Macroscopic and microscopic effects of various chemicals on the degreasing of bone

by

Marisca Meyer

Submitted in fulfilment of the requirements for the degree:

M.Sc. (Anatomy)

In the School of Medicine, Faculty of Health Sciences, University of Pretoria, South Africa

Supervisor:

Jolandie Myburgh

jolandie.myburgh@up.ac.za

Co-supervisor:

June Serem

june.serem@up.ac.za

September – 2021

DECLARATION

I, Marisca Meyer, declare that this thesis is my own work. It is being submitted for the degree of Master of Science in Anatomy at the University of Pretoria. It has not been submitted before for any other degree or examination at this or any other University.

Sign 

This__27____ Day of ____September____, 2021

ABSTRACT

Bone degreasing is a vital but challenging procedure which ensures that remains can be safely stored and handled. This practice is used in both anatomy and forensic anthropology, and trichloroethylene is the chemical commonly used as a degreasing agent in South Africa. However, this chemical poses a few difficulties. Not only is it highly carcinogenic and various safety precautions need to be taken when working with it, but specialized machinery and skilled operators are needed to operate them. Both trichloroethylene and the machinery are extremely expensive and most institutions in South Africa are not financially able to make use of this technique. A pilot study was previously done to analyse the degreasing effect of other chemicals (acetic acid, ammonia, bleach, ethanol and peroxide) on bone trying to find a suitable alternative which degreases the fastest, most effectively, with the least amount of destruction of bone, while taking into consideration price and safety. However, this study did not include the effect of these chemicals on the microscopic morphological characteristics of bone.

Therefore, the aim of this study was to investigate the effect of these alternative degreasing methods on the microscopic bone morphology in order to identify the most suitable alternative to trichloroethylene.

Bone slides were made of human metacarpal and phalangeal bones used in the pilot study to analyse microscopic damage caused by the abovementioned chemicals. A scoring system with various criteria i.e., microfractures, flaking of small particles of bone from the bone surface, bone loss in the cortex and medullary borders and overall changes in bone, was used to quantify the amount of damage caused by the chemicals.

In contrast to the pilot study where peroxide visually seemed the best degreaser, when viewed microscopically, peroxide scored the worst during this study and caused extensive microscopic damage to the bone. Ammonia and bleach also caused elaborate microscopic damage at all concentrations. Acetic acid did not cause significant damage with the low concentration but caused substantial damage with medium and high concentrations. Ethanol at low concentrations is regarded the most successful in this study, as it caused minimal microscopic damage to the bone while still being a suitable degreasing agent. However, higher concentrations of this chemical still caused extensive damage.

Therefore, ethanol should be considered as an alternative degreasing method as it has not only shown to be a successful degreaser but caused low levels of microscopic damage while also being considered less expensive and much safer to handle, store and dispose of than trichloroethylene.

However, other factors such as time taken to degrease and other concentrations of these chemicals should still be explored in order to establish an optimum degreasing method. This study only made use of metacarpal and phalangeal bones and thus the effect of these chemicals on other bones of the human body remains unknown and further research should be done accordingly.

Keywords: forensic anthropology, bone, degreasing, trichloroethylene, microscopy, macroscopic damage, microscopic damage, metacarpals, forensic analysis, human remains.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr Jolandie Myburgh and Dr June Serem for their support, time and patience throughout this project.

To the body donors and their families, thank you for giving the most generous and selfless gift that allowed me to conduct research and further my knowledge.

I would also like to thank the staff from the Department of Anatomy, Dr Helena Taute and Mr Marius Loots, for their support and help with the microscopic work.

A special thanks goes out to my beloved family and friends who have supported me throughout my studies and motivated me each day. Thank you for giving me the ability to never give up and to achieve anything I set my mind to.

Lastly, I would just like to thank the Department of Anatomy at the University of Pretoria for the use of the student bone collection and for the opportunity to further my knowledge.

Table of Content

List of Tables ..... **IV**

List of Figures ..... **IV**

CHAPTER 1 : INTRODUCTION **1**

CHAPTER 2 : LITERATURE REVIEW **2**

2.1 BONE MORPHOLOGY AND HISTOLOGY **2**

2.2 BACKGROUND ON MACERATION **5**

2.3 MACERATING TECHNIQUES **6**

2.3.1 Hot water maceration 6

2.3.2 Cold water maceration 7

2.3.3 Physical maceration 7

2.3.4 Chemical (detergent) maceration 7

2.3.5. Maceration with insects 8

2.4 DEGREASING TECHNIQUES **8**

2.4.1 Degreasing with ammonia 9

2.4.2 Degreasing with Xyol 9

2.4.3 Degreasing with organic solvents 10

2.4.4 Degreasing with TCE 10

2.4.5 Degreasing with compost 11

2.4.6 Degreasing with hydrogen peroxide 11

2.4.7 Degreasing with water 12

2.4.8 Degreasing with white gas (Coleman fuel) 12

2.5 DEGREASING IN SOUTH AFRICA **13**

2.6 LIGHT MICROSCOPY AND BONE **13**

CHAPTER 3 : MATERIALS AND METHODS **15**

3.1 SKELETAL SAMPLE **15**

3.2 SAMPLING PROCEDURE AND SECTION PREPARATION **15**

3.2.1 Degreasing technique 15

3.2.2 Sampling and preparation of bone slides 16

3.3 MICROSCOPIC ANALYSIS OF BONE SLIDES **18**

3.4 STATISTICAL ANALYSIS **20**

3.4.1 Intra- and Interobserver error (repeatability tests) 20

CHAPTER 4 : RESULTS **21**

4.1 Microfractures **21**

4.2 Flaking **23**
4.3 Bone loss in cortex **24**
4.4 Bone loss in medullary border **26**
4.5 Overall changes in bone **28**
4.6 Chemical breakdown **30**
4.6.1 Acetic acid 32
4.6.2 Ammonia 33
4.6.3 Bleach 34
4.6.4 Ethanol 34
4.6.5 Peroxide 35
4.6.6 Distilled water and TCE 36
4.6.7 Interchemical comparison 36
4.7 Intra-observer and interobserver error **38**
CHAPTER 5 : DISCUSSION **40**
5.1 Microfractures **40**
5.2 Flaking **41**
5.3 Bone loss in cortex **41**
5.4 Bone loss in medullary border **42**
5.5 Overall morphological changes **43**
5.5.1 Acetic acid 43
5.5.2 Ammonia 44
5.5.3 Bleach 45
5.5.4 Ethanol 45
5.5.5 Peroxide 46
5.5.6 Distilled water and TCE 47
5.6 Chemical degreasing vs destruction ability **47**
5. 7 Discrepancies in scores **50**
CHAPTER 6 : CONCLUSION **53**
6.1 Limitations and future recommendations **53**
6.1.1 Difficulty in preparation of slides 53
6.1.2 Repeatability and reliability of proposed method 54
6.1.3 Shortage of samples 54
CHAPTER 7 : REFERENCES **55**
CHAPTER 8 : APPENDICES **60**

APPENDIX 1: Bone images 60
1.1 Acetic acid 60
1.2 Ammonia 64
1.3 Bleach 67
1.4 Ethanol 70
1.5 Hydrogen peroxide 73
1.6 Distilled water (negative control) 76
1.7 TCE (positive control) 77
APPENDIX 2: Scoring sheets 78
APPENDIX 3: Meyer et. al. (unpublished honours report) 82
APPENDIX 4: Turnitin report 96
APPENDIX 5: Ethics approval letters 97

List of Tables

Table 3.1: Microscopic scoring of damage to the bone. 20
Table 4.1: Composite scores for all chemicals. 31
Table 4.1 (continued): Composite scores for all chemicals. 32
Table 4.2: Intra-observer error results. 39
Table 4.3: Interobserver error results. 39
Table 5.1: Discrepancies in the scores of particular chemicals 50

List of Figures

Figure 2.1: Structure of bone[10]. Image obtained from ClinicalKey. 3
Figure 2.2: Microscopic structure of bone[15]. Image obtained from ClinicalKey. 5
Figure 2.3: Example of light microscopy bone slide used in this study (Appendix.1.7 A1) 5
Figure 3.1: Process followed for degreasing of metacarpals/phalanges. 16
Figure 3.2: Process for bone slide preparation. 18
Figure 3.3: Only midsection (M) area was used for scoring as damage could be caused by preparation to superior (S) and inferior (I) edges 19
Figure 4.1: Appearance of microfractures on bones after 1, 2 and 3 months. 22
Figure 4.2: Examples of microfractures on bones. 22
Figure 4.3: Appearance of flaking on bones after 1, 2 and 3 months. 24
Figure 4.4: Examples of flaking on bones. 24
Figure 4.5: Cortical bone loss after 1, 2 and 3 months. 26
Figure 4.6: Examples of bone loss in cortical area of bone. 26
Figure 4.7: Medullary bone loss after 1, 2 and 3 months. 28
Figure 4.8: Examples of bone loss in medullary area of bone. 28
Figure 4.9: Overall morphological changes after 1, 2 and 3 months. 30
Figure 4.10: Examples of overall morphological changes in bone. 30
Figure 4.11: Composite score for acetic acid concentrations. 33
Figure 4.12: Composite score for ammonia concentrations. 33
Figure 4.13: Composite score for bleach concentrations. 34
Figure 4.14: Composite score for ethanol concentrations. 35
Figure 4.15: Composite score for peroxide concentrations. 35
Figure 4.16: Composite score for distilled water and TCE. 36

Figure 4.17: Composite scores for all chemicals after 1 month. 37
Figure 4.18: Composite scores for all chemicals after 2 months. 37
Figure 4.19: Composite scores for all chemicals after 3 months. 38
Figure 5.1: Crystal formation on the distal end of bone caused by acetic acid 30% during month 2 and 3 (Appendix 1.1 H2-I2).....44
Figure 5.2: Peroxide 30% exhibiting a clean, white degreased bone after two months (Appendix 1.5 H2). 47
Figure 5.3: Composite score of degreasing chemicals tested in the pilot study after 1 and 2 months.....48
Figure 5.4: Composite score of degreasing chemicals tested in the current study after 1, 2 and 3 months.....49

CHAPTER 1 : INTRODUCTION

Bone collections are important as they give physical access to both human and non-human skeletons which is vital for training, practice, and research[1]. Variation seen in these collections add to knowledge of estimation of sex, ancestry and stature[1]. Remains in these collections need to be properly processed prior to storage to ensure longevity of the bones as well as preventing a biohazardous environment. Furthermore, the remains need to be processed in such a way as to limit damage which can hide any pre-existing conditions or trauma. Although the bones are processed prior to storage, they continue to decompose over time. Therefore, processing methods should not accelerate this natural breakdown process, but rather preserve the bone by removing all agents (i.e., grease) which can possibly contribute to the breakdown process.

Bone degreasing can be defined as the procedure by which fatty elements inside the bone are removed after maceration[2]. If proper degreasing is not performed, bones will continue to leak a layer of grease and remain yellow in colour. Grease can damage the structure of bones over time, cause disease in the individuals working with the bones and make the bone difficult to handle[2]. The oil must, therefore, be drawn out not only to preserve the bone but to also make analysis and storage more efficient[2]. Numerous chemicals are used worldwide to degrease bone, including trichloroethylene (being the most common and considered as the gold standard), organic solvents, enzymes and aqueous ammonia[3]. Trichloroethylene (TCE) is a colourless, volatile liquid and is not only used as an extraction solvent for greases, oils, fats, waxes, and tars, but also to remove grease from metal parts. It can also be used to make other chemicals for instance hydrofluorocarbon-134a (HFC-134a), which is a refrigerant[2,4]. The main disadvantages of TCE is that it is a highly carcinogenic chemical; the machinery needs to be operated and maintained by specialized individuals; the degreaser machine and TCE are both tremendously expensive and the machinery needs to be housed in specialised facilities[5].

Although various institutions around the world degrease skeletal remains, no international standards exist for the degreasing of bones. Therefore, the need for a technique that is equally as effective as TCE, but presents less health risks and is more affordable, is greatly increasing. There is a need to find an alternative degreasing method and create international standards for degreasing that can be used both in South Africa, and any other country, to simplify the degreasing procedure and make it safer and more cost effective.

CHAPTER 2 : LITERATURE REVIEW

2.1 BONE MORPHOLOGY AND HISTOLOGY

Bone can be defined as a rigid form of specialized connective tissue which assists in support, protection and locomotion of the body and consists of both a cortex and matrix[6]. Bone tissue can be distinguished from the rest of the body tissues by a mineralization process of the matrix[7]. Human bones can be classified into five different groups based on its shape and structure, namely short bones, long bones, flat bones, irregular bones and sesamoid bones[6]. While short bones are almost equal in breadth and length, long bones are lengthier in one dimension than in the other and comprise of distal and proximal ends (also known as epiphyses) and a shaft (also known as the diaphysis). Flat bones are plate-like and thin. Irregular bones do not exhibit any of the features displayed in the categories mentioned above and the shape can be quite intricate. Lastly, sesamoid bones are typically present in a tendon where it passes over a joint which serves to protect the tendon, such as the patella[6]. Bone development takes place in two ways, namely endochondral and intramembranous ossification[7]. Endochondral ossification is the process by which growing cartilage is systematically replaced by bone to form the growing skeleton and is responsible for development of all bones except for the flat bones of the skull, the mandible and the clavicles[8-9]. The three aforementioned bones are formed through intramembranous ossification, which is the process of bone development from fibrous membranes[9].

Bone can either be dense (compact) or spongy (cancellous). Dense/compact bone contains very dense bone layers which is peripherally located while spongy/cancellous bone is centrally located and contains a rich blood supply and the bone marrow (Figure 2.1)[6].

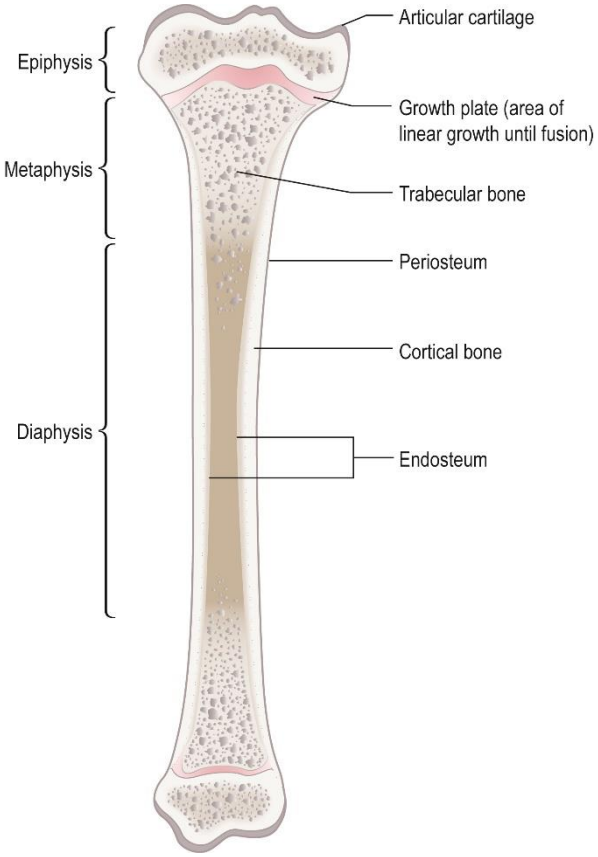


Figure 2.1: Structure of bone[10]. Image obtained from ClinicalKey.

Bone is laminar and the way in which the layers/lamellae are arranged are dependent on the course of blood vessels[11]. There are four laminar systems that can be distinguished namely Haversian systems (osteons), outer and inner circumferential systems and interstitial systems[11]. The Haversian system, or osteon, is known as the basic microscopic unit of bone (Figure 2.2). Osteons are roughly cylindrical structures and consist of concentric layers known as lamellae that surround a central canal called the Haversian canal[12]. The Haversian canal contains the blood as well as nerve supplies. Volkmann canals connect adjacent osteons and also connect the blood vessels of the Haversian canals with the periosteum, the tissue covering the bone's outer surface[12]. Both Haversian and Volkmann canals can be viewed with light microscopy (Figure 2.3). Lacunae are found within the lamellae and are oval hollows containing one or more osteocytes[11]. Many canaliculi radiate from the lacunae in all directions, connecting them to adjacent lacunae as well as to the Haversian canal[11].

The outer and inner circumferential systems are found beneath the periosteum and against the marrow cavity, respectively[11]. Each of these systems is composed of a number of lamellae. The interstitial systems are found in sites between the Haversian systems. Bone is not

a static structure and Haversian systems are continuously being removed and replaced with new ones and during this process, a small section of Haversian system remains and becomes the interstitial system[11].

Bone is an extensively complex form of tissue and consists of an osteoid and various cells such as osteoclasts, osteoblasts, osteoprogenitor cells and osteocytes. Osteoclasts are large and multi-nucleated and serve to resorb dentine, calcified cartilage and mineralized bone[13]. Osteoblasts are cuboidal, single-nucleated cells and are responsible for laying down bone matrix as well as the successive calcification of that matrix[7]. Osteoblasts can be found near the surface of developing bone tissue[8]. The osteoid (initial unmineralized bone) deposited by osteoblasts are deposited on prevailing mineralized surfaces and mineralization can only occur if the phosphate and calcium supplies in the extracellular fluid are adequate[6].

Osteoprogenitor cells are spindle-like in shape and are labeled ‘resting’ cells. They derive from mesenchymal tissue and line the marrow cavities, the innermost layer of the periosteum, the Volkmann’s canals and the Haversian canals[7]. These cells have the ability to change into either osteoblasts or osteoclasts[7,9]. Osteocytes derive from osteoblasts and are abundantly found in mature bone[14]. Osteocytes are located within the lacunae and have cytoplasmic processes that extend towards other osteocytes through canaliculi, which aid in the exchange of nutrients and waste products that maintain the viability of the osteocyte[14]. These cells are capable of both bone deposition and resorption and is involved in bone remodelling[9,14]. Osteocytes transmit signals to other osteocytes in response to slight deformations of bone caused by muscular activity[7]. By doing this, bone can either become stronger when additional stress is placed on it or weaker when relieved from stress[14].

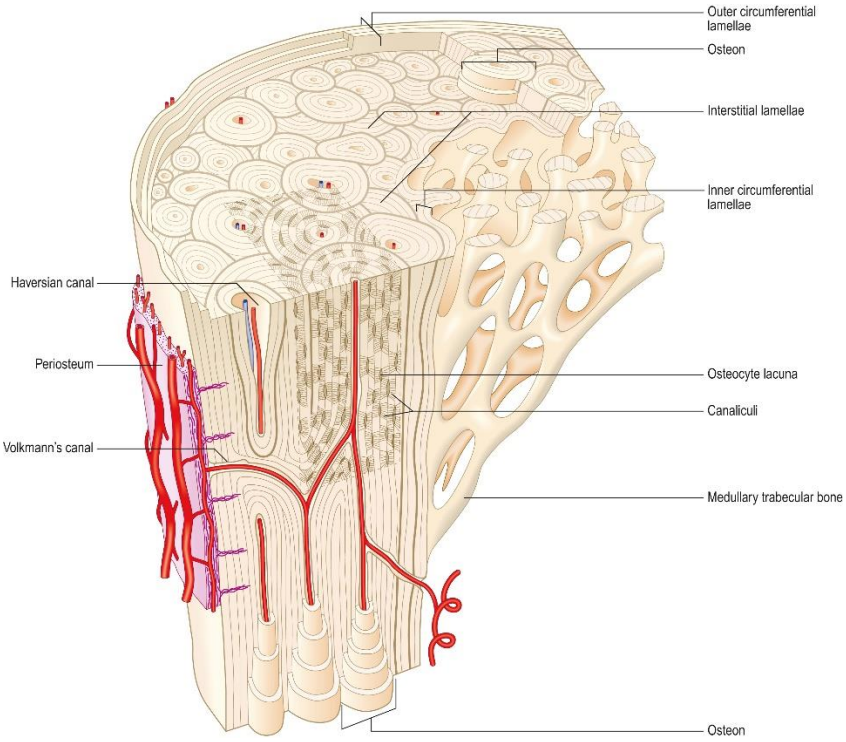


Figure 2.2: Microscopic structure of bone[15]. Image obtained from ClinicalKey.

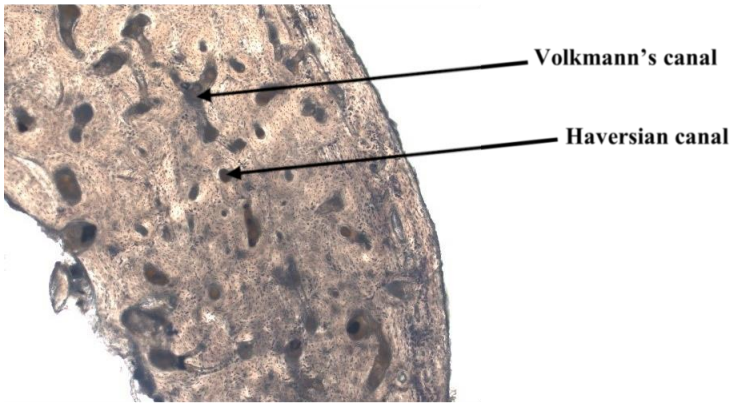


Figure 2.3: Example of light microscopy bone slide used in this study (Appendix.1.7 A1).

2.2 BACKGROUND ON MACERATION

Maceration can be defined as the removal of all soft tissue from bone in order to allow further examination[16]. Proper cleaning and processing of bone is performed in laboratories, either at universities, museums or morgues, across the world.

Maceration, as a preparation technique of bone, is generally practiced in fields such as zoology, anatomy, museum conservation, taxidermy and forensic anthropology. Although reasons may differ for applying maceration, the main goal remains the same for each of these

fields - skeletonized remains are essential for teaching, research and display purposes and need to be processed in order to allow for safe handling and storage[17].

Bone collections are essential for both teaching and research purposes in anatomy and medical sciences[18]. Disarticulated skeletons are used by zoologists to compare with fragmentary or incomplete remains recovered from archaeological sites to estimate identification of the unknown remains[19]. Museum curators use articulated as well as disarticulated skeletons for displays and collections in museums[19]. Forensic anthropologists require clean, skeletonised remains for assessment and evidential reasons in medico-legal cases[20]. These skeletonised remains need to be seen clearly to be inspected for possible causes of death due to perimortem trauma or factors of identification without the introduction of taphonomic hindrances during preparation[16]. Bone collections are of utter importance in these disciplines and consequently methods such as maceration and degreasing, which ensure the long-term storage and safety of bone, are of equal importance, as these bone collections are valuable and must be preserved for as long as possible to assist in these various disciplines worldwide.

2.3 MACERATING TECHNIQUES

Several maceration methods are used including hot and cold water maceration, physical maceration, chemical maceration or insects[21]. A combination of these methods is often used to macerate the remains e.g., hot water maceration or detergent maceration is often followed by physical maceration to remove any residing soft tissues. Advantages and disadvantages exist for each maceration method and several aspects such as time period for macerating, skeletal damage and health and safety issues need to be taken into account when selecting a method[21].

2.3.1 Hot water maceration

Boiling is the most commonly used maceration technique[22]. Remains are boiled at a temperature of between 40°C and 100°C for a variable period of time until the bone is clear of all soft tissue[23]. This method includes advantages such as that no harmful chemicals are used, and it is relatively inexpensive. Disadvantages include that prolonged boiling softens the bone which can compromise bone integrity[16] and heating the specimens might damage the bone morphology and compromise the retrieval of deoxyribonucleic acid (DNA)[24]. Hot water maceration would be the preferred maceration technique when remains need to be macerated frequently and on a large scale, eg. at the Forensic Anthropology Research Unit (FARC) at the

University of Pretoria where forensic skeletal analyses are done on a regular basis.

2.3.2 Cold water maceration

During cold water maceration, remains are added to a sealed container filled with water and left to decompose at ambient temperature. Advantages are similar to those of hot water maceration as it is also a relatively inexpensive method, and no harmful chemicals are used. However, disadvantages include that the maceration process will be lengthy and results may only be seen after weeks or months, and the water containers have to be replaced often to prevent excessive bacterial growth[25]. Cold water maceration would be the preferred maceration technique for the general public wanting to macerate the remains of a small animal, not needing complex machinery or rapid results.

2.3.3 Physical maceration

Manual cleaning is attained by using scalpels, tweezers, scissors, brushes, knives or by hand to remove soft tissue adhering to the bone surface. Although this is considered the easiest method, extensive experience is needed to maintain the condition of the bones, being cautious not to create artificial damage such as postmortem scratch or cut marks, or even fractures[21]. Physical maceration is often used secondary to other maceration techniques to complete the macerating process by removing any residing soft tissue. This technique is used at FARC after boiling maceration is completed.

2.3.4 Chemical (detergent) maceration

Any material that assists in removing dirt from substrates such as surfaces, material, or in this case, bone, is defined as a detergent[26]. Detergents are not only used in a domestic environment for dishwashing and laundry, but also for effective maceration[16]. Macerating with detergents can be equated to macerating with enzymes; however it contains biological enzymes instead of synthetic enzymes, which in turn presents with less health and safety risks[27].

Detergents contain deodorants which eradicates foul odours, subsequently offering a safe and effective macerating technique compared to other maceration methods. A temperature of between 40°C and 60°C has shown to be the optimal temperature for active biological enzymes found in detergents[16]. Although the impact of detergents on fragile bones remain unknown, detergent maceration has lower damaging effects on DNA than boiling[16,27]. Using harsh chemicals such as chlorine bleaches and hydrogen peroxide during maceration have been

rejected over the years due to their destructive effect on bones[17]. Chemical/detergent maceration will be the preferred maceration technique when no other technique is possible and reasonable results need to be obtained.

2.3.5. Maceration with insects

Dermestid beetles are beetles that feed on decomposed flesh[25]. The use of dermestid beetles has a relatively long history in American museums and the first uses of these insects have been dated back to 1937[28]. Using dermestid beetles can be advantageous as they macerate the bones quite rapidly without damaging the bone and no fleshy waste products are formed that needs to be disposed of, as is the case with other macerating techniques[25]. However, a few disadvantages are linked with this technique including the need of a special container to keep the beetles, maintaining the beetle colony between macerations as well as removing shedded exoskeletons or dead beetles from foramina or other openings in the bone[1,25]. Maceration with dermestid beetles would be the preferred maceration technique in a museum setting where the beetle colony can be maintained in a special container.

2.4 DEGREASING TECHNIQUES

After maceration, bones have to be degreased in order to remove any remaining oils. Because bone is porous and bone marrow is fatty, it will remain yellow in colour and leakage of grease from the decomposing fats within the bone marrow will continue over time. If no degreasing takes place, an oily layer will form on the surface of the bone causing it to become tacky to touch[2]. This oily layer makes it challenging to work with the bone and can harm bone structure over time. Also, the decomposing grease may possibly form a biohazard due to bacterial and fungal colonization. To avoid this, the oil must be removed from the bone marrow cavity. Some maceration processes may act as a degreaser, but is not adequate to completely degrease the bone[2]. For example, the boiling process will not always be able to eliminate all the fats before the bone surfaces starts to degrade. Consequently, other degreasing methods have to be considered[29]. The maceration method used will influence the amount of grease still present on the bones after maceration is completed. Chemicals such as ammonia, TCE and bleach are generally used for degreasing in practice[2]. Some chemicals can, however, be detrimental to the bone or to the wellbeing of the macerator and great caution should be taken when using these chemicals.

2.4.1 Degreasing with ammonia

Boiling alone is not always sufficient to remove the fats before degradation of bone surfaces starts and therefore facilities such as the University of Indianapolis Archaeology & Forensics Laboratory soak the bones in different solutions of degreasing chemicals for a limited amount of time[29]. Household ammonia is generally used in a dilution of two to three cups per gallon (3.8 litre) of water. The bones are left to soak in a degreasing bath containing the diluted ammonia solution at room temperature for up to a week which enable the little traces of remaining grease on the bone to liquify[29]. The University Museum of Bergen also degreased whale bones with ammonia at a solution of 25%[3]. Ammonia is an aqueous alkali and can break ester molecule groups present in fats into their fatty acid and glycerol components[3]. During the breakdown of the ester group, soluble soaps (sodium or potassium salts) are produced through saponification and the resulting foam can be wiped from the surface[30]. Any extra ammonia off-gases and low-molecular ammonium salts will leave the bone structure through sublimation[30].

Ammonia has been shown to be fairly effective at degreasing bone surfaces, however, it can be dangerous to health if not handled appropriately. Irritation to the nose and throat as well as coughing can be caused by inhalation of low concentrations of ammonia[31]. The odour of ammonia provides an early warning of its presence, yet ammonia can cause olfactory fatigue, possibly reducing awareness of lengthy exposure at low concentrations[32]. Additionally, rapid eye or skin irritation may occur due to exposure to low concentrations of ammonia. Severe injury such as skin burns, permanent eye damage and even blindness can be caused by higher concentrations of ammonia (e.g. before diluted for degreasing)[33].

2.4.2 Degreasing with Xyol

Other degreasing methods that have shown encouraging results include the use of xyol. Xyol is a solution consisting of 60% industrial grade alcohol and 40% xylenes, which can be used when the remains are very fragile[29]. The University of Indianapolis Archaeology & Forensics Laboratory has explored the use of this method for degreasing. Before the degreasing process can commence, the bones need to be air-dried for one or two days, presumably to remove excess water from the bone as xyol does not mix well with water, and then placed into a sealable glass container. The xyol solution is then poured over the bones so that the solution covers the top of the bones completely. The container is then sealed with silicone stopcock grease and placed in a fume hood. Throughout the degreasing process, the xylenes dissolve the fats in the bone while the alcohol enters the bone and removes the remaining water from the

bone. The bones can then be air-dried on paper for approximately two days after soaking[29].

A benefit of this method includes that xyol can be used repeatedly until the solution is completely saturated by fats, however, disadvantages include the extreme toxicity of xyol which needs to be mixed and used in a fume hood only and be disposed of in a manner consistent with local regulations; pouring the toxic substance down a drain will be extensively problematic as it cannot be removed from water during the water recycling process[29]. This means that xyol is not a suitable chemical to use in order to find cheaper, safer alternatives to TCE as the risks and costs of storage, handling and disposal poses the same problems.

2.4.3 Degreasing with organic solvents

Other organic solvents have also demonstrated to be useful in the degreasing process. The University Museum of Bergen investigated the use of paper bandages drenched with organic solvents to degrease oily historic whale skeletons[30]. Curators expected the oils to be soluble in non-polar solvents such as cyclohexane, toluene, xylene and methyl chloride, but these solvents proved ineffective[30]. Instead, polar solvents such as ethanol, isopropyl alcohol and acetone had a greater cleaning effect[30]. This discovery could be explained by the conversion of non-oxidised oils to oxidation degradation products and cross-linked oil films on the bone surfaces, which are polar compounds and thus will dissolve only in polar solvents[30]. This indicates that polar solvents such as ethanol, isopropyl alcohol and acetone could possibly be useful as degreasing agents as they are less costly and easier to handle and dispose of than TCE.

Another type of organic solvent has also been explored by the South Australian Museum, where a glycol ether was used as the degreasing agent[34]. Although the degreasing effects were promising, some difficulties arose that cannot be ignored, such as a loss of bone density and bone mass[34]. While organic compounds are generally safe to use, health issues can occur by accidental ingestion, exposure, or inhalation. Inhaling large amounts of the above-mentioned organic compounds can cause nausea, vomiting, mucous membrane and throat irritation as well as breathing difficulties[35]. Headache, dizziness and confusion can also be caused by prolonged exposure to acetone[36].

2.4.4 Degreasing with TCE

TCE, which is a colourless, volatile liquid that can be used not only for degreasing bones, but also as an extraction solvent for fats, greases, oils, tars and waxes, is considered the

most successful at degreasing[4-5]. The use of TCE has been questioned in recent years due to extensive health risks, even though it is remarkably effective in cleaning of bones. Additionally, specific machinery and facilities for the use of TCE is necessary and requires highly trained technicians. Not only is the machinery itself expensive, but its maintenance is also extensive and only certain specialised individuals can repair it. TCE itself is also considered to be a dangerous compound as it is extremely carcinogenic[5,37].

Numerous undesirable health effects such as dizziness, slowed reaction time, eye irritation, drowsiness and headache can be caused by exposure to the vapour[5,37]. Some universities who are privileged enough to own the necessary equipment and facilities to handle and dispose of TCE, such as the University of Pretoria, still use this as the main method of degreasing, as no other successful degreasing technique equal to that of TCE has been established yet.

2.4.5 Degreasing with compost

Compost has been used by the Smithsonian Institution for both the cleaning and degreasing of whale skeletons, in an attempt to find a substitute for boiling maceration[38]. According to the results, the method was effective and rapid in most cases. When a skull was brought back to the Smithsonian lab for cleaning and integration into the skeletal collection, it was surrounded by a mixture of elephant manure and hay. As a result, the specimen was cleaned and well degreased in less than three weeks[38]. Various other specimens were consequently cleaned and degreased using compost[38].

Compost degreasing is an admirable illustration of recycling, as no extra resources need to be bought, which lowers the cost quite reasonably. However, drawbacks include a need for a designated area to keep the compost, foul odour, challenges in collecting the compost and limited knowledge of the long-term effects of bacteria and other chemicals present within the manure on bone.

2.4.6 Degreasing with hydrogen peroxide

Another method the Smithsonian Institution has occasionally made use of involves hydrogen peroxide, to degrease oily specimens[38]. Hydrogen peroxide is highly effective in degreasing specimens and can also be used as a bleaching agent. However, the Institution hesitates to recommend it because of the highly destructive nature of hydrogen peroxide, that can not only cause damage to the structural integrity of the bones, but also be harmful to human

skin[2,38]. However, even though hydrogen peroxide is thought to be destructive and dangerous, contrary to other chemical substances, hydrogen peroxide does not produce gasses or residues, thus decreasing the health risks accompanying inhalation[39]. It is also unclear whether lower concentrations will cause less damage to the bone while still acting as an effective degreasing agent.

