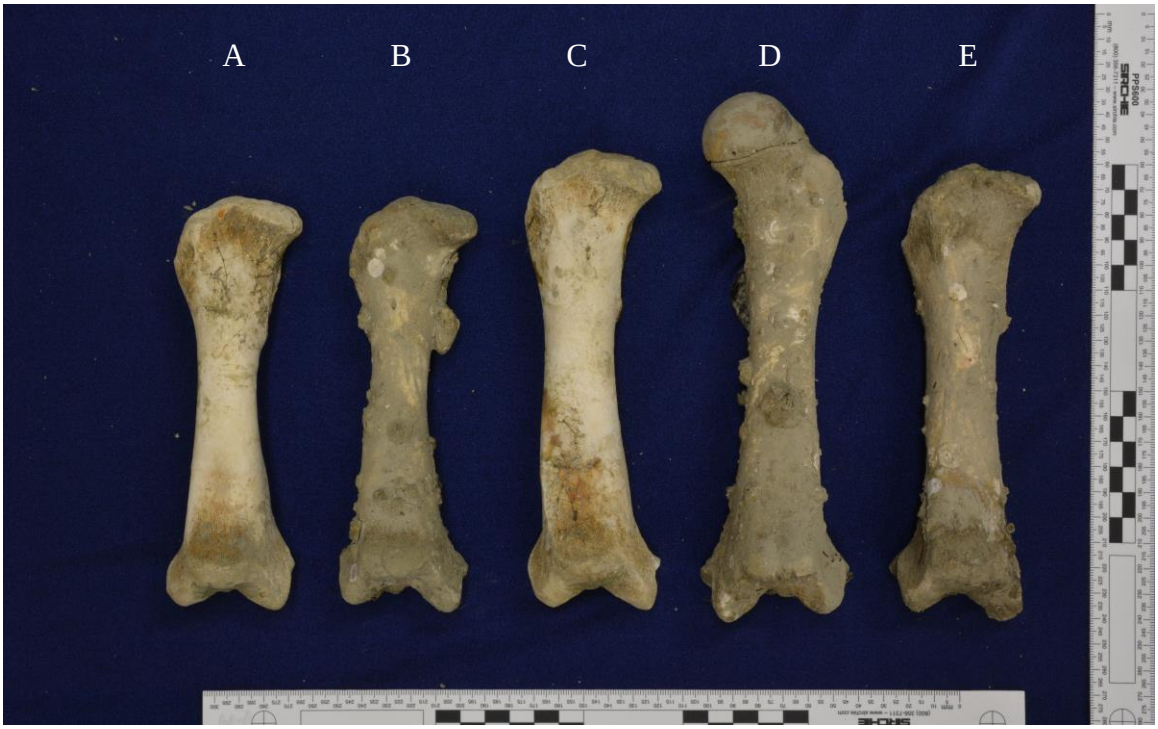

Figure 3.16: Seventh experimental group for the time trials that were submerged for 14 months (removed February 2018); Samples A-E.

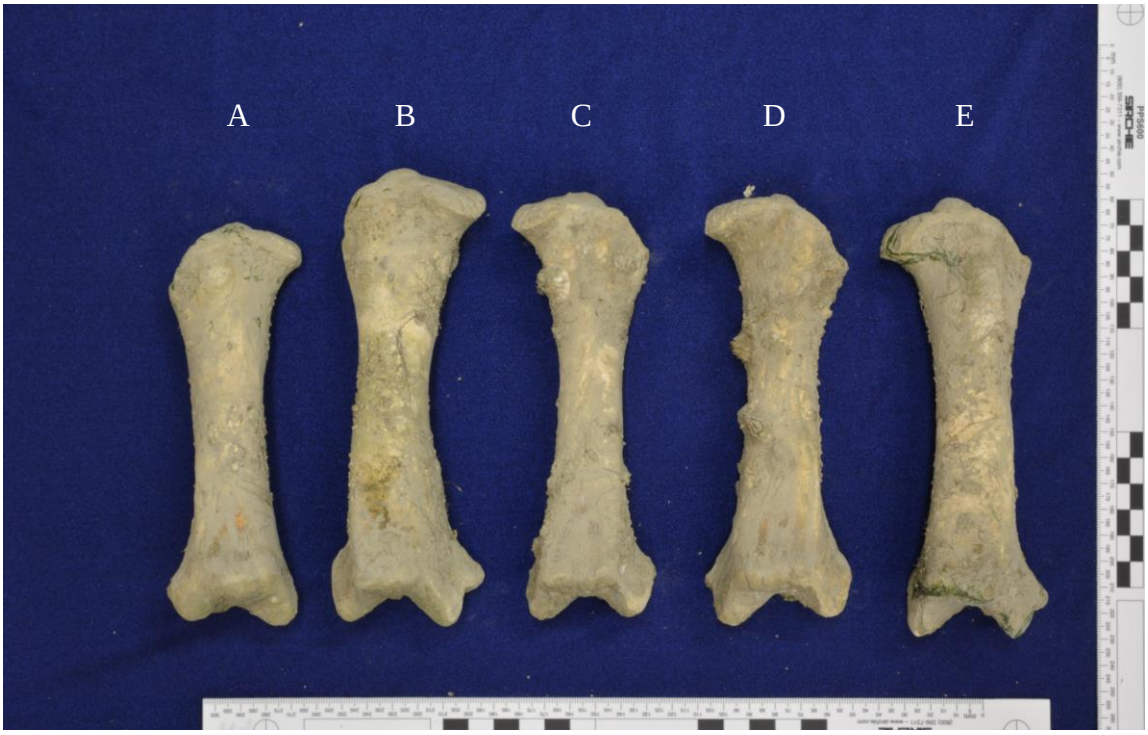

Figure 3.17: Eighth experimental group for the time trials that were submerged for 16 months (removed April 2018); Samples A-E.

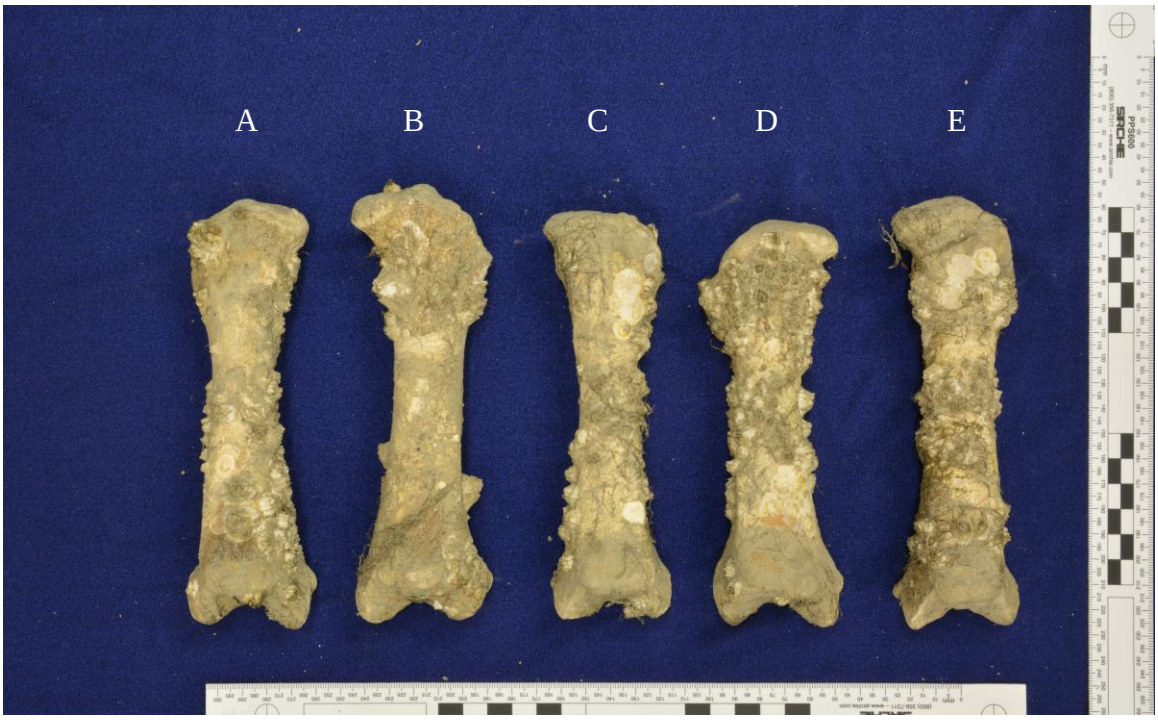

Figure 3.18: Ninth experimental group for the time trials that were submerged for 18 months (removed June 2018); Samples A-E.

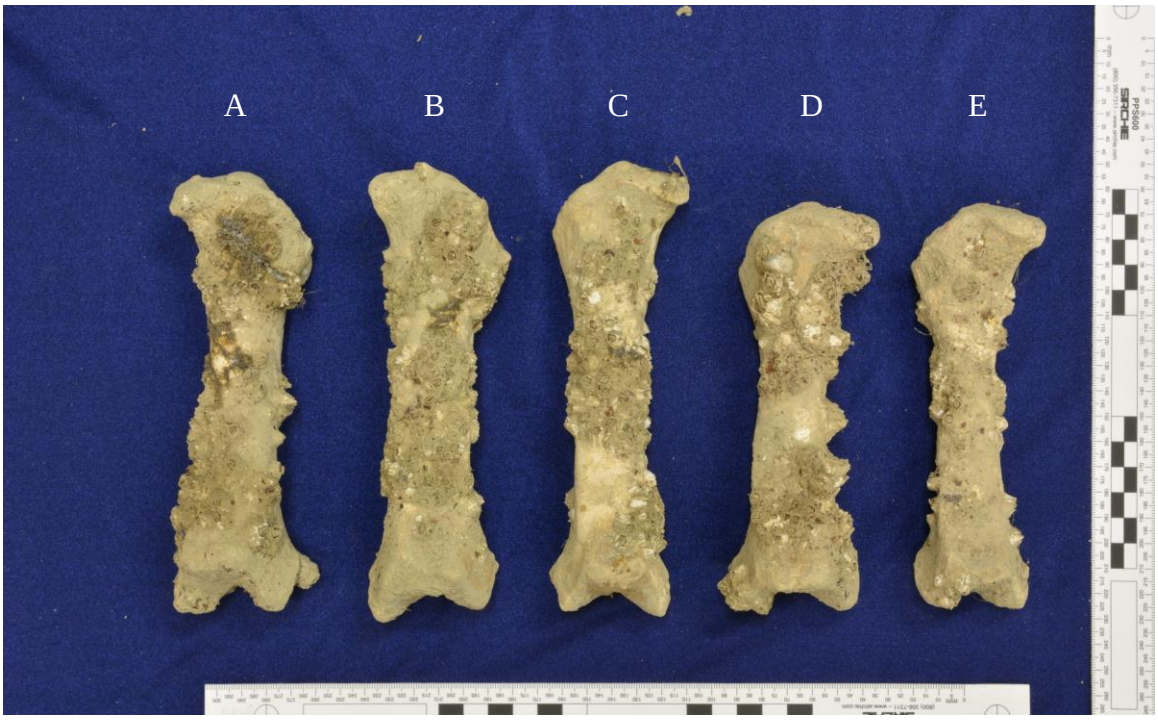

Figure 3.19: Tenth experimental group for the time trials that were submerged for 20 months (removed August 2018); Samples A-E.

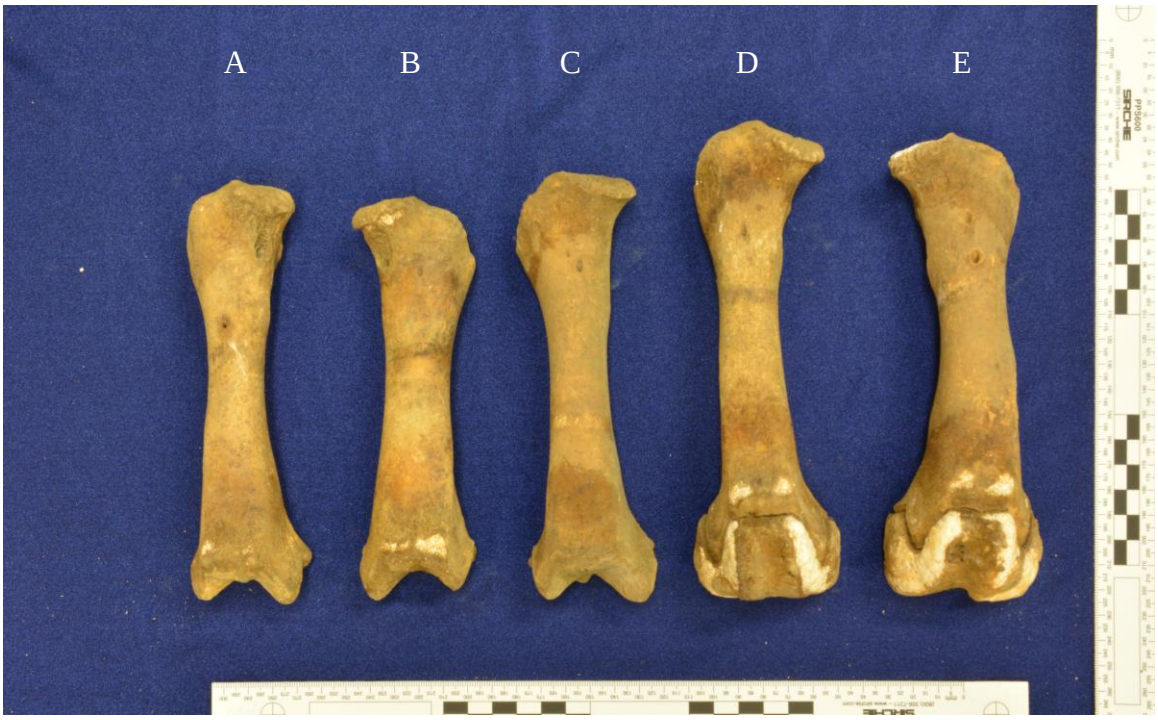

Figure 3.20: Experimental group submerged in the Holliston pond for 18 months for the salinity trials; Samples A-E.

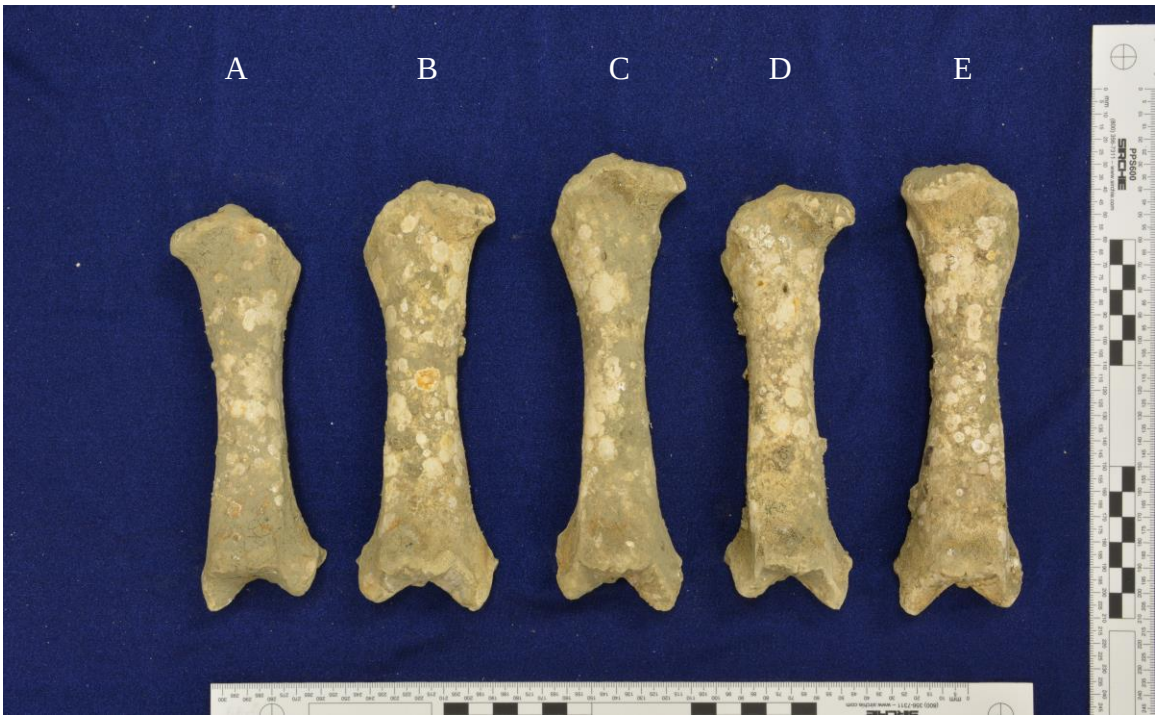

Figure 3.21: Experimental group submerged 3 m in the Boston Inner Harbor for 18 months for the salinity trials; Samples A-E.