2.4.7 Degreasing with water

Boiling maceration is often used to remove soft tissue from bones, and this technique has also been applied to attempt to degrease skeletonised remains. Water degreasing is the simplest of the degreasing methods. The process involves boiling skeletal remains in hot water tanks and has been used by the Smithsonian Institution in the 1980's to 1990's to not only clean, but also degrease whale skeletons[38]. Even though the boiling procedure was effective for both cleaning and degreasing of the specimens, it was not energy or time efficient. Another drawback was the build-up of coagulated lipids (also known as adipocere) on the bone surface, which were often challenging to clean off[38].

Overall, degreasing with water has not been found to be as efficient as other techniques and is consequently not usually considered when deciding on a degreasing method to produce bone that are completely dry and free of oil.

2.4.8 Degreasing with white gas (Coleman fuel)

White gas - also known as Coleman fuel - is a blend of heptane, cyclohexane, octane, pentane and nonane and is exceptionally explosive[2]. White gas may release harmful fumes thus extreme caution needs to be taken during usage and a highly ventilated area, with a fume hood, if possible, is necessary[2]. To avoid combustion, the fuel should also not be exposed to excessive amounts of heat[2]. A thick and strong container, such as a glass or metal, is needed to store the fuel to avoid excessive warping, as most plastics will be warped by the gas[2]. Small holes are drilled in both ends of the bone, which will help draw out the grease. The chosen container is then filled with enough white gas/Coleman fuel to cover the entire bone[2]. Subsequently, the lid is sealed, and the bones are soaked for four days. When a visible grease layer is present on the surface of the gas, the grease is transferred out of the container and disposed of appropriately as hazardous waste[2]. If no grease is present, the bones are left for another three to five days, at the most. The bones should then be removed and allowed to air dry for one day[2].

The cost of Coleman fuel ranges from cheap to expensive, depending on the quality and

purity of the Coleman fuel. However, this process needs to be monitored closely, as white gas can damage the bones and, if overdone, may leave a chalky residue[2]. Furthermore, the risks involved in using this chemical may render it useless in large scale degreasing as temperature controlled and specialized facilities are required during use[2]. The process of drilling holes into bone is also destructive and less than ideal and can be prevented by using an alternative degreasing method.

2.5 DEGREASING IN SOUTH AFRICA

Numerous forensic departments exist all over the country, yet not all of them are equipped to degrease skeletal remains. Except for the University of Pretoria, the University of the Witwatersrand is the only other institution with the ability to degrease bone.

According to Mr Jacob Mekwa, principal technician in the mortuary at the University of the Witwatersrand, the university makes use of TCE in a similar manner as the University of Pretoria. In addition to all the disadvantages of TCE usage already mentioned, Mr Mekwa states that he has noticed that the TCE tends to crack bones as well, which is inconvenient. Mr Mekwa stated that in the 27 years he has worked for the School of Anatomical Sciences, they have never used or even considered using an alternative degreasing method to TCE, but if the opportunity arose and a new degreasing method could be proved to be equally effective, they would not hesitate to appraise it.

Although a wide range of degreasing techniques have been tried and tested by various establishments, no specific method has shown to be the preferred method used worldwide regarding safety, cost efficiency, time and bone preservation.

2.6 LIGHT MICROSCOPY AND BONE

Light microscopy is an important instrument in modern cell biology and has numerous features that make it perfectly suited for the imaging of biology in living cells, e.g. the resolution corresponds to the sizes of subcellular structures, a range of fluorescent probes are available that makes it possible to mark organelles, proteins and other structures for imaging, and the fairly non-perturbing nature of light allows for living cells to be imaged for long periods of time[40]. Light microscopy can also be used to view bone, as bone consists of various subcellular structures that cannot be seen with the naked eye. Various other features that can also only be seen with a microscope include microfractures and breakdown of inner bone morphology.

Light microscopy can assist in detecting microscopic damage that is not evident macroscopically, but which may become greater with passage of time and cause extensive damage. Light microscopy is also used in the field of forensic anthropology for various reasons including age estimation, pathology, bone healing and trauma[41]. It is useful to assess bone morphology with light microscopy as factors such as fractures, pathological marks and indicators and age-related characteristics might be missed with the naked eye. Fractures can be thoroughly investigated with the microscope in order to determine whether it was caused by sharp- or blunt-force trauma, determine the direction of a bullet, or more specifically where areas of tension or compression can be found. Pathological and age-related marks such as weakened bone areas, osteophytes or healed fractures can be investigated in order to make a diagnosis or estimation of age. Light microscopy is also often used in the field of physical anthropology to assess bone taphonomy such as bite or puncture marks from scavengers, trauma or weathering[41-42].

The aim of this study was to establish an alternative degreasing method that is equally as effective as TCE but presents less health risks and is more affordable; as well as to create international standards for degreasing that can be used both in South Africa, and any other country, to simplify the degreasing procedure and make it safer and more cost effective.

These aims were achieved by:

- identifying five different chemicals with the potential to degrease in a pilot study
- comparing the effectiveness of these five different chemicals with regards to degreasing
- correlating effectiveness of chemicals vs. affordability
- correlating effectiveness of chemicals vs. safety
- comparing the destructive potential of chemical on the macroscopic structure of bone reviewed in the pilot study and microscopic structure of bone reviewed in the current study.

CHAPTER 3 : MATERIALS AND METHODS

3.1 SKELETAL SAMPLE

Fifty-one metacarpals and phalanges (three for each chemical concentration and three for both the positive and negative controls) from unknown cadavers used in dissection by the Department of Anatomy, University of Pretoria were used as samples in this study. These bones were selected at random, and no previous knowledge was acquired on whether these bones are left- or right-sided, the age of the individual(s) these bones were taken from or whether these bones were taken from one or more individuals. The metacarpals and phalanges had already been macerated by boiling in water and all soft tissue had been removed. The bones were allowed to dry before being degreased. The bones were degreased using five different degreasing chemicals in a pilot study (Honours project) as seen in Appendix 3 (Ethics reference number 185/2018). Although both the metacarpals and phalanges were degreased during the pilot study, it was decided that only the bones for month 1 and 2 (being the metacarpals) would be included in the honours study. However, in order to acquire a full data set, the bones for month 3 (being the phalanges) were also included in the MSc study. This research falls under the Human Tissue Act 65 of 1983 as well as the National Health Act 61 of 2003, specifying that an institution may use tissues of an individual who has donated his/her body for research purposes. A proposal was submitted, and the project was approved by the Research Ethics Committee of the Faculty of Health Sciences at the University of Pretoria (Ethics reference number 318/2020). As damage could possibly be caused to the bones during the process, only metacarpals and phalanges which are not designated for the Pretoria Bone Collection were used. These bones were accessioned back to the Pretoria Student Bone Collection after the study.

3.2 SAMPLING PROCEDURE AND SECTION PREPARATION

3.2.1 Degreasing technique

Five chemicals were tested as degreasing agents in a pilot study (Honours study, Ethics reference number 185/2018), namely: acetic acid, pure ammonia, bleach, ethanol and peroxide. Each chemical was diluted to three different concentrations, in a volume/volume ratio as follows: acetic acid (3%, 10%, 30%), pure ammonia (10%, 25%, 50%), bleach (10%, 25%, 50%), ethanol (50%, 70%, 100%) and peroxide (3%, 10%, 30%), which are regarded as low, medium and high concentrations for each. These concentrations are exploratory concentrations based on user experience in the laboratory and were calculated grounded on the strength of

each chemical. Bones were left in each solution for one, two and three months, respectively. Controls included: positive control – TCE and a negative control, distilled water. The bones degreased in the pilot study were allowed to dry and stored in clear plastic bags for 18 months until they were used for bone slide preparation as shown in Figure 3.1. This allowed the researchers to observe the possible damaged caused by the degreasing agents over time to determine the effect the chemicals will have on the longevity of the remains.

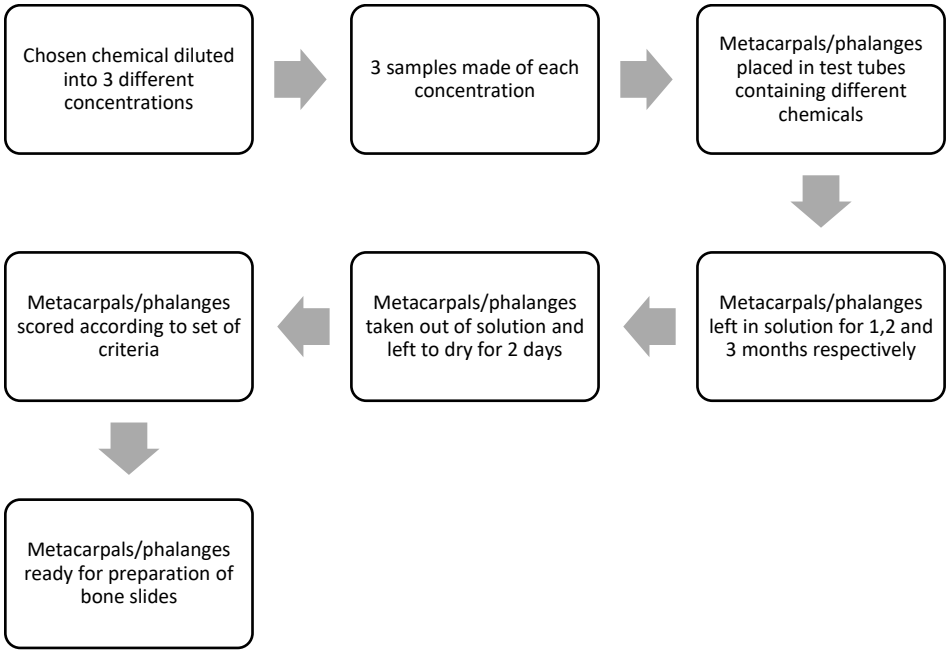


Figure 3.1: Process followed for degreasing of metacarpals/phalanges.

3.2.2 Sampling and preparation of bone slides

The thickest point of the midshaft of each metacarpal and/or phalange was marked with a pencil and used to take bone samples from. The bone was placed on a glass slab and secured with Prestik to reduce any extra movement.

A small slice of bone was removed from the midshaft of each metacarpal/phalange, using a GRIP handsaw fitted with a 1.5 mm bit. The smallest possible sample was taken from each bone in order to limit destruction to bone, which allows the bones to be further used for demonstration purposes.

Bone slides were made using the procedure proposed by Maat et al. 2005[43], to view the microscopic morphology of the metacarpals/phalanges using light microscopy. Half sections of a waterproof abrasive paper sheet (grit no. 220) were cut. Vaseline was used to grease a glass slab and one of the abrasive paper sheet halves was stuck on the slab with the abrasive

side up. The required section of bone was then removed.

Tap water was used to moisten a central area of the abrasive paper. The section was ground by hand with a rotating motion using a “Frost’s gripping device” until both sides were flat and smooth. Care was taken not to let the slice topple and moderate pressure was applied when grinding.

The “Frost’s gripping device” (a section holder), was constructed by folding a slip of abrasive paper across the central part of the one side of a glass microscope slide, with the abrasive side outward. The device was then held by the two free ends of the slip and the section was then placed beneath the device and ground in a circular motion.

The section was ground down on both sides alternately by using light to medium pressure with the “Frost’s device” during the rotating motion. The edges of the abrasive sheet were avoided as it could have been contaminated with Vaseline, which might have caused damage to the section as the edges would have turned up. The bone section was considered ready when it was translucent to the point that it was hardly visible.

In order to prevent the final section from curling up at the sides, it was kept moist at all times. The section was then cleaned by dipping it in distilled water and a small paintbrush was used to turn the section over underwater, ensuring it is cleaned on both sides. Cleaning underwater keeps the section in a state of suspension and avoid any rapid movements that may damage the section. The cleaning process was repeated three times, with refreshed distilled water each time. The cleaned section was then lifted out of the water and placed on a piece of filter paper.

A glass microscope slide (76 mm x 26 mm) was cleaned with 90% alcohol and placed on a glass slab. Each slide was numbered with a pencil according to the documented label of the metacarpal/phalange. A small amount of mounting medium was applied to the centre of the slide. The section was lifted with a pair of tweezers and positioned on top of the mounting medium. To ensure that the section was completely covered, more mounting medium was added immediately.

The section was repositioned with a needle if it accidentally moved off centre. A cover slip was then slowly lowered over the immersed section using a pair of tweezers. The mounting medium automatically spreads itself beneath the cover slip. The slide was left to dry horizontally for 24-48 hours before storing it in a slide box. A summary of the above procedure is explained in Figure 3.2 below.

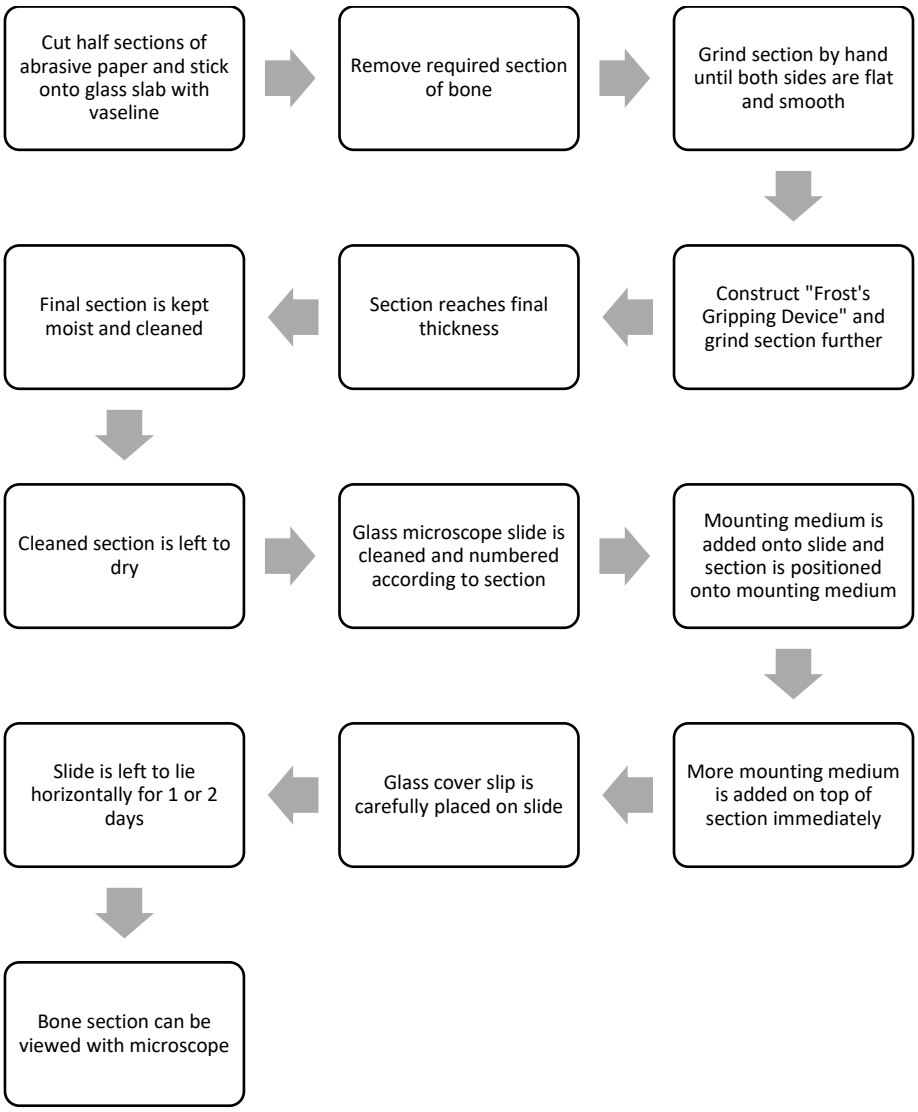


Figure 3.2: Process for bone slide preparation.

3.3 MICROSCOPIC ANALYSIS OF BONE SLIDES

A transmission light microscope (Zeiss Axio Imager M2) with a 5x objective was used to examine the bone slides. The Zeiss microscope was also fitted with a camera and a polarizing plate and digital photographs of each of the bone slides were taken for observations. The program, ZEISS ZEN Imaging Software, was used to take the digital photos. The benefits of using photographs to analyse the bone sections are well described in Ericksen's (1991)[44] article and include that: the field is arbitrarily defined, and the slide can be shifted to observe structures on the periphery without losing the field; the same field can repeatedly be found, and the photos can be kept as a permanent record; structures can be outlined and labelled on the photos and photos can supplement direct vision[44].

In order to record the level of damage, the midsection of the bone slides (Figure 3.3) was analyzed using a scoring method where a specific level of damage was assigned a point value. The midsection of the bone slide was used since the preparation process may have caused additional damage to the free superior and inferior edges of the bone (Figure 3.3). The amount of damage was recorded for all chemicals after one month, two months and three months in order to determine the effect of not only the various concentrations of the chemicals on bone but also the influence of duration of exposure on bone. The features analysed included the amount of microfractures, flaking, loss of cortical bone, loss of bone in medullary border and overall morphological changes (Table 3.1).

Microfractures refer to small fractures found on the bone surface either on the cortical or medullary side of the bone. Flaking refers to small particles of bone flaking off the bone surface on both the medullary and cortical side of the bone. Bone loss in cortex refers to noticeable bone loss on the cortical side of the bone. Bone loss in medullary border refers to noticeable bone loss on the medullary side of the bone. Overall changes in bone refer to the general microscopic morphological changes that can be seen on the bone slides, representing a combination of the four scores mentioned above. A score of zero represents no damage seen on bone, while a score of one represents minimal damage (enough damage to be noticeable, but not enough to cause extensive changes to bone morphology and render the bone unusable) and a score of two represents extensive damage (enough damage to cause extensive changes to bone morphology and render the bone unusable). These are exploratory scores based on user experience in the laboratory and have not been reviewed by other literature.

These scores were recorded on a data sheet (Appendix 2) and the values of the various features were then added to produce a composite or total bone score for each chemical concentration and month after exposure.

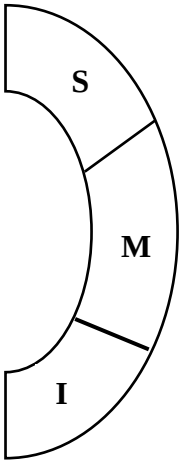


Figure 3.3: Only midsection (M) area was used for scoring as damage could be caused by preparation to superior (S) and inferior (I) edges.

Table 3.1: Microscopic scoring of damage to the bone.

Characteristic/Score	0 = no damage	1 = minimal damage	2 = extensive damage
Microfractures	No microfractures are present	Between 1 and 5 microfractures present	6 or more microfractures are present
Flaking	No flaking present	Minimal flaking present	Extensive flaking present
Bone loss in cortex	No bone loss present	Minimal bone loss present	Extensive bone loss present
Bone loss in medullary border	No bone loss present	Minimal bone loss present	Extensive bone loss present
Overall morphological changes	No noticeable changes to overall morphology	Minimal changes to morphology	Extensive changes to morphology

3.4 STATISTICAL ANALYSIS

Composite scores for each chemical concentration were made by adding scores of microfractures, flaking, bone loss in cortex and medullary border together. The score for overall morphological changes was left out as it is dependent on the above-mentioned scores. Plots for each microscopic characteristic, as well as each chemical separately, were created from these composite scores in order to compare and analyse the data.

3.4.1 Intra- and Interobserver error (repeatability tests)

The purpose of intra- and interobserver error tests is to test the repeatability of the proposed scoring method. Intra-observer error determines whether the observer is able to repeat his/her own results and thus was performed by the primary observer once and again after 33 days (Appendix 2). Interobserver error tests whether the results can be reproduced by anyone trying to make use of the proposed scoring method and was done by two external observers. To test the repeatability, slides were randomly chosen and re-analysed and compared to the original results. Intraclass correlation coefficient (ICC) tests on the statistical program IBM SPSS were used to assess intra- and interobserver error.

CHAPTER 4 : RESULTS

4.1 Microfractures

4.1.1 Month one

As can be seen in Figure 4.1, only four chemicals (acetic acid 3%, bleach 10%, distilled water and TCE) had no microfractures present after one month, while five chemicals (ammonia 25%, bleach 50%, both ethanol 50% and 70%, as well as peroxide 10%) had minimal microfractures present. Eight chemicals (acetic acid 10% and 30%, ammonia 5% and 10%, bleach 25%, ethanol 100% and peroxide 3% and 30%) had extensive microfractures present after one month of being submerged.

4.1.2 Month two

Six chemicals (distilled water, TCE, ammonia 10%, bleach 25% and ethanol 50% and 100%) showed no microfracturing after being submerged in their respective chemicals for two months (Figure 4.1). While only four chemicals (acetic acid 3%, ammonia 25%, bleach 50% and peroxide 10%) had minimal microfractures present, seven out of the 17 chemicals (acetic acid 10% and 30%, ammonia 5%, bleach 10%, ethanol 70% and peroxide 3% and 30%) showed extensive microfracturing on the bones.

4.1.3 Month three

After being submerged in chemicals for three months, five chemicals (acetic acid 3%, distilled water, ethanol 50% and 70%, as well as peroxide 10%) showed no microfracturing on the bone surface (Figure 4.1). Four chemicals (acetic acid 30%, ammonia 5% and 10%, and bleach 10%) showed minimal microfractures while eight chemicals (acetic acid 10%, ammonia 25%, bleach 25% and 50%, ethanol 100%, peroxide 3% and 30% as well as TCE) showed extensive microfracturing on the bone surface.

4.1.4 Overall period

Acetic acid 3% and ethanol 50% showed the least amount of microfracturing during all three months as well as the negative control (distilled water). TCE, serving as a positive control, also caused no microfracturing up until month three, where an extensive number of fractures could be seen on the bone surface. Acetic acid 10% and 30%, ammonia 5%, 10% and 25%, ethanol 100%, as well as peroxide 3% and 30% consistently showed extensive microfracturing

on the bone surface throughout all three months. Examples of morphology of no, minimal and extensive microfracture damage are shown in Figure 4.2.

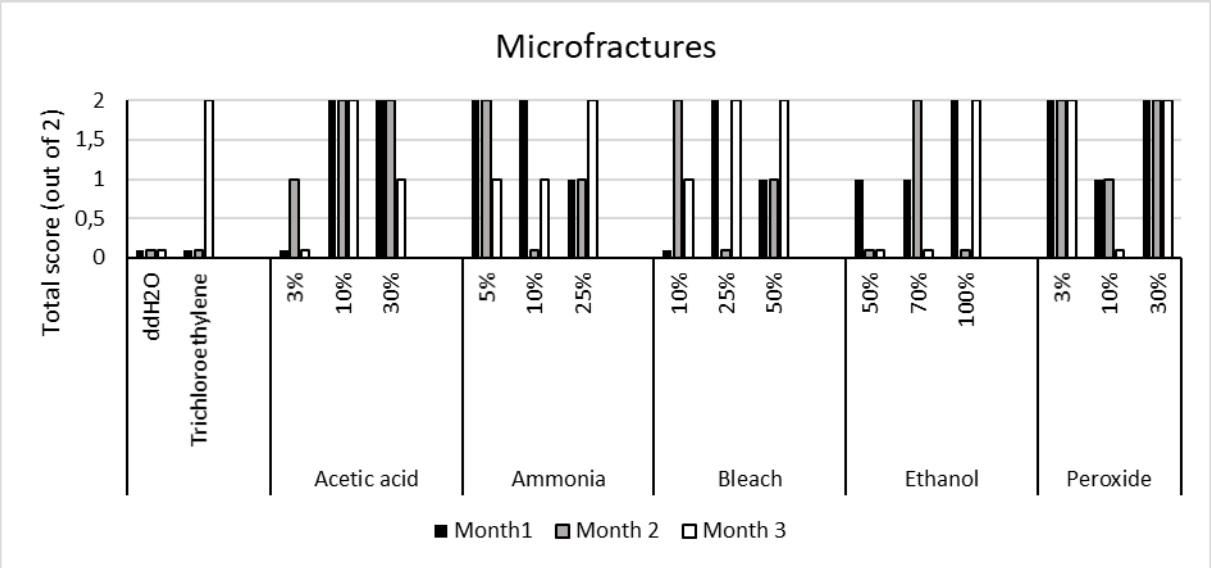
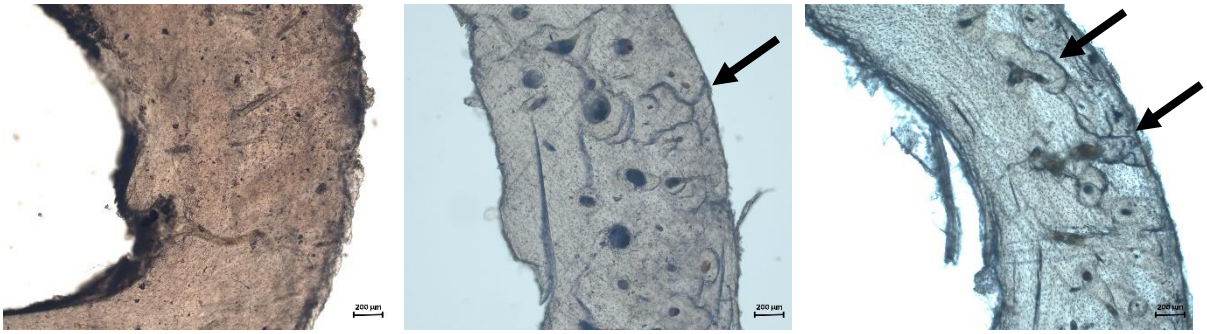


Figure 4.1: Appearance of microfractures on bones after 1, 2 and 3 months.

Negative control (no damage)

Minimal damage

Extensive damage



A: Distilled water Month 3 (Appendix 1.6 C1)

B: Ammonia 10% Month 3 (Appendix 1.2 F1)

C: Ammonia 25% Month 3 (Appendix 1.2 I1)

Figure 4.2: Examples of microfractures on bones (Scale bar: 200µm at 5x magnification). **A**: Light microscopy micrograph of bone slide exposed to distilled water for 3 months exhibiting no microfracturing. **B**: Light microscopy micrograph of bone slide exposed to 10% ammonia for 3 months exhibiting minimal microfracturing. **C**: Light microscopy micrograph of bone slide exposed to 25% ammonia for 3 months exhibiting extensive microfracturing.

4.2 Flaking

4.2.1 Month one

After one month, only three chemicals (bleach 10%, distilled water and TCE) showed no flaking. While 11 chemicals (acetic acid 3% and 30%, ammonia 5% and 25%, bleach 25% and 50%, all three ethanol concentrations as well as peroxide 10% and 30%) exhibited minimal flaking, only three chemicals (acetic acid 10%, ammonia 10% and peroxide 3%) showed extensive flaking (Figure 4.3).

4.2.2 Month two

Most chemicals continued to show signs of flaking after two months, except for three chemicals (bleach 25% and 50% as well as distilled water) which showed no flaking. Three chemicals (acetic acid 10% and 30% as well as ammonia 10%) showed extensive flaking while 11 out of the 17 chemicals (TCE, acetic acid 3%, ammonia 5% and 25%, bleach 10%, all ethanol and all three peroxide concentrations) showed only minimal flaking (Figure 4.3).

4.2.3 Month three

Four out of the 17 chemicals (acetic acid 10%, bleach 25% and ethanol 50% and 70%) showed no flaking after three months (Figure 4.3). Only three chemicals (bleach 10% and 50% as well as peroxide 30%) showed extensive flaking, while the remaining 10 chemicals (distilled water, TCE, acetic acid 3% and 30%, all 3 ammonia concentrations, ethanol 100% and peroxide 3% and 10%) showed only minimal flaking (Figure 4.3).

4.2.4 Overall period

No chemicals except for five (acetic acid 3%, ammonia 5% and 25%, ethanol 100% and peroxide 10%) were consistent throughout all three months in terms of flaking. Acetic acid 10% as well as ammonia 10% caused extensive flaking during the first two months, but no damage during the last month. Acetic acid caused more flaking during the second month than during months one and three. Peroxide 3% caused extensive flaking during the first month, but only minimal flaking during the following two months. Examples of the morphology of no, minimal and extensive flaking damage are shown in Figure 4.4.

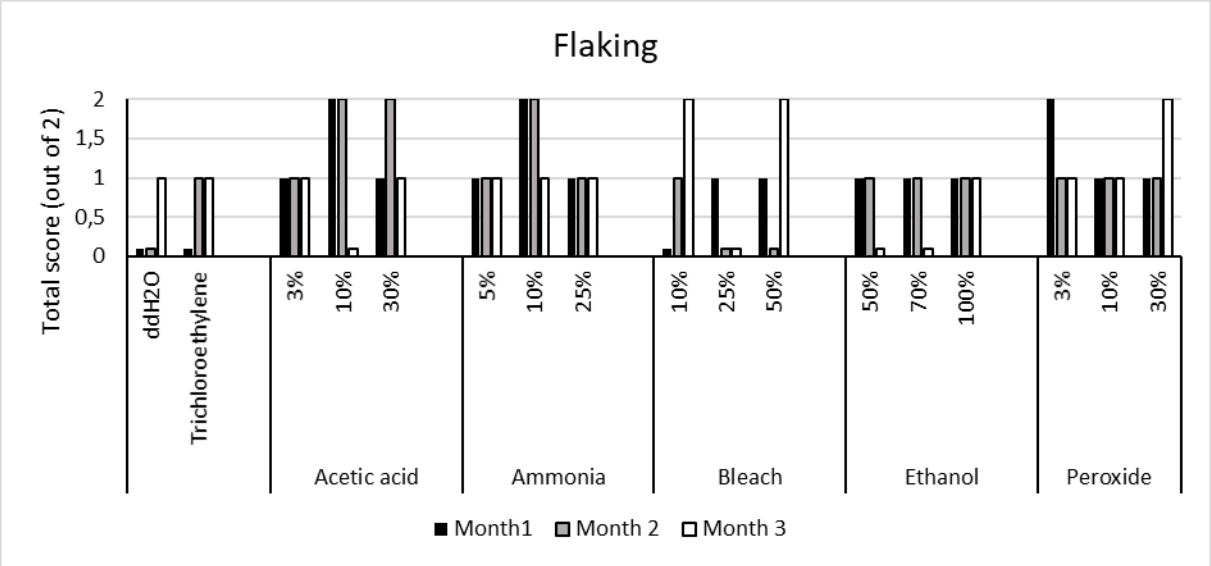


Figure 4.3: Appearance of flaking on bones after 1, 2 and 3 months.

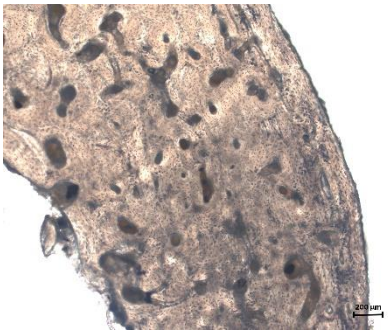
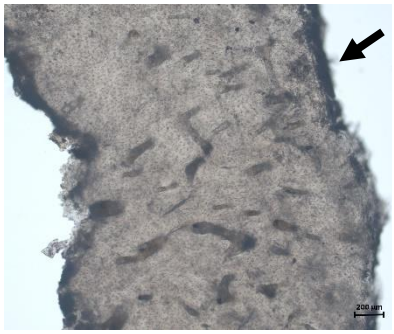
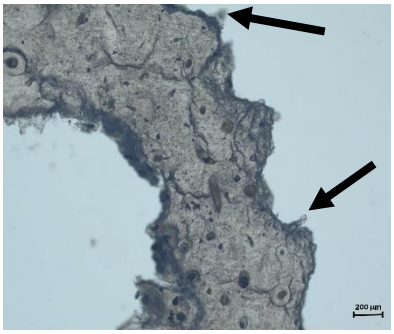
Positive control (no damage)	Minimal damage	Extensive damage
		
A: TCE Month 1 (Appendix 1.7 A1)	**B**: Acetic acid 3% Month 1 (Appendix 1.1 A1)	**C**: Ammonia 10% Month 1 (Appendix 1.2 D1)

Figure 4.4: Examples of flaking on bones (Scale bar: 200µm at 5x magnification). **A**: Light microscopy micrograph of bone slide exposed to TCE for 1 month exhibiting no flaking. **B**: Light microscopy micrograph of bone slide exposed to 3% acetic acid for 1 month exhibiting minimal flaking. **C**: Light microscopy micrograph of bone slide exposed to 10% ammonia for 1 month exhibiting extensive flaking.

4.3 Bone loss in cortex

4.3.1 Month one

Eight out of the 17 chemicals showed no bone loss (distilled water, TCE, acetic acid 10% and 30%, bleach 10% and all three concentrations of ethanol), while eight other chemicals (acetic acid 3%, ammonia 5% and 25%, bleach 25% and 50% and all three peroxide

concentrations) showed only minimal bone loss. Only one chemical, ammonia 10%, showed extensive bone loss during the first month (Figure 4.5).

4.3.2 Month two

More bone loss occurred during month two compared to month one (Figure 4.5), with eight out of the 17 chemicals (acetic acid 10% and 30%, ammonia 5%, bleach 10% and 50%, ethanol 100%, peroxide 30% and TCE) showing minimal bone loss while only three chemicals (ammonia 10% and 25% as well as ethanol 70%) showed extensive bone loss. Six chemicals (distilled water, acetic acid 3%, bleach 25%, ethanol 50% and peroxide 3% and 10%) showed no signs of bone loss in the cortical area during the second month.

4.3.3 Month three

Bone loss occurred in almost all chemicals after three months (Figure 4.5), with minimal bone loss seen in eight chemicals (acetic acid 3%, ammonia 10% and 25%, bleach 10% and 25% and all 3 ethanol concentrations) and extensive bone loss seen in six chemicals (ammonia 5%, bleach 50%, all three peroxide concentrations as well as TCE). Only three out of the 17 chemicals did not display any bone loss, including acetic acid 10% and 30% and distilled water.

4.3.4 Overall period

Ammonia caused the highest amount of bone loss out of all the chemicals. Peroxide caused bone loss mainly in month three, with only little bone loss occurring in the previous two months. While distilled water did not cause any bone loss, the bone loss caused by TCE increased each month. All bleach and ethanol concentrations caused bone loss at some stage, with bleach 50% causing extensive bone loss during month three and ethanol 70% during month two. Acetic acid caused the least bone loss throughout the experiment. Examples of the morphology of no, minimal and extensive cortical bone loss are shown in Figure 4.6.

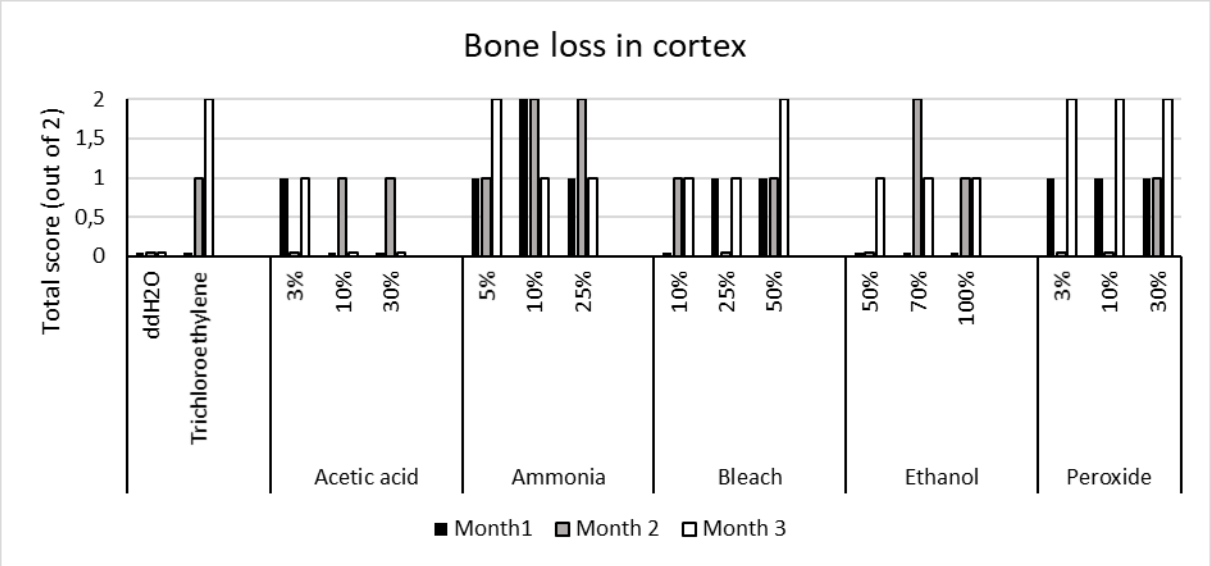
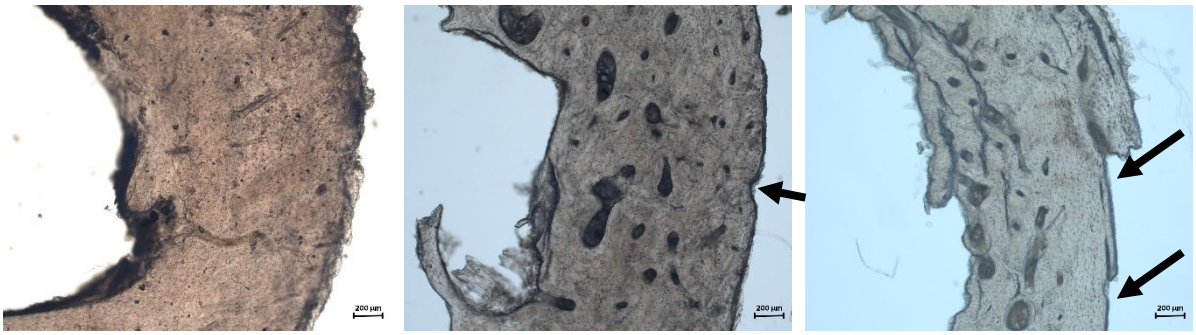


Figure 4.5: Cortical bone loss after 1, 2 and 3 months.

Positive control (no damage) | Minimal damage | Extensive damage



A: Distilled water Month 3 (Appendix 1.6 C1)

B: Ethanol 70% Month 3 (Appendix 1.4 F1)

C: Ammonia 5% Month 3 (Appendix 1.2 C1)

Figure 4.6: Examples of bone loss in cortical area of bone (Scale bar: 200µm at 5x magnification). **A**: Light microscopy micrograph of bone slide exposed to distilled water for 3 months exhibiting no bone loss. **B**: Light microscopy micrograph of bone slide exposed to 70% ethanol for 3 months exhibiting minimal bone loss. **C**: Light microscopy micrograph of bone slide exposed to 5% ammonia for 3 months exhibiting extensive bone loss.

4.4 Bone loss in medullary border

4.4.1 Month one

As seen in Figure 4.7, only two out of the 17 chemicals showed no bone loss (TCE and ethanol 50%). All other chemicals showed bone loss to some extent with 12 chemicals (distilled water, all 3 acetic acid concentrations, ammonia 5% and 25%, bleach 25% and 50%, ethanol

100% and all 3 peroxide concentrations) showing minimal bone loss and three chemicals (ammonia 10%, bleach 10% and ethanol 70%) showing extensive bone loss.

4.4.2 Month two

All 17 chemicals showed bone loss to some extent after two months (Figure 4.7), including both the positive and negative control. Ten out of the 17 chemicals (distilled water, TCE, acetic acid 3%, all three ammonia concentrations, bleach 10%, ethanol 50% and 100% and peroxide 30%) showed only minimal bone loss, while the other seven (acetic acid 10% and 30%, bleach 25% and 50%, ethanol 70% and peroxide 3% and 10%) showed extensive bone loss.

4.4.3 Month three

After three months, all chemicals showed signs of bone loss except for the positive control, TCE which showed no bone loss (Figure 4.7). Ten chemicals (distilled water, all three acetic acid concentrations, ammonia 10% and 25%, bleach 25%, ethanol 50% and 100%, and peroxide 30%) showed minimal bone loss while six chemicals (ammonia 5%, bleach 10% and 50%, ethanol 70% and peroxide 3% and 10%) showed extensive bone loss.

4.4.4 Overall period

Ethanol 70%, peroxide 3% and 10% and bleach 50% caused the most extensive bone loss during the three-month period while distilled water, TCE, acetic acid 3%, ammonia 25%, ethanol 50% and 100% as well as peroxide 30% caused minimal bone loss. Acetic acid 10% and 30% as well as bleach 25% was inconsistent as more bone loss occurred during month two than in month one or three. Ammonia 10% also caused more damage in month one than during month two or three. Examples of the morphology of no, minimal and extensive medullary bone loss are shown in Figure 4.8.

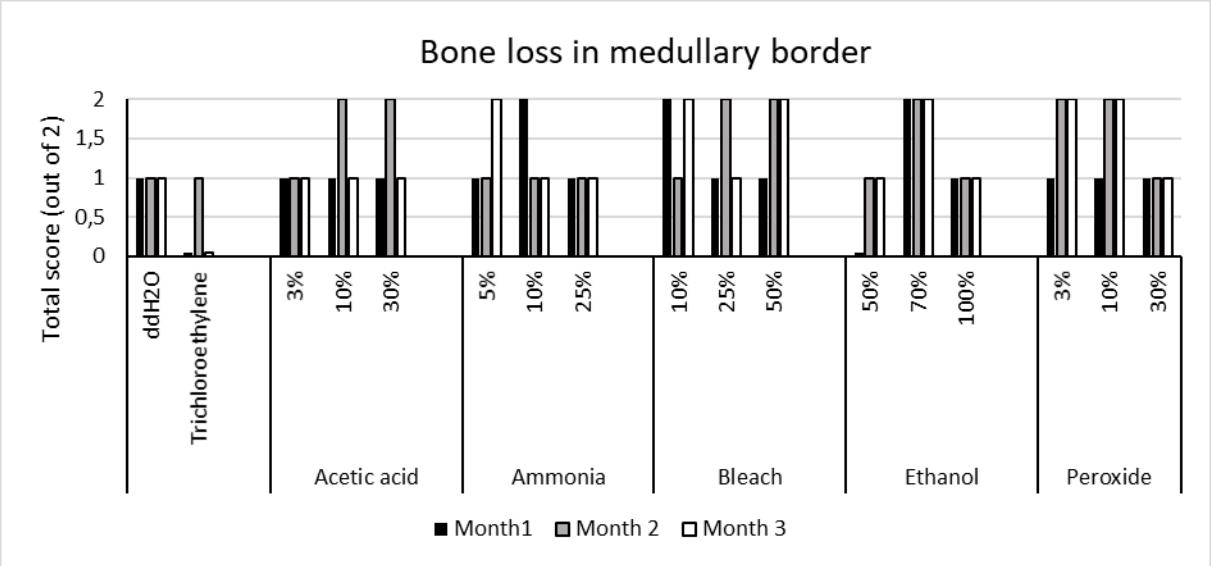
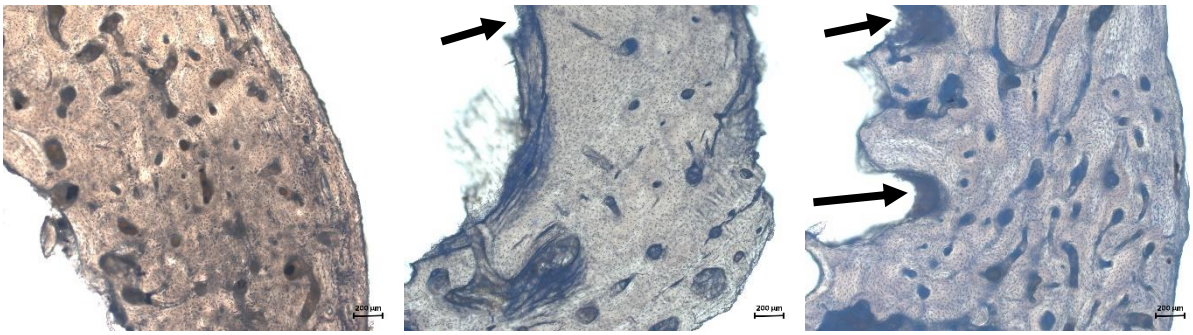


Figure 4.7: Medullary bone loss after 1, 2 and 3 months.

Positive control (no damage) | Minimal damage | Extensive damage



A: TCE Month 1 (Appendix 1.7 A1)

B: Ammonia 25% Month 1 (Appendix 1.2 G1)

C: Bleach 10% Month 1 (Appendix 1.3 A1)

Figure 4.8: Examples of bone loss in medullary area of bone (Scale bar: 200µm at 5x magnification). **A**: Light microscopy micrograph of bone slide exposed to TCE for 1 month exhibiting no bone loss. **B**: Light microscopy micrograph of bone slide exposed to 25% ammonia for 1 month exhibiting minimal bone loss. **C**: Light microscopy micrograph of bone slide exposed to 10% bleach for 1 month exhibiting extensive bone loss.

4.5 Overall changes in bone

4.5.1 Month one

After being submerged in chemicals for one month (Figure 4.9), all except for three chemicals (distilled water, ethanol 50% and TCE) showed signs of morphological changes. Nine out of the 17 chemicals (acetic acid 3% and 30%, ammonia 5% and 25%, bleach 10% and

50%, ethanol 100% and peroxide 10% and 30%) showed minimal changes to morphology while the other five chemicals (acetic acid 10%, ammonia 10%, bleach 25%, ethanol 70% and peroxide 3%) showed extensive changes.

4.5.2 Month two

Only two out of the 17 chemicals (distilled water and TCE) showed no morphological changes. Eight chemicals (acetic acid 3%, all 3 bleach concentrations, ethanol 50% and 100% and peroxide 3% and 30%) showed minimal morphological changes while the other seven (all three ammonia concentrations, acetic acid 10% and 30%, ethanol 70% and peroxide 10%) showed extensive changes to morphology (Figure 4.9).

4.5.3 Month three

After three months, all 17 chemicals caused morphological changes, including the positive and negative controls (Figure 4.9). Nine of the 17 chemicals (distilled water, all three acetic acid concentrations, ammonia 10%, bleach 10% and 25% and ethanol 50% and 100%) caused minimal morphological changes while the other eight (TCE, ammonia 5% and 25%, bleach 50%, ethanol 70% and all three peroxide concentrations) caused extensive morphological changes during this time.

4.5.4 Overall period

All ammonia and peroxide concentrations as well as ethanol 70% and acetic acid 10% has shown to cause extensive morphological changes throughout all three months. Acetic acid 3%, bleach 10% and ethanol 50% and 100% caused minimal morphological changes. Distilled water and TCE caused no changes to morphology expect during month three. Examples of overall no, minimal and extensive morphological changes are shown in figure 4.10 below.

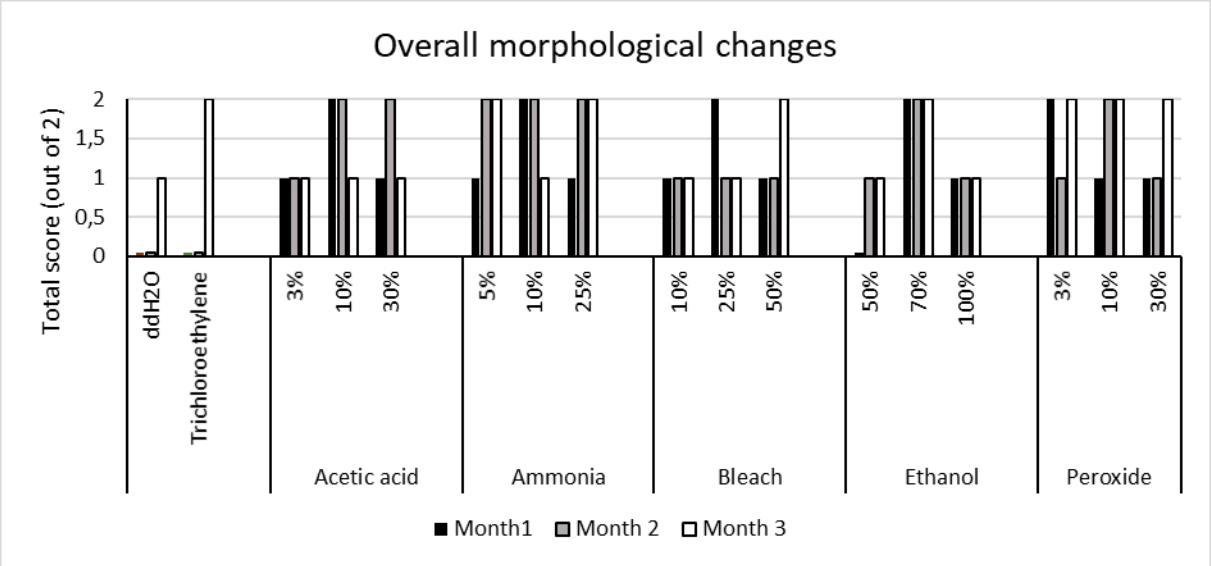


Figure 4.9: Overall morphological changes after 1, 2 and 3 months.

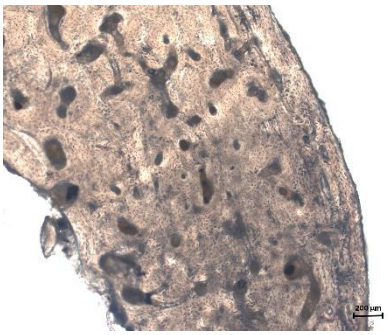
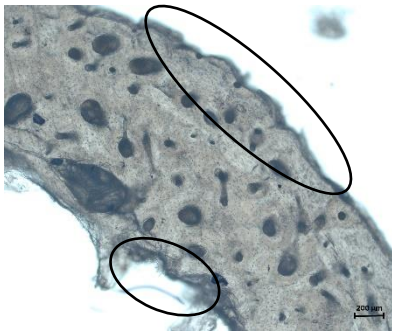
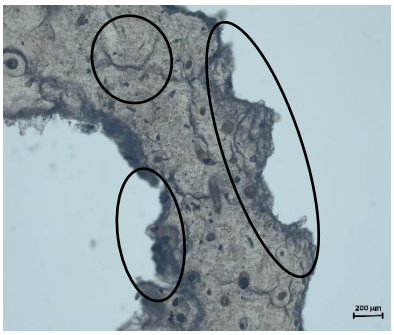
Positive control (no damage)	Minimal damage	Extensive damage
		
A: TCE Month 1 (Appendix 1.7 A1)	**B**: Bleach 50% Month 1 (Appendix 1.3 G1)	**C**: Ammonia 10% Month 1 (Appendix 1.2 D1)

Figure 4.10: Examples of overall morphological changes in bone (Scale bar: 200µm at 5x magnification). **A**: Light microscopy micrograph of bone slide exposed to TCE for 1 month exhibiting no overall microscopic morphological changes. **B**: Light microscopy micrograph of bone slide exposed to 50% bleach for 1 month exhibiting minimal microscopic morphological changes. **C**: Light microscopy micrograph of bone slide exposed to 10% ammonia for 1 months exhibiting extensive microscopic morphological changes.

4.6 Chemical breakdown

The composite or total bone score (Table 4.1) was used to analyse the combined amount of damage to the bones caused by the various concentrations over the 1-, 2- and 3-month periods.

Table 4.1: Composite scores for all chemicals.

Chemical	Microfractures	Flaking	Bone loss in cortex	Bone loss in medullary border	**Composite score**
Acetic acid 3% Month 1	0	1	1	1	**3**
Acetic acid 3% Month 2	1	1	0	1	**3**
Acetic acid 3% Month 3	0	1	1	1	**3**
Acetic acid 10% Month 1	2	2	0	1	**5**
Acetic acid 10% Month 2	2	2	1	2	**7**
Acetic acid 10% Month 3	2	0	0	1	**3**
Acetic acid 30% Month 1	2	1	0	1	**4**
Acetic acid 30% Month 2	2	2	1	2	**7**
Acetic acid 30% Month 3	1	1	0	1	**3**
Ammonia 5% Month 1	2	1	1	1	**5**
Ammonia 5% Month 2	2	1	1	1	**5**
Ammonia 5% Month 3	1	1	2	2	**6**
Ammonia 10% Month 1	2	2	2	2	**8**
Ammonia 10% Month 2	0	2	2	1	**5**
Ammonia 10% Month 3	1	1	1	1	**4**
Ammonia 25% Month 1	1	1	1	1	**4**
Ammonia 25% Month 2	1	1	2	1	**5**
Ammonia 25% Month 3	2	1	1	1	**5**
Bleach 10% Month 1	0	0	0	2	**2**
Bleach 10% Month 2	2	1	1	1	**5**
Bleach 10% Month 3	1	2	1	2	**6**
Bleach 25% Month 1	2	1	1	1	**5**
Bleach 25% Month 2	0	0	0	2	**2**
Bleach 25% Month 3	2	0	1	1	**4**
Bleach 50% Month 1	1	1	1	1	**4**
Bleach 50% Month 2	1	0	1	2	**4**
Bleach 50% Month 3	2	2	2	2	**8**
Ethanol 50% Month 1	1	1	0	0	**2**
Ethanol 50% Month 2	0	1	0	1	**2**
Ethanol 50% Month 3	0	0	1	1	**2**

Table 4.1 (continued): Composite scores for all chemicals.

Chemical	Microfractures	Flaking	Bone loss in cortex	Bone loss in medullary border	**Composite score**
Ethanol 70% Month 1	1	1	0	2	**4**
Ethanol 70% Month 2	2	1	2	2	**7**
Ethanol 70% Month 3	0	0	1	2	**3**
Ethanol 100% Month 1	2	1	0	1	**4**
Ethanol 100% Month 2	0	1	1	1	**3**
Ethanol 100% Month 3	2	1	1	1	**5**
Peroxide 3% Month 1	2	2	1	1	**6**
Peroxide 3% Month 2	2	1	0	2	**5**
Peroxide 3% Month 3	2	1	2	2	**7**
Peroxide 10% Month 1	1	1	1	1	**4**
Peroxide 10% Month 2	1	1	0	2	**4**
Peroxide 10% Month 3	0	1	2	2	**5**
Peroxide 30% Month 1	2	1	1	1	**5**
Peroxide 30% Month 2	2	1	1	1	**5**
Peroxide 30% Month 3	2	2	2	1	**7**
Distilled water Month 1	0	0	0	1	**1**
Distilled water Month 1	0	0	0	1	**1**
Distilled water Month 1	0	1	0	1	**2**
TCE Month 1	0	0	0	0	**0**
TCE Month 2	0	1	1	1	**3**
TCE Month 3	2	1	2	0	**5**

4.6.1 Acetic acid

Although acetic acid 3% caused the least damage to bone throughout all three months with a low composite score of 3 (Table 4.1, Figure 4.11, Appendix 1.1 A-C), it still caused noticeable changes. Both acetic acid 10% and 30% caused moderate damage after one month with composite scores of 5 and 4 (Table 4.1, Figure 4.11, Appendix 1.1 D and G), and the least damage after three months (Appedix 1.1 F and I), with the most damage to bone being caused after two months in these chemical concentrations (Appendix 1.1 E and H), respectively.

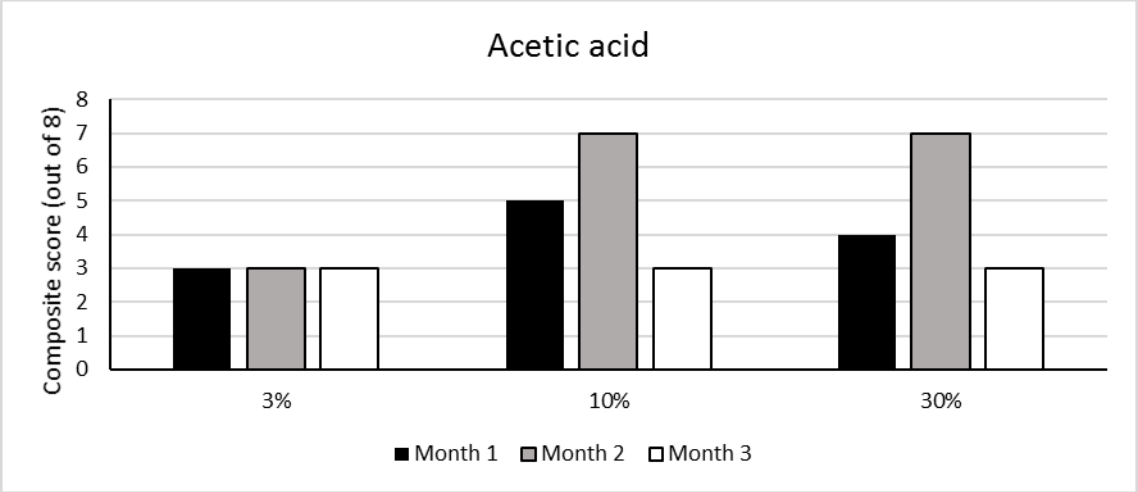


Figure 4.11: Composite score for acetic acid concentrations.

4.6.2 Ammonia

Ammonia caused noticeable damage during all three months and with all concentrations (Figure 4.12 and Table 4.1). Ammonia 5% caused a similar amount of damage during all three months, only slightly increasing in the last month (Appendix 1.2 A-C). Ammonia 10% caused extensive damage during its first month with a composite score of 8, however this damage decreased during the following two months with composite scores of 5 and 4, respectively (Table 4.1, Figure 4.12, Appendix 1.2 D-F). Ammonia 25% caused the least damage (composite score of 4) out of all three concentrations (Appendix 1.2 G-I), with the damage only slightly increasing during the second and third months.

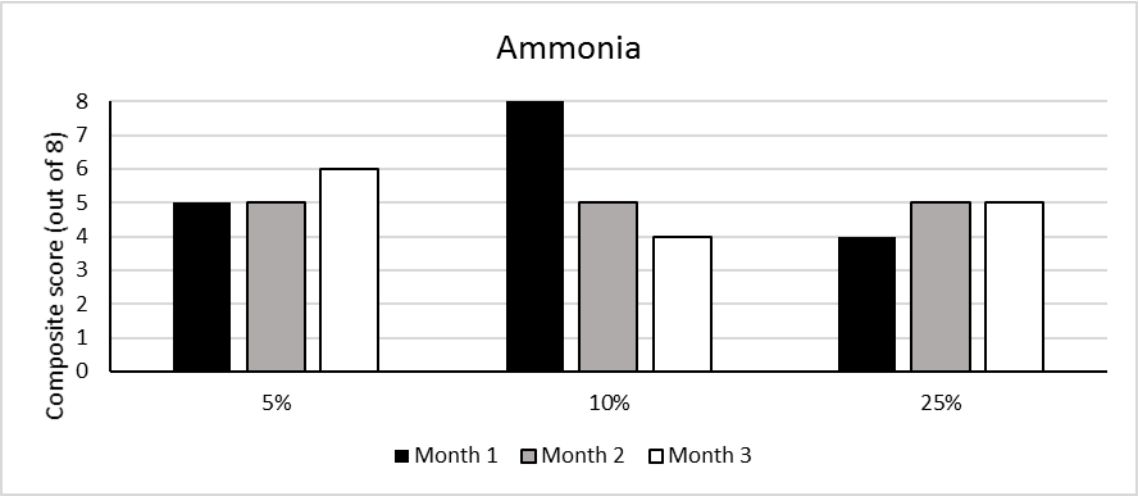


Figure 4.12: Composite score for ammonia concentrations.

4.6.3 Bleach

Bleach 10% caused minimal damage during the first month with a low composite score of 2 (Table 4.1, Figure 4.13, Appendix 1.3 A), with the damage increasing extensively during the following two months with composite scores of 5 and 6 respectively (Table 4.1, Figure 4.13, Appendix 1.3 B-C). Bleach 25% caused noticeable damage during month one and three (Appendix 1.3 D and F), but as little damage as bleach 10% during the second month (Appendix 1.3 E). Bleach 50% caused similar moderate damage during the first two months with composite scores of 4 each (Appendix 1.3 G and H), with damage increasing to the highest point during the last month with 8 as composite score (Table 4.1, Figure 4.13, Appendix 1.3 I).

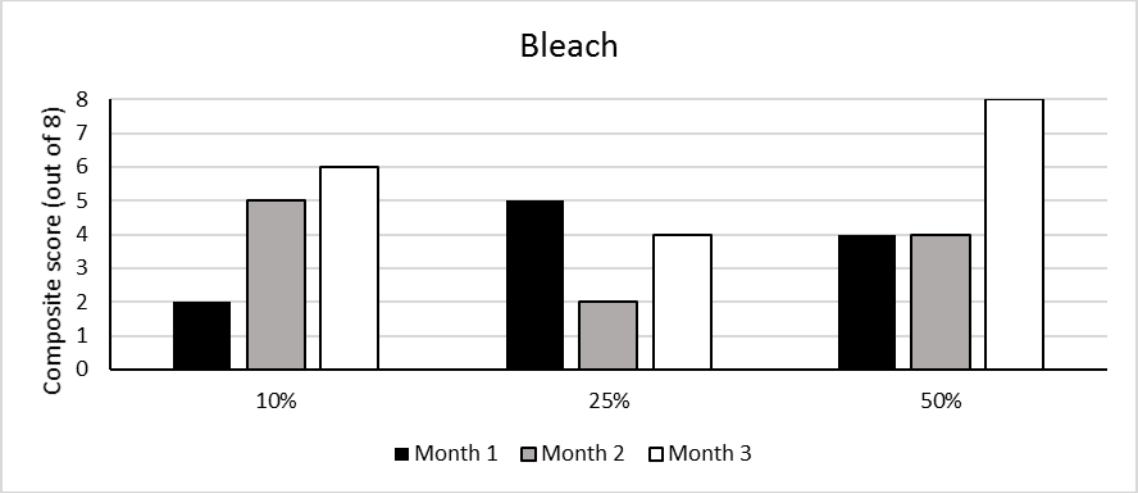


Figure 4.13: Composite score for bleach concentrations.

4.6.4 Ethanol

Ethanol 50% did not cause extreme changes to bone during any month, with a low composite score of 2 for all three months (Table 4.1, Figure 4.14, Appendix 1.4 A-C). Ethanol 70% caused noticeable damage during all three months (Appendix 1.4 D-F), with the most extensive damage being caused during month two with a composite score of 7 (Table 4.1, Figure 4.14). Although ethanol 100% caused noticeable damage to bone (Appendix 1.4 G-I), it was not as great as the damage caused overall by ethanol 70%.

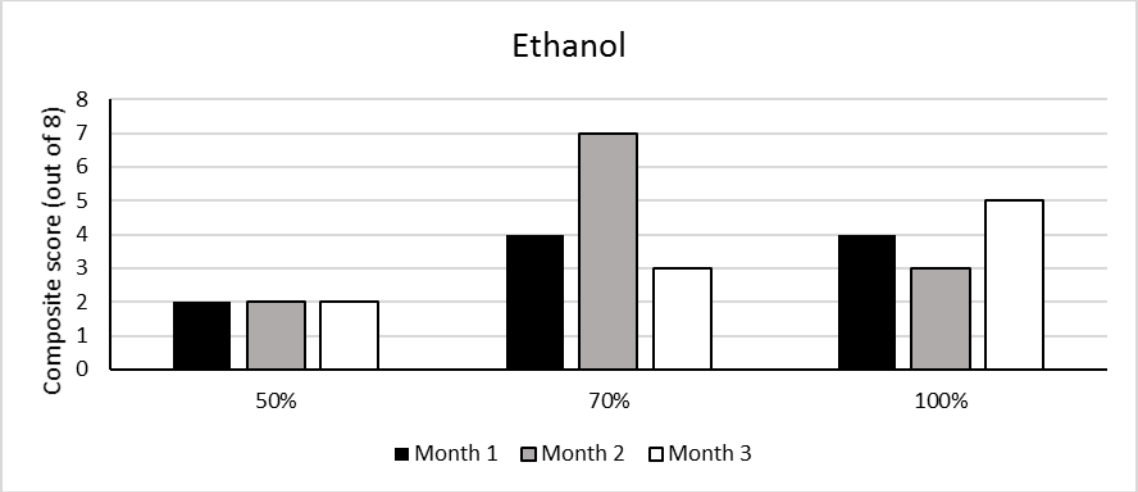


Figure 4.14: Composite score for ethanol concentrations.

4.6.5 Peroxide

Peroxide 3% caused extensive damage during all three months (Appendix 1.5 A-C), with the most damage being caused during the last month with a composite score of 7 (Table 4.1, Figure 4.15). Although peroxide 10% did not cause as extensive damage as peroxide 3%, it still caused noticeable damage during all three months with a composite score ranging between 4 and 5 (Table 4.1, Figure 4.15, Appendix 1.5 D-F). Peroxide 30% caused extensive damage during all three months (Appendix 1.5 G-I), with the most damage being caused in the last month with a composite score of 7, similar to peroxide 3% (Table 4.1, Figure 4.15).

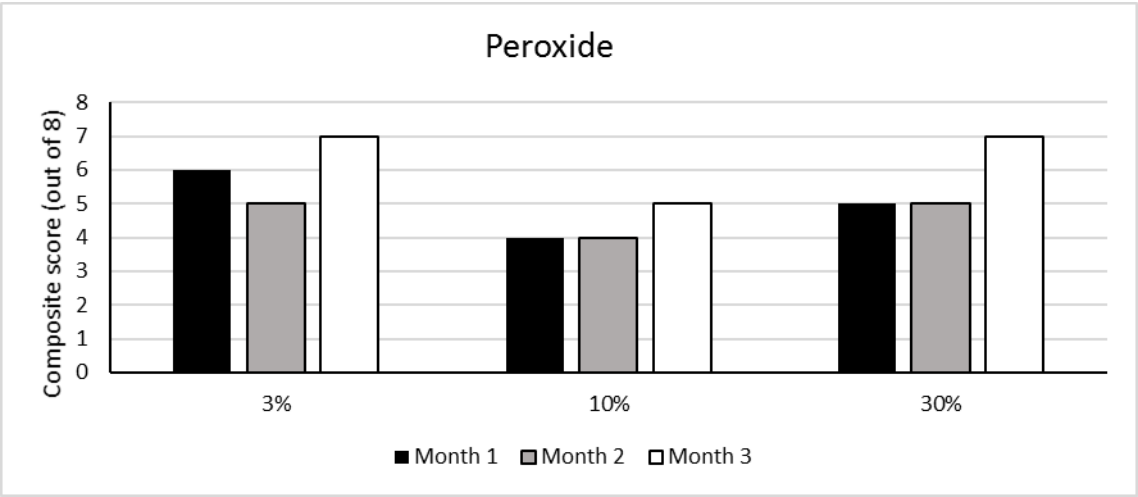


Figure 4.15: Composite score for peroxide concentrations.

4.6.6 Distilled water and TCE

As can be expected from a negative control, distilled water caused little to no changes during all three months (Appendix 1.6 A-C) with the exception of moderate damage during the last month, where the composite score was 2 (Table 4.1, Figure 4.16). TCE served its purpose as positive control by causing little to no changes during month one with a low composite score of 0 (Table 4.1, Figure 4.16, Appendix 1.7 A), however during month two and three noticeable damage occurred and the composite score increased to 3 and 5 respectively (Table 4.1, Figure 4.16, Appendix 1.7 B-C).