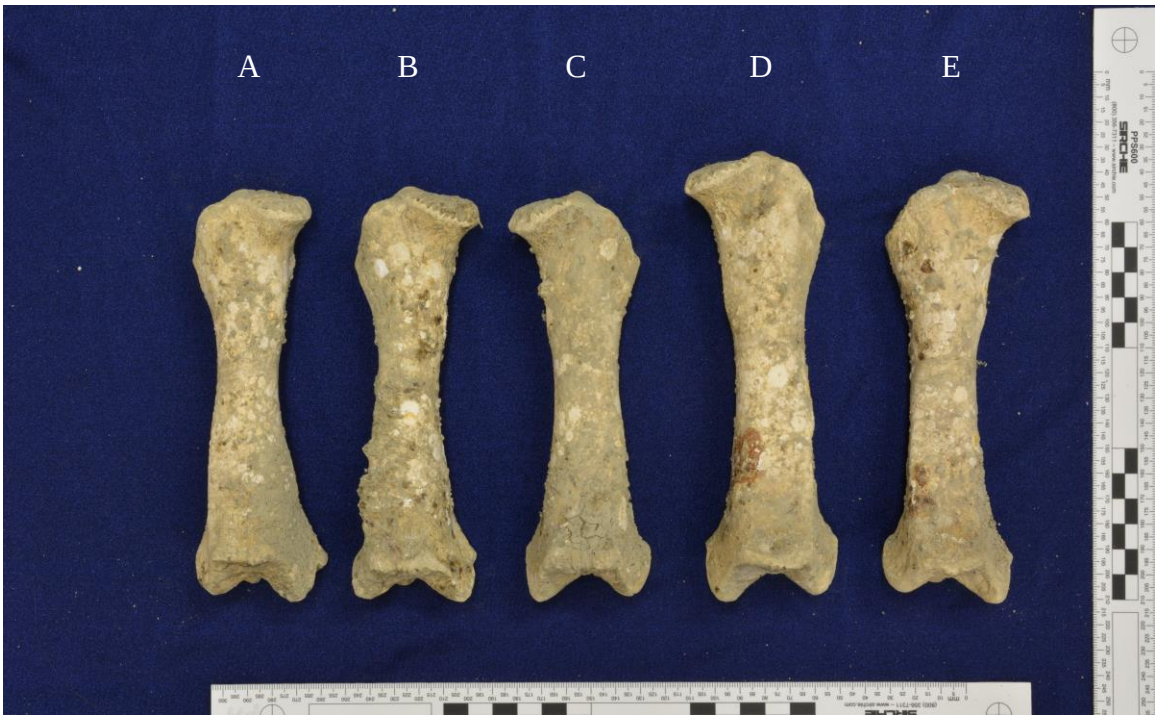

Figure 3.22: Experimental group submerged 6 m in the Boston Inner Harbor for 18 months for the salinity trials; Samples A-E.

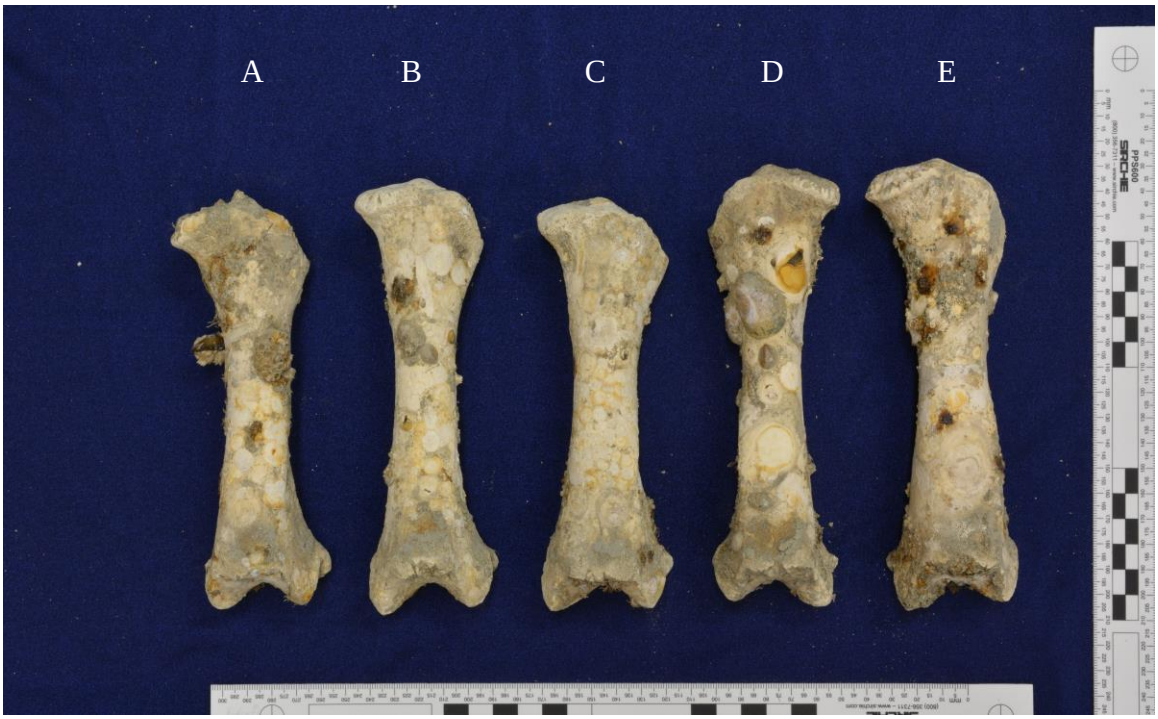

Figure 3.23: Experimental group submerged 10 m at Woods Hole for 18 months for the salinity trials; Samples A-E.

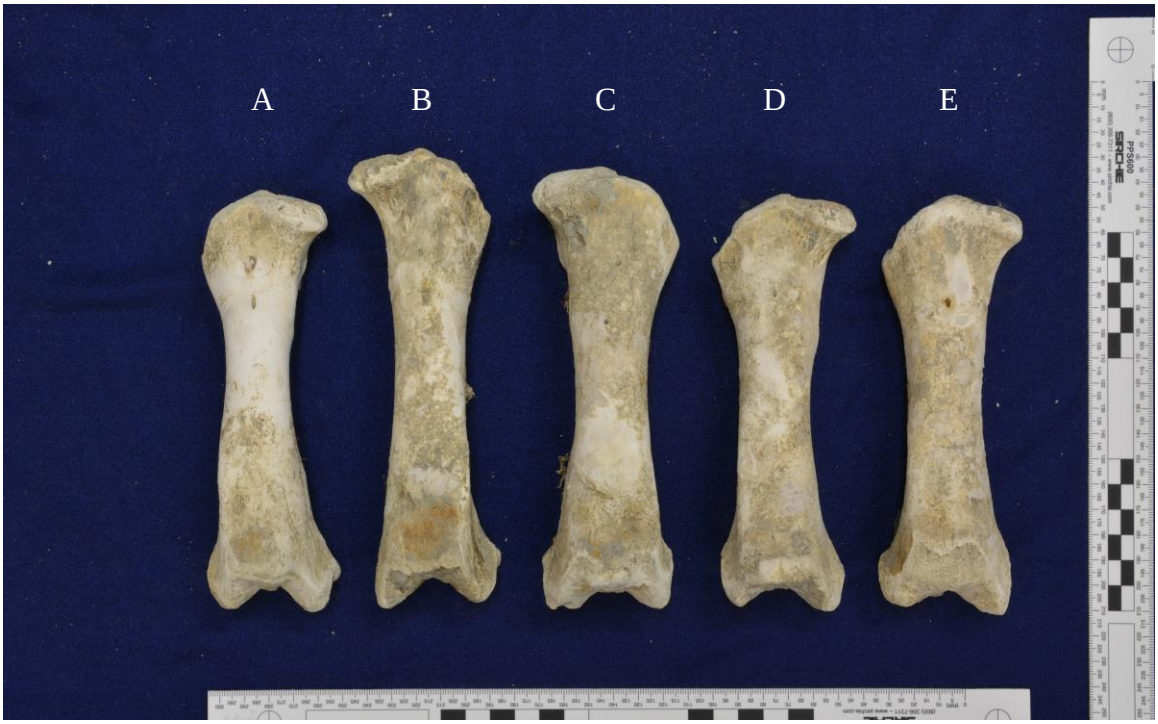

Figure 3.24: Experimental group submerged 18 m at Woods Hole for 18 months for the salinity trials; Samples A-E.

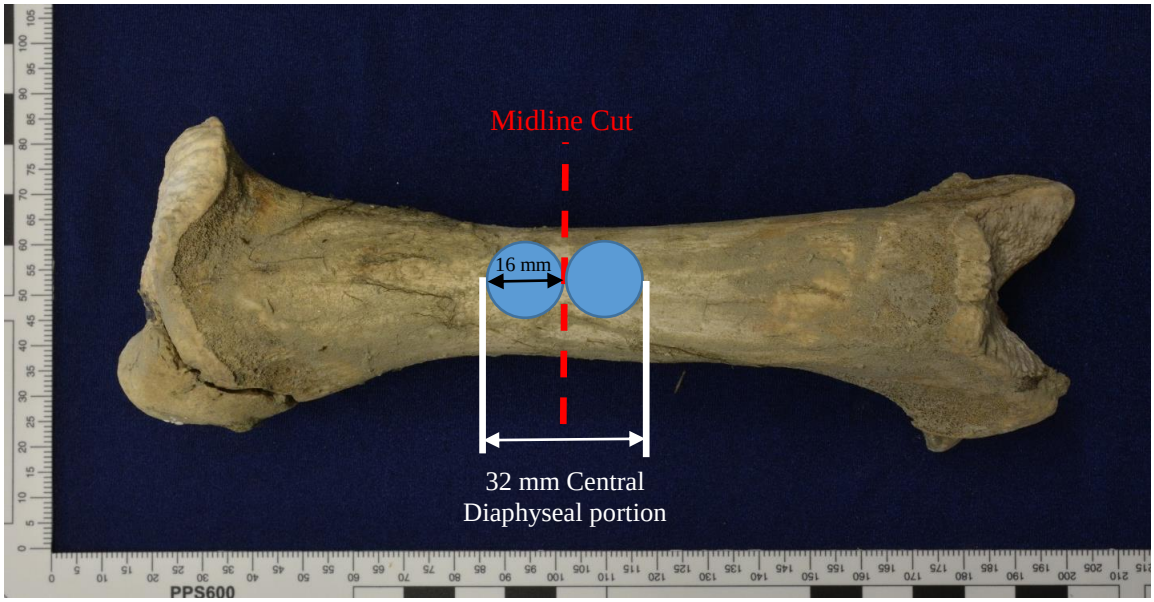

Figure 3.25: Diagram representing the coring process on the anterior surface of a bone sample. The red dashed line represents the midline cut done by the band saw. The blue circles represent the location of the 16 mm cores cut using the drill press, which sample an approximate 32 mm section of the central diaphysis.

RESULTS

Precision based upon the replicated measurements (accounting for the 9 sample runs and the three replicates measured each run) was determined to be less than 6% for all elements, with the exception of Cr and Pb, which were within 14 and 23%. Accuracy was based upon how close the measured concentration was to the certified value for each element in the SRMs; the most accurate concentration measurements were for P, Zn, Si, Sr, Ti, and Mn, which were within 3 and 12 percent error of the certified values (Table 3.1). The most inaccurate measurements were for Mg, which is evident from the high MC ratio in Figure 4.1; this was most likely due to the fact that Mg is on the cusp of the instrument's detection capabilities (the element with the lowest atomic number that the instrument detects).

Experiment 1: TT Results

For the time trials, the average and standard deviation of the 20 elemental concentrations are summarized in Table 4.1. The concentrations that show the lowest variation between the five bones analyzed each time interval were for Ca, Mg, P, Al, Si, Ni, S, Cl, and Ta (coefficient of variation [CV] <15%); three of the most abundant elements in the bones (Ca, P, Mg) had even lower variations (<5%). In contrast, Sr, Fe, and W had the largest variability, with CVs ranging from 28-106%, 8-67%, and 12-56% respectively; however, none of the bone samples from the time trials have high concentrations of Sr (<1.2 g/kg), Fe (<0.4 g/kg), or W (<0.19 g/kg). The other elements

are also represented in the bones at low concentrations, yet these have moderate variation among the bone samples for each time interval (<40%).

Table 4.1. Average (± standard deviation) bone concentrations for each two month interval of the time trials. All concentrations are the corrected value, except for those of Cl, Br, Ta, and W which are uncorrected (italicized).

Elem.	Amount of Time Submerged (in Months)										
	0	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**
Mg (g/kg)	4.45 ± 0.12	4.56 ± 0.18	4.51 ± 0.11	4.61 ± 0.14	4.60 ± 0.09	4.60 ± 0.03	4.56 ± 0.09	4.72 ± 0.05	4.56 ± 0.09	4.54 ± 0.11	4.51 ± 0.11
P (g/kg)	114.2 ± 4.0	111.9 ± 4.7	112.3 ± 4.6	115.1 ± 4.9	117.1 ± 1.8	114.0 ± 0.8	114.5 ± 1.9	118.4 ± 2.3	114.7 ± 2.8	114.6 ± 3.0	112.8 ± 3.0
K (mg/kg)	1284 ± 141	256 ± 16	263 ± 31	321 ± 66	247 ± 29	415 ± 89	304 ± 61	298 ± 95	317 ± 55	242 ± 38	298 ± 37
Ca (g/kg)	248.5 ± 5.2	244.2 ± 7.0	243.5 ± 7.8	249.1 ± 8.1	249.7 ± 3.5	245.7 ± 0.5	245.3 ± 3.4	251.5 ± 3.5	246.1 ± 5.2	244.9 ± 4.1	245.5 ± 5.7
Fe (mg/kg)	43.0 ± 4.2	62.6 ± 22.6	86.1 ± 16.9	156.7 ± 45.2	77.5 ± 6.3	244.4 ± 98.5	162.5 ± 61.9	131.0 ± 88.4	132.5 ± 48.6	82.1 ± 31.9	111.9 ± 49.9
Zn (mg/kg)	162 ± 20	210 ± 45	201 ± 41	203 ± 42	206 ± 32	204 ± 54	203 ± 56	200 ± 60	212 ± 47	262 ± 105	216 ± 60
Sr (mg/kg)	100 ± 85	484 ± 357	315 ± 231	729 ± 380	689 ± 733	530 ± 448	319 ± 200	520 ± 357	936 ± 270	560 ± 382	663 ± 397
Pb (mg/kg)	0.91 ± 0.18	1.04 ± 0.18	0.78 ± 0.09	0.95 ± 0.25	0.92 ± 0.20	1.28 ± 0.24	1.15 ± 0.11	1.08 ± 0.22	0.75 ± 0.08	0.84 ± 0.26	0.89 ± 0.28
Si (g/kg)	11.5 ± 0.3	12.7 ± 0.9	12.8 ± 0.8	14.3 ± 1.2	13.5 ± 1.3	14.0 ± 1.1	13.5 ± 0.7	13.7 ± 0.5	14.5 ± 0.4	13.1 ± 0.6	13.5 ± 1.2
S (g/kg)	0.81 ± 0.08	1.37 ± 0.19	1.33 ± 0.09	1.47 ± 0.15	1.53 ± 0.06	1.88 ± 0.16	2.06 ± 0.24	2.10 ± 0.22	2.22 ± 0.19	1.98 ± 0.19	1.85 ± 0.26
Ni (mg/kg)	18.8 ± 0.8	18.9 ± 0.9	18.9 ± 0.6	19.9 ± 2.0	19.7 ± 1.7	20.0 ± 0.7	19.2 ± 0.7	19.7 ± 1.3	19.1 ± 1.0	19.0 ± 0.7	19.0 ± 0.7

Elem.	Amount of Time Submerged (in Months)										
	0	**2**	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**
Th (mg/kg)	2.53 ± 0.21	2.34 ± 0.45	2.74 ± 0.33	2.44 ± 0.23	2.35 ± 0.59	2.80 ± 0.31	2.69 ± 0.43	2.75 ± 0.44	2.32 ± 0.35	2.48 ± 0.29	2.64 ± 0.38
Al (g/kg)	11.9 ± 0.5	12.4 ± 0.3	12.5 ± 0.3	13.1 ± 0.8	12.9 ± 0.3	13.9 ± 1.0	13.2 ± 0.6	13.3 ± 0.6	12.7 ± 0.2	12.5 ± 0.5	12.5 ± 0.5
Ti (g/kg)	0.022 ± 0.005	0.034 ± 0.010	0.037 ± 0.008	0.054 ± 0.013	0.035 ± 0.004	0.071 ± 0.023	0.048 ± 0.019	0.047 ± 0.019	0.050 ± 0.010	0.031 ± 0.003	0.036 ± 0.014
Cr (mg/kg)	21.3 ± 3.3	22.2 ± 2.1	22.1 ± 2.7	23.8 ± 2.9	20.1 ± 1.5	21.9 ± 3.1	25.4 ± 4.6	23.8 ± 1.3	20.5 ± 1.9	24.2 ± 1.9	26.0 ± 3.2
Mn (mg/kg)	14.5 ± 2.0	15.6 ± 2.5	17.2 ± 2.0	19.6 ± 3.2	17.4 ± 2.1	26.2 ± 6.7	24.9 ± 4.1	22.1 ± 6.8	23.1 ± 5.0	18.5 ± 3.2	18.3 ± 3.0
Cl (g/kg)	0.45 ± 0.05	3.36 ± 0.18	3.16 ± 0.24	3.24 ± 0.36	3.08 ± 0.34	3.49 ± 0.43	3.48 ± 0.37	3.51 ± 0.70	3.58 ± 0.38	3.06 ± 0.18	3.67 ± 0.23
Br (mg/kg)	1.5 ± 0.3	21.8 ± 1.4	21.0 ± 1.4	22.6 ± 2.1	20.2 ± 1.4	25.0 ± 3.2	23.2 ± 1.1	22.5 ± 4.2	23.5 ± 3.0	21.7 ± 1.9	24.6 ± 1.3
Ta (mg/kg)	23.9 ± 3.1	29.6 ± 4.3	29.8 ± 2.7	29.4 ± 2.6	28.4 ± 3.6	29.2 ± 2.7	29.7 ± 2.4	31.8 ± 3.3	30.6 ± 2.8	29.5 ± 2.6	32.1 ± 1.0
W (mg/kg)	73.8 ± 20.7	111.7 ± 47.0	91.4 ± 51.6	72.6 ± 26.2	46.1 ± 10.2	74.3 ± 16.3	63.4 ± 21.1	66.0 ± 20.3	65.2 ± 14.9	49.2 ± 15.4	53.9 ± 6.5

In order to visualize any trends in the elemental composition data from the time trials, scatter plots with lines of best fit were created (Figures 4.2-4.16). Prior to statistical analysis (see below), the author noted the general trends seen in the graphical data. The average concentrations for Mg, P, Ca, Pb, Ni, Th, Al, and Ti seem to remain relatively constant over the 20 month period (Figures 4.2-4.4). The concentrations for Ta, Cr, S, Si and Mn increase slightly over time, while the concentrations for W decrease over time (Figures 4.5-4.10). The concentrations for Sr, Zn, Fe also steadily increase over the 20 month submergence period; however, as noted earlier, there is a lot of variance in the

concentrations among the five bones for each time interval (Figures 4.11-4.13). There is a large increase in concentration of Cl and Br within the first two months of submergence; however, the rate of change in those concentrations slows to a more gradual increase after the first two months (Figures 4.14 and 4.15). In contrast, there is a large decrease in K within the first two months of submergence; however, that rate of change levels off after the first two months (Figure 4.16).