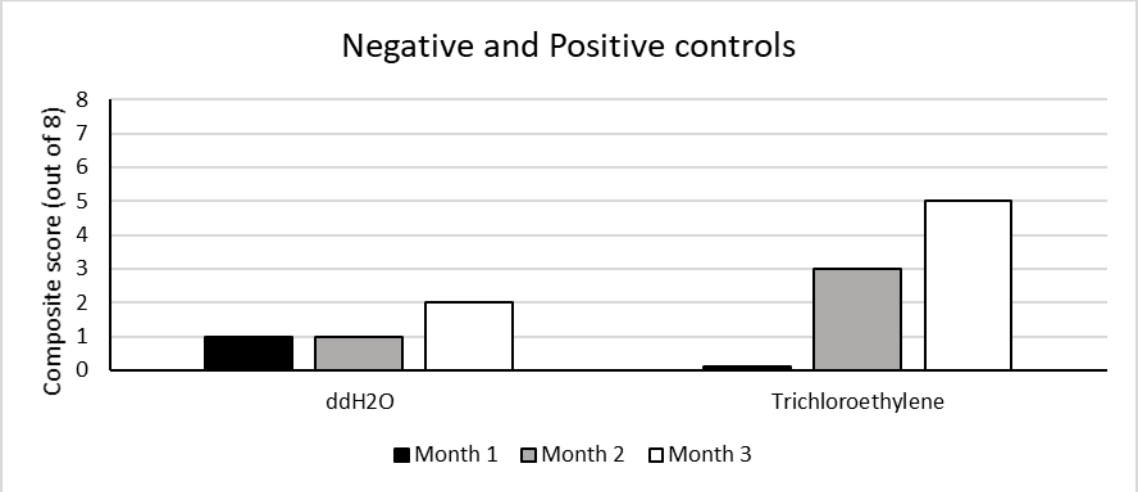


Figure 4.16: Composite score for distilled water and TCE.

4.6.7 Interchemical comparison

The composite scores for month one (Figure 4.17) indicate that both ethanol 50% and bleach 10% scored the lowest (both with composite scores of 2), thus causing the least amount of damage to bone. Ammonia 10% showed to be the least successful chemical with the highest score (composite score of 8) during the first month and caused the most damage to bone.

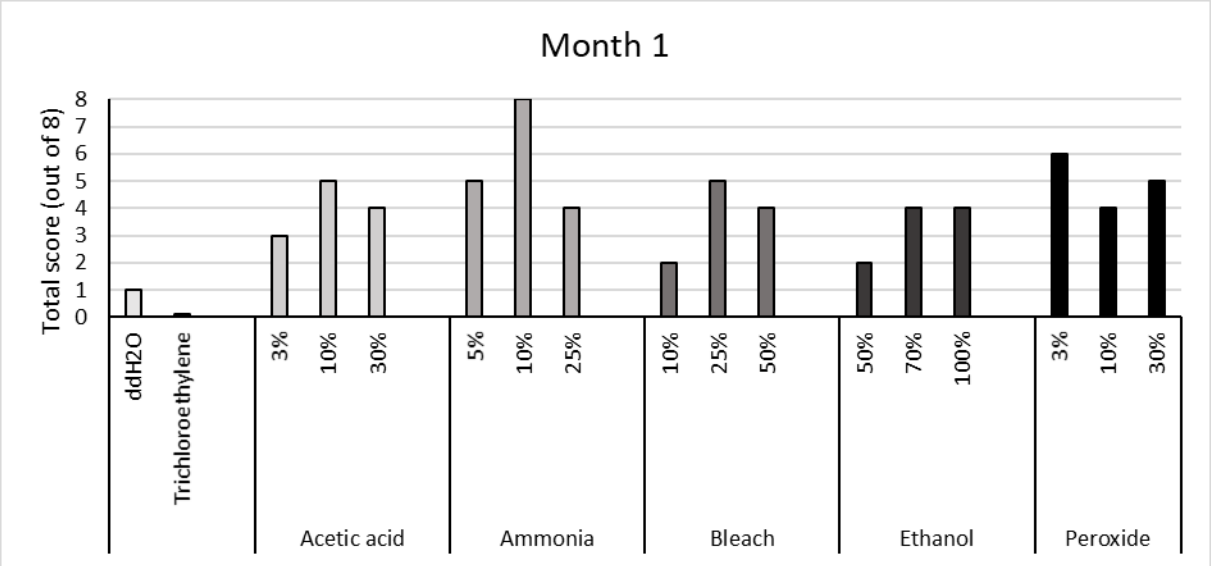


Figure 4.17: Composite scores for all chemicals after 1 month.

Most chemicals showed increased damage after two months (Figure 4.18), however, ethanol 50% still showed to be the lowest scoring chemical during this time (composite score of 2). Acetic acid 10% and 30% caused extensive damage, as well as ethanol 70% (all with composite scores of 7).

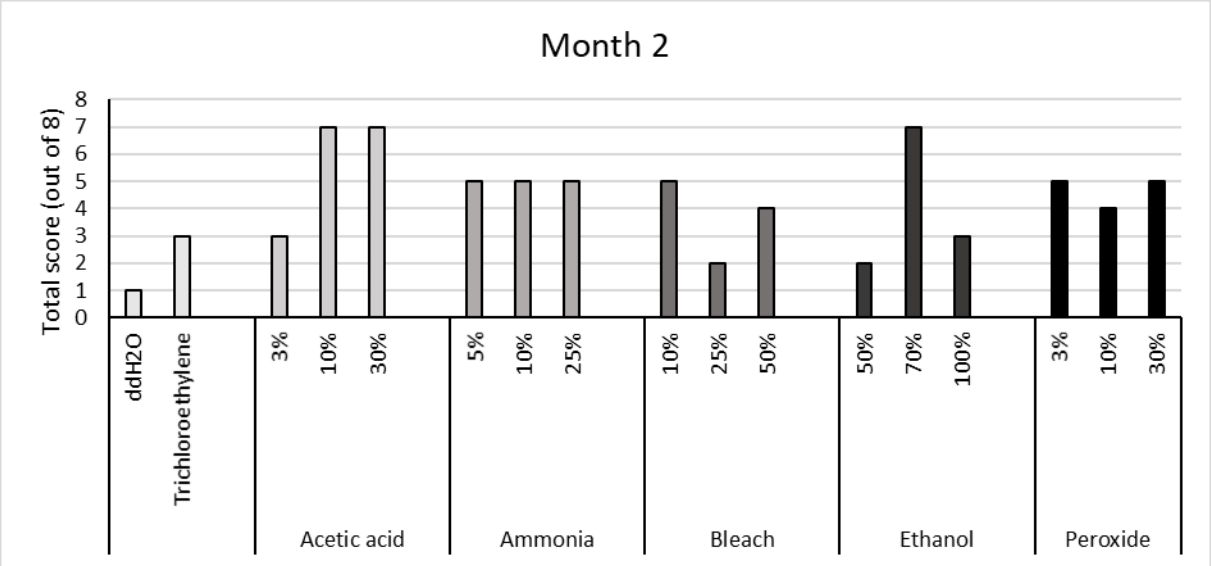


Figure 4.18: Composite scores for all chemicals after 2 months.

Although acetic acid caused the most damage during month two, it seemed to cause the least overall amount of damage after three months (Figure 4.19), making it the second-best overall chemical during this time (composite score of 3 for all concentrations). However,

ethanol 50% still scored the lowest out of all the chemicals (composite score of 2). Bleach and peroxide caused noticeable damage with bleach 50% having a high composite score of 8 and peroxide 3% and 30% both scoring a 7 making these the least successful chemicals during this time.

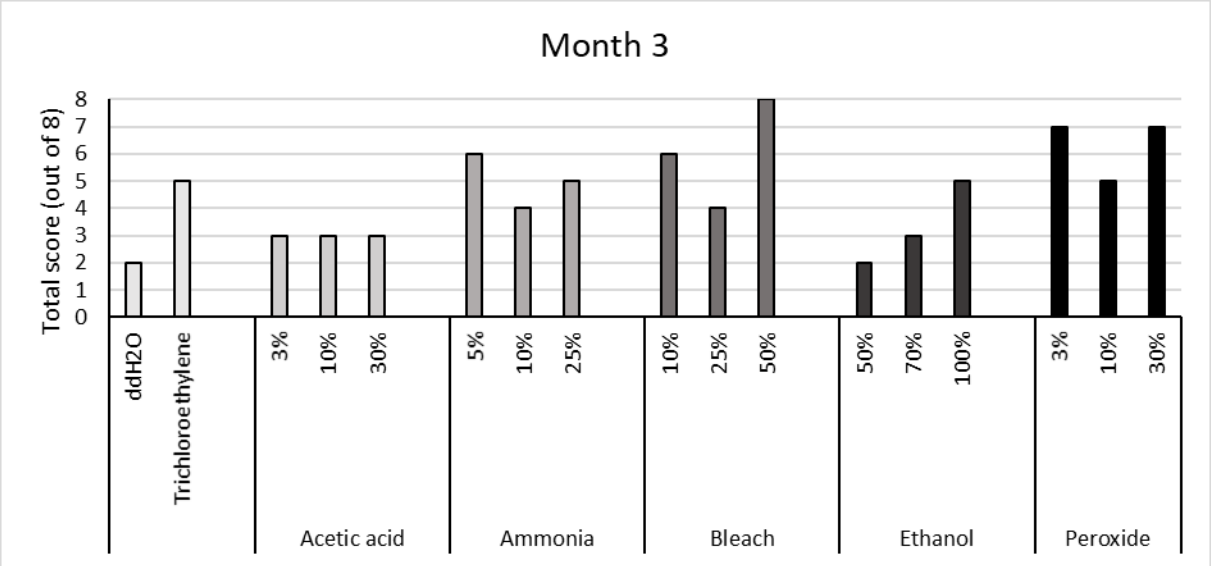


Figure 4.19: Composite scores for all chemicals after 3 months.

4.7 Intra-observer and interobserver error

In all categories for intra-observer error (Table 4.2), the intraclass correlation (ICC) was >0.9, indicating excellent reliability[45]. Interobserver error ranged from moderate to excellent reliability depending on the score in question. As can be seen in Table 4.3, the ICC value of 0.925 indicates excellent reliability for the scoring of microfractures while the ICC value of 0.793 indicates good reliability[45] for the scoring of flaking of bones. Bone loss in cortical area scored an ICC value of 0.880, which indicates good reliability[45]. Bone loss in medullary border scored an ICC value of 0.693, indicating moderate reliability[45]. Lastly, overall morphological changes scored an ICC value of 0.772, which indicates good reliability[45].

Table 4.2: Intra-observer error results.

		Intraclass Correlation[b]	95% Confidence Interval		F Test with True Value 0			
			Lower Bound	Upper Bound	Value	df 1	df2	Sig
Microfractures	Single measures	.947[a]	.908	.969	35.75	50	50	.000
	Average measures	.973[c]	.952	.984	35.75	50	50	.000
Flaking	Single measures	.824[a]	.711	.895	10.40	50	50	.000
	Average measures	.903[c]	.831	.945	10.40	50	50	.000
Bone loss in cortex	Single measures	.877[a]	.794	.928	15.03	50	50	.000
	Average measures	.935[c]	.885	.963	15.03	50	50	.000
Bone loss in medullary border	Single measures	.866[a]	.776	.921	13.69	50	50	.000
	Average measures	.928[c]	.874	.959	13.69	50	50	.000
Overall morphological changes	Single measures	.870[a]	.784	.924	14.66	50	50	.000
	Average measures	.931[c]	.879	.960	14.66	50	50	.000

Table 4.3: Interobserver error results.

		Intraclass Correlation[b]	95% Confidence Interval		F Test with True Value 0			
			Lower Bound	Upper Bound	Value	df1	df2	Sig
Microfractures	Single measures	.805[a]	.712	.876	13.31	50	100	.000
	Average measures	.925[c]	.881	.955	13.31	50	100	.000
Flaking	Single measures	.561[a]	.376	.712	5.75	50	100	.000
	Average measures	.793[c]	.643	.881	5.75	50	100	.000
Bone loss in cortex	Single measures	.710[a]	.561	.818	9.78	50	100	.000
	Average measures	.880[c]	.793	.931	9.78	50	100	.000
Bone loss in medullary border	Single measures	.429[a]	.173	.633	4.67	50	100	.000
	Average measures	.693[c]	.386	.838	4.67	50	100	.000
Overall morphological changes	Single measures	.530[a]	.299	.703	5.83	50	100	.000
	Average measures	.772[c]	.561	.877	5.83	50	100	.000

CHAPTER 5 : DISCUSSION

Degreasing is a crucial part of the preparation of remains for analysis and storage. Efficiency, accuracy and reproducibility of degreasing methods are an important factor to take into account when choosing a method. This study investigated different chemicals in search of a replacement for TCE which although accurate, is a highly toxic and expensive chemical. The following paragraphs will discuss the results obtained from the chemicals in terms of efficacy using various factors (microfractures, flaking, bone loss in cortex as well as medullary border and overall morphological changes) and composite scores compared to TCE.

5.1 Microfractures

Microfractures are small fractures on the bone surface that compromise bone integrity. Acetic acid and ammonia caused minimal to extensive microfractures during all three months (Figure 4.1, Appendix 1.1 A-I and 1.2 A-I), while bleach mainly caused extensive microfracturing in month three (Appendix 1.3 C, F, I). Ethanol caused the least amount of microfractures on the bone during all three months (Figure 4.1, Appendix 1.4 A-I), making it the most successful chemical considering fracturing. Peroxide caused extensive microfracturing during all three months (Figure 4.1, Appendix 1.5 A-I). Distilled water, acting as negative control, did not cause any fracturing during any month (Figure 4.1, Appendix 1.6 A-I), while the positive control TCE caused a substantial amount of fracturing in the last month (Figure 4.1, Appendix 1.7 C). It is expected that distilled water will not cause microscopic fractures on bone, however, microscopic fractures caused by TCE indicates that it still causes damage at a microscopic level, demonstrating that it still has drawbacks even though TCE is identified as the gold standard of degreasing.

Microfractures on bone could be due to the effect of the chemical, or due to mechanical error while the bone slides were prepared. These fractures may seem insignificant at first but could possibly grow larger with time and cause cracks in the bone, which in turn may affect its use. Bones that are cracked or have macroscopic fractures, will not be able to be used in museum exhibitions or in a research environment. It will also negatively impact a forensic analysis as these macroscopic fractures could mimic trauma and influence the forensic report. Therefore, microfractures are undesirable in degreased bones and should be taken into account when a degreasing method is chosen.

5.2 Flaking

All the chemicals caused bone flaking to some extent, with some chemicals being worse than others. During the first two months, acetic acid and ammonia caused an extensive amount of flaking (Figure 4.3, Appendix 1.1 A-B, D-E, G-H and 1.2 A-B, D-E, G-H) whereas bleach and peroxide was more likely to cause flaking during the third month (Figure 4.3, Appendix 1.3 C, F, I and 1.5 C, F, I). Distilled water caused minimal flaking after the third month (Figure 4.3, Appendix 1.6 C), while TCE started to cause minimal flaking during month two (Figure 4.3, Appendix 1.7 B). Ethanol caused minimal to no flaking during all three months (Figure 4.3, Appendix 1.4 A-I), making it the least destructive chemical considering flaking.

As with microfractures, flaking of bone could be due to the effect of the chemical, or due to mechanical error while the bone slides were prepared. When flaking occurs, it does not only compromise bone integrity, but also makes the bone difficult to work with as it can result in sections of the outer layer of bone being lost during handling. Storage may also be a problem as flaking will only get worse with time, making it impossible to handle and analyse the bone without further damaging the bone. Flaking is, therefore, also undesirable in many contexts such as museum exhibition, research and educational purposes as well as in a forensic context where the bones are often handled or moved and should be taken into account when deciding on a degreasing method.

5.3 Bone loss in cortex

Cortical bone loss was not as eminent during month one, except for ammonia (Figure 4.5, Appendix 1.2 A, D, G) and peroxide (Figure 4.5, Appendix 1.5 A, D, G) which caused minimal bone loss. During month two, a higher amount of bone loss was noted in all chemicals, with ammonia (Appendix 1.2 B, E, H) and ethanol 70% (Appendix 1.4 B, E, H) causing extensive bone loss (Figure 4.5). All chemicals caused bone loss to some extent during the last month, with ammonia, bleach and peroxide having extensive bone loss (Figure 4.5). Although acetic acid caused minimal to no bone loss during all three months, ethanol 50% still showed the lowest amount of cortical bone loss (no loss during the first two months and only minimal loss during the last month) which makes it the least destructive chemical regarding bone loss in the cortical area (Figure 45, Appendix 1.4 A-I). The gold standard TCE caused no bone loss during the first month, however, minimal to extensive bone loss occurred during the second and third month (Figure 4.5, Appendix 1.7 A-C).

Bone loss in the cortex cannot be ignored as it is the outer layer which protects the bone and when loss occurs there, the bones are compromised and may easily be damaged and can ultimately influence measurements taken from the bones in the forensic or research setting. Furthermore, the overall appearance and diameters of the bone may change over time due to extensive loss of the cortex.

5.4 Bone loss in medullary border

Bone loss in the medullary border proved to be a difficult area to score as bone loss could be caused by both the chemical itself and/or mechanical error during the preparation of the slides. The medullary area contains spongy bone and is weaker and less compact than cortical bone, making it easily influenced by external factors such as chemical damage or slide preparation. It has also shown to be a very subjective score which makes it difficult to get consensus amongst scorers, as can be seen with the ICC score of only 0.693 in the interobserver error analysis.

All chemicals showed bone loss during all three months, with the loss gradually increasing each month. It can be noted that ethanol 70% caused extensive amounts of bone loss during each month (Figure 4.7, Appendix 1.4 D-F) while the other ethanol concentrations caused minimal to no bone loss (Figure 4.7, Appendix 1.4 A-C and G-I). Ammonia and bleach caused minimal to extensive bone loss during month one, while acetic acid, bleach and peroxide caused extensive loss during month two. Finally, during the last month, ammonia, bleach and peroxide caused extensive bone loss (Figure 4.7). Ethanol 50% is regarded the most successful chemical considering medullary bone loss, as it caused no bone loss during month one and only minimal bone loss during the following two months (Figure 4.7, Appendix 1.4 A-C). Distilled water caused minimal bone loss during all three months (Figure 4.7, Appendix 1.6 A-C). TCE caused no bone loss during the first and third month, with only minimal bone loss occurring during month two, which makes it slightly more successful than ethanol 50% (Appendix 1.7 A-C).

Since ethanol 70% caused substantial bone loss during all three months, it can be regarded as the least successful chemical concerning medullary bone loss, together with bleach and peroxide.

Bone loss in the medullary border may not be as extreme as bone loss in the cortical area but should still be taken into account. When looking at outer bone morphology, medullary

bone loss may seem insignificant. However, it can weaken the bone internally, making it easier for the bone to break when handled incorrectly.

5.5 Overall morphological changes

Overall morphological changes are influenced by all the scores mentioned above and is dependent on how well the chemical scored on all the above-mentioned factors. Therefore, it can be seen that ethanol caused the least morphological changes – with the exception of ethanol 70% which caused extreme morphological changes during all three months (Figure 4.9, Appendix 1.4 A-I).

Acetic acid 10% and 30%, ammonia 5% and 25% and peroxide 10% and 30% caused only minimal morphological changes during the first month but caused extensive changes thereafter (Figure 4.9). Other than expected, bleach caused minimal morphological changes in the bone (Figure 4.9, Appendix 1.3 A-I). Both distilled water and TCE, the negative and positive controls respectively, only showed morphological changes during month three with distilled water exhibiting minimal morphological changes and TCE exhibiting extensive changes (Figure 4.9, Appendix 1.6 and 1.7).

5.5.1 Acetic acid

While acetic acid 3% caused minimal to no microfractures, both the 10% and 30% concentrations caused an extensive number of fractures. All the concentrations caused extensive flaking to occur, which is disadvantageous as it will complicate the storage process and make bone difficult to work with. Although minimal bone loss occurred in the cortex with all three concentrations, extensive bone loss occurred in the medullary border. All of the factors mentioned above can be explained by a chemical reaction between the acetic acid and the bone itself. Acetic acid reacts with calcium carbonate in bones to produce a soluble salt called calcium acetate, as well as carbonic acid[46]. Calcium acetate diffuses out of the bones and into the water component of the solution when it is formed, which causes the bone to become soft and less rigid[46]. Carbonic acid is unstable at room temperature and breaks down into water and carbon dioxide gas instantly, which can be seen as small bubbles in the solution if the bones are watched closely over time[46]. The calcium acetate can possibly explain the formation of crystals that were seen on the distal end of these bones in the pilot study (Figure 5.1) as well as the overall softness. During preparation of the bone slides, it was easy to cut sections of bone as the bone was not brittle, but not as hard as the bone was before acetic acid was used. Acetic

acid is considered to be a reasonably cheap chemical which can be easily acquired. It is also safe to use as it can be poured down the drain after use with no hazardous effects. However, the amount of damaged caused, may not make it a viable option for degreasing bone in the long run.

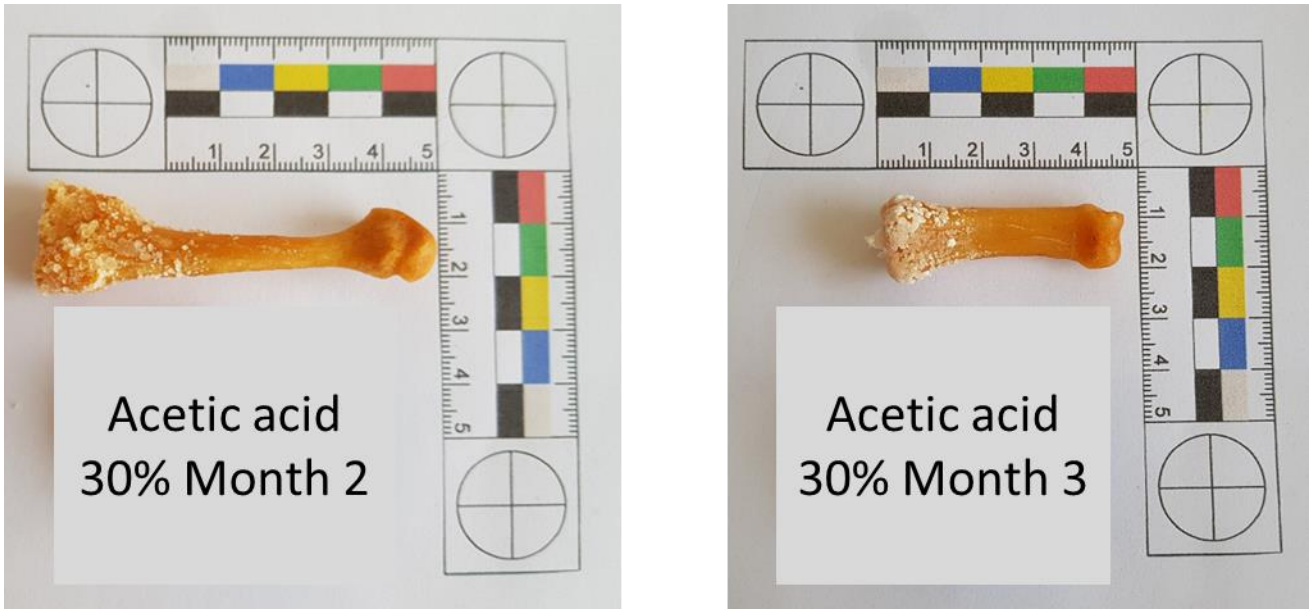


Figure 5.1: Crystal formation on the distal end of bone caused by acetic acid 30% during month 2 and 3 (Appendix 1.1 H2-I2).

5.5.2 Ammonia

Not only did all ammonia concentrations cause extensive microfracturing as well as flaking on the bones during all three months, but it also caused extensive bone loss in both the cortex and medullary side of the bone, which makes it less ideal to use as degreaser. During the slide preparation it was noticed that the bone was exceptionally brittle, making it difficult to cut a section as the bone broke easily. It has previously been demonstrated that ammonia salts may cause bone demineralization as well as bone resorption in rats[47], which may explain the abovementioned observations.

Ammonia is quite expensive to acquire and can also be hazardous to health when not handled correctly. Inhalation of lower concentrations of ammonia may cause coughing and throat irritation[3-4,9]. Ammonia presents with a strong odour and can cause olfactory fatigue, making it dangerous to work with over a prolonged period of time[3-4,9]. Furthermore, rapid skin or eye irritation may also occur due to exposure to ammonia. Higher concentrations of ammonia, for example before diluted for degreasing, may cause severe injury such as skin

burns, permanent eye damage and even blindness[3-4]. Ammonia also needs to be discarded of using laboratory protocols and cannot be poured down the drain, which adds an inconvenient and expensive extra step to the degreasing process. Considering these issues and the amount of damage caused to the bone, ammonia is not a suitable chemical to use in bone degreasing.

5.5.3 Bleach

Bleach 10% presented positive results during the first month, as almost no microfracturing, flaking or bone loss in the cortex occurred. Extensive bone loss still occurred in the medullary border. During the second and third months, however, extensive microfracturing, flaking, and bone loss in both areas was noted.

Both bleach 25% and 50% caused extensive microfracturing and flaking in all three months. Bone loss in the cortex seemed to be worse during month one and three, with a small amount occurring during the second month. Bone loss in the medullary border was extensive in all three months with all three concentrations. Bleach has shown to cause demineralisation and loss of bone integrity, which can explain the bone loss mentioned above[46-48]. Taxidermists also never use bleach as a degreaser/whitener, as it has been shown to cause the bone to become flaky and chalk-like, which is an undesirable effect[24].

During slide preparation, it was noted that the bones were oily and no longer degreased, indicating that the pilot study degreasing was not successful for these bones. However, bleach is easily accessible and very low-priced, thus making it a good economical choice as degreaser. Although it can be hazardous when accidentally ingested, bleach is considered to be a relatively safe chemical to use as it can be poured down the drain after use and no special equipment is needed to work with it. Considering these issues and the amount of damage caused to the bone, bleach is not a suitable chemical to use in bone degreasing.

5.5.4 Ethanol

Ethanol 50% showed to be very successful during this experiment, as almost no microfracturing occurred during all three months. Minimal flaking was noted in the first two months, with almost no flaking present during month three. Minimal bone loss occurred in both the cortex and medullary border, except for a noticeable amount of bone loss in the medullary border during month three.

Ethanol 70% deemed to be less successful, with extensive microfracturing and flaking occurring during the first two months, that seemed to decrease during the third month. Bone

loss was not as prominent during month one, but in the following two months extensive bone loss occurred in both the cortex and medullary border.

Ethanol 100% caused extensive microfractures on the bones during month one and three, with month two as an exception as only a small amount of microfractures was present. Substantial flaking occurred during all three months. Only a small amount of bone loss was noted in the cortex during the three months, but substantial bone loss occurred in the medullary border.

During slide preparation, it was noted that the bones were slightly oily, but not as oily as the bleached bones. The bones were also slightly brittle but not to the extent as to where it breaks, and bone slides could be made with no difficulties. Ethanol is considered as one of the more expensive chemicals used in this study, however it is still less expensive than TCE. No special machinery is needed for the use of this chemical, and it is also considered relatively safe as it can be poured down the drain after use with no extra safety measurements needed (once any human tissue has been filtered out). Taking these factors into consideration, ethanol at lower concentrations may be a highly suitable chemical to use to degrease bone.

5.5.5 Peroxide

Peroxide as a chemical did not deem as successful as anticipated by the pilot study. During the pilot study, peroxide degreased the bone exceptionally well and did not cause a sediment or fatty layer, did not change bone morphology, and did not produce a foul odour after drying (Appendix 3). It also caused the bone to change from a dark brown colour to a clean, white colour (Figure 5.2). However, during the current study, it was seen that peroxide caused extreme microfracturing during all three months with all three concentrations. Flaking was more prominent with peroxide 3%, but peroxide 10% and 30% also showed substantial flaking. Bone loss in the cortex seemed to be worse during month one and three, with all concentrations showing little to no bone loss during month two. Bone loss in the medullary border was extensive for all three months with all three concentrations. Slide preparation was also difficult - the bones were degreased but extremely brittle, causing section slicing to be almost impossible. The bones either broke as slicing took place or broke afterwards when grinding with sandpaper.

Peroxide is one of the lowest priced chemicals used in this study, and it is easily accessible. Although it is considered a relatively safe chemical to use as it can be poured down the drain after use, hydrogen peroxide is highly destructive and can not only cause damage to

the structural integrity of the bones but can also be harmful to human skin[24,26]. Nevertheless, even though hydrogen peroxide is deemed destructive and dangerous, contrary to other chemical substances such as ammonia, hydrogen peroxide does not produce residues or gasses thereby decreasing the health risks accompanying inhalation[27].

Although peroxide appeared to be the best degreaser based on the macroscopic appearance, the amount of damage it produced on the microscopic level makes it not suitable as a degreaser in bone that needs to be stored or handled over a long period of time.

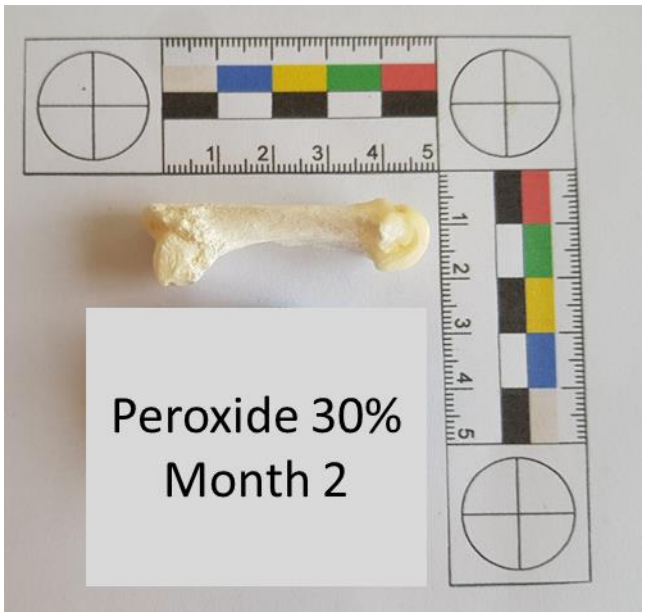


Figure 5.2: Peroxide 30% exhibiting a clean, white degreased bone after two months (Appendix 1.5 H2).

5.5.6 Distilled water and TCE

Distilled water caused little to no degreasing or damage to bone during the time of experiment, which is expected of a negative control. TCE, known as the gold standard of degreasing, acted as positive control during this experiment. TCE degreased the bones exceptionally well, as expected, however, it still caused damage to bone during the second and third month. This could be explained by the fact that TCE in practice is never used to degrease remains for longer than a few days, at most. Using TCE for long periods of time has shown to damage the bone and should not be done in practice.

5.6 Chemical degreasing vs destruction ability

As depicted in the pilot study (Appendix 3) shown in Figure 5.3, the chemical with the highest score indicated the most successful degreasing ability and lowest score the least

successful degreasing ability, determined using the following macroscopic parameters: bone mass; colour difference; sediment layer formed; fatty layer formed; bone morphology and odour after drying. Thus, peroxide 3% and peroxide 10% were equally effective degreasers as they scored the highest. This is in contrast to the current study and can be explained due to only microscopic parameters (microfractures; flaking; bone loss in cortex; bone loss in medullary border and overall morphological changes) that were used.

Ammonia 25% showed to be the least successful degreaser with the lowest score of all the chemicals, in agreement with the current study. Ethanol showed to be the second-best degreaser of all the chemicals, with its scores correlating closely to peroxide, however in the current study when microscopic morphology is taken into account ethanol is seen to be a much better degreaser than peroxide (Figure 5.4).

Acetic acid showed to be third most successful, with bleach correlating closely but having slightly lower scores, more or less similar to the results obtained in this study. Trichloroethylene, representing the gold standard of degreasing, had the second highest scores during both months demonstrating its successful degreasing ability, however in the current study it is seen that using TCE for more than one month causes detrimental microscopic changes to the bone (Figure 5.4).

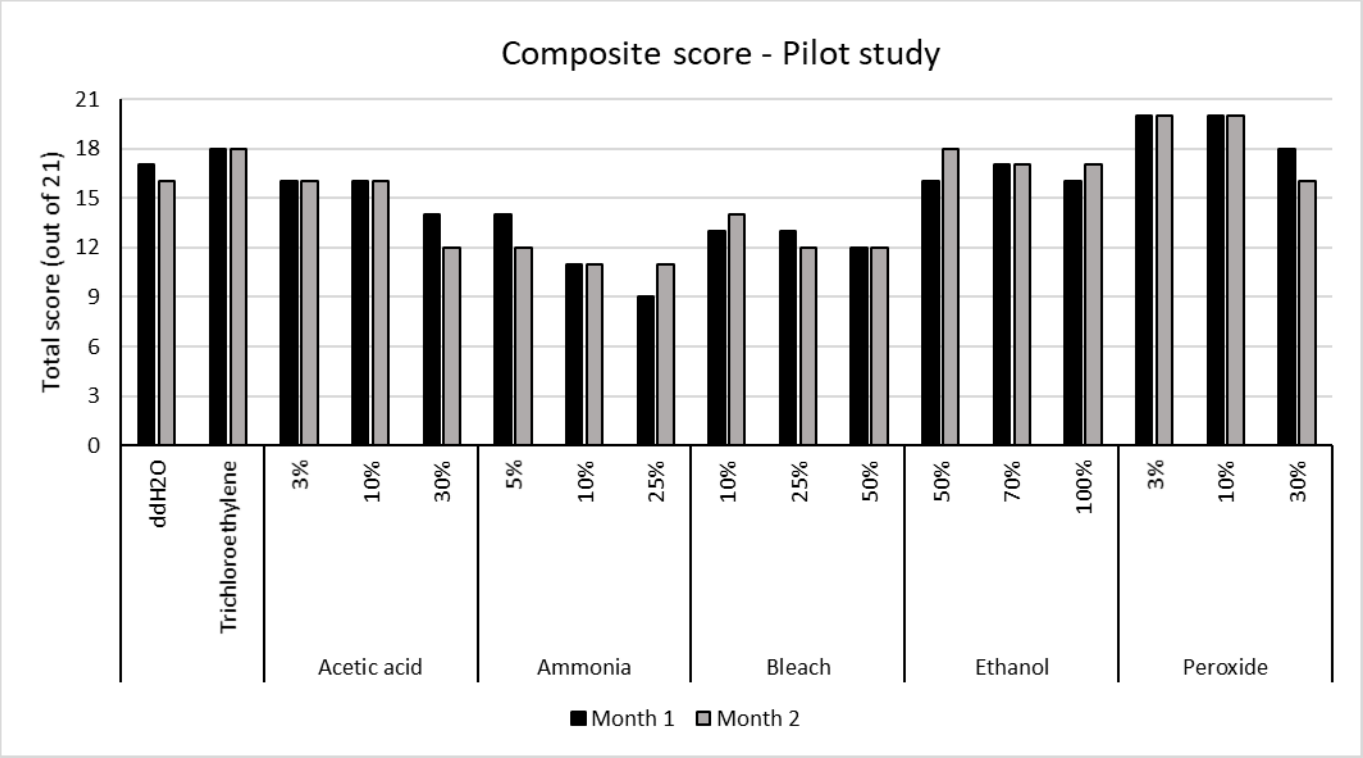


Figure 5.3: Composite score of degreasing chemicals tested in the pilot study after 1 and 2 months.

In the current study (Figure 5.4), the chemical with the lowest score indicates the least destruction and highest score the most severe destruction. Thus, ethanol and acetic acid showed to have the least overall destructive ability, while both ammonia and peroxide showed to be the most destructive. Bleach ranged from low destructive ability in lower concentrations, to higher destruction in the higher concentrations. TCE, representing the gold standard of degreasing, did not cause any damage during the first month, but was increasingly destructive during the second and third months.

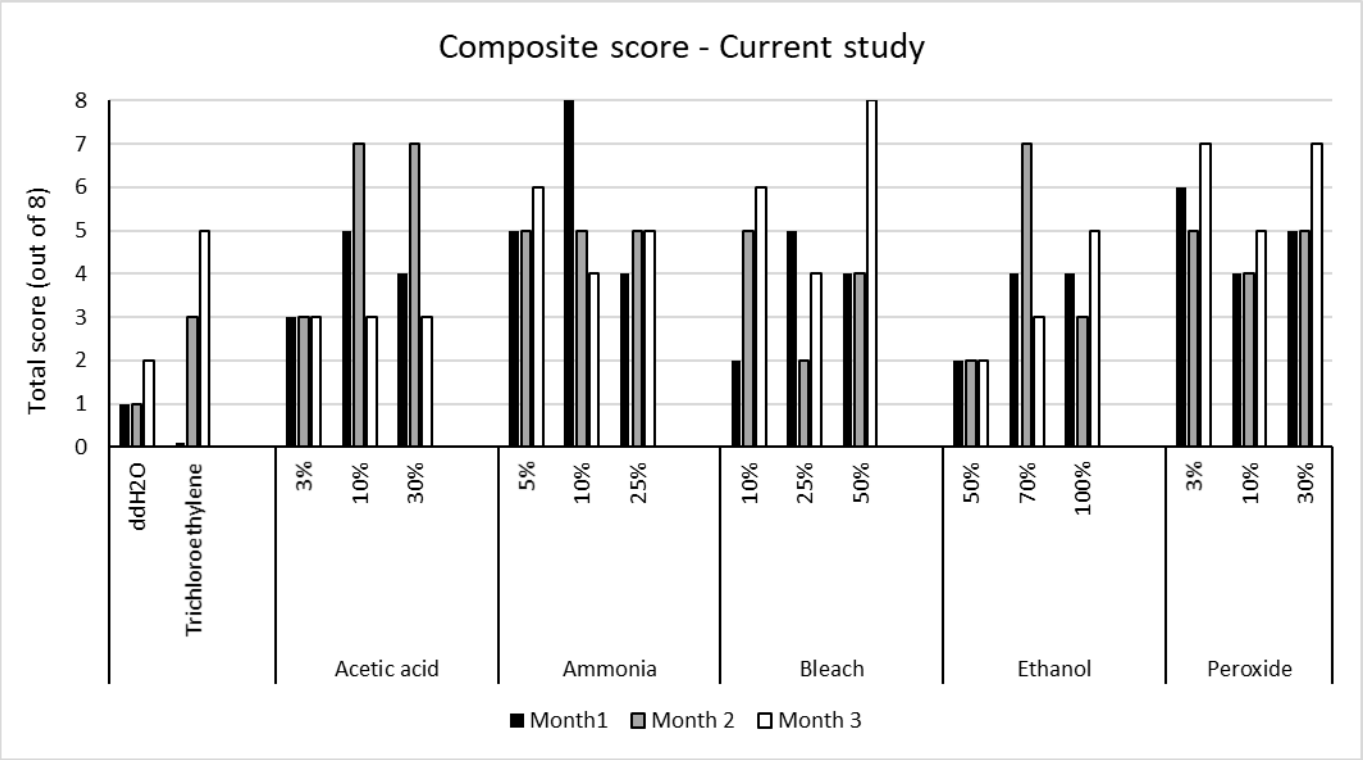


Figure 5.4: Composite score of degreasing chemicals tested in the current study after 1, 2 and 3 months.

Considering both studies, it can be said that ammonia was the least successful chemical as it did not degrease well on a macroscopic level, but also caused extensive damage on a microscopic level. Acetic acid also did not perform well macroscopically or microscopically. Not only did it form crystals on the bone itself during degreasing (Figure 5.1), but it also did not degrease exceptionally well and caused extensive damage on a microscopic level.

Bleach achieved better results both macroscopically and microscopically, as it degreased bone relatively well and did not cause extensive microscopic damage during the first month. Thereafter, more damage was noticed and therefore bleach is not considered to be the best degreasing agent. Peroxide was considered the best degreaser when taking into account

macroscopic factors but caused extensive damage microscopically. Thus, even though the chemical showed promising degreasing properties, it was at the expense of microscopic characteristics. Although ethanol did not achieve the best results as a degreasing agent, it still degreased well enough to be taken into account. Ethanol 50% performed very well on a microscopic level as it did not cause extensive damage in any form. Ethanol 70% and 100% caused increased amounts of damage, but still not as much as the other chemicals in the study. Ethanol degreased the bone successfully, but the bone still contained enough oil to prevent further damage, which caused protection of the bone on a microscopic level.

Compared to TCE, ethanol at low concentrations can be considered a suitable alternative as it degreases sufficiently while causing low levels of bone destruction, is safe to use in a laboratory environment and costs significantly less. Hydrogen peroxide has excellent degreasing abilities and costs less than ethanol, but the high levels of bone destruction it causes makes it unsuitable as an alternative to TCE. It also proposes some safety hazards which can be avoided by rather using ethanol. Ammonia should not be considered as an alternative degreaser as it is not only unsuccessful in degreasing, but also causes high levels of bone destruction. It is more expensive than ethanol and poses many unwanted health and safety hazards which can easily be avoided by rather using ethanol. Both bleach and acetic acid are deemed average degreasers, as they do not degrease exceptionally well, but also do not cause extensive levels of bone destruction. Both are inexpensive (even more so than ethanol) and are safe to use in a laboratory environment as they mostly do not propose any safety hazards. However, ethanol should still be favoured over these chemicals since they do not cost significantly less than ethanol and cause unwanted side-effects that are not prevalent with ethanol usage.

5. 7 Discrepancies in scores

Of interest to note is that some chemicals did not follow an expected pattern.

Table 5.1: Discrepancies in the scores of particular chemicals.

Chemical	Discrepancy
Acetic acid 3%	Showed microfractures at month two and not month one and three as well as reduced bone loss from month one to month two and increased bone loss in month three.

Acetic acid 10% and 30%	Showed increased cortical bone loss from month one to two and decreased bone loss in month three, as well as increases in month two and not month three in medullary bone loss.
Acetic acid 30%	Showed increased flaking at month two and reduced flaking in month three.
Bleach 10%	Showed increases in medullary bone loss in month one and three and not month two.
Bleach 25%	Showed increases in medullary bone loss in month two and not month three, also showed microfractures at month one and three and not month two, as well as flaking in month one then reduced flaking in months two and three.
Bleach 50%	Showed flaking in months one and three and not month two.
Ethanol 50% and 70%	Showed flaking in month one then reduced amount of flaking in months two and three.
Peroxide 3% and 10%	Both chemicals showed reduced bone loss from month one to two which then increased in month three. Peroxide 3% also showed flaking in month one then reduced flaking in months two and three and peroxide 10% showed microfractures at month one and two and not month three.
Ammonia 10%	Showed increases of medullary bone loss in month one and not month two and three.

These discrepancies could be explained by either mechanical error by the researcher when the bone slides were prepared as the abrasive paper used could cause damage mimicking microfractures, flaking or bone loss, or due to all the bones not being morphologically identical. Both metacarpals and phalanges were used in this study and although both are bones of the hand, the thickness of the cortex of the two bones could differ, which in effect changes the thickness of the spongy bone on the medullary border side and can possibly influence scores of different months.

It should also be considered that these bones came from different individuals, and it is possible that the bones could differ in thickness or strength due to variation between these individuals. Different ages of these individuals could also influence the general histological appearance of the bones.

Other environmental influences on chemicals should also be taken into account for example, hydrogen peroxide which is sensitive to light[49]. Hydrogen peroxide spontaneously decomposes into water and oxygen and UV light from the sun catalyses this reaction[49]. For this reason, peroxide is mostly kept in brown bottles in a cool, dark place however in this research experiment, all the test tubes were kept in the same conditions (a work bench in a laboratory filled with sunlight) which could have influenced the successful working of this chemical.

CHAPTER 6 : CONCLUSION

Bone degreasing remains an important procedure to ensure that skeletal remains can be safely stored and handled and although TCE is the chemical commonly used as a degreasing agent in South Africa, it poses too many difficulties and disadvantages.

The aim of this study was to therefore investigate the effect on the microscopic bone morphology of alternative degreasing methods, previously identified in a pilot study to degrease the fastest, most effectively, with the least destruction of bone, while taking into consideration price and safety.

Taking all factors into account, ethanol 50% is the most successful degreasing chemical tested in this study. This chemical caused the least amount of microfractures and flaking, as well as minimal amounts of bone loss in both the cortical and medullary area of bone. This influenced the overall morphological changes, which were limited in the case of ethanol 50%. Ethanol is also considered to be relatively low-priced and safe, as it can be poured down the drain and no extra safety measures need to be in place for its usage.

Peroxide 30% is the least successful chemical tested in this study. Multiple microfractures were caused by this chemical, as well as extensive flaking. The bone was very brittle, and substantial bone loss occurred in both the cortical and medullary area. Extensive morphological changes could be seen, which confirms that peroxide 30% is not the best choice for degreasing bone.

However, although ethanol 50% showed positive results it is not yet possible to declare this a viable degreasing method, as more research still needs to be done. This research project only tested degreasing of metacarpals and phalanges, and the success of these chemicals on other bones are yet to be determined.

6.1 Limitations and future recommendations

6.1.1 Difficulty in preparation of slides

Numerous aspects could have influenced the appearance of the slides during slide preparation, which could possibly have influenced the results. Grinding of the bone sections during slide preparation was the most significant factor. The histological structures will not be visible with the microscope if the sections are ground too thinly which could lead to misinterpretation of structures and cause inaccurate results. The same issue could be applied for sections being too thick, but this can fortunately be corrected by re-grinding the slide to the correct thickness. Some of the structures could also be lost if the bone slice is not perfectly

horizontally ground and cracks could occur which could modify the structures or be misinterpreted as fractures.

Another factor that influenced the quality of the slides was the sandpaper used to grind down the sections. Although the sandpaper was of an exceptionally fine grit, “scratch” lines were visible on the bone sections after grinding. The images were slightly distorted by these lines which could have influenced the scores given to each bone. Another aspect to consider when grinding the sections is that the grinding motion should be circular as to prevent unevenness and scratches.

Some of the bones were also very brittle and difficult to slice and retrieve bone sections. Some samples had to be taken multiple times before a decent slice could be obtained, as the bone fractured and flaked off even before the grinding process started. In an effort to prevent fracturing during slicing, superglue was added to the bone slice before grinding started, but this proved ineffective as the superglue obscured the image taken with the microscope and scoring of the sample couldn’t take place efficiently.

However, taking all these factors taken into consideration; the quality of the bone slides produced in this study were still adequate.

6.1.2 Repeatability and reliability of proposed method

It is difficult to identify structures correctly by interpreting the definitions of the various structures described in the literature when a picture reference is not given. Some of the scores in this study has proven to be somewhat objective, causing moderate interobserver error. To increase the repeatability of this method, it is suggested that picture references are included in the scoring table so that all observers have the same understanding. Furthermore, the number of observers need to be increased in order to test the validity of the scoring system to define and convert the qualitative features seen on the bone into quantitative scores.

6.1.3 Shortage of samples

This study made use of the samples which were used in the pilot study, which is 51 in total. This posed difficulties during statistical analysis, as the sample size was too small since there was only one sample for each chemical concentration. Therefore, statistics could not be performed on the data. In an ideal setting, at least three samples for each chemical would be needed and thus regarding future research, a larger sample size using the same bone type is suggested.

CHAPTER 7 : REFERENCES

1. Smithsonian National Museum of Natural History. [Internet]. Skeletal research collections. 2020. [cited 2020 Feb 10]. Available from: https://naturalhistory.si.edu/education/teaching-resources/written-bone/forensic-anthropology/skeletal-research-collections.

2. Rowley B. Protocols for Cleaning and Articulating Large Mammal Skeletons. 2015. Symposium 2(1): 1-27.

3. Aboe G. [Internet]. We are not alone and not the first! Museum of Natural History; 2013. [cited 2020 Feb 13] Available from: https://onceinawhale.com/2013/07/02/we-are-not-alone-and-not-the-first/.

4. Agency for Toxic Substances and Disease Registry. [Internet]. Public health statement: Trichloroethylene, 2016. Division of Toxicology and Human Health Sciences. [cited 2020 Mar 3]. Available from: https://www.atsdr.cdc.gov/ToxProfiles/tp19-c1-b.pdf

5. van der Spuy T. Degreasing with Trichloroethylene: A Look at the Latest Developments around Degreasing with Chlorinated Solvents [Unpublished]. SA Metal Finishers; 2008.

6. Freemont A.J., Bone. Current Orthopaedics. 1998; 12(3):181-192. doi:https://doi.org/10.1016/S0268-0890(98)90023-8

7. Ross M.H, Romrell L.J., Kaye G.I., Chapter 8 - Bone. In: Histology: A Text and Atlas. Philadelphia: Williams & Wilkens; 1995. p. 150-187.

8. Junqueira L.C., Carneiro J., Kelley R.O., Chapter 8 - Bone. In: Basic Histology (ninth edition). Stanford, Connecticut: Appleton & Lange; 1998. p. 134-151.

9. Leeson T.S., Leeson R.C., Chapter 7 - Specialized Connective Tissue: Cartilage and Bone. In: Histology (fourth edition). Philadelphia, Pennsylvania: Saunders Company; 1981. p. 137-164.

10. Feather A., Randall D., Waterhouse M., Chapter 19 – Bone disease. In: Kumar and Clark's Clinical Medicine (tenth edition). Elsevier, 2021. p. 471-485.

11. Coetzee H., Loots G., Meiring J., Chapter 5 - Bone. In: Human Histology. Pretoria, South Africa: Van Schaik; 2003. p. 94-97.

12. Cowin S.C., Cardoso L., Blood and Interstitial Flow in the Hierarchical Pore Space Architecture of Bone Tissue. Journal of Biomechanics. 2015; 48(5):842-854. doi:10.1016/j.jbiomech.2014.12.013

13. Väänänen K., Zhao H., Chapter 8 - Osteoclast Function: Biology and Mechanisms. In: Bilezikian J.P., Raisz L.G., Rodan G.A., editors. Principles of Bone Biology (second edition). San Diego: Academic Press; 2002. p. 127-139.

14. Florencio-Silva R., Rodrigues da Silva Sasso G., Sasso-Cerri E., Simões M.J., Cerri P.S., Biology of Bone Tissue: Structure, Function, and Factors that Influence Bone Cells. BioMed Research International. 2015; 2015:421746. doi:10.1155/2015/421746

15. Standring S., Chapter 5 - Functional Anatomy of the Musculoskeletal System. In: Gray's Anatomy (42nd edition). Elsevier, 2021. p. 85-126.

16. Mairs S., Swift B., Rutty G.N., Detergent: An Alternative Approach to Traditional Bone Cleaning Methods for Forensic Practice. The American Journal of Forensic Medicine and Pathology. 2004; 25(4):276-284. doi:10.1097/01.paf.0000147320.70639.41

17. Couse T., Connor M., A Comparison of Maceration Techniques for Use in Forensic Skeletal Preparations. Journal of Forensic Investigation. 2015; 3(1):1-6

18. Dayal M.R., Kegley A.D., Strkalj G., Bidmos M.A., Kuykendall K.L., The History and Composition of the Raymond A. Dart collection of Human Skeletons at the University of the Witwatersrand, Johannesburg, South Africa. American Journal of Physical Anthropology. 2009; 140(2):324-335. doi:10.1002/ajpa.21072

19. Causey D., Trimble J., Old Bones in New Boxes: Osteology Collections in the New Millennium. The Auk. 2005; 122(3):971-979. doi:10.1642/0004-8038(2005)122[0971:OBINBO]2.0.CO;2

20. Steadman D.W., DiAntonio L.L., Wilson J.J., Sheridan K.E., Tammariello S.P., The Effects of Chemical and Heat Maceration Techniques on the Recovery of Nuclear and Mitochondrial DNA from Bone. Journal of Forensic Sciences. 2006; 51(1):11-17. doi:10.1111/j.1556-4029.2005.00001.x

21. Mann R.W., Berryman H.E., A Method for Defleshing Human Remains Using Household Bleach. Journal of Forensic Sciences. 2012; 57(2):440-442. doi:10.1111/j.1556-4029.2011.01987.x

22. Eliopoulos C., Lagia A., Manolis S., A Modern, documented Human Skeletal Collection from Greece. Homo - Journal of Comparative Human Biology. 2007; 58(3):221-228. doi:10.1016/j.jchb.2006.10.003

23. King C., Birch W., Assessment of Maceration Techniques used to Remove Soft Tissue from Bone in Cut Mark Analysis. Journal of Forensic Sciences. 2015; 60(1):124-135. doi:10.1111/1556-4029.12582

24. Eriksen A.M., Simonsen K.P., Rasmussen A.R., Conservation of Mitochondria DNA in Fast Enzyme Macerated Skeletal Material. International Journal of Conservation Science. 2013; 4(2):127-132.

25. McGowan-Lowe J. [Internet]. How to Clean Animal Bones - The Complete Guide. 2021. [cited 2021 Mar 25]. Available from: http://www.jakes-bones.com/p/how-to-clean-animal-bones.html.

26. Bourne M.C., Jennings W.G., Definition of the word "Detergent". Journal of the American Oil Chemists Society. 1963; 40(5):212. doi:10.1007/BF02632586

27. Ajayi A., Edjomariegwe O., Iselaiye O.T., A Review of Bone Preparation Techniques for Anatomical Studies. Malayan Journal of Bioscience. 2016; 3(2):76-80.

28. Hinshaw S.H. [Internet]. Dermestarium: University of Michigan Museum of Zoology. 2020. [cited 2020 Apr 16]. Available from: https://webapps.lsa.umich.edu/ummz/mammals/dermestarium/default.asp

29. Nawrocki S.P. [Internet]. Cleaning Bones: University of Indianapolis Archeology & Forensics Laboratory. 2006. [cited Apr 18]. Available from: http://archlab.uindy.edu/documents/CleaningBones.

30. Turner-Walker G., The Nature of Cleaning: Physical and Chemical Aspects of Removing Dirt, Stains and Corrosion. International Symposium on Cultural Heritage Conservation; 2012; National Yunlin University of Science & Technology; Tainan, Taiwan.

31. Agency for Toxic Substances and Disease Registry. [Internet]. Public Health Statement: Ammonia, 2004. Division of Toxicology and Human Health Sciences. [cited 2020 Apr 29]. Available from: https://www.atsdr.cdc.gov/toxprofiles/tp126-c1-b.pdf

32. New York State Department of Health [Internet]. The Facts About Ammonia. 2004. [cited 2020 Apr 29]. Available from: https://www.health.ny.gov/environmental/emergency/chemical_terrorism/ammonia_tech.htm

33. Canadian Centre for Occupational Health and Safety [Internet]. Ammonia. 2017. [cited 2020 Apr 29]. Available from: https://www.ccohs.ca/oshanswers/chemicals/chem_profiles/ammonia.html.

34. Stemmer D., Kehagias O., Bone Degreasing – Finding a New Solution to an Old Problem. Biodiversity Information Science and Standards. 2018; 2:1-2.

35. Meadows M. [Internet]. The Dangers of Isopropyl Alcohol. Production Automation Corporation; 2016. [cited 2020 May 19]. Available from: https://blog.gotopac.com/2016/01/06/the-dangers-of-isopropyl-alcohol/.

36. Agency for Toxic Substances and Disease Registry. [Internet]. Public health statement: Acetone, 1994. Division of Toxicology and Human Health Sciences. [cited 2020 May 28]. Available from: https://wwwn.cdc.gov/TSP/PHS/PHS.aspx?phsid=3&toxid=1

37. Cadet J., Bolla K., Chapter 111 - Environmental Toxins and Disorders of the Nervous System. In: Neurology and Clinical Neuroscience. Philadelphia: Elsevier; 2007. p. 1477-1488.

38. Ososky J., History of Collecting, Preparing and Degreasing Whale Skeletons at the Smithsonian Institution [Unpublished]. Smithsonian Institution; 2013. p. 1-8.

39. Solver Chem Publications [Internet]. Advantages and Disadvantages of Hydrogen Peroxide use. 2016. [cited 2020 Jun 15]. Available from: https://www.solverchem.com/articles/disinfectant-products-articles/advantages-and-disadvantages-of-hydrogen-peroxide-use/details.

40. Thorn K., A Quick Guide to Light Microscopy in Cell Biology. Molecular Biology of the Cell. 2016; 27(2):219-222. doi:10.1091/mbc.E15-02-0088

41. Moore S. [Internet]. Uses of Microscopy in Forensics. 2020. [cited 2020 Jun 26]. Available from: https://www.azolifesciences.com/article/Uses-of-Microscopy-in-Forensics.aspx.

42. Cerutti E., Spagnoli L., Araujo N., Gibelli D., Mazzarelli D., Cattaneo C., Analysis of Cutmarks on Bone: Can Light Microscopy Be Of Any Help? American Journal of Forensic Medicine and Pathology. 2016; 37(4):248-254. doi:10.1097/paf.0000000000000260

43. Maat G.J.R., Aarents M.J., Nagelkerke N.J.D., Age Prediction from Bone Replacement : Remodeling of Circumferential Lamellar Bone Tissue in the Anterior Cortex of the Femoral

Shaft of the Present Dutch Population. Leiden, The Netherlands: Barge's Anthropologica, Leiden University Medical Center; 2005; 10:1-19

44. Ericksen M. F., 1991. Histologic Estimation of Age at Death using the Anterior Cortex of the Femur. American Journal of Physical Anthropology. 84:171-179.

45. Koo T.K., Li M.Y., A Guideline of Selecting and Reporting Intraclass Correlation Coefficients for Reliability Research. Journal of Chiropractic Medicine, 2016. 15:155-163

46. Bush J. [Internet]. The Effect of Vinegar on Chicken Bones. Sciencing; 2018. [cited 2020 Aug 11]. Available from: https://sciencing.com/effect-vinegar-chicken-bones-5946177.html.

47. U.S. Department of Health and Human Services. Chapter 3 – Health Effects. In: Toxicological Profile for Ammonia. 2004. p. 66-68.

48. Kerbl F.M., De Villiers P., Litaker M., Eleazer P.D., Physical Effects of Sodium Hypochlorite on Bone: An Ex Vivo Study. Journal of Endodontics. 2012; 38(3):357-359. doi:10.1016/j.joen.2011.12.031

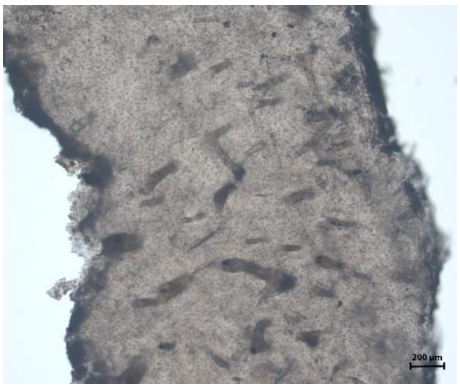
49. Bi L., Li D., Huang Z.,Yuan Z., Effects of Sodium Hydroxide, Sodium Hypochlorite, and Gaseous Hydrogen Peroxide on the Natural Properties of Cancellous Bone. Artificial Organs, 2013. 37(7), p.629-636.

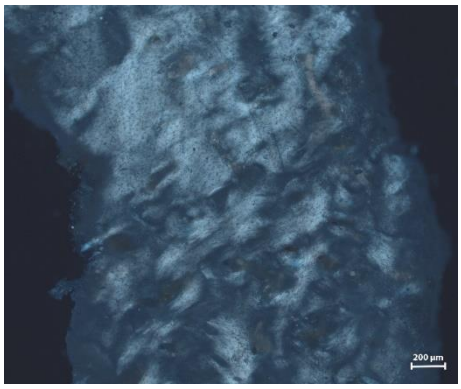
CHAPTER 8 : APPENDICES

APPENDIX 1: Bone images

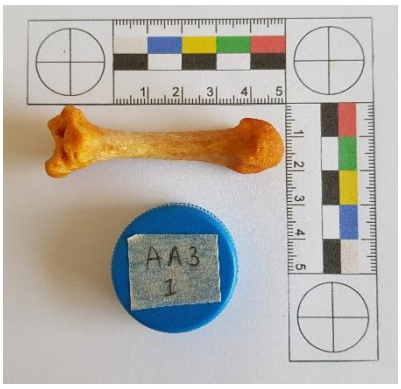
1.1 Acetic acid

A1



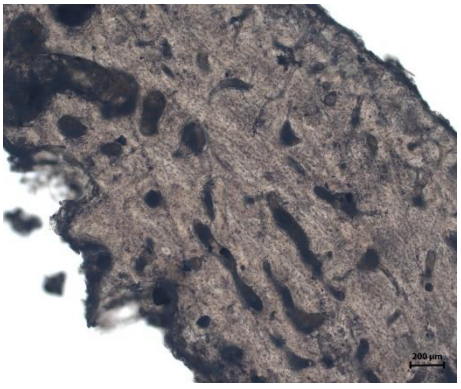


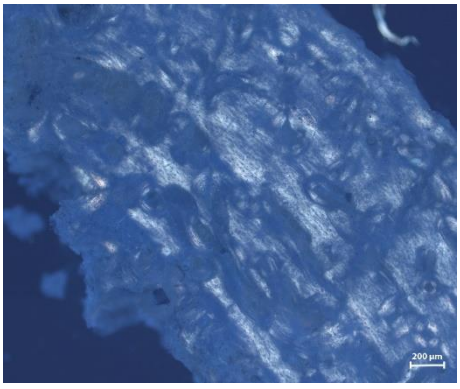
A2



A1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 3% acetic acid for 1 month (Scale bar: 200µm at 5x magnification).
A2: Bone morphology photograph of bone exposed to 3% acetic acid for 1 month.

B1

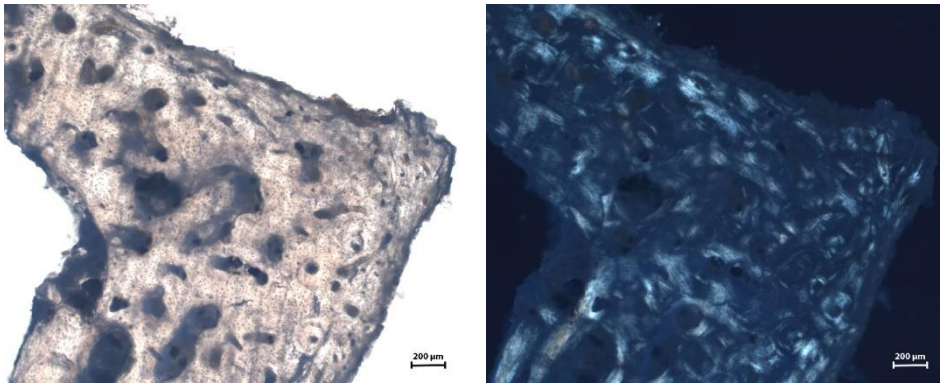




B2

B1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 3% acetic acid for 2 months (Scale bar: 200µm at 5x magnification).
B2: Bone morphology photograph of bone exposed to 3% acetic acid for 2 months.

C1 C2

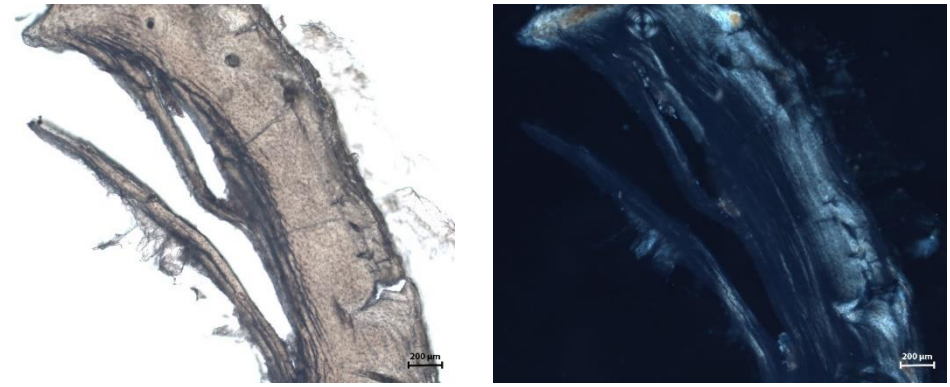


C1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 3% acetic acid for 3 months (Scale bar: 200µm at 5x magnification).
C2: Bone morphology photograph of bone exposed to 3% acetic acid for 3 months.

D1 D2

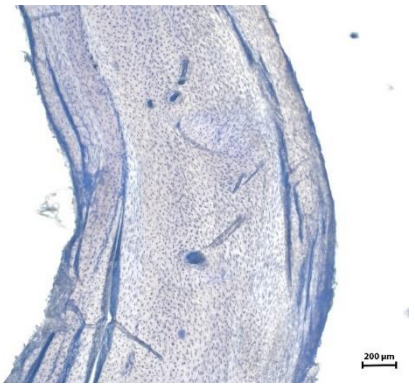
D1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 10% acetic acid for 1 month (Scale bar: 200µm at 5x magnification).
D2: Bone morphology photograph of bone exposed to 10% acetic acid for 1 month.

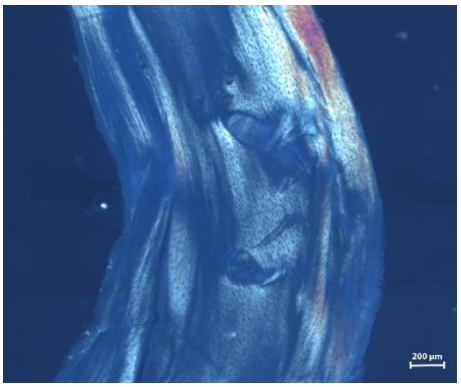
E1 E2



E1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 10% acetic acid for 2 months (Scale bar: 200µm at 5x magnification).
E2: Bone morphology photograph of bone exposed to 10% acetic acid for 2 months.

F1

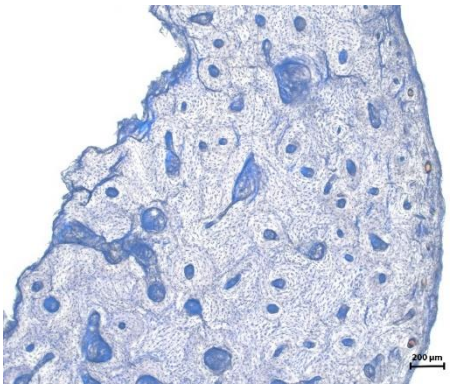


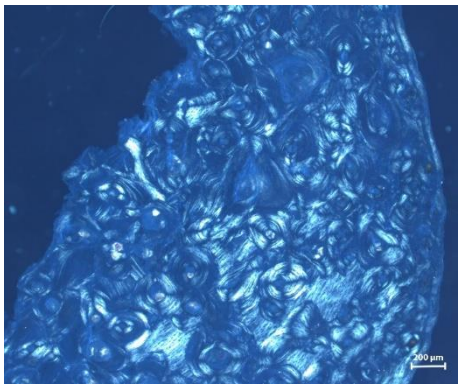


F2

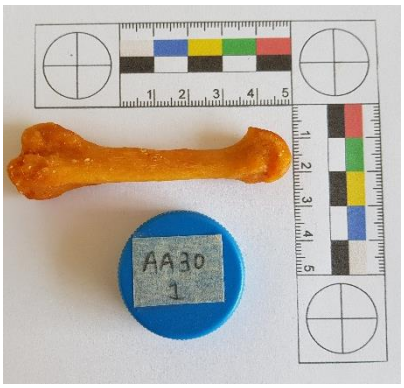
F1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 10% acetic acid for 3 months (Scale bar: 200µm at 5x magnification).
F2: Bone morphology photograph of bone exposed to 10% acetic acid for 3 months.

G1



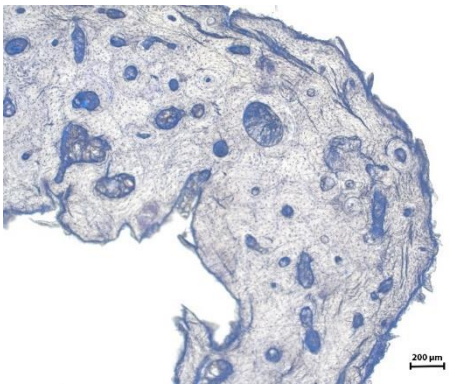


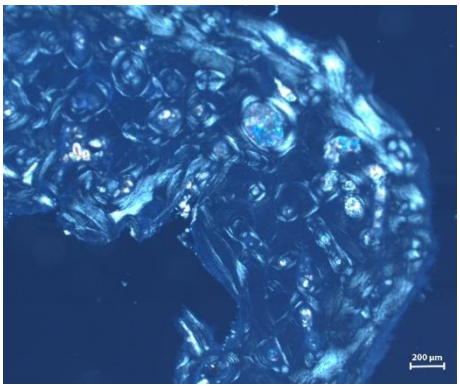
G2



G1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 30% acetic acid for 1 month (Scale bar: 200µm at 5x magnification).
G2: Bone morphology photograph of bone exposed to 30% acetic acid for 1 month.