A Pearson's correlation was used to determine the relationships between the elements within the submerged bones and the amount of time in water (Table 4.2). A number of elements (K, Fe, Zn, Sr, Si, S, Cr, Mn, Cl, Br, Ta, W) have a significant correlation with the amount of time spent submerged in the water in regards to a linear relationship. The transformed concentrations of K and W were negatively correlated with time submerged, while the other variables were positively correlated. The strongest correlation to time submerged were the concentrations of S, followed by Cl and Br. Zn had the weakest correlation to time submerged.

Table 4.2. Pearson's correlation coefficients for the relationship between the natural log of the elemental concentrations and the number of months submerged. Italicized variables represent those whose concentrations were uncorrected before the natural log transformation. **Correlation is significant at the 0.01 level (2-tailed) and *Correlation is significant at the 0.05 level (2-tailed).

	Number of Months Submerged
Ln(Mg)	0.147
Ln(P)	0.127
Ln(K)	-.423**
Ln(Ca)	-0.017
Ln(Fe)	.333*
Ln(Zn)	.266*
Ln(Sr)	.406**
Ln(Pb)	-0.081
Ln(Si)	.425**
Ln(S)	.793**
Ln(Al)	0.219
Ln(Ti)	0.189
Ln(Cr)	.302*
Ln(Mn)	.363**
Ln(Ni)	0.034
Ln(Th)	0.060
Ln(Cl)	.526**
Ln(Br)	.526**
Ln(Ta)	.430**
Ln(W)	-.413**

An ANCOVA was run in order to determine the effect of submergence (control group that was never submerged vs. all of the submerged experimental groups) on the transformed elemental concentrations after controlling for the amount of time submerged.

After adjusting for the amount of time submerged, there was a significant difference in transformed concentrations of K, Fe, Sr, Si, S, Cl, Br, and Ta between the control samples and the submerged samples (Table 4.3).

Table 4.3. ANCOVA results for elements found to be significantly correlated with time according to the Pearson's test. Significant p values (< 0.05) are bolded. Italicized variables represent those whose concentrations were uncorrected before the natural log transformation.

	Independent Variable		**Covariate**	
	Control vs. Submerged Groups		**Amount of Time Submerged**	
Dependent Variable	**F**	**Sig. (p)**	**F**	**Sig. (p)**
Ln(K)	164.939	**0.000**	0.222	0.639
Ln(Fe)	8.263	**0.006**	0.878	0.353
Ln(Zn)	2.422	0.126	0.966	0.330
Ln(Sr)	11.808	**0.001**	1.839	0.181
Ln(Si)	12.995	**0.001**	2.165	0.147
Ln(S)	41.582	**0.000**	58.901	**0.000**
Ln(Cr)	0.037	0.848	3.541	0.065
Ln(Mn)	2.602	0.113	2.857	0.097
Ln(Cl)	1033.82	**0.000**	2.44	0.124
Ln(Br)	1660.03	**0.000**	3.162	0.081
Ln(Ta)	12.067	**0.001**	2.462	0.123
Ln(W)	1.291	0.261	11.804	**0.001**

Post hoc testing determined that the transformed K concentrations were significantly lower in the submerged group than those in the control group (M_{diff} = -1.515 mg/kg, 95% CI [-1.752, -1.279], $p < 0.0005$). The transformed Fe concentrations were significantly greater in the submerged group than those in the control group (M_{diff} = 0.795

mg/kg, 95% CI [0.240, 1.351], p = 0.006). The transformed Sr concentrations were significantly greater in the submerged group than those in the control group (M_{diff} = 1.409 mg/kg, 95% CI [0.586, 2.233], p = 0.001). The transformed Si concentrations were significantly greater in the submerged group than those in the control group (M_{diff} = 0.138 g/kg, 95% CI [0.061, 0.214], p = 0.001). The transformed S concentrations were significantly greater in the submerged group than those in the control group (M_{diff} = 0.482 g/kg, 95% CI [0.332, 0.631], p < 0.0005). The transformed Cl concentrations were significantly greater in the submerged group than those in the control group (M_{diff} = 1.972 g/kg, 95% CI [1.849, 2.095], p < 0.0005). The transformed Br concentrations were significantly greater in the submerged group than those in the control group (M_{diff} = 2.652 mg/kg, 95% CI [2.521, 2.782], p < 0.0005). The transformed Ta concentrations were significantly greater in the submerged group than those in the control group (M_{diff} = 0.187 mg/kg, 95% CI [0.079, 0.294], p = 0.001).

The covariate, amount of time submerged, significantly predicted the transformed concentrations of S and W (Table 4.3); therefore, the transformed concentrations of S and W were significantly influenced by the number of months the samples were submerged. According to this linear model, the adjusted means for Zn, Cr, and Mn were not significantly different between the control and submerged groups; there was also no significant relationship between the number of months submerged and the transformed concentrations of these elements (Table 4.3).

The elements with significant correlations according to the Pearson's test were then applied to a multivariable linear regression in an attempt to identify a regression

equation that could predict the amount of time submerged for an unknown bone. The regression analysis did not produce any outliers and passed the Durbin Watson test for the independence of observances; however, the normality of the data was still slightly skewed even after the natural log transformation. According to an ANOVA analysis the resulting model was significantly better at predicting the amount of time submerged than simply predicting using the mean ($F [12, 42] = 14.187$, $p < 0.0005$). The coefficient of determination (R^2) for this sample was 0.802, which means that 80.2% of the variance in time submerged was predicted from the concentrations of the selected elements above; the adjusted R^2, which is a more accurate value of what is expected in a population, was 74.6%. Although 12 elements were used as predictors to create the regression model, only five elements (Sr, Si, S, Cr, and W) were determined to be significant unstandardized coefficients ($p < 0.05$), meaning the addition of those elements as predictors improved the predictability of the model. The model was redefined to only include the elements that had significant unstandardized coefficients (Sr, Si, S, Cr, W) or those with relatively large coefficients that were close to being significant ($p < 0.1$) (K, Zn, Cl, Br). The R^2 for the redefined model was the same as the original model (80.2%), but this adjusted R^2 was slightly higher than the original (76.2%). The transformed concentrations of K, Zn, Sr, Si, S, Cr, Cl, Br, and W significantly predicted the number of months submerged ($F [9, 45] = 20.234$, $p < 0.0005$). Out of the nine transformed elemental concentrations that were used as predictors for the redefined model, only one (Zn) was determined to not be a significant unstandardized coefficient; therefore, the

predictability of the model was improved by using the transformed concentrations of K, Sr, Si, S, Cr, Cl, Br, and W. The final regression equation was:

Amount of Time Submerged = (34.832 ± 26.566) + (7.230 ± 2.671)ln[K] + (4.455 ± 1.128)ln[Sr] + (-56.108 ± 14.224)ln[Si] + (19.982 ± 2.665)ln[S] + (7.838 ± 3.278)ln[Cr] + (-12.163 ± 5.923)ln[Uncorrected Cl] + (11.308 ± 4.961)ln[Uncorrected Br] + (-5.365 ± 1.526)ln[Uncorrected W]

when the amount of time submerged is in months, the concentrations for K, Sr, Cr, Br, and W are in mg/kg, and the concentrations for Si, S, and Cl are in g/kg.

Experiment 2: ST Results

The averages and standard deviations of the 20 elemental concentrations for each submergence location from the salinity trials (Holliston Pond, Boston Harbor [3 m], Boston Harbor [6 m], Woods Hole [10 m], Woods Hole [18 m]) are summarized in Table 4.4. The concentrations that show the lowest variation between the five bones analyzed in each group were for Ca, Mg, P, Al, Si, Ni, S, Cr and Ta (<15%); four of the most abundant elements in the bones (Ca, Mg, Si, Al) had even lower variations (<6%). In contrast, Sr and W had the largest variability, with CVs ranging from 14-89% and 23-66% respectively; however, none of the bone samples from any of the submergence locations had high concentrations of Sr (<0.7 g/kg) or W (<0.09 g/kg). The other elements are also represented in the bones at low concentrations, yet these have more moderate variations (CVs of <36%) among the five bone samples tested for each time interval.

Table 4.4. Average (± standard deviation) bone concentrations for each submergence location of the salinity trials. All concentrations are the corrected value, except for those of Cl, Br, Ta, and W which are uncorrected (italicized). "BDL" = Below detection limit.

Elem.	Submergence Location				
	Fresh	Brackish		Salt	
	Holliston Pond	Boston Harbor (3 m)	Boston Harbor (6 m)	Woods Hole (10 m)	Woods Hole (18 m)
Mg (g/kg)	4.30 ± 0.28	4.53 ± 0.14	4.69 ± 0.23	4.59 ± 0.02	4.57 ± 0.15
P (g/kg)	112.5 ± 9.1	110.9 ± 1.7	112.3 ± 6.1	114.0 ± 1.2	112.1 ± 4.5
K (mg/kg)	BDL	427 ± 82	443 ± 39	361 ± 82	242 ± 21
Ca (g/kg)	241.3 ± 14.1	233.4 ± 4.1	238.6 ± 8.2	243.0 ± 1.9	236.4 ± 7.6
Fe (mg/kg)	401.3 ± 24.0	105.4 ± 37.3	115.3 ± 26.0	49.7 ± 6.2	44.9 ± 9.8
Zn (mg/kg)	195 ± 22	216 ± 38	185 ± 25	213 ± 36	200 ± 33
Sr (mg/kg)	208 ± 113	615 ± 437	386 ± 344	278 ± 41	317 ± 45
Pb (mg/kg)	0.88 ± 0.18	0.93 ± 0.19	0.98 ± 0.19	0.85 ± 0.15	0.86 ± 0.18
Si (g/kg)	11.8 ± 0.7	13.2 ± 0.8	13.3 ± 0.5	12.3 ± 0.3	12.4 ± 0.7
S (g/kg)	0.30 ± 0.02	1.97 ± 0.18	2.04 ± 0.24	2.14 ± 0.08	2.21 ± 0.10
Ni (mg/kg)	17.8 ± 0.4	18.5 ± 1.2	19.5 ± 0.6	18.2 ± 0.7	19.1 ± 0.9
Th (mg/kg)	2.74 ± 0.44	2.64 ± 0.28	2.84 ± 0.29	2.69 ± 0.44	2.63 ± 0.23
Al (g/kg)	11.5 ± 0.7	12.5 ± 0.3	13.1 ± 0.7	12.4 ± 0.3	12.2 ± 0.5
Ti (g/kg)	0.031 ± 0.003	0.039 ± 0.012	0.039 ± 0.009	0.035 ± 0.006	0.024 ± 0.002
Cr (mg/kg)	21.1 ± 2.0	20.0 ± 3.6	26.4 ± 2.9	21.1 ± 1.6	24.1 ± 2.3
Mn (mg/kg)	132.9 ± 3.8	32.9 ± 6.0	30.0 ± 6.1	16.8 ± 2.7	17.7 ± 3.1
Cl (g/kg)	BDL	5.60 ± 1.70	6.62 ± 1.35	3.52 ± 0.90	3.16 ± 0.60
Br (mg/kg)	0.9 ± 0.2	35.4 ± 9.0	43.1 ± 7.1	25.8 ± 4.5	23.8 ± 3.8
Ta (mg/kg)	29.7 ± 4.2	28.3 ± 3.8	27.8 ± 3.4	28.9 ± 0.7	27.1 ± 3.6
W (mg/kg)	87.2 ± 33.7	81.9 ± 19.3	88.6 ± 59.1	53.3 ± 14.6	57.6 ± 17.1

Prior to running a one-way ANOVA, a Levene's test determined that equal variances were not assumed for Mg, P, K, Fe, Sr, and Mn; therefore, these elements were

analyzed by a Welch's ANOVA and a Games-Howell post hoc test, rather than an ANOVA followed by a Tukey post hoc test. There was a significant difference among the five submergence locations for the natural log concentrations of K, Fe, Si, S, Al, Ti, Cr, Mn, Ni, Cl, and Br (Table 4.5).

Table 4.5. ANOVA results for the salinity trials. Significant p values (< 0.05) are bolded. *Results obtained from Welch's ANOVA. Italicized variables represent those whose concentrations were uncorrected before the natural log transformation.

Dependent Variable	F	Sig. (p)
Ln(Mg)*	1.369	0.325
Ln(P)*	2.373	0.128
Ln(K)*	57.561	**0.000**
Ln(Ca)	1.006	0.428
Ln(Fe)*	312.043	**0.000**
Ln(Zn)	0.854	0.508
Ln(Sr)*	1.633	0.246
Ln(Pb)	0.432	0.784
Ln(Si)	5.354	**0.004**
Ln(S)	636.639	**0.000**
Ln(Al)	5.933	**0.003**
Ln(Ti)	4.335	**0.011**
Ln(Cr)	4.804	**0.007**
Ln(Mn)*	404.078	**0.000**
Ln(Ni)	3.585	**0.023**
Ln(Th)	0.295	0.877
Ln(Cl)	307.766	**0.000**
Ln(Br)	315.463	**0.000**
Ln(Ta)	0.424	0.789
Ln(W)	1.679	0.194

The post hoc tests determined the homogenous subsets (Table 4.6). Games-Howell post hoc testing revealed significant differences in K concentration between the bones submerged in the Holliston Pond and Woods Hole (10 m)/Woods Hole (18 m) and Woods Hole (10 m)/Boston Harbor (3 m)/Boston Harbor (6 m). All three major salinity locations (Holliston Pond, Boston Harbor, and Woods Hole) produced bones with significantly different concentrations of Mn and Fe. Depth had no significant effect on K, Fe, or Mn concentrations for neither the Boston Harbor samples nor the Woods Hole samples.

Tukey post hoc testing revealed significant differences in Si and Al concentrations between bone groups from Holliston Pond/Woods Hole (10 m)/Woods Hole (18 m) and Boston Harbor (3 m)/Boston Harbor (6 m)/Woods Hole (10 m)/Woods Hole (18 m); therefore, the Holliston pond samples had significantly different Al and Si concentrations than the Boston Harbor (3 m and 6 m) samples. However, depth did not cause a significant difference between the two sets of Boston Harbor samples, as well as the two sets of Woods Hole samples. The bones from Holliston Pond had significantly lower concentrations of S than the bones from all other salinity groups; however, there was no significant difference between the S concentrations of the 10 m and 18 m Woods Hole samples or between the 3 m and 6 m Boston Harbor samples. For Ti, one homogenous subset included Woods Hole (10 m)/Woods Hole (18 m)/Holliston Pond, while the other included Holliston Pond/Woods Hole (10 m)/Boston Harbor (3 m)/Boston Harbor (6 m); therefore, there was a significant difference between the Ti concentrations of the Woods Hole (18 m) samples and the Boston Harbor (3 m and 6 m) samples. There

was no significant difference in Ti concentrations due to depth for the Boston Harbor samples, as well as for the Woods Hole samples.

There was a significant difference in Cr concentrations between the bones submerged at 3 m in the Boston Harbor and those submerged at 6 m, but it was harder to differentiate between the bones from Holliston Pond, Woods Hole (10 m), and Woods Hole (18 m). There was a significant difference the Ni concentrations between the bones from the Holliston Pond and those from the 6 m depth of the Boston Harbor; however, neither of those samples could be distinguished from the Woods Hole samples and the Boston Harbor (3 m) samples when just analyzing Ni concentrations. The Br concentrations within the bones significantly differed among the Holliston Pond samples and the Boston Harbor (3 m and 6 m) samples and the Woods Hole (10 m)/Woods Hole (18 m)/Boston Harbor (3 m) samples; however, the different depths for both the Woods Hole and Boston Harbor samples could not be separated. When looking at the Cl concentrations, the submergence groups could be separated into four homogenous subsets: 1. Holliston Pond, 2. Woods Hole (10 and 18 m), 3. Woods Hole (10 m)/Boston Harbor (3 m), 4. Boston Harbor (3 and 6 m). Yet again, depth did not significantly influence the Cl concentrations in the Boston Harbor and Woods Hole samples.