H1

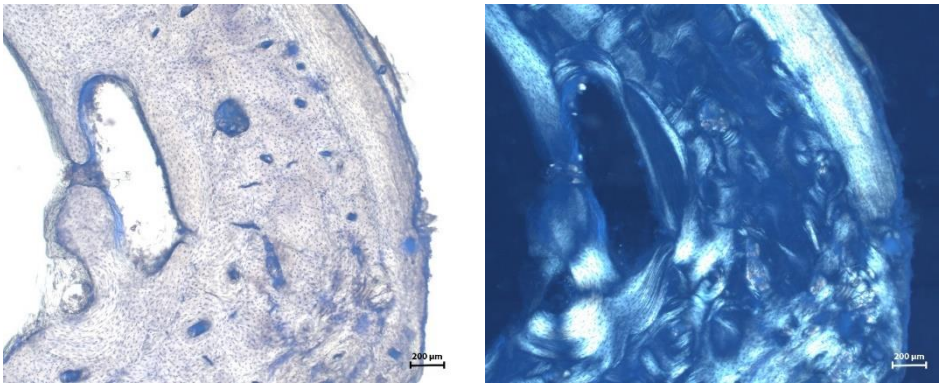




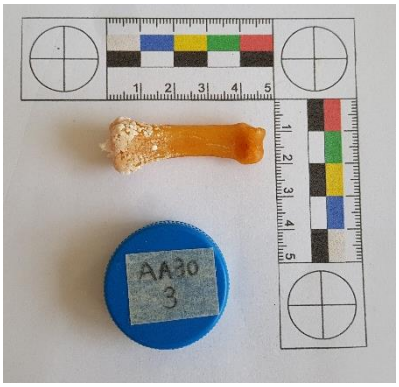
H2

H1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 30% acetic acid for 2 months (Scale bar: 200µm at 5x magnification).
H2: Bone morphology photograph of bone exposed to 30% acetic acid for 2 months.

I1



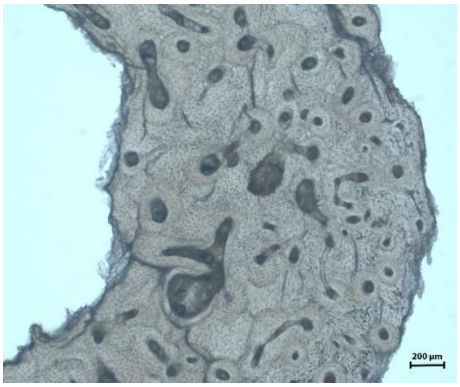
I2

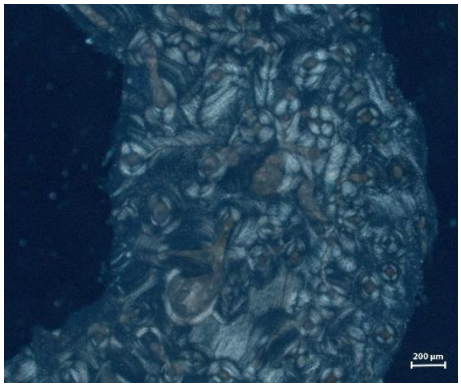


I1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 30% acetic acid for 3 months (Scale bar: 200µm at 5x magnification).
I2: Bone morphology photograph of bone exposed to 30% acetic acid for 3 months.

1.2 Ammonia

A1

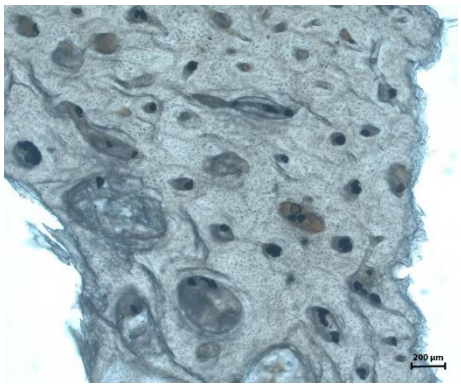


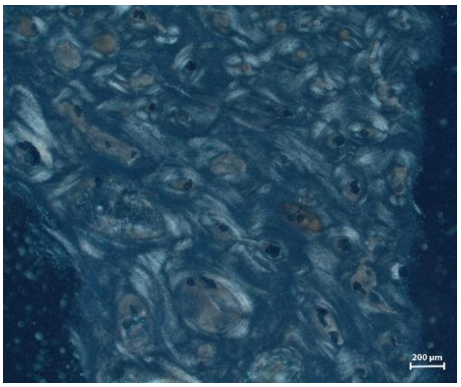


A2

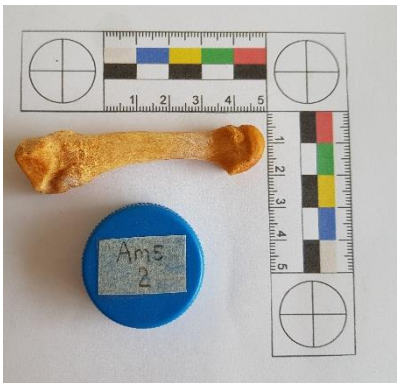
A1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 5% ammonia for 1 month (Scale bar: 200µm at 5x magnification).
A2: Bone morphology photograph of bone exposed to 5% ammonia for 1 month.

B1



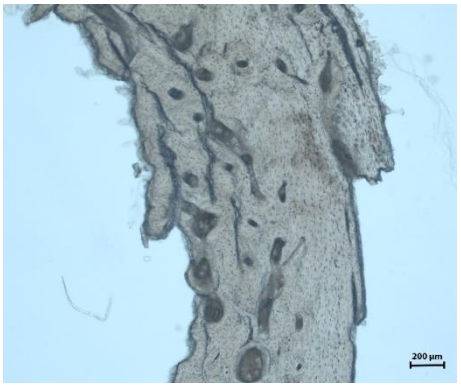


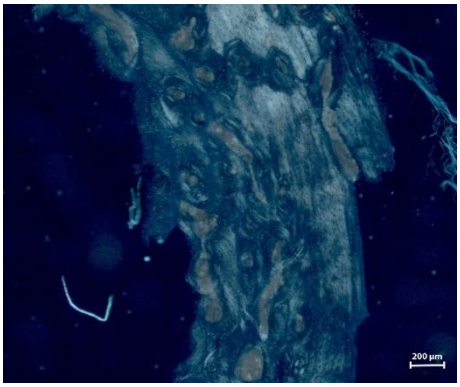
B2



B1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 5% ammonia for 2 months (Scale bar: 200µm at 5x magnification).
B2: Bone morphology photograph of bone exposed to 5% ammonia for 3 months.

C1

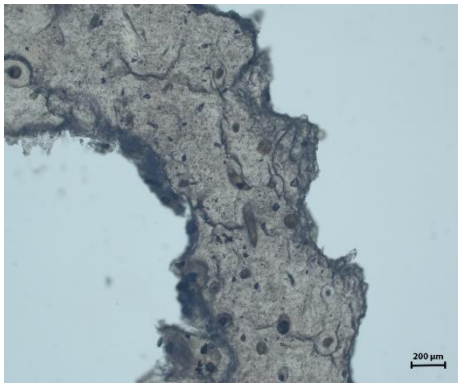


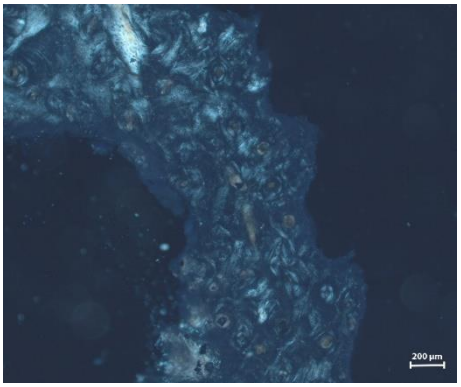


C2

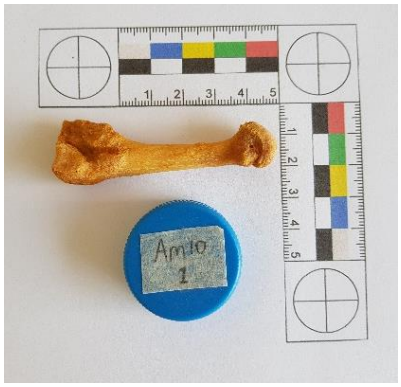
C1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 5% ammonia for 3 months (Scale bar: 200µm at 5x magnification).
C2: Bone morphology photograph of bone exposed to 5% ammonia for 3 months.

D1



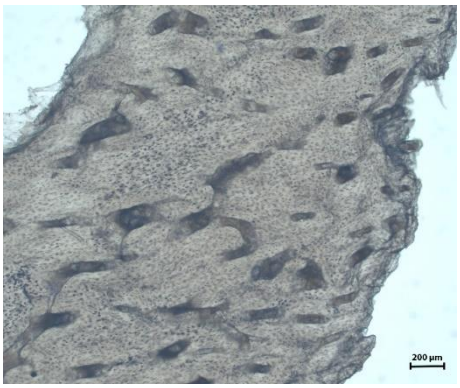


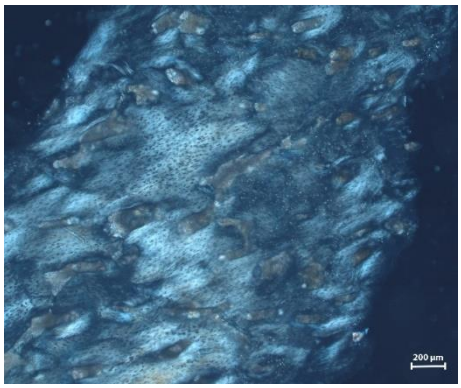
D2



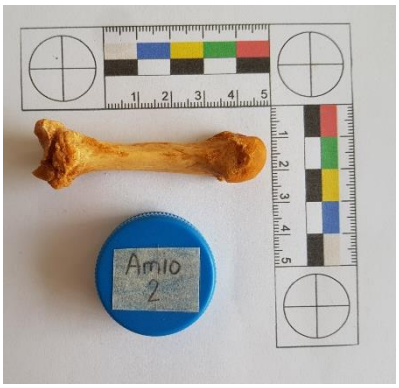
D1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 10% ammonia for 1 month (Scale bar: 200µm at 5x magnification).
D2: Bone morphology photograph of bone exposed to 10% ammonia for 1 month.

E1



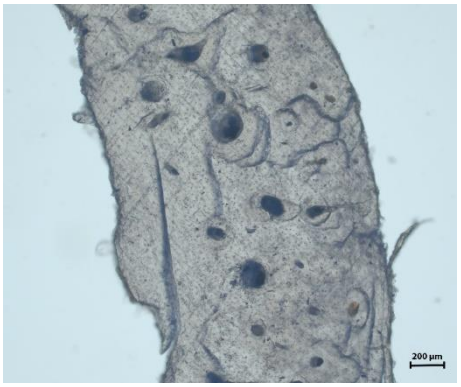


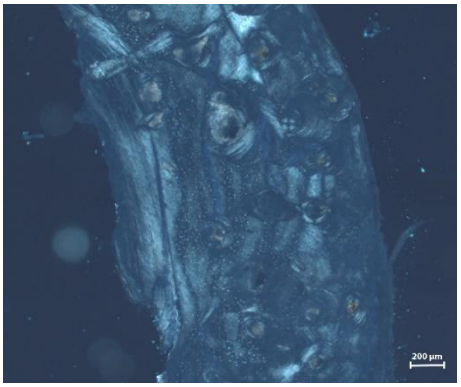
E2



E1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 10% ammonia for 2 months (Scale bar: 200µm at 5x magnification).
E2: Bone morphology photograph of bone exposed to 10% ammonia for 2 months.

F1

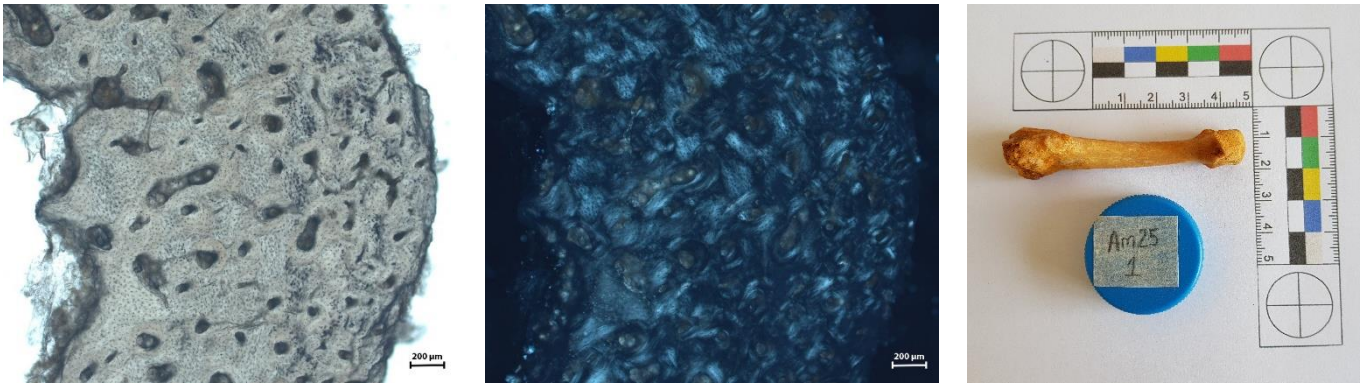




F2

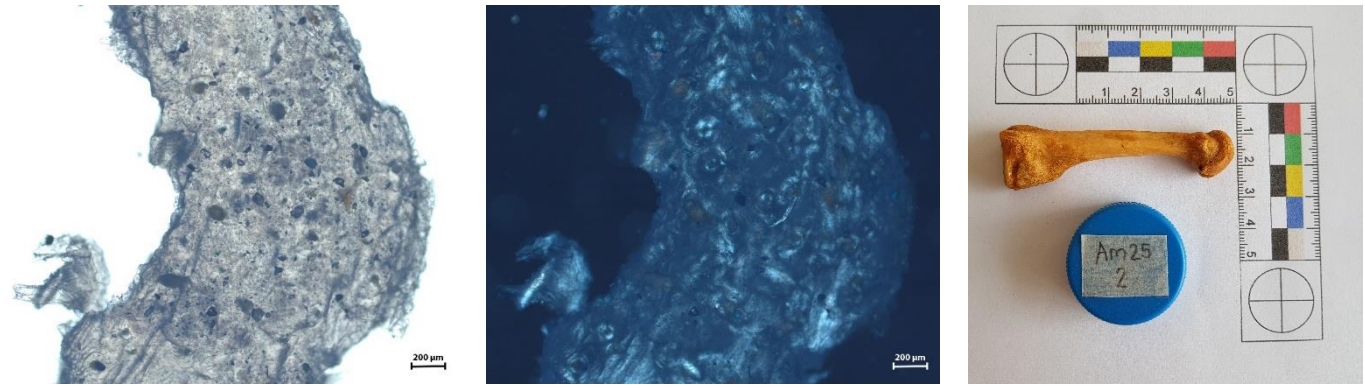
F1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 10% ammonia for 3 months (Scale bar: 200µm at 5x magnification).
F2: Bone morphology photograph of bone exposed to 10% ammonia for 3 months.

G1 G2



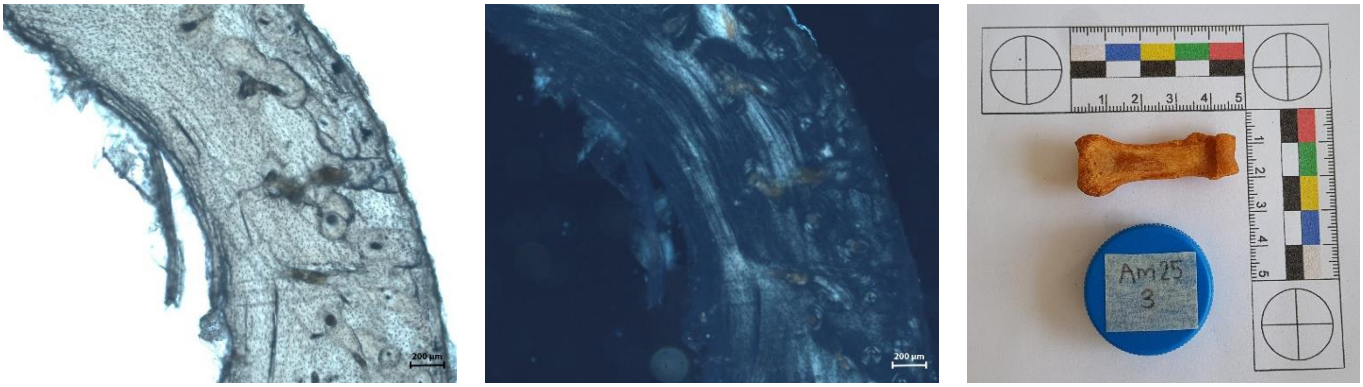
G1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 25% ammonia for 1 month (Scale bar: 200µm at 5x magnification).
G2: Bone morphology photograph of bone exposed to 25% ammonia for 1 month.

H1 H2



H1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 25% ammonia for 2 months (Scale bar: 200µm at 5x magnification).
H2: Bone morphology photograph of bone exposed to 25% ammonia for 2 months.

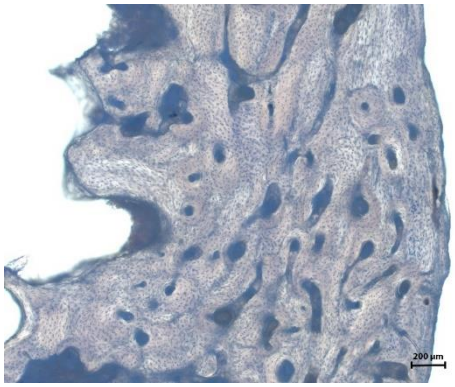
I1 I2

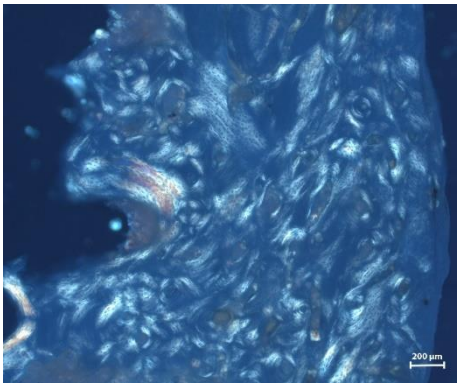


I1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 25% ammonia for 3 months (Scale bar: 200µm at 5x magnification).
I2: Bone morphology photograph of bone exposed to 25% ammonia for 3 months.

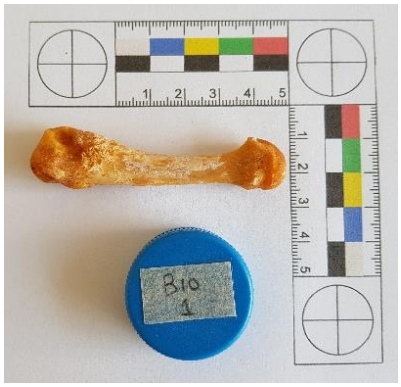
1.3 Bleach

A1



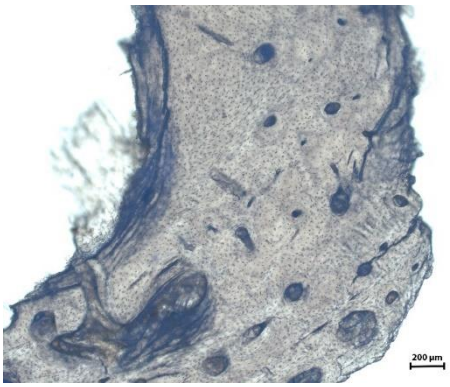


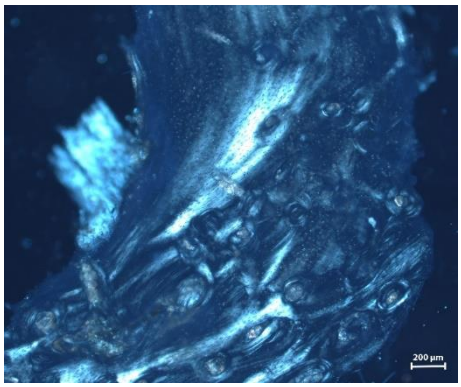
A2



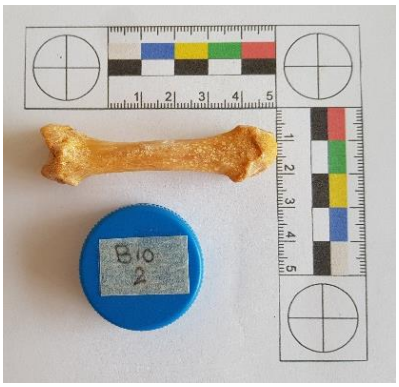
A1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 10% bleach for 1 month (Scale bar: 200µm at 5x magnification).
A2: Bone morphology photograph of bone exposed to 10% bleach for 1 month.

B1



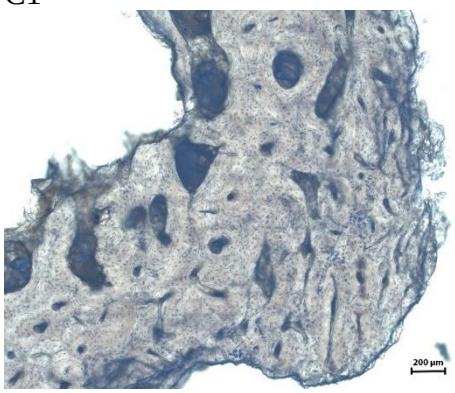


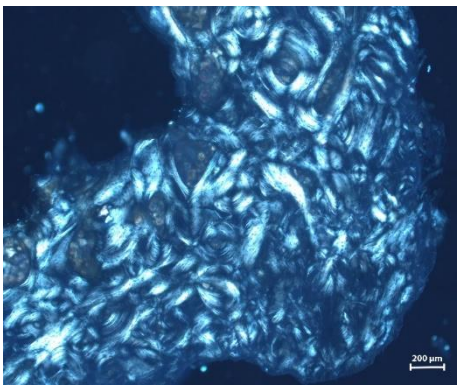
B2



B1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 10% bleach for 2 months (Scale bar: 200µm at 5x magnification).
B2: Bone morphology photograph of bone exposed to 10% bleach for 2 months.

C1

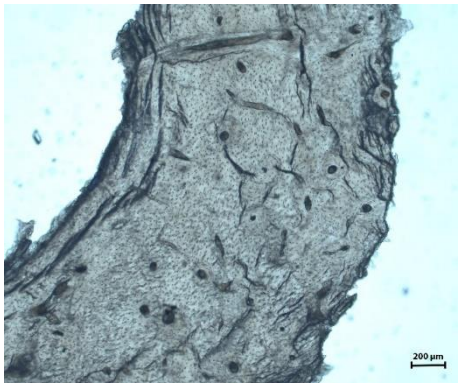
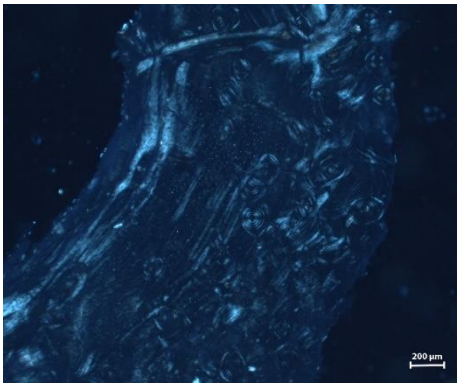
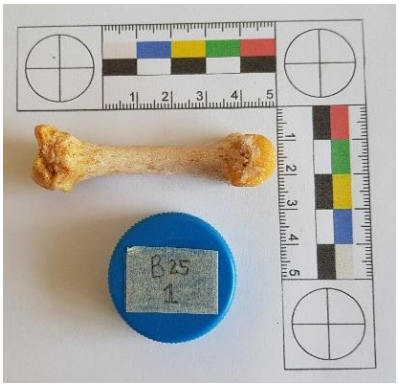




C2

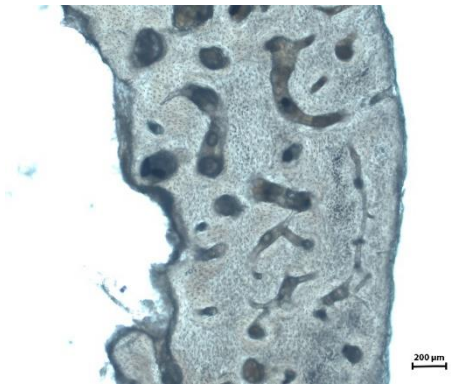
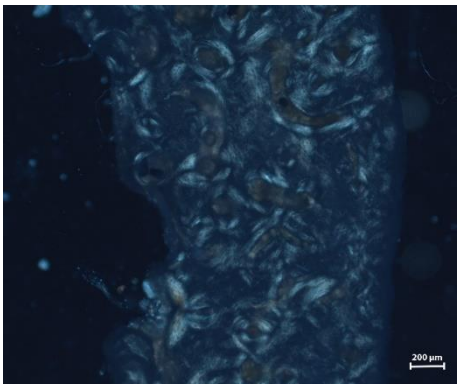
C1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 10% bleach for 3 months (Scale bar: 200µm at 5x magnification).
C2: Bone morphology photograph of bone exposed to 10% bleach for 3 months.

D1 D2

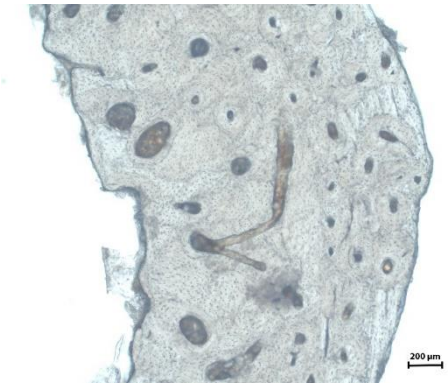
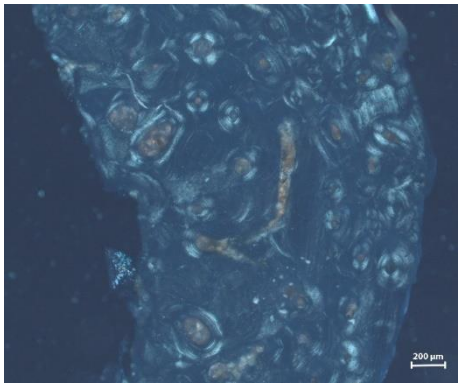
D1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 25% bleach for 1 month (Scale bar: 200µm at 5x magnification).
D2: Bone morphology photograph of bone exposed to 25% bleach for 1 month.

E1 E2

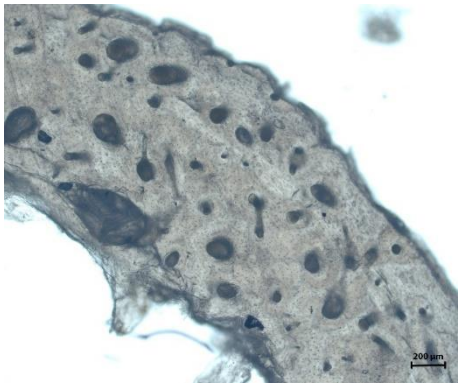

E1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 25% bleach for 2 months (Scale bar: 200µm at 5x magnification).
E2: Bone morphology photograph of bone exposed to 25% bleach for 2 months.

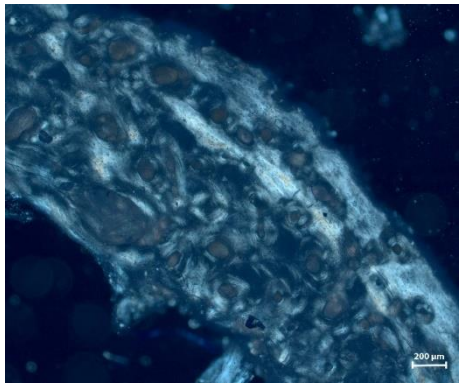
F1 F2


F1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 25% bleach for 3 months (Scale bar: 200µm at 5x magnification).
F2: Bone morphology photograph of bone exposed to 25% bleach for 3 months.

G1

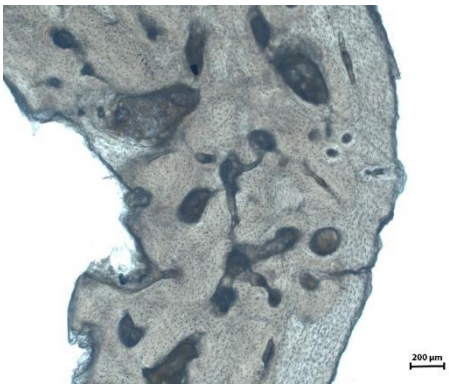


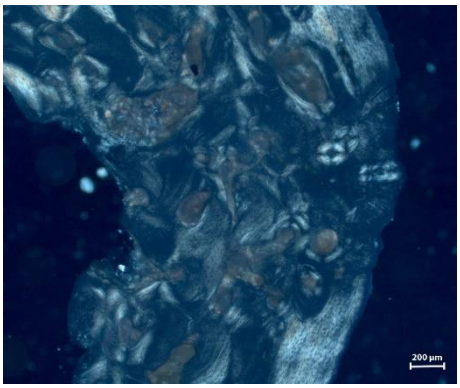


G2

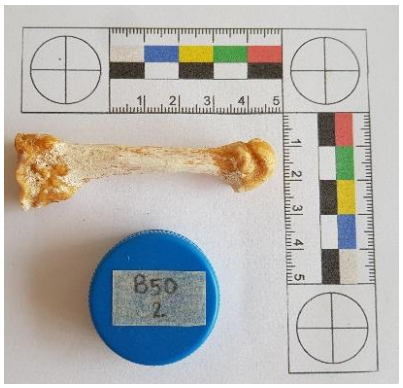
G1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 50% bleach for 1 month (Scale bar: 200µm at 5x magnification).
G2: Bone morphology photograph of bone exposed to 50% bleach for 1 month.

H1



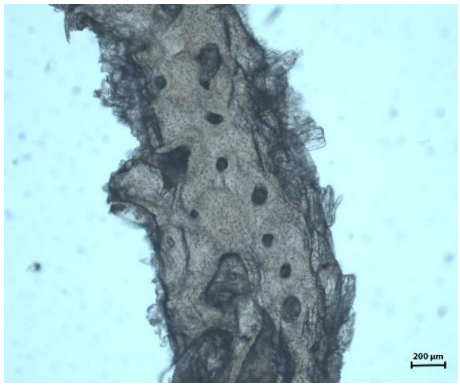


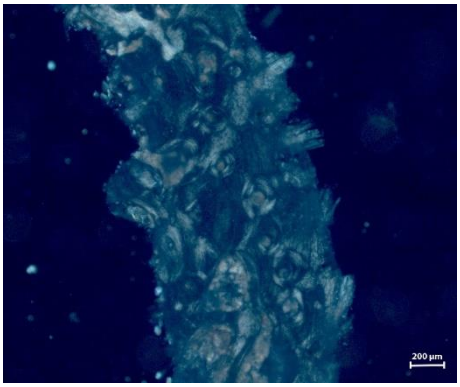
H2



H1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 50% bleach for 2 months (Scale bar: 200µm at 5x magnification).
H2: Bone morphology photograph of bone exposed to 50% bleach for 2 months.

I1



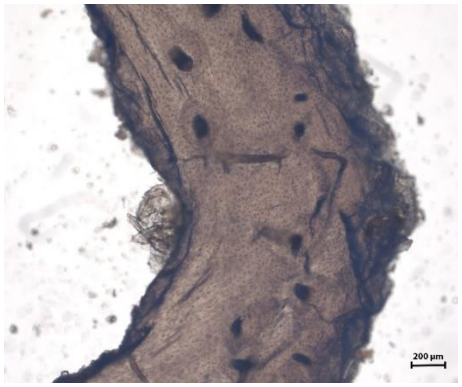


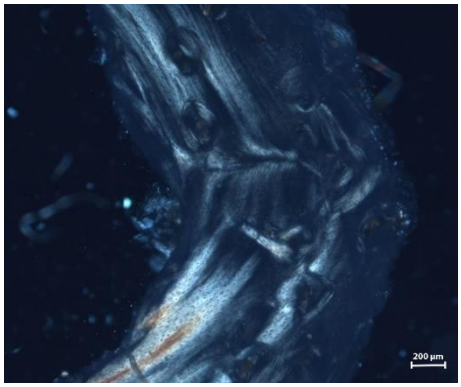
I2

I1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 50% bleach for 3 months (Scale bar: 200µm at 5x magnification).
I2: Bone morphology photograph of bone exposed to 50% bleach for 3 months.

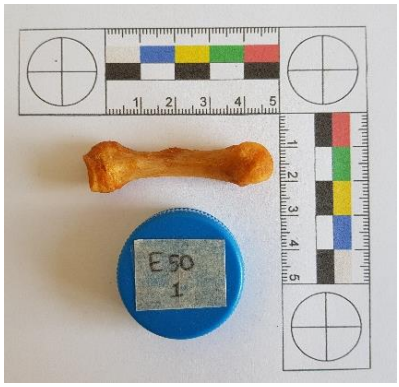
1.4 Ethanol

A1



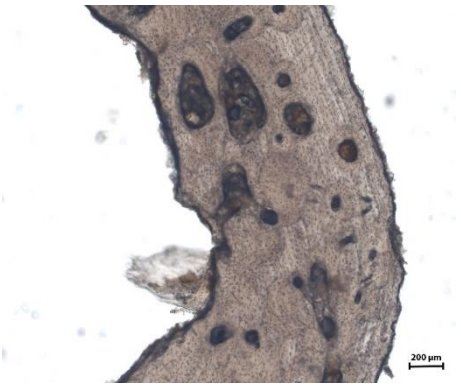


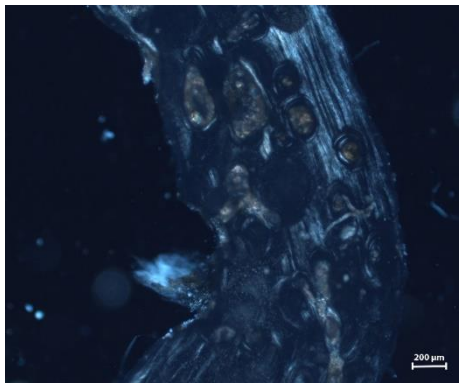
A2



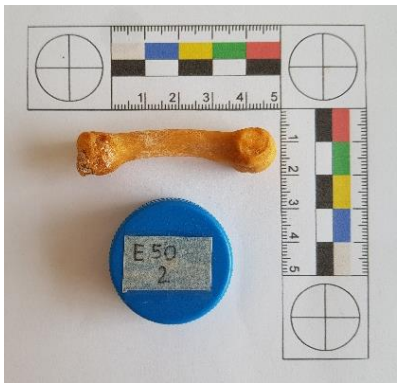
A1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 50% ethanol for 1 month (Scale bar: 200µm at 5x magnification).
A2: Bone morphology photograph of bone exposed to 50% ethanol for 1 month.

B1



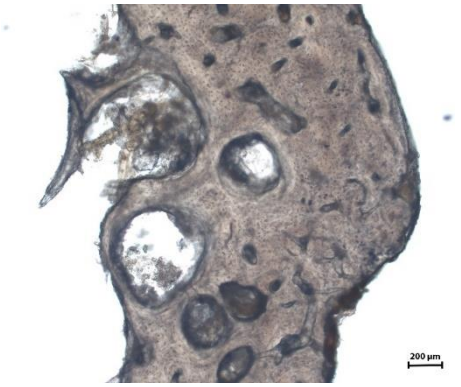


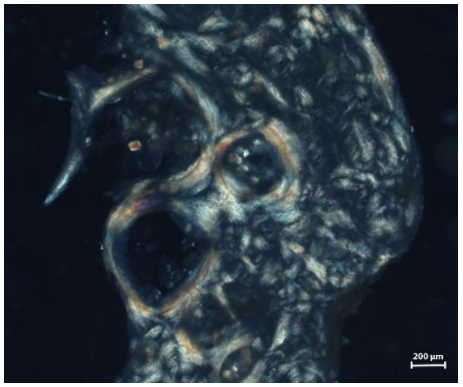
B2



B1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 50% ethanol for 2 months (Scale bar: 200µm at 5x magnification).
B2: Bone morphology photograph of bone exposed to 50% ethanol for 3 months.

C1

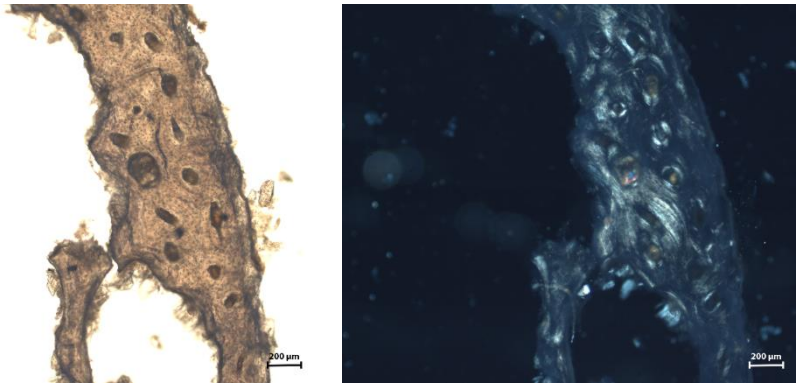




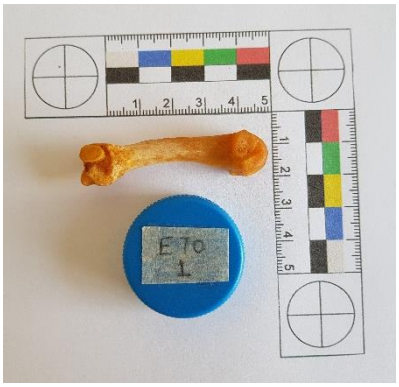
C2

C1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 50% ethanol for 3 months (Scale bar: 200µm at 5x magnification).
C2: Bone morphology photograph of bone exposed to 50% ethanol for 3 months.

D1

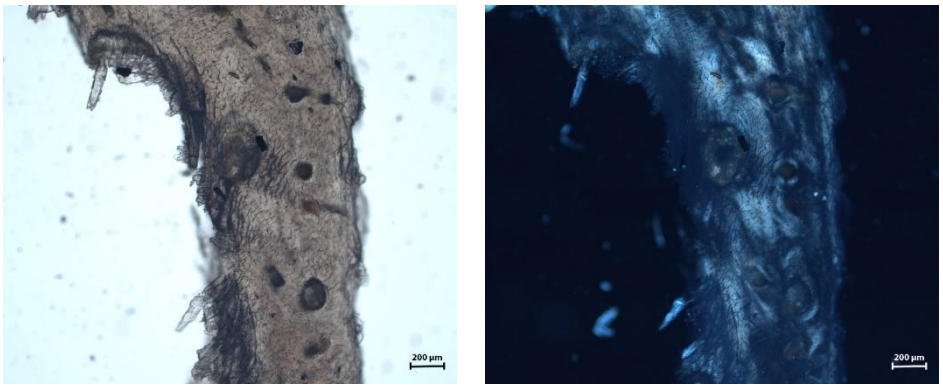


D2



D1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 70% ethanol for 1 month (Scale bar: 200µm at 5x magnification).
D2: Bone morphology photograph of bone exposed to 70% ethanol for 1 month.

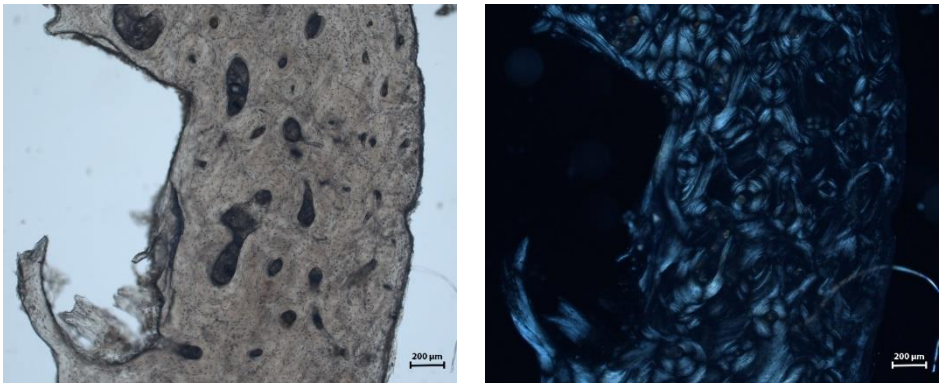
E1



E2

E1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 70% ethanol for 2 months (Scale bar: 200µm at 5x magnification).
E2: Bone morphology photograph of bone exposed to 70% ethanol for 3 months.

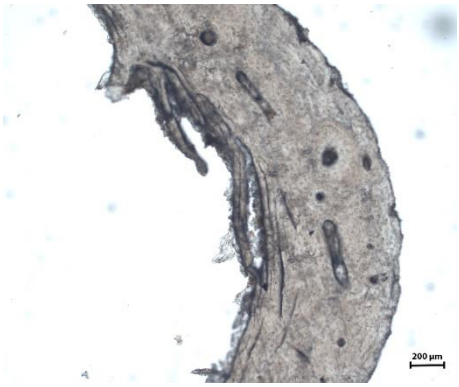
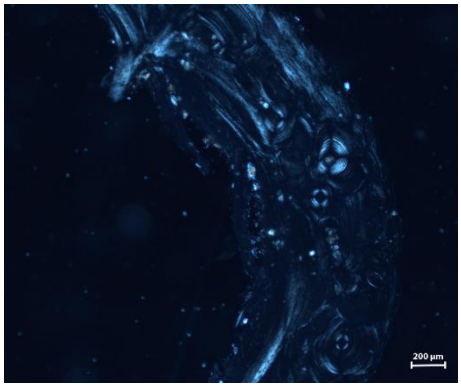
F1



F2

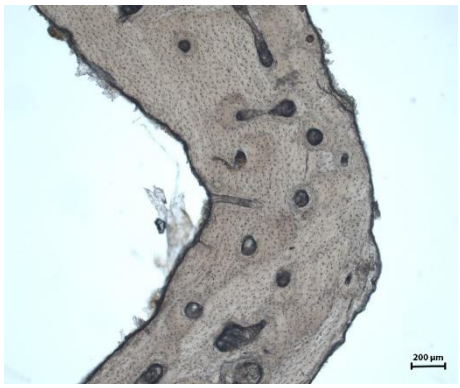
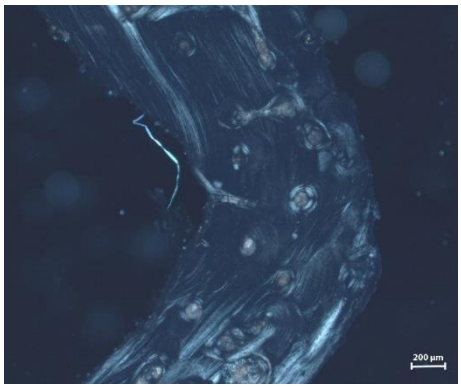
F1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 70% ethanol for 3 months (Scale bar: 200µm at 5x magnification).
F2: Bone morphology photograph of bone exposed to 70% ethanol for 3 months.

G1 G2

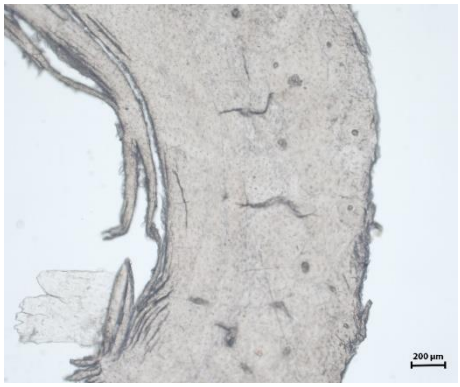
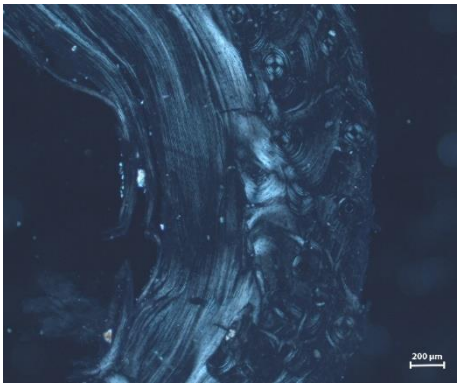
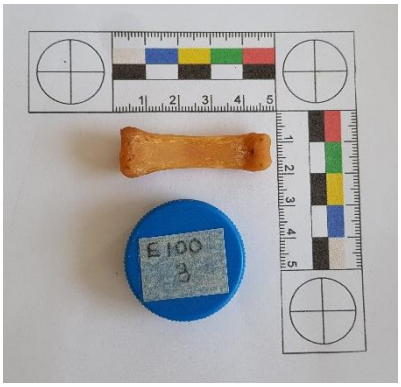

G1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 100% ethanol for 1 month (Scale bar: 200µm at 5x magnification).
G2: Bone morphology photograph of bone exposed to 100% ethanol for 1 month.

H1 H2


H1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 100% ethanol for 2 months (Scale bar: 200µm at 5x magnification).
H2: Bone morphology photograph of bone exposed to 100% ethanol for 2 months.

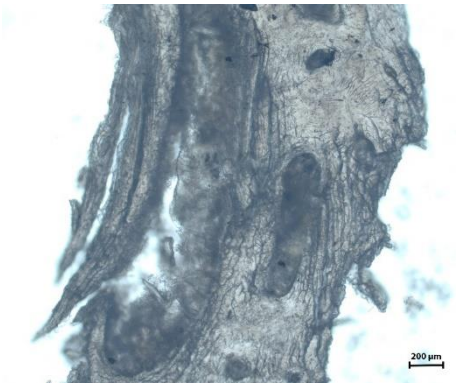
I1 I2

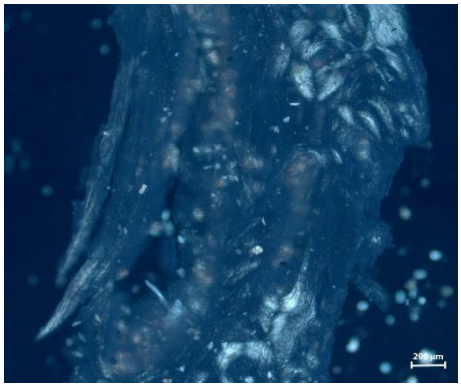




I1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 100% ethanol for 3 months (Scale bar: 200µm at 5x magnification).
I2: Bone morphology photograph of bone exposed to 100% ethanol for 3 months.

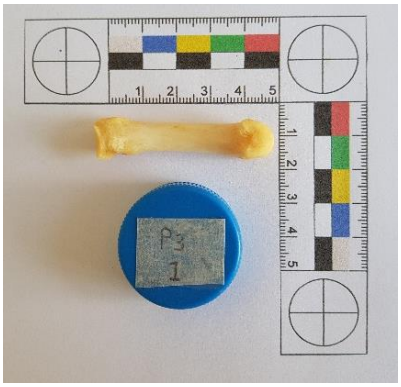
1.5 Hydrogen peroxide

A1



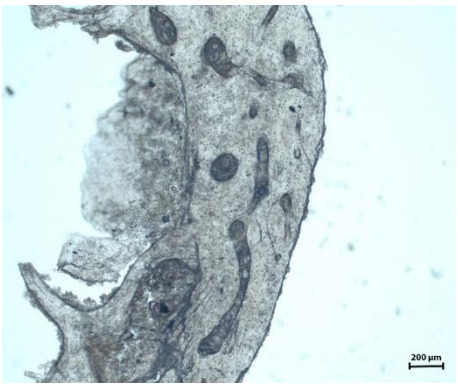


A2



A1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 3% peroxide for 1 month (Scale bar: 200µm at 5x magnification).
A2: Bone morphology photograph of bone exposed to 3% peroxide for 1 month.

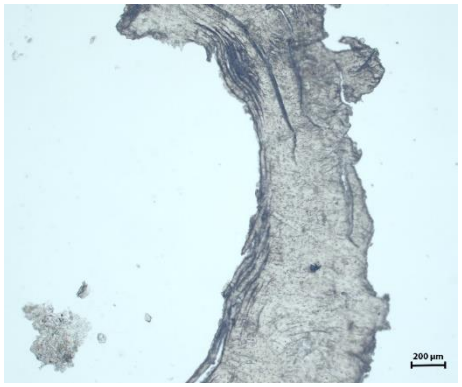
B1

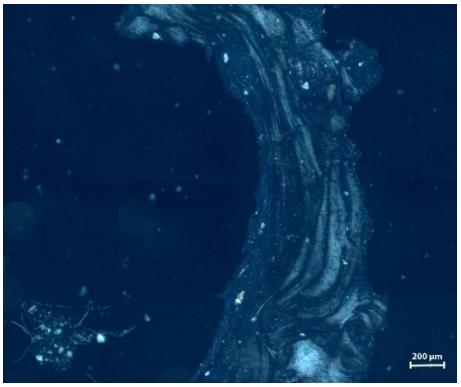


B2

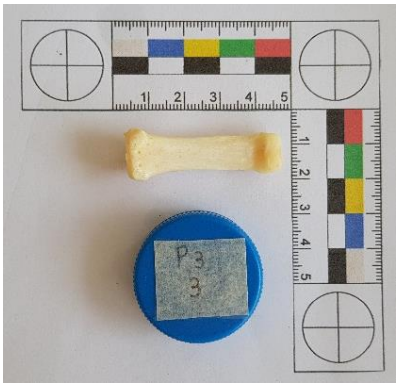
B1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 3% peroxide for 2 months (Scale bar: 200µm at 5x magnification).
B2: Bone morphology photograph of bone exposed to 3% peroxide for 2 months.

C1



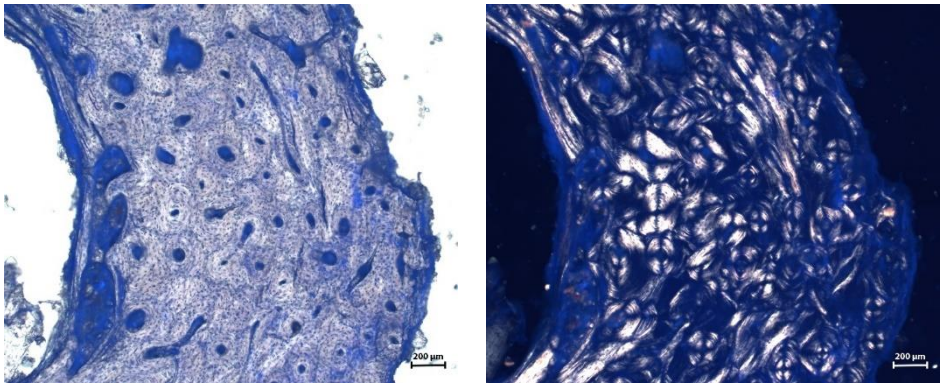


C2

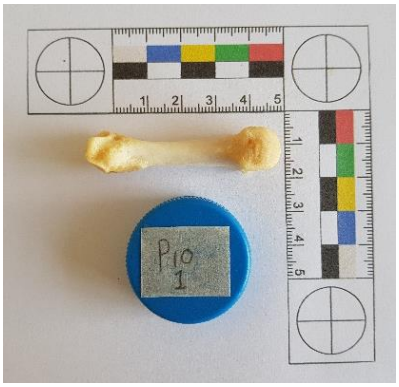


C1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 3% peroxide for 3 months (Scale bar: 200µm at 5x magnification).
C2: Bone morphology photograph of bone exposed to 3% peroxide for 3 months.

D1

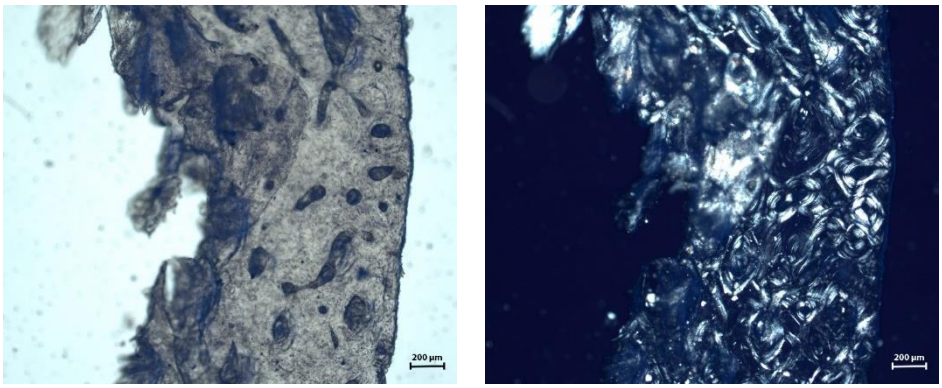


D2

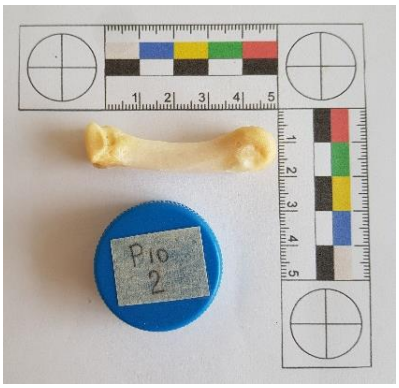


D1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 10% peroxide for 1 month (Scale bar: 200µm at 5x magnification).
D2: Bone morphology photograph of bone exposed to 10% peroxide for 1 month.

E1

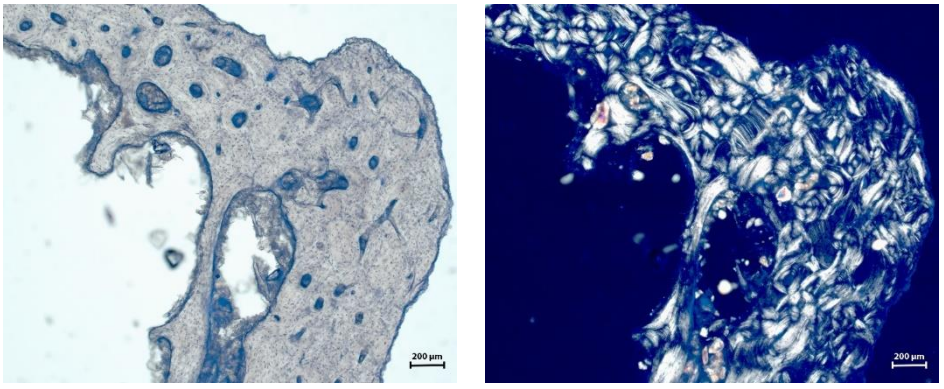


E2



E1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 10% peroxide for 2 months (Scale bar: 200µm at 5x magnification).
E2: Bone morphology photograph of bone exposed to 10% peroxide for 2 months.

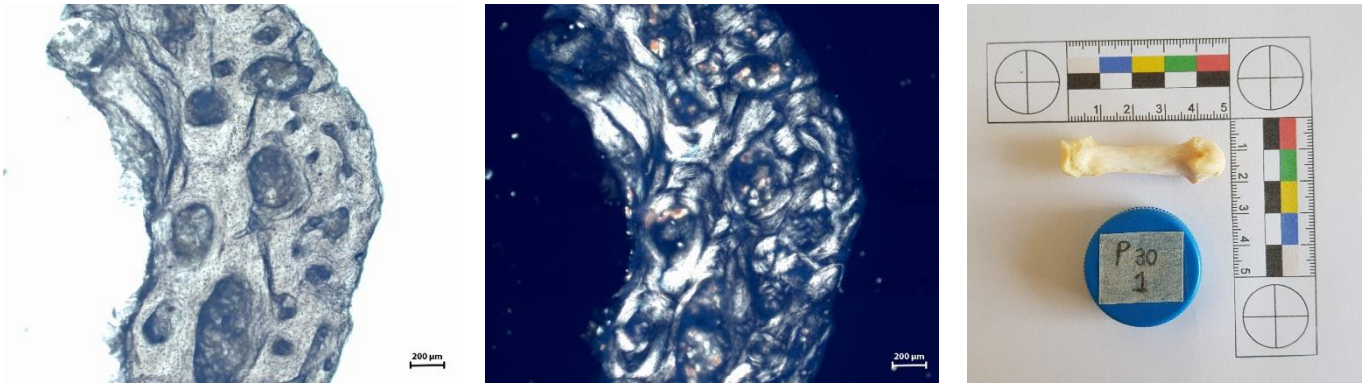
F1



F2

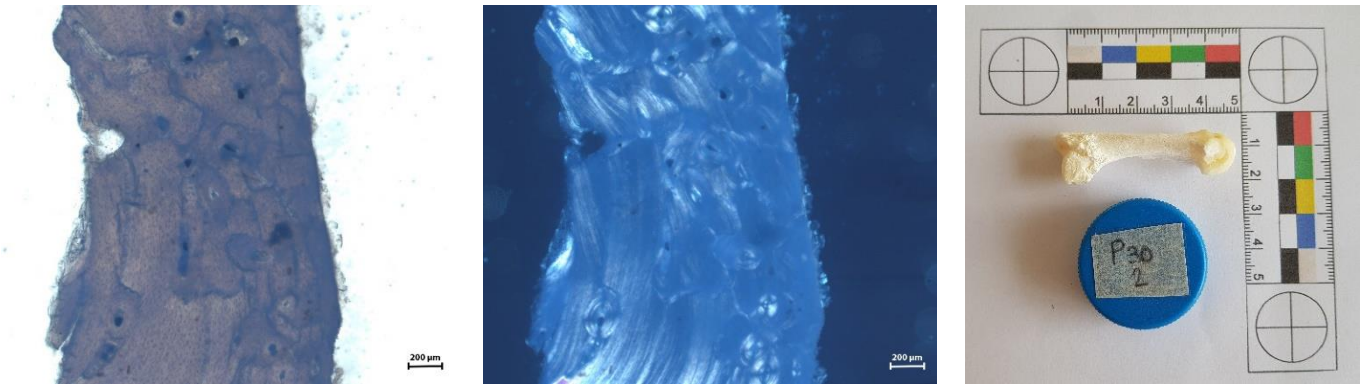
F1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 10% peroxide for 3 months (Scale bar: 200µm at 5x magnification).
F2: Bone morphology photograph of bone exposed to 10% peroxide for 3 months.

G1 G2



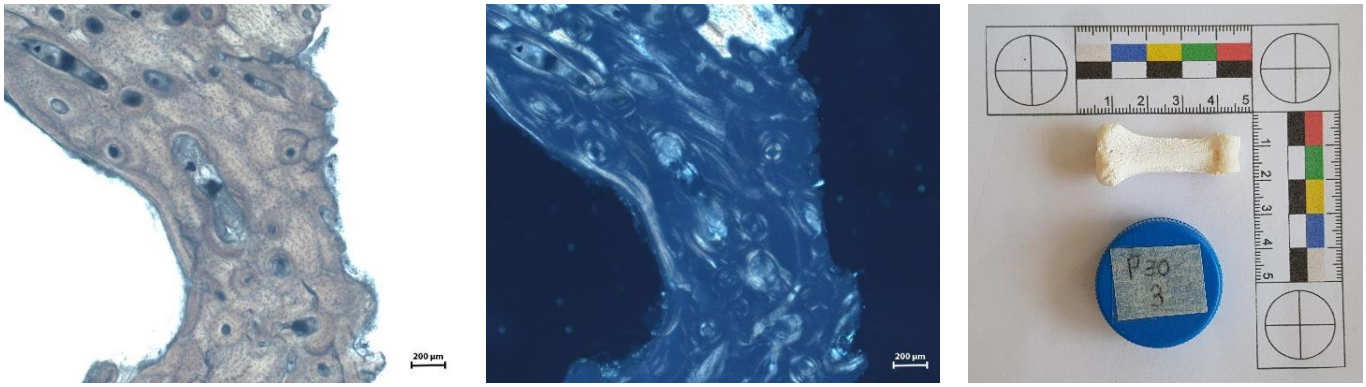
G1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 30% peroxide for 1 month (Scale bar: 200µm at 5x magnification).
G2: Bone morphology photograph of bone exposed to 30% peroxide for 1 month.

H1 H2



H1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 30% peroxide for 2 months (Scale bar: 200µm at 5x magnification).
H2: Bone morphology photograph of bone exposed to 30% peroxide for 2 months.

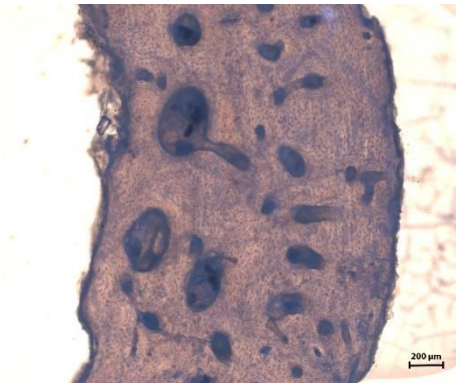
I1 I2

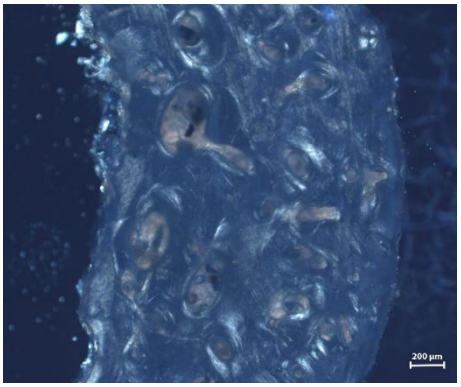


I1: Light microscopy micrograph and polarised micrograph of bone slide exposed to 30% peroxide for 3 months (Scale bar: 200µm at 5x magnification).
I2: Bone morphology photograph of bone exposed to 30% peroxide for 3 months.

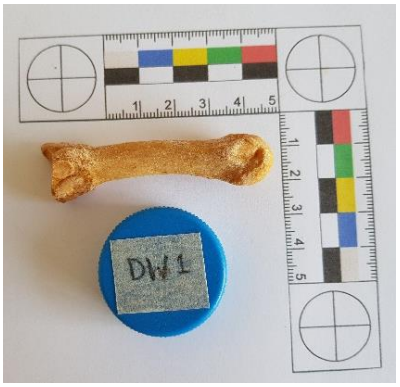
1.6 Distilled water (negative control)

A1



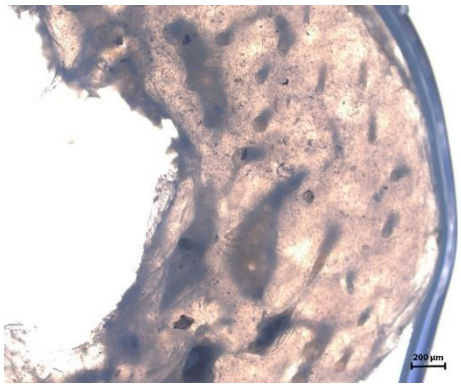


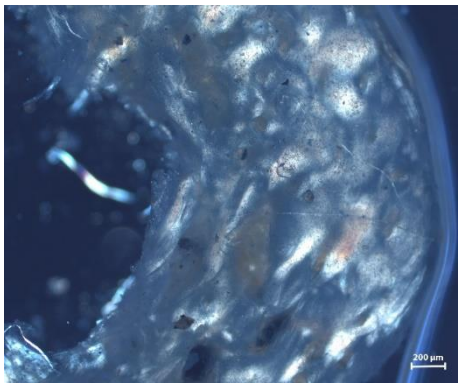
A2



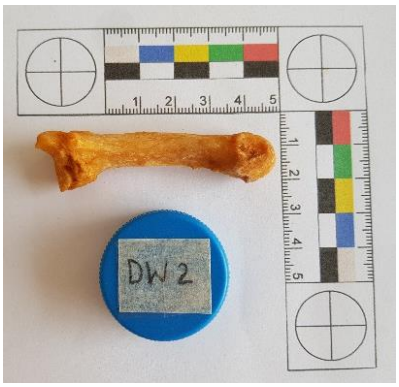
A1: Light microscopy micrograph and polarised micrograph of bone slide exposed to distilled water for 1 month (Scale bar: 200µm at 5x magnification).
A2: Bone morphology photograph of bone exposed to distilled water for 1 month.

B1



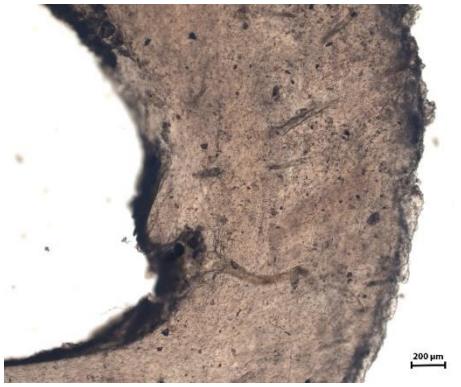


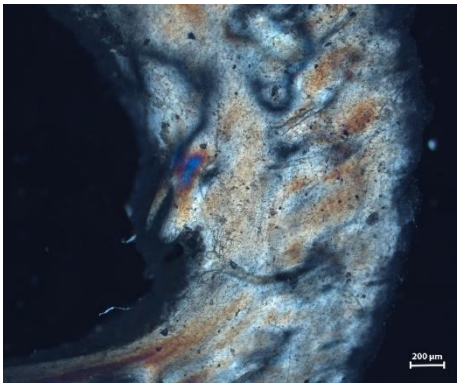
B2



B1: Light microscopy micrograph and polarised micrograph of bone slide exposed to distilled water for 2 months (Scale bar: 200µm at 5x magnification).
B2: Bone morphology photograph of bone exposed to distilled water for 2 months.

C1



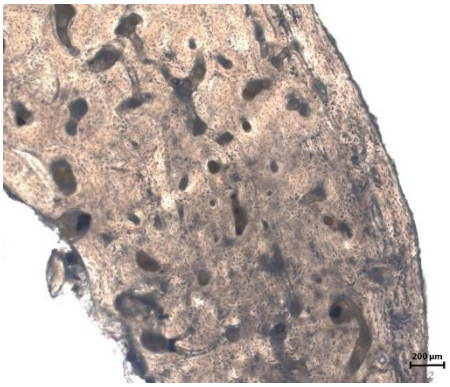


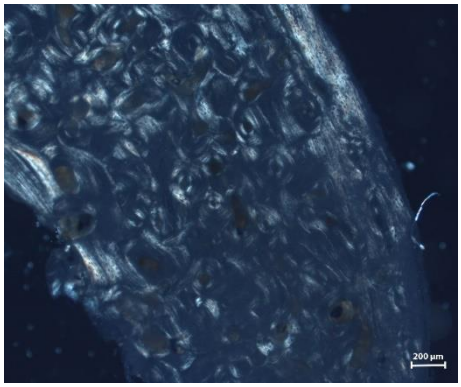
C2

C1: Light microscopy micrograph and polarised micrograph of bone slide exposed to distilled water for 3 months (Scale bar: 200µm at 5x magnification).
C2: Bone morphology photograph of bone exposed to distilled water for 3 months.