Table 4.6. Homogenous subsets of submergence locations determined by post hoc testing of elements with significant differences among the groups.

Elem.	Homogenous Subsets			
	1	2	3	4
S	Holliston Pond	Boston Harbor (3 m) Boston Harbor (6 m) Woods Hole (10 m) Woods Hole (18 m)		
Fe	Holliston Pond	Boston Harbor (3 m) Boston Harbor (6 m)	Woods Hole (10 m) Woods Hole (18 m)	
Mn	Holliston Pond	Boston Harbor (3 m) Boston Harbor (6 m)	Woods Hole (10 m) Woods Hole (18 m)	
Cl	Holliston Pond	Woods Hole (18 m) Woods Hole (10 m)	Woods Hole (10 m) Boston Harbor (3 m)	Boston Harbor (3 m) Boston Harbor (6 m)
K	Holliston Pond	Woods Hole (18 m) Woods Hole (10 m)	Woods Hole (10 m) Boston Harbor (3 m) Boston Harbor (6 m)	
Br	Holliston Pond	Woods Hole (10 m) Woods Hole (18 m) Boston Harbor (3 m)	Boston Harbor (3 m) Boston Harbor (6 m)	
Si	Holliston Pond Woods Hole (10 m) Woods Hole (18 m)	Woods Hole (10 m) Woods Hole (18 m) Boston Harbor (3 m) Boston Harbor (6 m)		
Al	Holliston Pond Woods Hole (10 m) Woods Hole (18 m)	Woods Hole (10 m) Woods Hole (18 m) Boston Harbor (3 m) Boston Harbor (6 m)		

Elem.	Homogenous Subsets			
	1	2	3	4
Ni	Holliston Pond Woods Hole (10 m) Boston Harbor (3 m) Woods Hole (18 m)	Woods Hole (10 m) Boston Harbor (3 m) Woods Hole (18 m) Boston Harbor (6 m)		
Ti	Woods Hole (18 m) Holliston Pond Woods Hole (10 m)	Holliston Pond Woods Hole (10 m) Boston Harbor (3 m) Boston Harbor (6 m)		
Cr	Boston Harbor (3 m) Holliston Pond Woods Hole (10 m) Woods Hole (18 m)	Holliston Pond Woods Hole (10 m) Woods Hole (18 m) Boston Harbor (6 m)		

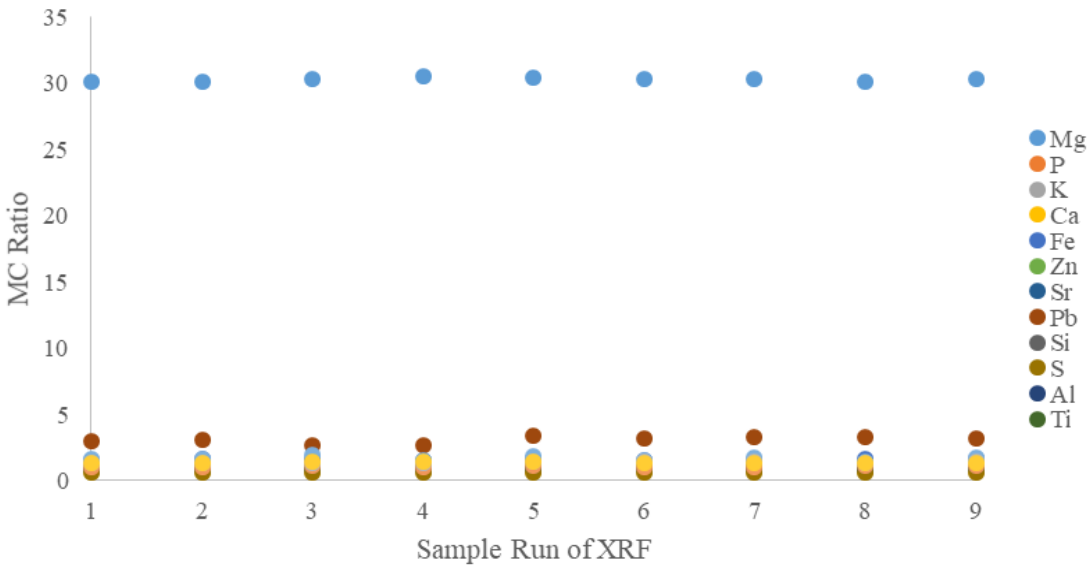

Figure 4.1: Run-specific Measured-to-Certified (MC) Ratios for each analyzed element in the SRMs (NIST 1486, NIST 1646a, and NIST 2702).

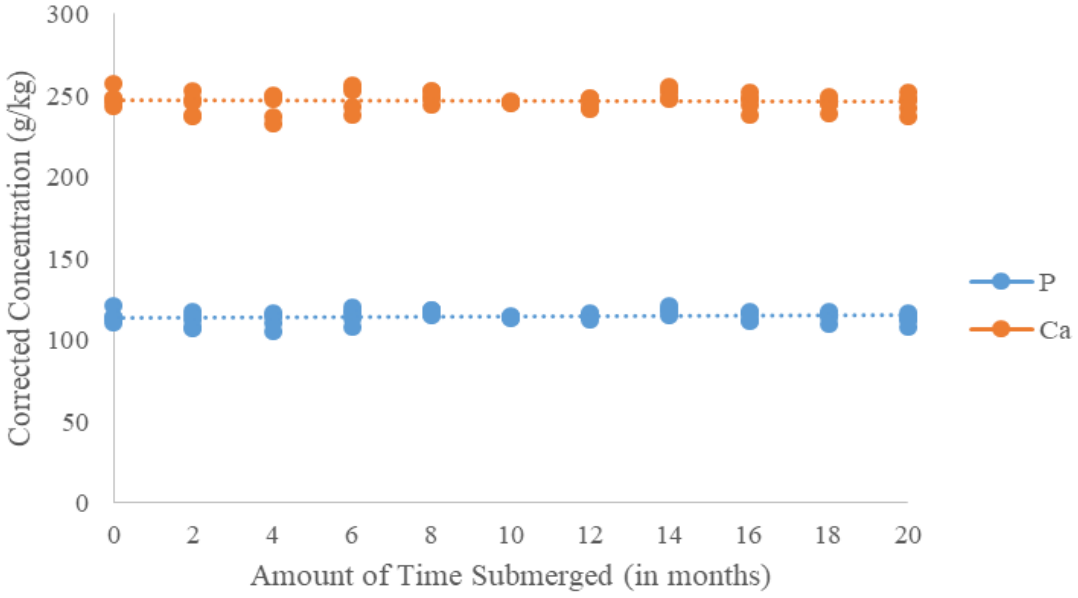

Figure 4.2: Corrected concentrations of P and Ca over the 20 month time trial period for Samples A-E of each experimental group.

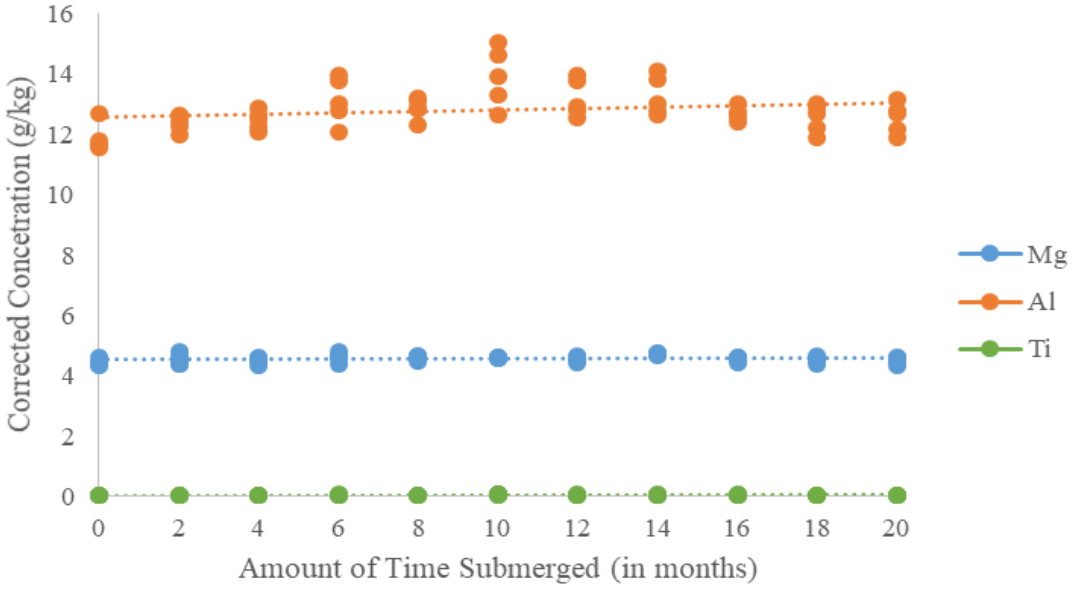

Figure 4.3: Corrected concentrations of Mg, Al, and Ti over the 20 month time trial period for Samples A-E of each experimental group.

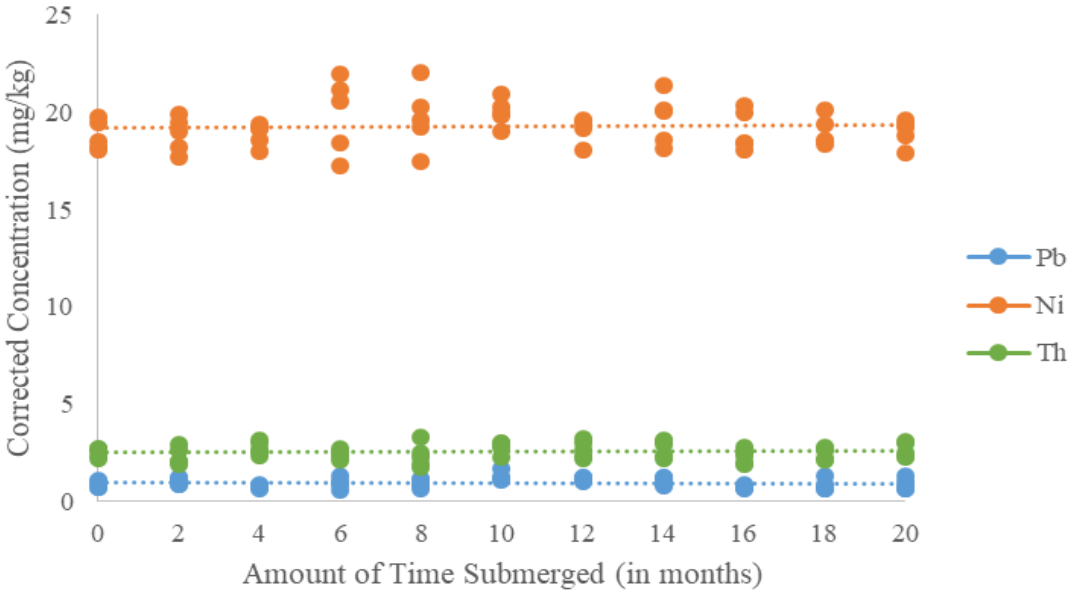

Figure 4.4: Corrected concentrations of Pb, Ni, and Th over the 20 month time trial period for Samples A-E of each experimental group.

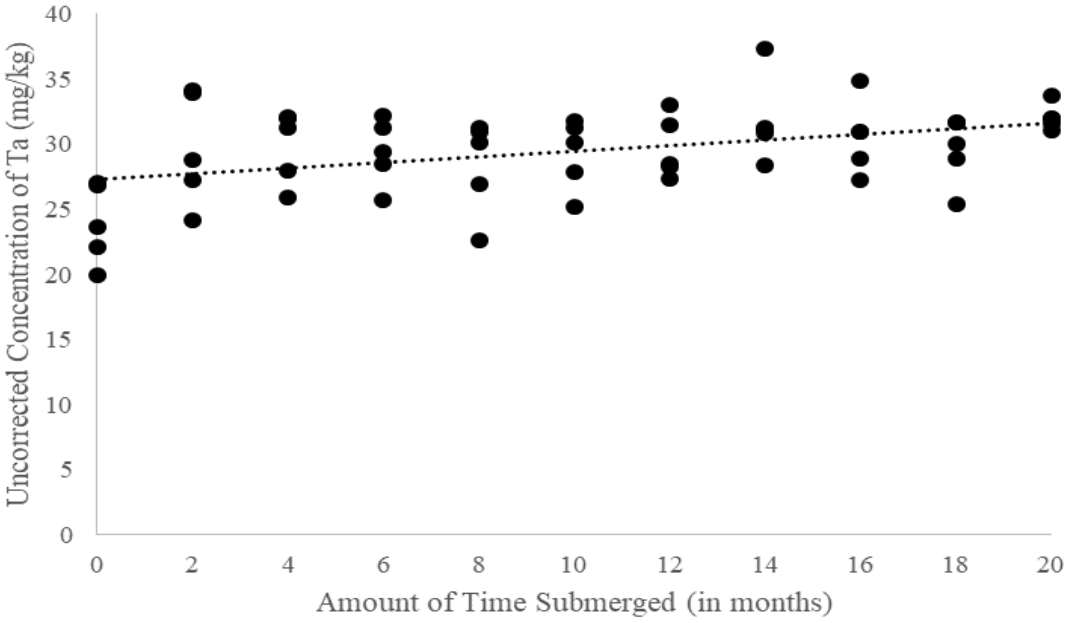

Figure 4.5: Uncorrected concentrations of Ta over the 20 month time trial period for Samples A-E of each experimental group.

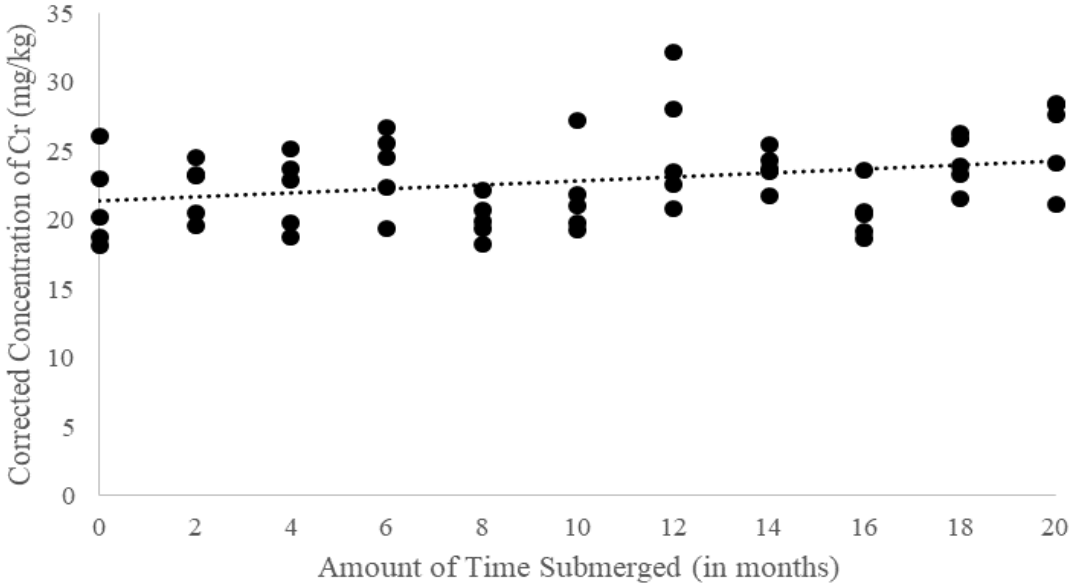

Figure 4.6: Corrected concentrations of Cr over the 20 month time trial period for Samples A-E of each experimental group.

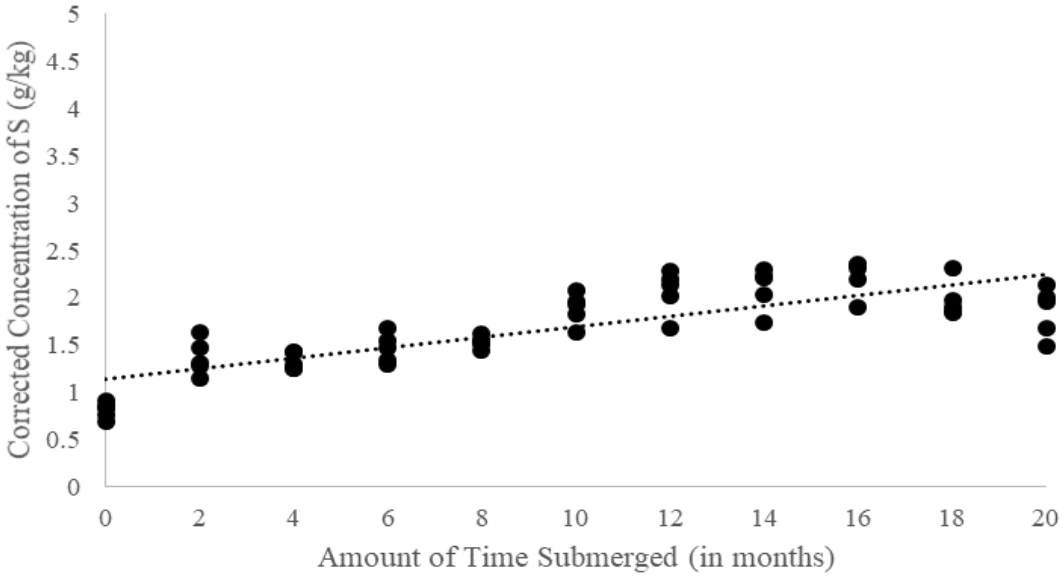

Figure 4.7: Corrected concentrations of S over the 20 month time trial period for Samples A-E of each experimental group.

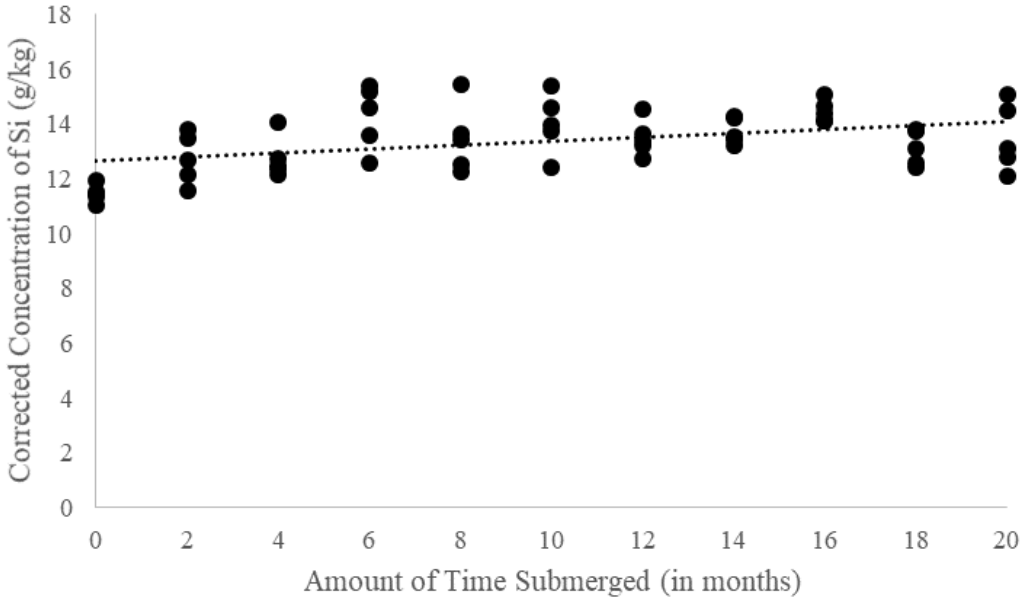

Figure 4.8: Corrected concentrations of Si over the 20 month time trial period for Samples A-E of each experimental group.

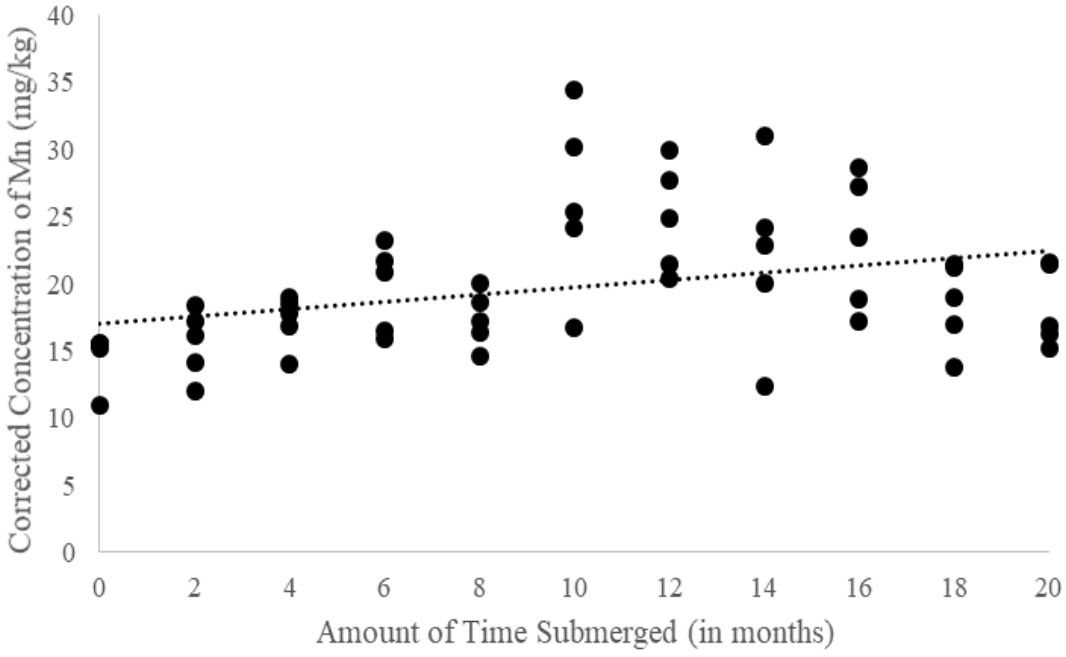

Figure 4.9: Corrected concentrations of Mn over the 20 month time trial period for Samples A-E of each experimental group.

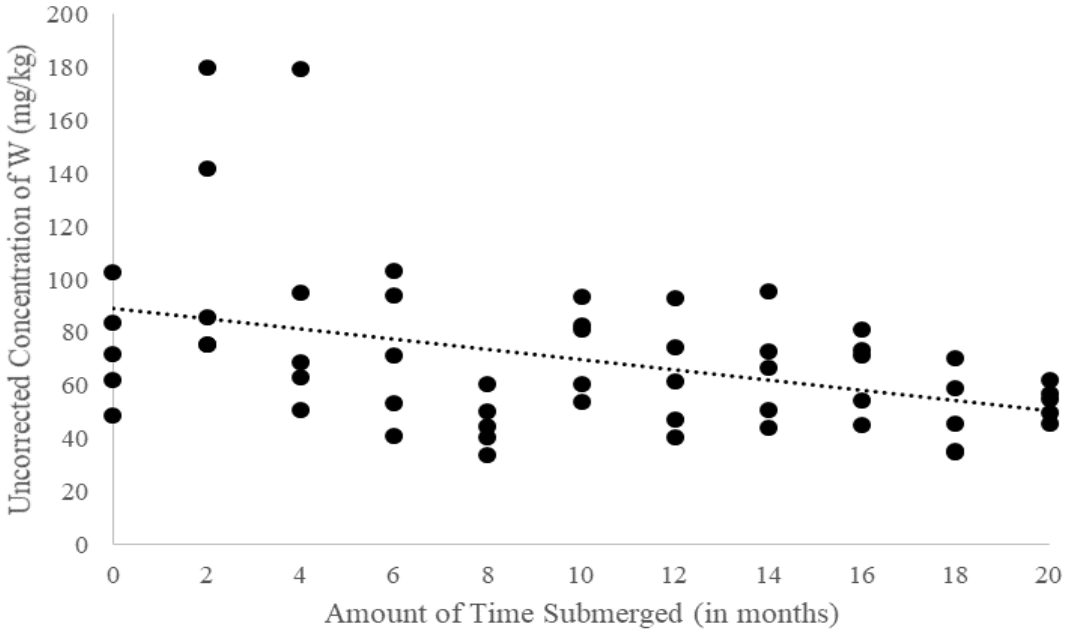

Figure 4.10: Uncorrected concentrations of W over the 20 month time trial period for Samples A-E of each experimental group.

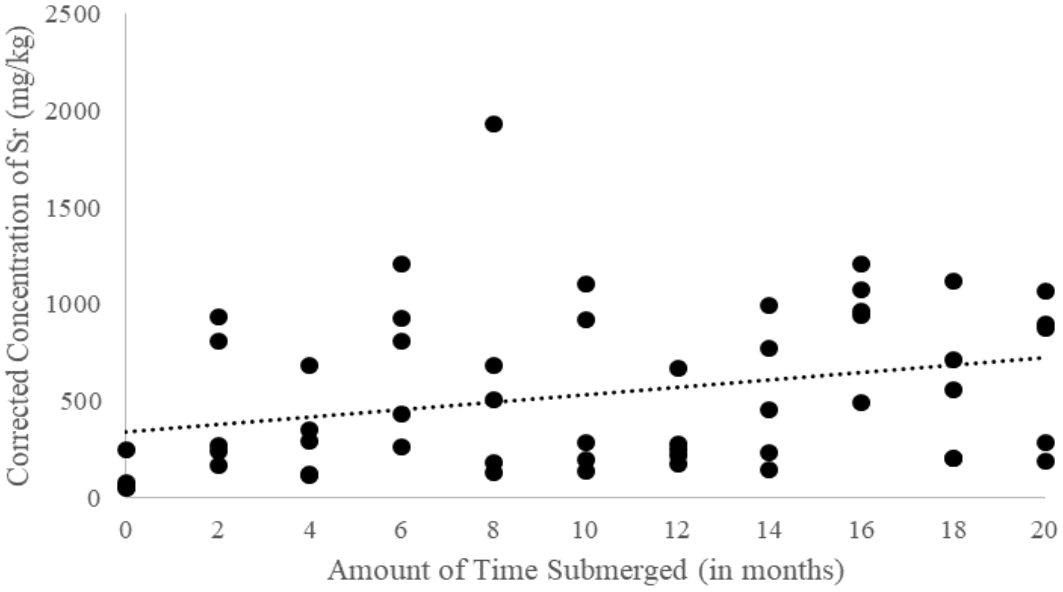

Figure 4.11: Corrected concentrations of Sr over the 20 month time trial period for Samples A-E of each experimental group.

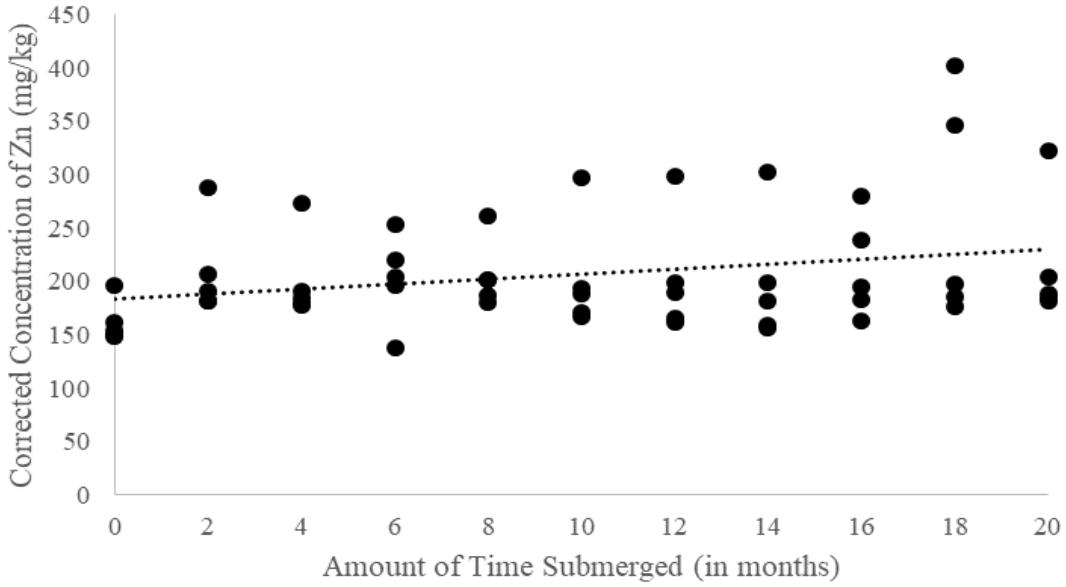

Figure 4.12: Corrected concentrations of Zn over the 20 month time trial period for Samples A-E of each experimental group.

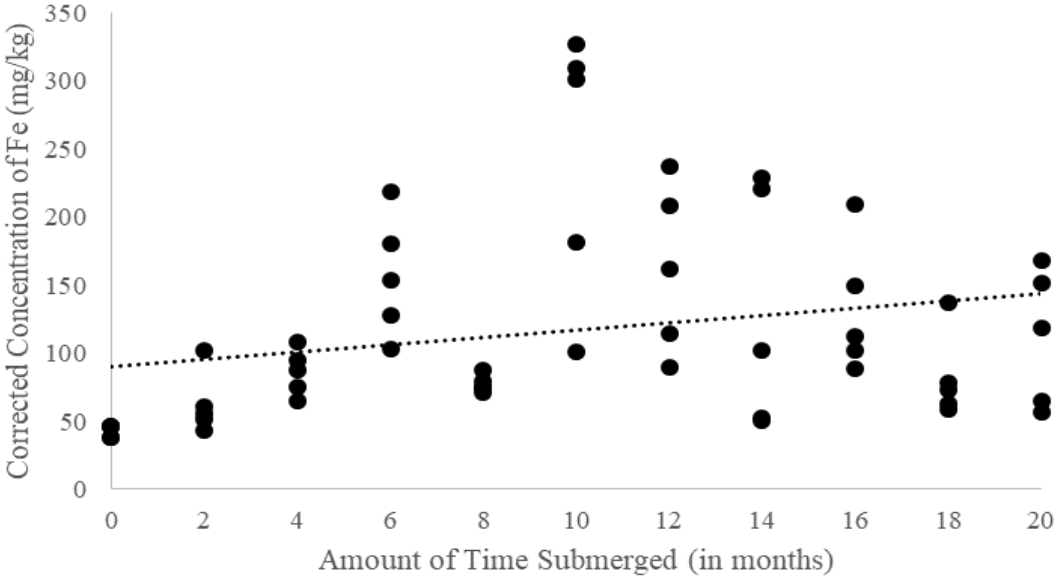

Figure 4.13: Corrected concentrations of Fe over the 20 month time trial period for Samples A-E of each experimental group.

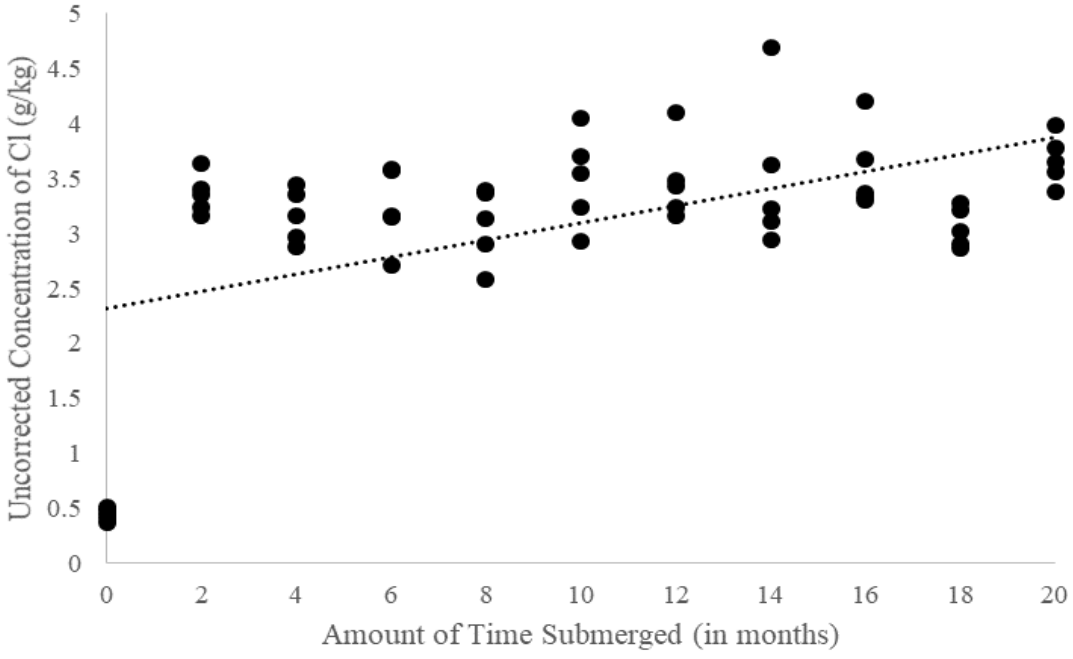

Figure 4.14: Uncorrected concentrations of Cl over the 20 month time trial period for Samples A-E of each experimental group.

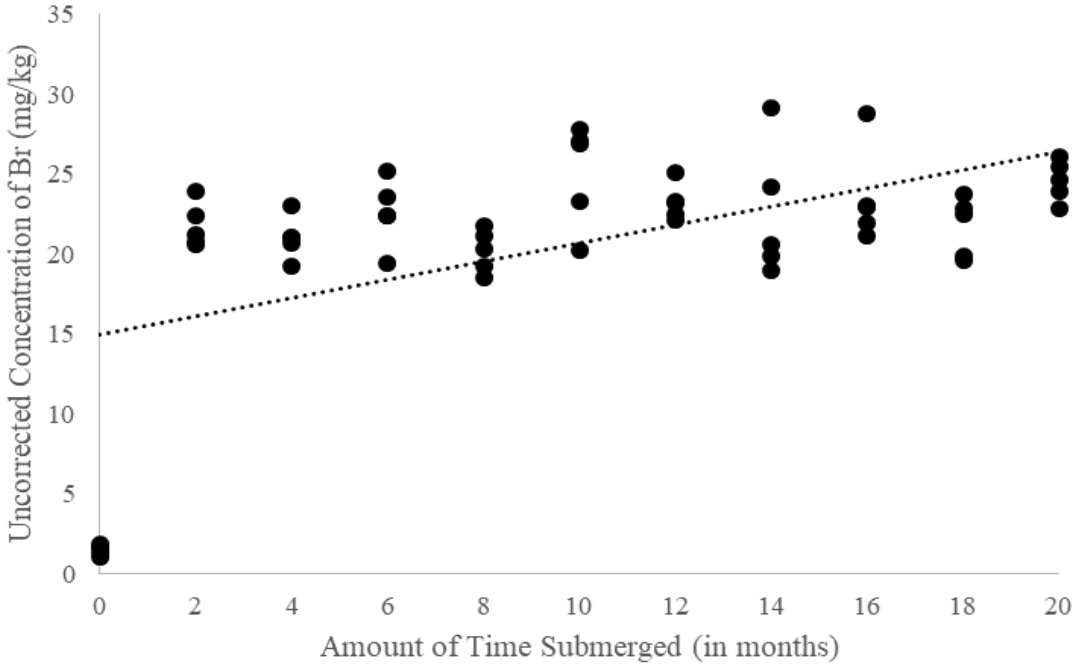

Figure 4.15: Uncorrected concentrations of Br over the 20 month time trial period for Samples A-E of each experimental group.

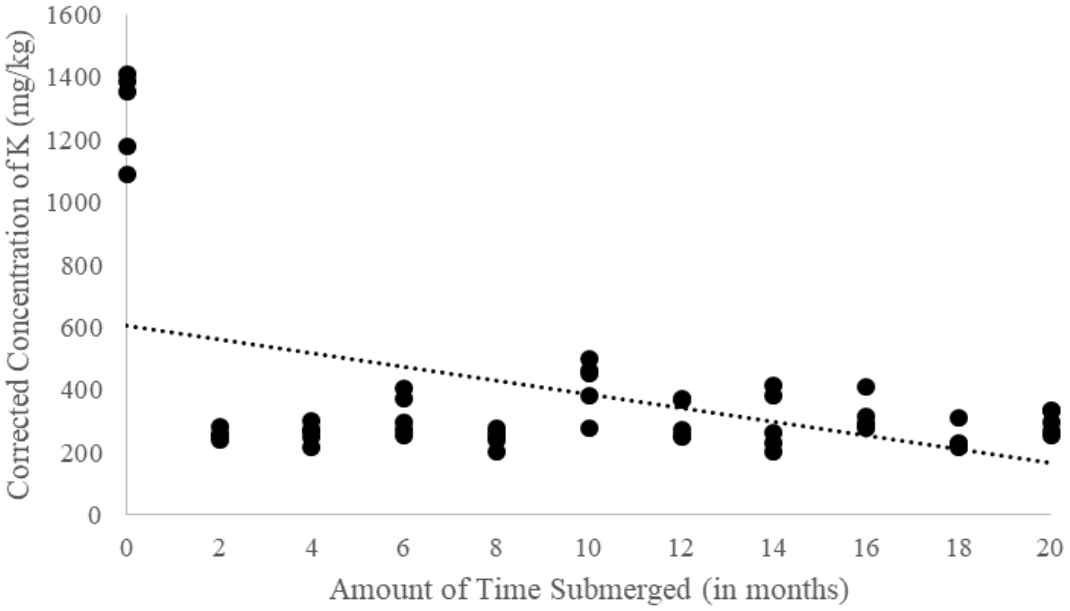

Figure 4.16: Corrected concentrations of K over the 20 month time trial period for Samples A-E of each experimental group.

DISCUSSION

The major elements (i.e., Ca, P, Mg) of bone showed the lowest variability among the bone samples within each experimental group (CV < 6%); these concentrations also did not significantly differ over time (Table 4.2) and or when exposed to a different aquatic environment (Table 4.5). This suggests that those largest components of bone remained relatively stable at least during the first 20 months of complete submergence in any aquatic environment. In contrast, some of the minor elements, such as Sr, Fe, and W, were found to vary greatly among individuals within each time interval (CV < 106%) and salinity locale (CV < 89%). These variations could have occurred if the bones were sampled in different locations or different depths each time; however, this was not the case in the present study due to the fact that sampling location on the central diaphysis was controlled for. The samples were also thoroughly homogenized in order to obtain an overall representation of bone composition rather than a depth-dependent analysis.

Another explanation for the variability in the minor elements within the sample groups is that these elemental concentrations were different in the living individuals. For example, Sr and Fe both can replace Ca in the bony matrix during life (Pfretzschner 2004; Trueman 1999), and W is a contaminant in drinking water that accumulates within bones when consumed (VanderSchee *et al*. 2018). Therefore, variations in these elements could all be caused by environmental exposures during life. The only way to control for this factor would be to obtain every pig femur from the same farm around the same time. For the current study, it was not necessary to control for, because then the experiment

would not reflect a real world scenario in which a victim's lifelong environmental exposures are completely or partially unknown. Antemortem accumulation and poor sampling techniques are not the only explanation for the variability in some of the elemental concentrations; diagenesis also impacts bone and its elemental concentrations.

Experiment 1: TT Discussion

The results from the TT portion of the study supports the claims that diagenesis impacts the chemical composition of bone, since various statistical analyses determined significant relationships between a number of elemental concentrations and time spent submerged in the harbor waters. For example, the Pearson's correlation test determined a significant relationship between time submerged and the transformed concentrations for K, Fe, Zn, Sr, Si, S, Cr, Mn, Cl, Br, Ta, and W (Table 4.2). The ANCOVA analysis determined a significant difference in concentrations of K, Fe, Sr, Si, S, Cl, Br, and Ta between the control and submerged groups; also according to the ANCOVA results, the concentrations of S and W were significantly related to the covariate, specifically the number of months spent submerged (Table 4.3).

McElreath (2018) analyzed the composition of bones from a number of animals that were submerged in the ocean from 0 - 427 days; it was suggested that the bones' compositions were affected by what elements were readily available in the surrounding environment. The elements S, Cl, Br, and Sr, are some of the major and minor components of seawater (Culkin and Cox 1966; Kennish 2001; Millero 2006; Wilson 1975); Si is commonly found in fresh, brackish, and saltwaters due to terrestrial erosion

and the breakdown of skeletal materials of diatoms (Kennish 2001; Tréguer and De La Rocha 2013). Fe, Zn, Cr, Mn, and W, are known local contaminants of the Boston Harbor (Breault *et al*. 2004; Buchholtz ten Brink *et al*. 2002; Hubbard and Bellmer 1989). The natural process of diffusion could explain why there was a significant difference in K, Fe, Sr, Si, S, Cl, Br, Ta concentrations between the bones that were never submerged and those submerged for any amount of time, when excluding the effect of the amount of time submerged. The surrounding water has an excess of K, Fe, Sr, Si, S, Cl, Br, and Ta; therefore, the intake, deposition into crevices, and the structural substitution of these elements can occur as soon as the bone samples are exposed to this environment (Keenan and Engel 2017). This explains the significantly higher concentrations of Fe, Sr, Si, S, Cl, Br, and Ta within the submerged bones when compared to the control bones. McElreath (2018) also found elevated levels of S and Si within animal bones submerged in the ocean compared to those of the baseline samples that were never exposed to the water.

However, this diffusion theory cannot explain the significant decrease in the K concentrations of the submerged bones compared to the control bones; K is a major component in seawater and therefore should not be leaching from the bone and entering the surrounding environment. McElreath (2018) also noted lower concentrations of K within the bones submerged in the ocean when compared to the baseline samples. McElreath (2018) suggested that the overall mean percentage for this element decreased as other elements were incorporated into the bony matrix over time. The instrumentation utilized in the current study and in McElreath (2018) produced semi-quantitative data in the form of mass percents; a mass percent represents the percentage of a component in a

mixture relative to all the other components within that mixture, rather than the actual element's concentration that is present in the sample. In the case of the decrease in K concentrations once submerged in the water, K may not be leaching into the environment once it has entered the water; as the other environmental elements are incorporated into the bone, their mass percentages increase, while the mass percentage of K decreases.

Once submerged, K, Fe Sr, Si, Cl, Br, and Ta did not significantly change over time, meaning after the initial deposition of environmental elements into the bone, the rate of this incorporation plateaued. This could be because the breakdown of the organic components of bone, which frees up physical space within the crevices of the bone as well as exposes more sites for ionic substitutions, requires a lot of time (i.e., thousands of years to complete early diagenesis depending upon the environment) (Pfretzschner 2004; Tütken *et al*. 2008).

The ANCOVA analysis determine that the concentration of S had a significant positive relationship with the amount of time submerged in the water. S is a major element in seawater and therefore, readily available for deposition within a submerged bone, potential in the form of pyrite. Pyrite (FeS_2) is formed during diagenesis and fossilization when Fe ions from the environment react with sulphides from degrading collagen; this process can occur faster in the ocean, because the two S molecules needed to combine with a single Fe molecule can also come from the readily available sulphate ions found within seawater (Pfretzschner 2004). The significant increase in S overtime suggests that S molecules are continually incorporated into the bone and are potentially forming increasing amounts of pyrite as diagenesis continues. Although the ANCOVA

analysis did not identify a significant relationship between Fe and the amount of time submerged, the Pearson's correlation test did.

The Pearson's test and the ANCOVA identified a significantly negative relationship between the concentrations of W and the amount of time submerged. W is not a major element found within the ocean; however, it can be deposited within cortical bone during life (VanderSchee *et al*. 2018). VanderSchee *et al*. (2018) determined that solubilized W (WO_4^{2-}) can be absorbed by mice from drinking water, leading to a buildup of W throughout the long bones. However, they noted that when the W source was removed from the water, concentrations were still detected in the outer cortical layers, but not the inner bone. This study supported the idea that W within bone can eventually leach out into the environment, although it may take a long period of time. The current study noted a significant decrease in W over time spent submerged, which could be due to the gradual diffusion of W out of the bones.

A regression equation based upon the concentrations of elements that correlate with submergence time would be useful to predict the PMSI of an unknown bone from the ocean environment. The model produced in the present study used the concentrations for K, Sr, Si, S, Cr, and Br. Although the R^2 was high (80.2%), suggesting that these predictors explained a large portion of the variance in the outcome, the results from this analysis should be used with caution. The compositional data in the present study were transformed using the natural log in order to aid in normalizing the data, a process used by Buikstra *et al*. (1989) and Lambert *et al*. (1991) during their analyses of archaeological bone composition. Even after this transformation, not all of the data fit a

normal distribution, which was evident in the Q-Q plots; the use of skewed data within certain statistical tests is a violation of some tests' assumptions and therefore, the results obtained from the statistical tests seem more significant than they actually are. López-Costas *et al*. (2016) and Krajcarz (2019) used a centered-log ratio transformation on their data, because the chemical composition that they obtained in their studies were part of a closed data set that needed to be open before analysis. A closed data set is one in which the concentrations of an element has an influence on the concentrations of other elements, because the values that are given do not represent the actual amount present in the bone, but rather a percentage of what is present (i.e., weight percent, ppm, or mg/kg); therefore, if the concentration of one element increases, than the concentrations of other elements will decrease. López-Costas *et al*. (2016) stated that failure to account for this constraint can lead to problems when analyzing data that are missing major trends; they noted how Buikstra *et al*. (1989) did not see any co-variation within the elements making up the bony matrix and therefore, led to them ignoring the major influences of diagenesis. López-Costas *et al*. (2016) emphasized this issue and noted that in some cases a log transformation can still produce the same results as a clr transformation.

Experiment 2: ST Discussion

The ANOVA for the salinity trials highlighted a number of significant relationships between the different submergence locations when looking at elemental concentrations. Firstly, there was no significant difference in the concentrations of Mg, P, Ca, Zn, Sr, Pb, Th, Ta, and W between the bones of the different submergence

environments. Although found in seawater as major, minor, and trace elements (Brewer 1975; Kennish 2001; Millero 2006; Wilson 1975), Ca, P, Mg, Zn, and Sr are the major components of bone (Lambert *et al*. 1991; Price 1989). The inability to differentiate between environments using these elements suggested that when submerged for 18 months, the concentrations of these elements reflected the stable makeup of bone rather than the influence of the surrounding environments. These elements are stable within the bone structure and therefore, less mobile, so a longer period of time is necessary for these elements to exchange with others from the environment. The stage of diagenesis when elements within the lattice structure are substituted with those from the surrounding environment can take up to thousands of years, which is significantly longer than 18 months (Keenan and Engel 2017). Therefore, in order to be able to differentiate submergence environments by analyzing Mg, P, Ca, Zn, or Sr concentrations, a longer submergence interval is necessary. Pb, Th, Ta, and W are not major bone components nor are they commonly found in unpolluted seawater or freshwater (Brewer 1975; Huheey *et al*. 1993; Keenan and Engel 2017; Lambert *et al*. 1991; Millero 2006; Price 1989). Because there are lower concentrations of these elements in the surrounding environments, longer submergence times may be necessary to accumulate significant levels of these elements within bone, potentially resulting in the ability to differentiate between submergence environments.

The elements that were significantly different between groups were S, Fe, Mn, K, Cl, Br, Si, Al, Ni, Ti, and Cr. The bones from a freshwater environment were distinguished from the bones in the other environments (brackish and salt) when

comparing concentrations of S, Fe, Mn, Cl, K, and Br. The differences in concentrations of S most clearly differentiated between a freshwater environment (Holliston Pond) and an environment containing saltwater (Boston Harbor and Woods Hole). S is a major component of seawater (Brewer 1975; Millero 2006; Wilson 1975); therefore, the samples exposed to an environment containing any amounts of seawater are expected to have higher concentrations of S than an environment that contains no seawater. There was no significant difference between the Boston Harbor (brackish) samples and the Woods Hole (saltwater) samples; however, there were lower concentrations of S in the Boston Harbor samples than in the Woods Hole samples. Although the samples exposed to the purest seawater were expected to have significantly higher concentrations than those samples from diluted seawater (brackish waters), the Boston Harbor and Woods Hole samples could not be separated according to S concentrations, because the brackish water from the harbor is more similar to seawater than freshwater. The water from the harbor is composed of more saltwater from the Atlantic Ocean than freshwater from the surrounding rivers, which is evident from the salinity values of each location: < 0.5 PSU for freshwater, 35 PSU for open ocean water, and 27.76 PSU for the Inner Boston Harbor (Kennish 2001; MWRA 2017). This is further supported by the fact that the freshwater input flow into the harbor is approximately 350-500 ft^3/s, while the tidal input flow into the harbor is approximately 320,000 ft^3/s for a 6 hour period with volumes between 10.6 billion and 179.9 billion gallons of water, depending if it is low or high tide (USACE and Massport 2006). Neither the two saltwater samples nor the two brackish water samples could be separated by depth when comparing S concentrations; this is most likely do to

the fact that S is a conservative element within the ocean, meaning that its concentrations do not vary with depth (Kennish 2001; Millero 2006; Nozaki 1997).

The analysis of Fe and Mn differentiated between freshwater and water containing any amount of saltwater, as well as between all three salinity environments (Holliston Pond, Boston Harbor, and Woods Hole). The concentrations of Fe and Mn were the highest in the Holliston Pond samples, followed by the Boston Harbor samples, and then the lowest in the Woods Hole samples. This inverse relationship between the Fe or Mn concentrations and the amount of seawater present in the environment is due to the fact that there are naturally higher levels of these elements in freshwater compared to seawater. For example, there is approximately 0.002 mg/L Fe in seawater and approximately 0.7 mg/L in freshwater rivers (Brewer 1975; WHO 2003b). According to Brewer (1975), the average concentration of Mn in seawater is 0.2 µg/L, but WHO (2011) estimates the average to be 2 µg/L with a range from 0.4 to 10 µg/L; the concentration range of Mn in freshwater is 1-200 µg/L, but can reach up to 1300 µg/L when reducing conditions are present (WHO 2011). The erosion of the shallow Dedham granite bedrock, found within Holliston, introduces high amounts of Fe and Mn into the local freshwater; residents of Holliston have even complained about the smell and color of their drinking water, which is due to the high levels of Fe and Mn in the freshwater that comprises the local aquafers and the aquifer's capture zones (Henn *et al*. 2018). Although higher freshwater concentrations are explained by the erosion of the local bedrock, they can also be amplified by the addition of known local contaminants from the nearby Waste Transfer Station and the Combustion Research Center (Henn *et al*. 2018).

Generally, Mn and Fe in freshwater enter a brackish environment from the rivers and their concentrations are naturally diluted once mixed with seawater; the concentrations further decrease in the waters farthest from the freshwater source, therefore, explaining why the Woods Hole samples had the lowest concentrations of Fe and Mn (Rex and Connor 1997). Neither of the two saltwater samples nor the two brackish water samples could be separated by depth when comparing Fe or Mn concentrations; although neither of these elements are conservative elements within the ocean, the differences in depth between the shallower and deeper samples may not be large enough for there to be a significant difference in these elemental concentrations.

Samples exposed to a freshwater environment were differentiated from those exposed to some sort of saltier water (brackish and seawater) by their Cl, K, and Br concentrations; specifically, the Holliston Pond samples were differentiated from the Woods Hole and Boston Harbor samples. The bones exposed to the Holliston Pond had the lowest concentrations of Cl, K, and Br when compared to the brackish and saltwater environments. This is most likely due to the fact that Cl, K, and Br are major components of seawater with average concentrations around 18800-19870 mg/L, 380-416 mg/L, and 67-68 mg/L respectively; these elements are only small components of freshwater with average concentrations around 8 mg/L, 2.3 mg/L, and 0.02 mg/L (Brewer 1975; Huheey *et al*. 1993).

Because of these averages, it was expected that the saltwater samples would have the highest concentrations of these elements, followed by the brackish and then freshwater samples; however, the XRF results concluded that the Boston Harbor samples

had the highest levels of these elements. The influence of urban pollution is the best explanation for this trend, since the "anthropogenic input of large volumes of wastewater discharges, storm water runoff, and other pollutant components can mask contributions of certain chemical constituents from natural waters" (Kennish 2001, p. 51). Once urban pollutants are added to the harbor water, they are not easily flushed out of the surrounding area, because the water flow within the harbor is the slowest along the shoreline where most contaminants enter (Signell and Butman 1992). These areas of slow currents tend to maintain higher concentrations of the pollutants within the water and the sediments, which act as trace metal reservoirs that can re-release elements back into the overlying water (Kennish 2001; Signell and Butman 1992). Because the Boston Harbor is tidally dominated, the ebb and flow of the daily tides does help flush out pollutants from the harbor into the Massachusetts Bay; however, only about 40% of the harbor water at high tide is washed out into the Massachusetts Bay during low tide and 58% of this released water is brought back into the harbor during the following high tide (Signell and Butman 1992). Wind and density currents within the harbor can expedite the flushing process, but the rate of pollutant dispersion is also dependent upon the pollutant's specific location and time of release (Signell and Butman 1992).

The elevated concentrations of Cl within the harbor samples was specifically due to Cl-containing pollutants that were introduced to the water. Chlorinated pesticides, such as Dichlorodiphenyltrichloroethane (DDT), its byproducts (i.e. DDE), polychlorinated biphenyls (PCBs), heptachlor, alpha-Chlordane, and trans-Nanochlor, have been detected within the harbor water and sediments (Buchholtz ten Brink *et al*. 2002; Rex and Connor

1997; Werme *et al*. 2017); the sources of these pollutants include the Massachusetts Water Resources Authority's sewage services, other treatment plants, and river inputs (Rex and Connor 1997). Fertilizers and road salts also contain Cl and can be added to the harbor via storm water runoff (Hunt *et al*. 2012; WHO 2003a). In regards to K, the elevated concentrations within the bones from the harbor was most likely due to the influence of K-containing salts. These salts are commonly found in commercial deicers and potash fertilizers, which can enter the harbor water system via runoff (Hunt *et al*. 2012).

Br-containing pollutants present within the harbor most likely caused the higher concentrations of Br within the harbor bone samples when compared to the Woods Hole bone samples. For example, a group of chemicals that is becoming more of a concern for the Boston Harbor environment is brominated flame retardants (BFRs); BFRs are chemicals that make materials, such as plastics, textiles, foams, and circuit boards, more fire resistant (Hunt *et al*. 2006). Some BFRs are dissolved within a matrix, allowing for them to more easily leach out of the product and enter the surrounding environment; these types of BFRs include polybrominated diphenyl ethers (PBDEs) and polybrominated biphenyls (PBBs) (Yogui and Sericano 2009). Although the production of PBBs within the U.S. was discontinued in the 1970s, PBDE production has increased over the years (Hunt *et al*. 2006; Yogui and Sericano 2009). About 50% of the global demand for PBDEs came from North America, and more specifically, more than 90% of the production of the PBDE Penta-BDE occurs within the U.S. (Yogui and Sericano 2009). PBDEs are commonly released into the environment from technical

manufacturing plants, polymer manufacturing plants, electronic waste recycling facilities, effluent and sludge from sewage treatment plants, raw leachate from landfills, and the natural breakdown of products containing PDBEs. These chemicals accumulate within sediments, waters, and living organisms. In regards to PBDE flame retardants, the most contaminated areas within the U.S. are the Boston and San Francisco Harbors (Yogui and Sericano 2009). PBDEs have been detected within the soft tissue of Blue mussels (*Mytilus edulis*) from the Boston Harbor and the Massachusetts Bay, as well as in the blubber of Atlantic white-sided dolphins (*Lagenorhynchus acutus*) from the Massachusetts coastline (Yogui and Sericano 2009).

Even though the harbor samples consistently had higher concentrations of Cl, K, and Br than the saltwater samples, because of the presence of numerous pollutants, the differences in those concentrations were not always significant, making it harder to confidently differentiate between the brackish and saltwater samples. When analyzing Br concentrations, the two Woods Hole (10 and 18 m) samples were significantly different from the Boston Harbor (6 m) samples, but not the Boston Harbor (3 m) samples. The Cl concentrations for the Woods Hole (10 m) samples were not significantly different from the Cl concentrations for the Boston Harbor (3 m) samples. When analyzing K concentrations, the Boston Harbor (3 and 6 m) samples could be differentiated from the Woods Hole (18 m) samples, but not the Woods Hole (10 m) samples. The difficulty in separating the shallower harbor samples from the saltwater samples or the shallower saltwater samples from the harbor samples suggest that the Cl, Br, and K concentrations at these depths are too similar to one another and therefore the shallower harbor water

contains less contaminants like normal ocean water and the shallower ocean water contains more contaminates like the harbor water.

It was also more difficult to differentiate between the different depth samples within the Woods Hole environment, as well as the Boston Harbor environment. For example, there was no significant difference in the Cl, Br, or K concentrations between the different depth samples of the same locale; this was most likely because Cl, Br, and K are all conservative elements in seawater and either the sampling depths were too close to one another to see any concentration differences in the brackish samples for these elements or there was no vertical difference in the brackish water, because it is predominantly saltwater (Kennish 2001; Millero 2006; Nozaki 1997).

There was a significant difference between the Si concentrations of the freshwater pond samples and those of the brackish water samples; the Holliston samples had significantly lower concentrations of Si than the Boston Harbor samples (3 and 6 m). The Woods Hole samples that were exposed to more concentrated saltwater had Si levels in between the Holliston pond and Boston Harbor samples; however, there was no significant difference between the saltwater and the freshwater samples nor was there a significant difference between the saltwater and brackish water samples in regards to Si concentrations.

Si is an element commonly found within the Earth's crust, often in the form of silica. The weathering of these minerals via a number of pathways eventually leads to the addition of Si into the oceans, with rivers being the major source of marine silica (Tréguer and De La Rocha 2013). Particulate silica and silicic acid, a form of dissolved

silica, enters rivers as the minerals within rocks, such as quartz, feldspar, and numerous clays, mechanically and chemically breakdown; these forms of silica also re-enter the water as siliceous skeletal and structural remains of diatoms, radiolarians, silicoflagellates, some sponges and choanoflagellates, and numerous plant types decay (Kennish 2001; Tréguer and De La Rocha 2013). Rivers typically have the highest Si concentrations followed by estuaries and then the ocean (Bell 1994; Brewer 1975; Huheey *et al*. 1993). The highly concentrated terrestrial silica is first exported to the rivers and then continuously diluted as it enters the estuary and eventually disperses throughout the coastal and open ocean waters (Bell 1994); the fact that the input rates of Si into the ocean is less than the output rates also explains why oceanic Si concentrations remain lower (Tréguer and De La Rocha 2013). However, the Si concentrations from the freshwater samples of the present study did not reflect that trend, since they had the lowest concentrations when compared to the samples from the other aquatic environments. This was most likely because they were not exposed to a highly turbulent riverine environment, in which a lot of mineral weathering and diatom cycling occurred; the Holliston pond is a slow moving, shallow pond, most likely with lower particulate and dissolved Si concentrations. Since the Si concentrations of the pond samples were so low, they could not be distinguished from the low Si concentrations within the saltwater samples; however, they were significantly different from the higher Si concentrations within the samples from the brackish environment.

The samples from the brackish environment had the highest amount of Si among the different submergence locations, because this specific harbor is the convergence point

of three rivers, all of which continually replenish the estuary's supply of Si. The brackish water bone samples could not be distinguished from the saltwater bone samples, because although three rivers do converge within the Boston Harbor, the majority of the water resembles saltwater as seen by the high salinity levels and prominent tides. The presence of dams, such as those along the Charles and Mystic Rivers, have also been known to decrease the amount of dissolved silicon within estuaries, therefore, making the estuary's Si concentrations more similar to those of the open ocean (Tréguer and De La Rocha 2013).

The variations in Al, Ni, Ti, and Cr concentrations among the bone samples of the different submergence locations were less extreme; therefore, significant differences between the locations and distinct patterns were harder to determine. For example, the Al and Ni concentrations within the bone samples were highest in the harbor samples, followed by the saltwater samples and then the freshwater pond samples. Although there was this trend, the only significant difference in Al concentrations occurred between the freshwater samples and the harbor samples of both depths and the only significant difference in Ni concentrations occurred between the freshwater samples and the deeper harbor samples.

Al and Ni are known contaminants of the Boston Harbor; both metals are still found within the harbor sediments and Ni is one of the elements still measured in the MWRA effluent and outfall discharges (Buchholtz ten Brink *et al*. 2002; Hunt *et al*. 2006; Werme *et al*. 2017). The harbor waters most likely contain the highest amounts of these two metals when compared to the other submergence locations, because of its

proximity to numerous pollution sources. However, because the saltwater submergence location was so close to shore, metal pollution may also have impacted this environment to some degree. Therefore, it was more difficult to differentiate between the bone samples from the polluted harbor and those from the slightly less-polluted saltwater. Al and Ni are also naturally found within freshwater and riverine waters due to soil and mineral leaching (WHO 2005, 2010), which increased the difficulty in differentiating between the bone samples from the freshwater pond and those of the saltwater locale.

Although not significant enough to differentiate between the two depths, the deeper harbor samples had slightly elevated Ni and Al concentrations when compared to the shallower samples. This was most likely due to the fact that these metals follow a nutrient profile, which means they are scavenged by organisms, such as plankton, within the upper water levels while accumulating in the lower water levels as those sinking, organismal remains are broken down and as those sequestered in the lower sediments are re-suspended (Kennish 2001; Twining *et al*. 2012).

Ti and Cr are also known contaminants found throughout the harbor and its sediments (Buchholtz ten Brink *et al*. 2002; Hubbard and Bellmer 1989; Hunt *et al*. 2006; Werme *et al*. 2017). In respect to Ti levels, commercially engineered nanoparticles are common Ti-containing pollutants that enter the aquatic environment via effluent discharges and runoff containing sunscreens, paints, and battery acids (Asztemborska *et al*. 2018). In a laboratory setting, Asztemborska *et al*. (2018) determined that short-term TiO_2 nanoparticle pollution in an aquatic environment resulted in the bioaccumulation of Ti in fish, plants, and sediments within that environment. In the current study, the

brackish harbor samples of both depths were only significantly different from the deeper saltwater samples; the harbor samples contained significantly higher Ti concentrations than the 18m saltwater samples. This could have been due to the fact that the harbor samples were closer to the pollution source near the city and the shallower saltwater samples were closer to the entry point of any surface pollutants, resulting in lower concentrations of Ti in the deeper saltwater samples.

In regards to Cr concentrations, the only significant difference found among the samples of the different submergence locations was between the shallower and deeper brackish water samples. Cr is naturally found within fresh and saltwaters as well as being known as a specific contaminant within the Boston Harbor originating from the MWRA effluent and outfall discharges (Buchholtz ten Brink *et al*. 2002; Huheey *et al*. 1993; Hunt *et al*. 2006; Kimbrough *et al*. 1999; Werme *et al*. 2017). Depending upon the conditions of the surrounding environment (pH, redox potential, sunlight, available reducing and oxidizing agents), Cr-containing ions can be oxidized (Kimbrough *et al*. 1999). This reactions produces an insoluble form of Cr, which cannot dissociate throughout the water column; this form sinks to the bottom and adheres to the sediments (Kennish 2001). Similar to the cycle of other metal contaminants that bind to sediments, this insoluble form can potentially undergo a chemical reaction that transforms the metal back into a form that can be resuspended within the water at the sediment-water interface or within the underlying porewaters (Kalnejais *et al*. 2010). This resuspension near the harbor floor could explain the significantly greater amounts of Cr in the harbor samples from the 6m depth than those from the 3m depth.

Determination of Method's PMSI Potential

In the TT experiment, a linear regression equation was created in order to help predict an unknown bone sample's submergence time from the concentrations of specific elements found within the sample. The variables within this equation were the transformed concentrations of K, Sr, Si, S, Cr, Cl, and Br. This equation needs to be more thoroughly tested by repeating the present study at the same location, but with new sets of bones that potentially contain more samples per time interval. The results from numerous replications of the present study will test the accuracy of the linear regression equation for this specific environment. Therefore, until these trials are performed, this linear regression equation should not be used in the forensic community to determine a PMSI of an unknown bone sample. However, the present study was successful at creating an equation and there is hope for future development of an accurate linear regression equation that could determine an unknown bone's PMSI.

Once this specific equation is deemed accurate after multiple repetitions of the study at the specific location within the Boston Harbor, the equation's accuracy should be tested when using elemental concentrations of bones with known submergence periods from other aquatic environments. The ST experiment of the present study determined that the concentrations of K, Sr, S, Cl, and Br did not significantly differ among samples that were submerged in brackish water and saltwater for an 18 month period. Theoretically, the equation created in the TT experiment may be useful in determining PMSI for bones found in brackish or saltwater environments, because these more constant elements make

up the majority of variables in the equation. A linear regression equation that could calculate an accurate PMSI without being limited by the type of aquatic environment the bone was found in would be extremely useful. For instance, if this theoretical equation existed, a PMSI could still be calculated for bones found in the open ocean that may have originated from a brackish harbor, or for bones from the open ocean that were found washed ashore in a brackish environment.

The ST results unexpectedly identified a number of elements with significantly higher concentrations in the harbor samples than the saltwater or freshwater samples. In these cases, the brackish samples were usually expected to have elemental concentrations within the middle, because brackish water is a dilution of saltwater with freshwater, or conversely, a dilution of freshwater with saltwater. The unexpectedly high concentrations of Cl, Br, K, Al, Ni, Ti, and Cr in the harbor samples were most likely due to addition of pollutants into these waters. In recent years, restrictions on industrial processes and the overall awareness of pollution has resulted in a decline of pollution in the Boston Harbor water and sediments (Hunt *et al*. 2006). The elemental concentrations of the harbor should gradually return back to their expected values; although, it may take a while because pollutants accumulating on top of the seafloor can become resuspended into the water until they are buried by other sediments (Kalnejais *et al*. 2010). Once the concentrations return to normal, a researcher's ability to differentiate bone samples from different aquatic environments based upon their elemental concentrations may be affected, which then may improve a linear regression equation's ability to accurately predict a PMSI for a bone from an unknown aquatic environment.

Ideally, this equation would also be applicable to freshwater samples, however, this may not be necessary if enough contextual or taphonomic information is collected on the sample. The regression equation developed in the present study would not be able to accurately determine a PMSI of freshwater bone sample, because Sr was the only element within the equation that had no significant difference in concentrations among all three submergence locations (freshwater pond, brackish harbor, and saltwater).

Limitations and Future Research

Although the present research project was successful at identifying elements that were affected by diagenetic processes within different aquatic environments and over time, there are a number of limitations to the current study; many of these issues can be fixed in future studies. One limitation was the amount of sample necessary for analysis. In order to guarantee that the X-rays within the XRF did not pass through the entire sample, 5 g of powdered bone was necessary for each sample capsule. As stated earlier, these 5 g of bone were obtained from eight cores that were drilled at midshaft. This resulted in a significant portion of each diaphysis being cut and processed. If this processing methodology was applied to a human femur, a smaller portion of the overall bone would be affected, because of the difference in size of human and pig bones.

Another limitation of the current study, was that the bones did not remain physically intact, because coring and powdering was necessary for ED-XRF analysis. In some forensic laboratories, this degree of sample destruction may not be allowed; however the coring of bone is a widely accepted practice in the fields of archaeology and

biological anthropology. Bone sections are used for DNA analyses, isotope analyses, and even elemental analyses similar to the present one. New methodologies are being developed in order to decrease the amount of destruction this process can have on the remaining bone; some of these techniques were utilized in the present study, such as a drill press set to a slower speed, clamps, and a diamond-coated drill bit (Stein and Sander 2009). Although the ED-XRF used in the current study required the samples to be powdered, the bone samples were not completely destroyed. These samples are physically altered, but their chemical composition remained the same, and the powder could be removed from the XRF capsules if another form of analysis is necessary.

Other instrumentation may be considered if smaller amounts of a sample need to be collected or if less destructive methodologies are required; however, the preparation methods for these other instruments need to be thoroughly scrutinized. For example, when performing chemical analyses on bone, many researchers have physically scraped away the surface layers of bone to get a smoother surface for analysis (i.e., Christensen *et al.* 2012), chemically cleaned the bone to rid it from environmental contamination (i.e., Cáceres-Saez *et al.* 2016), or used chemical analyses that destroyed the bone (i.e., Cáceres-Saez *et al.* 2016; Lambert *et al*. 1991). Drying the bone in ovens at non-physiological temperatures can alter the bones' chemical composition (Keenan and Engel 2017). The present study dried the samples within an oven set to an appropriate physiological temperature (35°C) in order to prevent any adverse chemical alterations. Although this drying method was preferred, it took the bone cores longer to dry than studies that used higher temperatures; the bones needed to remain in the oven long

enough so that when the cores were ground in the mill, the powder would not clump, because it was still damp.

Other researchers (i.e., Trujillo-Mederos *et al.* 2012) have purposefully added reagents to their samples in order to remove the organic components of the bone, which would speed up the preparation process; however, this is not ideal, because these reagents are affecting the chemical composition of the bone even before the sample is initially analyzed. Less extreme protocols sometimes require the rinsing of bone samples with deionized or distilled water; this was done by McElreath (2018) before drying her representative sections of bone. In their study on suspended major elements and trace metals in estuary water samples from the Netherlands, Zwolsman and van Eck (1999) stated that rinsing the dried samples prior to analysis would not only remove precipitated salts that formed when drying, but also a number of elements, such as K, S, Cu, Ni, Zn, P, and Ba. When studying diagenesis, especially early diagenesis, those elements are extremely important to the final analysis as proven in the present study.

Other instruments can only analyze completely digested samples, which were physically and chemically altered by acids; these instruments then permanently destroy the samples during the analysis. For example, AAS is more sensitive method of analyzing chemical composition that requires the samples to be digested into a liquid form and then vaporized within the instrument (Cáceres-Saez *et al.* 2016). Although coring and milling the bones samples in the present study was tedious, the slightly more sensitive and accurate results acquired by another instrument did not justify the complete loss of the samples, especially in a preliminary study like the current one.

The detection limits for the ED-XRF was another limitation of the present study. Because the samples were loose powders that were run under He purge, there was more opportunity for ambient air to interact with the X-rays, leading to variations in the measured concentrations of elements (Smith 2007). The powdered samples could be pressed into a pellet, which would reduce pore space and necessitate running the XRF under vacuum. Analysis of a pellet within a vacuum would produce more accurate results for the lighter elements than analysis of a loose powder in He. The present study analyzed loose powder in He, because Smith (2007) determined that use of powdered samples significantly decreased the preparation time and still provided adequate results. Future research on bone elemental composition should compare these two preparation techniques, because slightly more accurate and less variable data would be beneficial, especially when statistical tests are later applied to the data.

Because such small changes in elemental concentrations occur during early diagenesis, inductively coupled plasma mass spectrometry (ICP-MS) should be used in future studies on early diagenesis and PMSI. ICP-MS is an instrument common in analytical or geological laboratories. These instruments are more expensive to use than the XRF spectrometers, however, the benefits of using ICP-MS can potentially justify the cost. This instrument can detect more elements than an XRF spectrometer, which may help identify more significant relationships between certain elemental concentrations and the amount of time submerged in the ocean (Krajcarz 2019). In regards to the physical size of a bone sample, only about 1 g of sample is necessary for analysis (Krajcarz 2019). A small cross-section of bone could be analyzed as a powder or in solid form; if in solid

form, laser ablation can be run from the exterior of the bone to the interior of the bone in order to obtain changes in elemental concentrations across the bone rather than analyzing one complete homogenized sample. This technique with ICP-MS could lead to research that determines the diffusion rate of environmental elements within bone, by detecting the variations in concentrations according to depth over the course of a specific time period; this can lead to another way of determining the PMSI.

The amount of bones analyzed in the present study and its effect on the performed statistical analyses were more limitations. In order to improve the results of the statistical analyses of this data, more samples from each time interval and from each submergence location is necessary. There was a lot of variance among bones for some elements, and this could have been fixed with more samples that would help normalize the data. Other than the fact that this was a preliminary study meant to create a useful methodology of studying variations in elemental concentrations of bones submerged in aquatic environments, there were also time and monetary restraints that required a reasonable limitation on the number of samples tested. Now that a methodology has been developed, the remaining dried bones from each salinity locale that were not selected for use in the present study could be analyzed with the same or different methods in a future study; the results from each of the studies could then be compared. In regards to the TT experiment, future studies could be performed on the remaining proximal and distal metaphyses, which were the remnants from the present study's coring procedure. Although this area of bone is less sturdy than the midshaft, more information about chemical changes due to an

aquatic environment could be obtained by studying the effects of sampling location on the elemental concentrations.

While expanding upon the present research in the future, the effects of submergence location should also be expanded upon. More locations should be analyzed that include fresh, salt, and brackish waters. The data collected from additional sites would help test the accuracy of the trends noted in the present study. Although depth was partially touched upon in the present study, further research on how depth affects elemental concentrations within bone is needed, since many components of saltwater are depth dependent (Nozaki 1997). While expanding to new locations, the elemental composition of the surrounding water and seafloor/sediment should also be analyzed. With this additional data correlations could be made between the elemental concentrations within the bones and those of the surrounding environment at a specific time; this will allow for more accurate explanations of trends in the data. Other properties of the surrounding environment, such as temperature and currents, could also be correlated with changes in bones' elemental concentrations at specific times.

Time is a major limitation of the present study. Diagenesis is the major process affecting the elemental composition of bones submerged in the ocean. Although it commences immediately after death, diagenesis continues for thousands of years at a relatively slow rate. Bone is dense, but it is still porous and permeable to external minerals. However, the diffusion of external elements into the inner layers of cortical bone are limited by the rate in which fluid can travel through the extremely narrow, not easily accessible canals within the bone. For example, Pfretzschner (2004) describes why

diffusion through the Haversian system is extremely slow; Haversian canals only run parallel to the bone axis, the intersecting Volkmann canals are rare, and the mineral wall surrounding secondary osteons prevents the passage of fluid from one osteon to the next. The effective diffusion constants were measured in fresh bone with different stains and determined to be 0.02 mm^2/day; that rate is approximately 500 times slower that the rate of the stains traveling in free water (Pfretzschner 2004). Clearly, time is an extremely important factor in studies about early diagenesis; therefore, future projects should expand the experimental period beyond two years. A longer experimental period would allow for higher concentrations of environmental elements to penetrate further into the bone, potentially resulting in the identification of significant relationships between more environmental elements and amount of time submerged.

Another way of expanding the experimental period would be to analyze bones that were already in the ocean for different periods of time; researchers would then not have to place samples their own samples into the environment and wait for a certain amount of years to pass before they could perform an analysis. Since these procedures are still in development, analysis on human bone from forensic cases or archaeological excavations is impractical. However, there are other bones in the ocean that are nonhuman. A number of marine archaeological shipwreck sites contain human remains. There has also been evidence of nonhuman remains at these sites from those that naturally died there over the years, those that were brought on board for food, and those that invaded; for example, 1058 animal bones were recovered from the wreck of the *San Diego*, which sank in 1600, while 825 animal bones were recovered from the *La Belle*

that sank in 1686 (Bruseth and Turner 2005; Migaud 2011). Historically, not all nonhuman remains are documented or even recovered from these sites. Although there is a newly developing area of research about nonhuman remains on board these ships, more research can come from those submerged remains. Rather than testing the PMSI/diagenesis methodologies on bones that have only been submerged for two years or on important archaeological human remains, they can be tried on these submerged, nonhuman archaeological remains.

CONCLUSIONS

The present study analyzed the effects of environmental contamination on submerged bone samples in a brackish harbor environment over a 20 month period, as well as samples submerged for 18 months in three aquatic environments (freshwater pond, brackish harbor, and saltwater inlet). The results for the time trials provided evidence that some elemental concentrations within the bone were significantly related to the duration of time spent in the water. The ED-XRF analysis determined that K, Fe, Zn, Sr, Si, S, Cr, Mn, Cl, Br, Ta, and W were significantly correlated with the amount of time submerged, according to a Pearson's correlation test. The elements that were not found to be significantly correlated with submergence time included the major components of bone (Mg, P, and Ca), as well as a number of trace metals (Pb, Al, Ti, Ni, Th). The fact that the major components of bone did not change over time while submerged demonstrated the stability of those elements during the early stages of diagenesis. The metals whose concentrations were not correlated with time are only trace elements in saltwater; this suggested that a longer amount of time spent submerged would be necessary to accumulate any significant amounts of these elements.

The ANCOVA analysis of the significant elements identified in the Pearson's test determined that the concentrations for K, Fe, Sr, Si, S, Cl, Br, and Ta were significantly different between the control group and the experimental group; the control samples were never submerged in the water, while the experimental group consisted of samples that were submerged for any period of time ranging from 2-20 months. The concentrations for

Fe, Sr, Si, S, Cl, Br, and Ta were significantly greater in the submerged samples than in the control samples. This is evidence that the more available elements in the surrounding environment diffused into the bone and either deposited within the crevices of the bone or substituted into the structure. Cl, S, Sr, Br, and Si are commonly found within saltwater, while the other elements are known local contaminants. The concentration of K was the only concentration that was greater in the control samples than in the submerged samples. Rather than this demonstrating a decrease in K concentration once submergence occurred, the author suggested that this reflected an decrease in overall mean mass percentage of K relative to the increases in mass percentage of other elements that were incorporated into the bone overtime; McElreath (2018) made similar conclusions about the decrease in K percentages within submerged cow bones.

The covariate, number of months submerged, significantly predicted the transformed concentrations of S and W. The concentration of S was positively influenced by the duration of submergence; this was most likely because S is a major element of seawater that was not only deposited into the bone as salts, but also as newly forming pyrite molecules, which gradually forms during diagenesis (Pfretzschner 2004). The concentration of W was negatively influenced by the duration of submergence, most likely due to the gradual diffusion of W out of the bone into the W-deficient aquatic environment.

The linear regression equation, which was developed from the results of the Pearson's correlation test, included variables for the transformed concentrations of K, Sr, Si, S, Cr, Cl, and Br. These predictors account for a major portion of the variance within

the outcome (R^2 = 80.2%); however, due to the fact that the transformed data was not completely normalized, the statistical results suggest the data trends are more significant than they actually are.

The results for the salinity trials provided evidence that some elemental concentrations within the bone were significantly different among the three submergence locations. The ANOVA analysis determined that there was no significant difference in concentrations of Mg, P, Ca, Zn, Sr, Pb, Th, Ta, and W between the bones from the different submergence locations; the elements making up this non-fluctuating group consisted of the major components of bone and a number of trace metals of seawater. The elements with significantly different concentrations between the submergence locations were S, Fe, Mn, K, Cl, Br, Al, Ni, Ti, and Cr. The freshwater samples were significantly different from the other two environments (brackish and salt) in regards to the concentrations of S, Fe, Mn, K, Cl, and Br. Differentiations between the environments based upon the concentrations of the remaining elements was more difficult, because of the compounding effects of pollution in specific environments.

Although the present study produced significant results, neither the regression equation nor the elemental trends among the different submergence environments are ready to be applied to real forensic data at the moment. Numerous replications of the present study need to occur in order to test the accuracy of the linear regression equation for this specific environment and its usefulness for analyzing bone from other submergence environments.

Theoretically, the present equation may be useful in determining PMSI for bones found in brackish or saltwater environments, because the elements that remained constant within the saltwater and brackish environments (K, Sr, S, Cl, Br) also make up the majority of variables in the linear regression equation. A linear regression equation that could calculate an accurate PMSI without being limited by the type of surrounding aquatic environment would be extremely useful. The conditions of aquatic environments vary greatly throughout the world and bones have the ability to travel between different types of aquatic environments that are adjacent to one another; these factors could complicate the usefulness of linear regression equations in determining PMSI, unless they were deemed irrelevant. Only future studies will be able to tell the accuracy and usefulness of the present equation.

Although terrestrial remains dominate the focus of archaeological and anthropological research on decomposition and the determination of a postmortem intervals, it is still important to study these aspects on human remains in aquatic environments. Homicides (DiBiase 2015; Ebbesmeyer and Haglund 2002), suicides (Byard *et al*. 2001; Kaliszan *et al*. 2013), accidental drownings (CDC 2016), shipwrecks (Broadwater 2012; Bruseth and Turner 2005; Cunningham Dobson and Tolson 2010; Lewis *et al*. 2004, Marchant 2016; Russell *et al*. 2006; Steptoe and Wood 2002; Stirland 2013), airplane crashes (Bureau d'Enquêtes et d'Analyses 2012; Ribéreau-Gayon *et al*. 2017; Stone *et al*. 2011), and burials at sea (EPA 2017; London *et al*. 2006) are all means in which human remains are introduced to the ocean.

Once these remains undergo decomposition, disarticulation, and marine scavenging, the skeletal remains are the only evidence left of an individual (Haglund and Sorg 2002; Sorg et a. 1997); just as fresh and decomposing remains can be discovered and delivered to the medical examiner's office, skeletal remains can just as easily wash ashore or be collected in a fishing net (Pokines and Higgs 2015). Therefore, a way of accurately estimating PMSI for each stage of the remains is necessary for the forensic field. Although numerous studies have utilized barnacles when estimating minimum PMSI (Bytheway and Pustilnik 2013; Dennison *et al*. 2004; Magni *et al*. 2015; Pirtle 2017), a more reliable method is required. Archaeologists and biological anthropologists have studied the changes in elemental concentrations within bones due to diagenesis (Pfretzschner 2004; Trueman 1999). Therefore, researchers from these fields can combine their knowledge with those in the forensic field to create a reliable and versatile way of calculating PMSI.

The present study has proven that some elemental concentrations within submerged bones are related to the amount of time submerged and that some concentrations are affected by the type of environment in which the bones were submerged. The present study needs to be replicated numerous times in order to test the accuracy of the calculated linear equation. Future studies need to include more bone samples, more submergence locations, more data about the condition of the surroundings, and more data on the surrounding environment's elemental concentrations. Future studies could focus on extending the submergence periods, by either placing their own samples or analyzing nonhuman samples that were already found within the aquatic environment.

Nonhuman remains found within dated shipwrecks have historically been undervalued; however, now these nonhuman samples can be put to use by potentially developing a universal linear regression equation to calculate time submerged. Researchers could look into other instrumentation that may be more affective at obtaining accurate elemental concentrations in bone samples.

Overall, the present study demonstrated the need for further research on elemental concentrations of submerged bone in order to determine a methodology of estimating an accurate PMSI. The present study specifically highlighted numerous ways in which future research could expand upon these topics. Experts with various backgrounds, such as anthropologists, geologists, analytical chemists, physiologists, zoologists, and oceanographers, could contribute significant knowledge to this area of study, because so many areas of study are involved in analyzing bone composition and interpreting its relationship with the surrounding environment.

LIST OF JOURNAL ABBREVIATIONS

Adv X-Ray Anal ..... Advances in X-Ray Analysis

Am J Forensic Med Pathol..... The American Journal of Forensic Medicine and Pathology

Am J Psychiatry ..... America Journal of Psychiatry

Am J Sci ..... American Journal of Science

Annu Rev Mar Sci ..... Annual Review of Marine Science

Archaeol Anthropol Sci ..... Archaeological and Anthropological Sciences

Aust Archaeol ..... Australian Archaeology

Biol Bull ..... Biological Bulletin

Can Soc Forensic Sci J ..... Canadian Society of Forensic Science Journal

Chem Ecol ..... Chemical Ecology

Commun Chem ..... Communications Chemistry

Crit Rev Environ Sci Technol ..... Critical Reviews in Environmental Science and Technology

C R Palevol ..... Comptes Rendus Palevol

Deep Sea Res ..... Deep-sea Research

Earth Planet Sci Lett ..... Earth and Planetary Science Letters

Environ Int ..... Environment International

Forensic Sci Int ..... Forensic Science International

Geochim Cosmochim Acta ..... Geochimica et Cosmochimica Acta

Global Biogeochem Cycles ..... Global Biogeochemical Cycles

Int Biodeterior Biodegradation ..... International Biodeterioration & Biodegradation

Integr Comp Biol ..... Integrative and Comparative Biology

Int J Adhes Adhes ..... International Journal of Adhesion and Adhesives

Int J Legal Med ..... International Journal of Legal Medicine

Int J Nautical Archaeol ..... The International Journal of Nautical Archaeology

Int J Osteoarchaeol ..... International Journal of Osteoarchaeology

J Anim Ecol ..... The Journal of Animal Ecology

J Archaeol Sci ..... Journal of Archaeological Science

J Cult Herit ..... Journal of Cultural Heritage

J Exp Biol ..... The Journal of Experimental Biology

J Forensic Sci ..... Journal of Forensic Sciences

J Geophys Res ..... Journal of Geophysical Research

J Hum Evol ..... Journal of Human Evolution

Leg Med (Tokyo) ..... Legal Medicine (Tokyo)

Mar Biol ..... Marine Biology

Mar Chem ..... Marine Chemistry

Mar Ecol Prog Ser ..... Marine Ecology Progress Series

Mar Pollut Bull ..... Marine Pollution Bulletin

Northeast Nat ..... Northeastern Naturalist

Nucl Instrum Methods Phys Res B ..... Nuclear Instruments in Physics Research B

N Z J Mar Freshwater Res ..... New Zealand Journal of Marine and Freshwater Research

Palaeogeogr ..... Palaeogeography

Palaeoclimatol ..... Palaeoclimatology

Palaeoecol ..... Palaeoecology

PLoS One ..... Public Library of Science One

Proc R Soc Lond B ..... Proceedings of the Royal Society of London, Series B

Sci Total Environ ..... Science of the Total Environment

URIWW ..... The University of Rhode Island Watershed Watch

Water Air Soil Pollut ..... Water, Air, and Soil Pollution

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