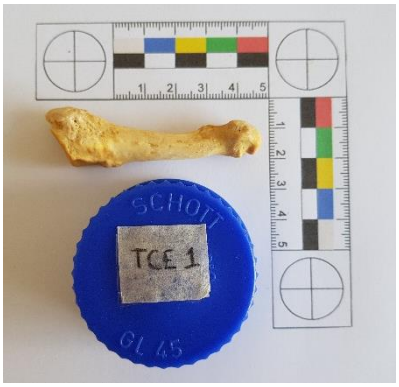
1.7 TCE (positive control)

A1



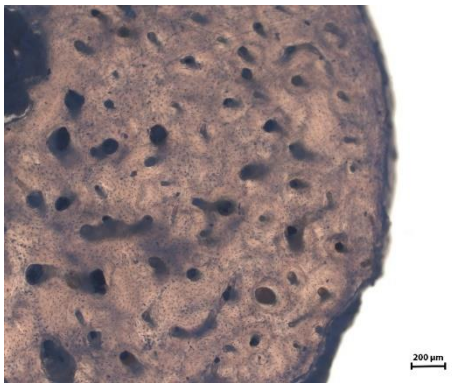


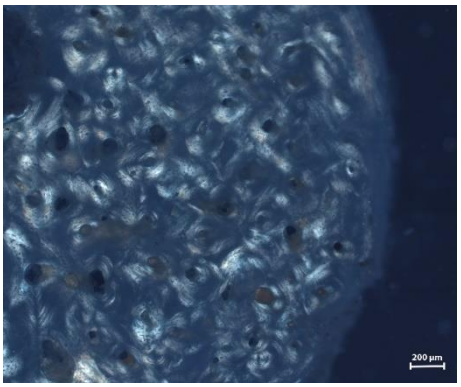
A2



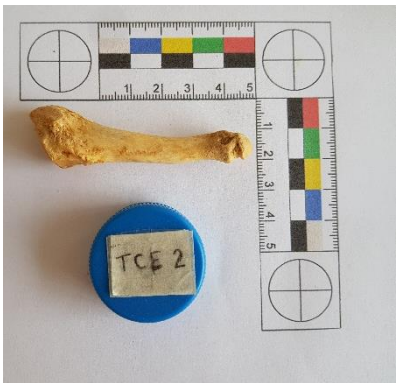
A1: Light microscopy micrograph and polarised micrograph of bone slide exposed to TCE for 1 month (Scale bar: 200µm at 5x magnification).
A2: Bone morphology photograph of bone exposed to TCE for 1 month.

B1



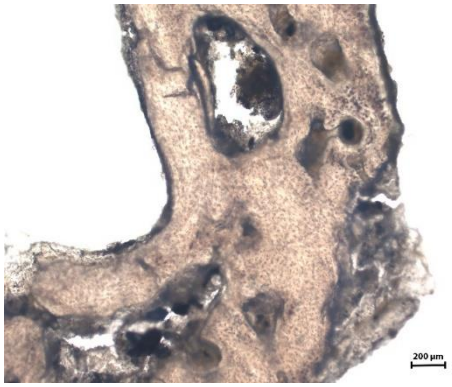


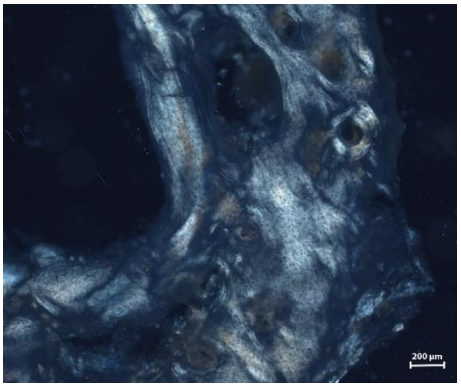
B2



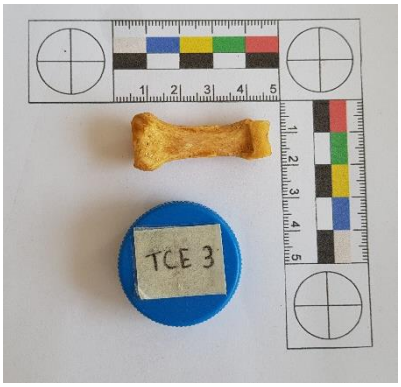
B1: Light microscopy micrograph and polarised micrograph of bone slide exposed to TCE for 2 months (Scale bar: 200µm at 5x magnification).
B2: Bone morphology photograph of bone exposed to TCE for 2 months.

C1





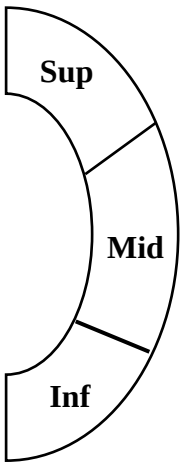
C2



C1: Light microscopy micrograph and polarised micrograph of bone slide exposed to TCE for 3 months (Scale bar: 200µm at 5x magnification).
C2: Bone morphology photograph of bone exposed to TCE for 3 months.

APPENDIX 2: Scoring sheets

KEY:			
Abbreviation	**Explanation**	**Abbreviation**	**Explanation**
DW1	Distilled water Month1	B10_3	Bleach 10% Month 3
DW2	Distilled water Month 2	B25_1	Bleach 25% Month 1
DW3	Distilled water Month 3	B25_2	Bleach 25% Month 2
TCE1	Trichloroethylene Month 1	B25_3	Bleach 25% Month 3
TCE2	Trichloroethylene Month 2	B50_1	Bleach 50% Month 1
TCE3	Trichloroethylene Month 2	B50_2	Bleach 50% Month 2
AA3_1	Acetic acid 3% Month 1	B50_3	Bleach 50% Month 3
AA3_2	Acetic acid 3% Month 2	E50_1	Ethanol 50% Month 1
AA3_3	Acetic acid 3% Month 3	E50_2	Ethanol 50% Month 2
AA10_1	Acetic acid 10% Month 1	E50_3	Ethanol 50% Month 3
AA10_2	Acetic acid 10% Month 2	E70_1	Ethanol 70% Month 1
AA10_3	Acetic acid 10% Month 3	E70_2	Ethanol 70% Month 2
AA30_1	Acetic acid 30% Month 1	E70_3	Ethanol 70% Month 3
AA30_2	Acetic acid 30% Month 2	E100_1	Ethanol 100% Month 1
AA30_3	Acetic acid 30% Month 3	E100_2	Ethanol 100% Month 2
Am5_1	Ammonia 5% Month 1	E100_3	Ethanol 100% Month 3
Am5_2	Ammonia 5% Month 2	P3_1	Peroxide 3% Month 1
Am5_3	Ammonia 5% Month 3	P3_2	Peroxide 3% Month 2
Am10_1	Ammonia 10% Month 1	P3_3	Peroxide 3% Month 3
Am10_2	Ammonia 10% Month 2	P10_1	Peroxide 10% Month 1
Am10_3	Ammonia 10% Month 3	P10_2	Peroxide 10% Month 2
Am25_1	Ammonia 25% Month 1	P10_3	Peroxide 10% Month 3
Am25_2	Ammonia 25% Month 2	P30_1	Peroxide 30% Month 1
Am25_3	Ammonia 25% Month 3	P30_2	Peroxide 30% Month 2
B10_1	Bleach 10% Month 1	P30_3	Peroxide 30% Month 3
B10_2	Bleach 10% Month 2		



KEY:

Bone scores as viewed with microscope.

Sup: Superior third of bone
Mid: Middle third of bone
Inf: Inferior third of bone

Date: 17/09/2020
Observer: MM

Chemical	Amount of microfractures			Flaking			Bone loss in cortex			Bone loss in medullary border			Overall morphological changes		
	SUP	MID	INF	SUP	MID	INF	SUP	MID	INF	SUP	MID	INF	SUP	MID	INF
DW1	0	0	0	0	0	1	0	0	0	1	1	2	1	0	1
DW2	0	0	0	1	0	1	1	0	0	1	1	1	1	0	1
DW3	0	0	0	1	1	0	0	0	0	0	1	0	0	1	0
TCE1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TCE2	0	0	0	0	1	0	0	1	1	1	1	1	0	0	0
TCE3	1	1	1	0	1	0	1	2	0	1	0	2	2	2	1
AA3_1	1	0	0	1	1	1	1	1	1	0	1	1	0	1	1
AA3_2	0	1	0	1	1	1	1	0	0	2	1	1	1	1	1
AA3_3	1	0	1	2	1	2	1	1	0	1	1	2	1	1	1
AA10_1	1	1	1	2	2	2	1	0	1	1	1	1	2	2	2
AA10_2	1	1	1	1	2	2	0	1	0	1	2	1	1	2	1
AA10_3	1	1	1	1	0	0	0	0	0	1	1	2	1	1	2
AA30_1	1	1	1	1	1	1	1	0	1	2	1	2	1	1	1
AA30_2	1	1	1	2	2	2	1	1	1	1	2	2	2	2	2
AA30_3	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1
Am5_1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1
Am5_2	1	1	1	1	1	2	0	1	2	1	1	2	1	2	2
Am5_3	1	1	1	1	1	0	1	2	1	1	2	1	1	2	1
Am10_1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2
Am10_2	1	0	0	1	2	1	1	2	1	1	1	1	1	2	1
Am10_3	1	1	1	1	1	1	1	1	1	1	1	2	1	1	2
Am25_1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Am25_2	1	1	1	2	1	2	2	2	1	2	1	2	2	2	2
Am25_3	1	1	1	2	1	1	2	1	0	1	1	1	2	2	1
B10_1	1	0	0	0	0	0	0	0	0	1	2	1	1	1	1
B10_2	1	1	1	1	1	1	0	1	1	1	1	2	1	1	2
B10_3	1	1	0	1	2	1	1	1	2	1	2	1	1	1	2
B25_1	1	1	1	2	1	2	1	1	1	1	1	1	1	2	1
B25_2	0	0	0	1	0	0	1	0	0	1	2	1	1	1	1
B25_3	1	1	1	1	0	2	2	1	1	2	1	1	2	1	1
B50_1	1	1	1	0	1	1	1	1	1	1	1	2	1	1	2
B50_2	1	1	0	0	0	0	0	1	0	1	2	2	1	1	1
B50_3	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2
E50_1	1	1	0	1	1	1	1	0	1	1	0	0	1	0	1
E50_2	0	0	0	1	1	1	1	0	1	0	1	0	1	1	1
E50_3	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1
E70_1	1	1	1	1	1	1	1	0	0	1	2	1	1	2	1
E70_2	1	1	1	2	1	2	1	2	2	1	2	1	2	2	2
E70_3	0	0	0	1	0	1	1	1	0	0	2	0	1	2	0
E100_1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1
E100_2	1	0	0	1	1	0	0	1	1	1	1	1	0	1	1
E100_3	1	1	1	1	1	1	0	1	1	1	1	1	2	1	1
P3_1	1	1	1	1	2	2	1	1	1	0	1	1	2	2	2
P3_2	1	1	1	1	1	1	0	0	0	1	2	2	1	1	2
P3_3	1	1	1	1	1	2	1	2	1	1	2	1	2	2	2
P10_1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1

P10_2	1	1	1	1	1	1	1	0	1	1	2	2	1	2	2
P10_3	0	0	0	1	1	1	1	2	1	2	2	2	2	2	2
P30_1	1	1	1	1	1	1	2	1	1	2	1	2	2	1	2
P30_2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	2
P30_3	1	1	1	1	2	1	2	2	2	1	1	2	2	2	2

Date: 20/10/2020
Observer: MM

Chemical	Amount of microfractures			Flaking			Bone loss in cortex			Bone loss in medullary border			Overall morphological changes		
	SUP	MID	INF	SUP	MID	INF	SUP	MID	INF	SUP	MID	INF	SUP	MID	INF
DW1	0	0	1	0	0	1	0	0	0	1	1	2	1	1	1
DW2	0	0	0	1	0	0	1	0	0	1	1	1	1	0	1
DW3	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0
TCE1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TCE2	0	0	0	0	1	1	0	0	1	1	1	1	0	0	0
TCE3	1	1	1	1	1	0	1	1	0	1	0	2	1	1	1
AA3_1	0	0	0	1	1	1	1	1	1	0	1	1	0	1	1
AA3_2	0	0	0	1	1	1	0	0	0	2	1	1	1	1	1
AA3_3	1	0	1	2	1	2	1	1	0	1	1	1	1	1	1
AA10_1	1	1	1	2	2	2	1	0	1	1	1	1	2	2	2
AA10_2	1	1	1	1	2	2	0	1	0	1	1	1	1	2	1
AA10_3	1	1	1	1	1	0	1	0	0	1	1	2	1	1	2
AA30_1	1	1	1	1	1	1	1	0	1	2	1	2	1	1	2
AA30_2	1	1	1	2	2	2	1	1	1	1	2	1	1	2	2
AA30_3	1	1	1	1	1	1	0	0	0	0	2	1	1	1	1
Am5_1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	1
Am5_2	1	1	1	1	1	2	0	1	2	1	1	2	0	2	2
Am5_3	1	1	1	1	1	1	1	2	1	1	2	1	1	2	1
Am10_1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2
Am10_2	1	0	0	1	2	1	1	2	1	1	1	1	1	1	1
Am10_3	1	1	1	1	1	1	1	1	1	1	1	2	1	1	2
Am25_1	0	1	1	2	1	2	1	1	1	1	1	1	1	1	1
Am25_2	1	1	1	2	1	1	2	1	1	2	2	2	2	2	2
Am25_3	1	1	1	2	1	1	2	1	1	1	1	1	2	1	1
B10_1	1	0	0	0	0	0	0	0	0	1	2	1	0	1	1
B10_2	1	1	1	1	1	1	0	1	1	1	1	2	1	1	1
B10_3	0	1	0	1	2	1	1	1	2	1	2	1	1	1	2
B25_1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1
B25_2	0	0	0	0	0	0	1	0	0	1	1	1	1	1	1
B25_3	1	1	1	1	1	1	1	1	1	2	1	1	2	1	1
B50_1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	2
B50_2	1	1	0	0	0	0	0	1	1	1	2	2	1	1	2
B50_3	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2
E50_1	1	1	0	1	1	0	1	0	1	1	1	0	1	0	1
E50_2	0	0	0	1	1	1	1	0	1	0	1	0	1	1	1
E50_3	0	0	0	0	0	1	0	1	0	1	1	1	1	1	1
E70_1	1	1	1	1	1	1	1	1	0	1	2	1	1	2	1
E70_2	1	1	1	2	1	2	2	2	2	1	2	1	2	2	2

E70_3	0	0	0	1	1	1	1	1	0	0	2	0	1	2	0
E100_1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1
E100_2	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1
E100_3	1	1	1	1	2	1	1	1	1	1	1	1	1	1	2
P3_1	0	1	1	1	2	2	1	1	1	0	1	1	2	2	2
P3_2	1	1	1	1	1	1	1	0	0	2	2	2	2	1	2
P3_3	1	1	1	1	1	2	1	2	1	1	2	2	2	2	2
P10_1	1	1	1	1	2	1	1	1	1	0	1	1	1	1	1
P10_2	1	1	1	1	1	2	1	1	1	1	2	2	1	2	2
P10_3	0	0	0	1	1	1	1	2	1	2	2	2	2	2	2
P30_1	1	1	1	1	1	1	1	1	1	2	1	2	2	1	2
P30_2	1	1	1	1	1	2	1	1	2	1	1	1	1	1	2
P30_3	1	1	1	1	2	1	2	2	2	1	1	1	1	2	2

APPENDIX 3: Meyer et. al. (unpublished honours report)

An exploratory study on the degreasing ability of various chemicals on long bones

ABSTRACT

Bone degreasing is a common and important technique used in Anatomy departments and forensic anthropology. Degreasing ensures that the remains can be safely stored and handled. Although important, the process of degreasing bone is challenging. In South Africa, TCE is commonly used to degrease bones. However, this chemical is a highly carcinogenic substance and various safety precautions need to be in place. The machinery used is specialized and there are a limited number of individuals with the necessary skills to run them. Finally, TCE and the machinery are very expensive and many institutions in South Africa are not in the financial position to make use of this technique. The need for a method that is as efficient as TCE but poses fewer health risks and is more affordable is greatly increasing.

This study therefore investigated alternative degreasing methods to see which of the chosen chemicals degreases the fastest, most effectively, with the least destruction of bone, while taking into consideration price and safety. The degreasing ability of five chemicals (ammonia, peroxide, bleach, acetic acid and ethanol) at various concentrations were tested by using cadaver metacarpals and scoring their abilities according to colour difference, bone morphology, odour, sediment layer and fatty layer formation and loss of bone mass. Although some of these chemicals such as ammonia and bleach did not seem efficient, peroxide showed the most promising results as a possible degreasing chemical in future.

LITERATURE REVIEW

Maceration is the act of removing all soft tissue from the bones for further examination and must be done without damaging or morphing the bone (Mairs *et al.* 2004). Maceration is done in laboratories either in the police department or at universities and these laboratories need to be equipped with all the necessary tools to perform macerations and protect the macerator. This equipment includes gloves, masks, gowns, and other tools such as macerating pots, scalpels, brushes and many more.

Various maceration techniques are used which include: hot water maceration, the manual removing of soft tissue using scissors, scalpels, brushes and/or knives and maceration using detergents (Mann & Berryman 2012). Often a combination of methods is used to macerate the remains e.g. hot or cold water maceration or detergent maceration is often followed by physical maceration to remove any remaining soft tissues. Each maceration method has its own benefits and drawbacks and various factors such as skeletal damage, time period for macerating, health and safety issues are to be taken into consideration when deciding on which technique to use (Mann & Berryman 2012).

After maceration is completed, the bone should be further degreased. Bone is porous, and bone marrow is greasy (Rowley 2015). If not degreased, the bones will remain a yellow colour and continue to leach a layer of oil over the entire specimen, making it difficult to work with and harming the structure of the bones over time. In order to prevent this, the oil must be drawn out from the bone marrow cavity. Maceration processes may act as a degreaser but is not sufficient to degrease bone completely (Rowley 2015). The boiling process will not always be able to eliminate the fats before mechanical degradation of the bone surfaces begin and therefore other degreasing agents have to be considered (Nawrocki 2006). The amount of grease present on the bones after maceration is completed, will depend on the maceration method used, as some of these methods are able to degrease the bones slightly. Chemicals such as TCE, ammonia and bleach are commonly used for degreasing in practice (Rowley 2015). These chemicals can be harmful to the bone or to the macerators health and great caution should be taken when using these chemicals.

MATERIALS AND METHODS

Fifty-one metacarpals from cadavers used in dissection was used as models in this study. The metacarpals were macerated by boiling in water and all soft tissue was removed whereafter they were allowed to dry prior to weighing and measuring. The metacarpals were measured and weighed and only metacarpals in a set range of length and weight were used to ensure validity of the study. Five chemicals were tested as degreasing agents, namely: acetic acid, bleach, peroxide, pure ammonia and ethanol. Each chemical was diluted to 3 different concentrations (v/v), as follows: acetic acid (3%, 10%, 30%), bleach (10%, 25%, 50%), peroxide (3%, 10%, 30%), pure ammonia (5%, 10%, 25%) and ethanol (50%, 70%, 100%).

Controls included: a positive control, TCE and a negative control, distilled water.

Bone mass was weighed prior to placing them in a tube filled with 45 mL solution of the selected chemical. These test tubes were stored uncovered in the cell biology laboratory at the University of Pretoria. The caps for acetic acid, bleach and ammonia were not turned on too tightly as a precautionary method because gas formation took place. After nine days, it was noted that some of the bones started floating on top of the solution. A small piece of plastic mesh was added to each test tube to prevent the bones from floating and keep the whole bone covered in solution. The bones were left unmoved and unhandled until it was time for scoring to take place.

After one month, the first set of bones were taken out of their test tubes using tweezers. These bones were placed in a tray lined with paper tissue. The remains were scored and weighed directly after taking them out of solution, as well as one week after they were taken out, to note any changes that occurred during that week.

This process was repeated after two months, where the set of bones that stayed in solution for two months were taken out and placed in a tray lined with paper tissue to dry. This set of bones were also scored and weighed directly after taking them out, as well as one week and two weeks after taking them out. A scoring system was used to evaluate the data and noted on a data collection sheet (Table 1)

Table 1: Summary of scoring method

Score	**Colour difference**	**Sediment layer**	**Fatty layer**	**Bone Morphology**	**Odour after drying**
1	Dark brown and sticky	Cloudy fluid	Clearly visible fatty layer present	Very brittle and fragile	A strong, unpleasant odour developed during the process and is still stuck on the bone
2	Light brown colour	Sediment layer is present	Vague fatty layer	Mostly brittle but intact	A bad, intolerable smell is produced.

			present		
3	Bright to dark yellow colour	No sediment layer present	No fatty layer present	Slightly brittle	An irritating odour is produced.
4	Light yellow colour			Slight change in morphology	A moderate scent is produced.
5	Completely white and clean			No change in bone morphology	A pleasant scent or no smell is produced.

After the bones were scored, they were covered with a sheet of paper tissue until the next scoring session took place to prevent any external factors influencing the bones. After the last scoring took place, each bone was placed in its own specifically marked sealable plastic bag and stored in a box.

The remaining fluid in the test tubes were filtered out into separate flasks for each chemical with filter paper to filter out any human tissue that remained in the test tubes. After filtration, the chemicals were disposed of in the correct manner following protocol- the bleach, acetic acid, ethanol and peroxide were carefully flushed down the drain while the ammonia was disposed of in a specific tank to be destroyed with other hazardous material. The used filter paper and paper tissue was disposed of in the correct manner, by discarding them into a bin in the dissection hall of the department of anatomy, together with other human tissue, where these materials will be disposed of accordingly as stated by the National Health Act.

RESULTS

Changes in bone variables in Month 1

The scores for all the various variables were combined in order to observe the combined score (Figure 1-2). A low score represents poor results in the five variables whereas a high score represents better results in the variables.

All three concentrations of peroxide have shown to have the best overall score at the end of month one, followed closely by TCE, while ammonia 25% showed to have the worst score. Ethanol 100% showed promising results, yet distilled water showed similar results to ethanol

70% and better results than ethanol 50%. All acetic acid and bleach concentrations showed similar overall results, with bleach 50% being the second lowest scored concentration.

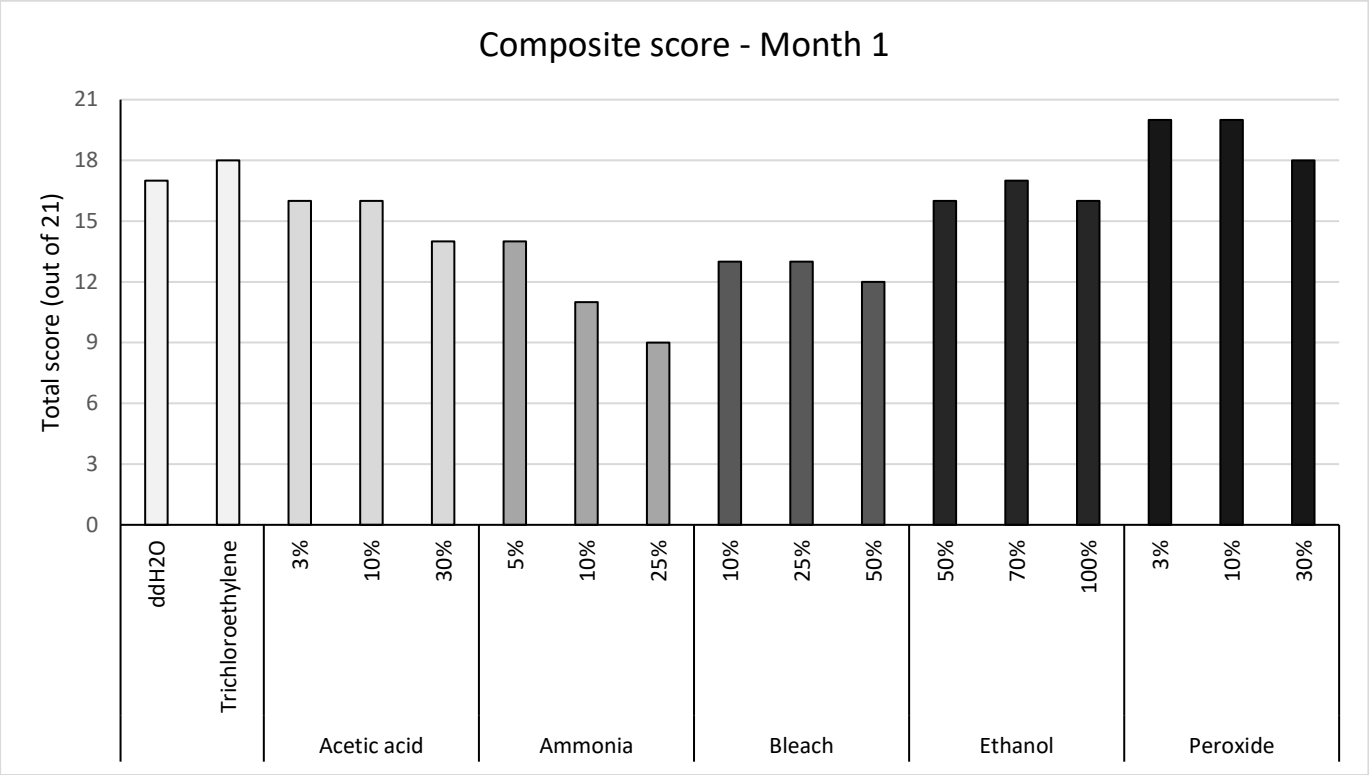


Figure 1: Month 1 combined score of colour difference, bone morphology, odour, sediment layer and fatty layer that occurred in the bones.

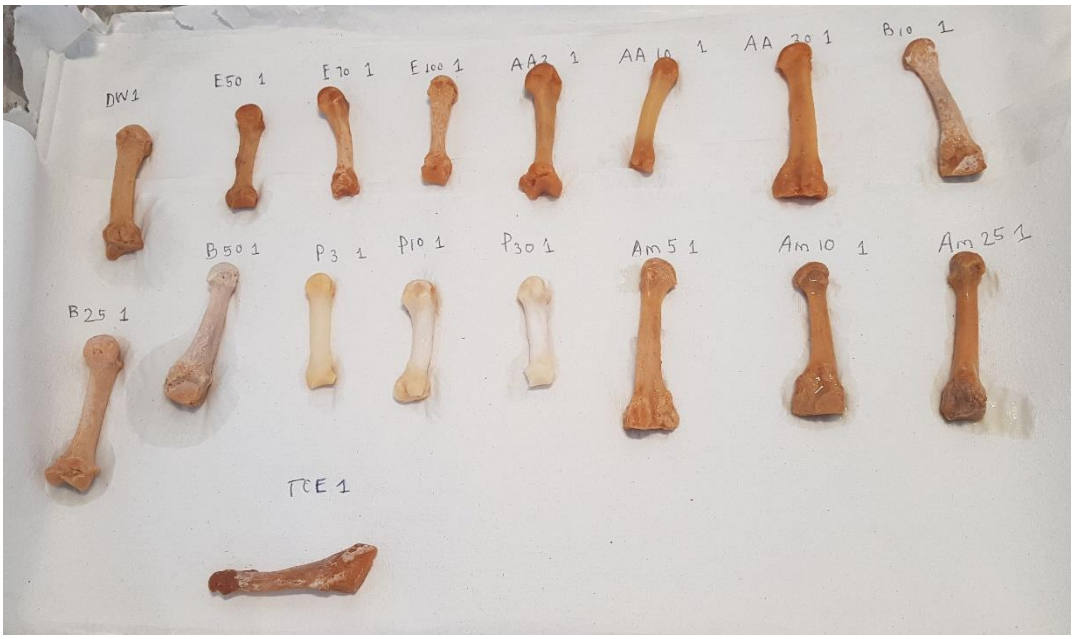


Figure 2: Results of bone changes after 1 month.

4.2. Changes in bone variables in Month 2

The scores for all the various variables were combined in order to observe the combined score (Figure 3-4). A low score indicates poor results in the five variables whereas a high score indicates better results in the variables.

After two months drenched in chemicals, peroxide 3% and peroxide 10% still degreased the most efficiently, with TCE and ethanol 50% following closely. Acetic acid 3%, acetic acid 10%, distilled water and peroxide 30% presented with similar overall scores. Acetic acid 30%, all ammonia concentrations and all bleach concentrations, degreased the least efficiently.

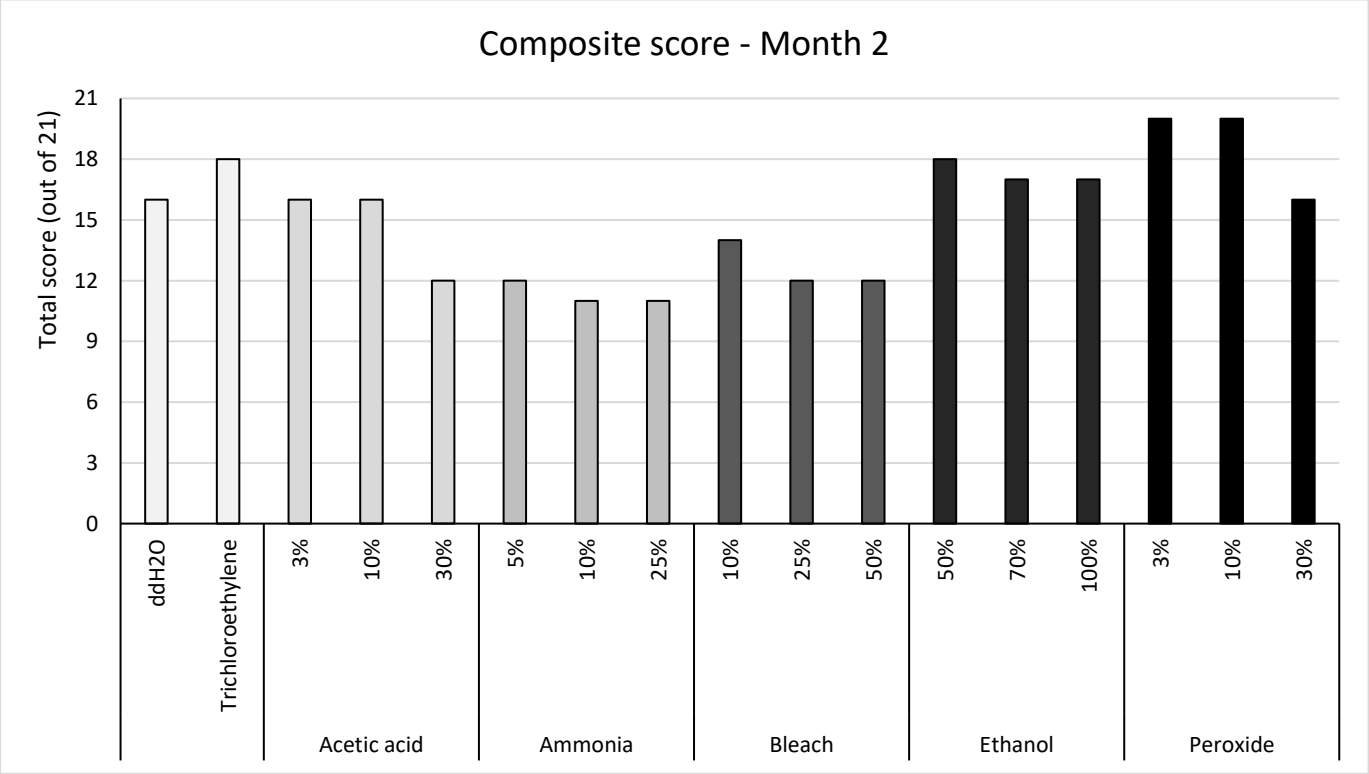


Figure 3: Month 2 Combined score of colour difference, bone morphology, odour, sediment layer and fatty layer that occurred in the bones.

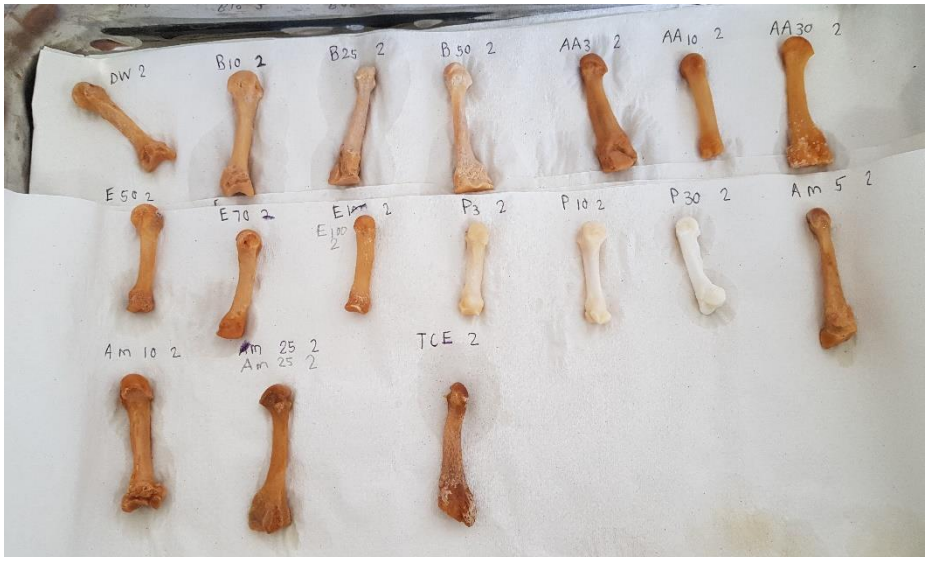


Figure 4: Results of bone changes after 2 months

4.3. Differences in bone variables in Month 1 vs Month 2

The combined scores for months 1 and 2 were added together to compare the results of each chemical during these two months (Figure 3). A low score indicates poor results in all five variables whereas a high score indicates better results concerning all five variables.

Peroxide 3% and peroxide 10% showed the best results after both month 1 and month 2. TCE following close second. Although peroxide 30% showed promising results in month 1, this result worsened after month 2. Ammonia 25% showed the worst results during month 1 but improved when bones were put in for two months. Acetic acid 30%, bleach 25% and distilled water showed better results after one month than after two months, while bleach 10%, ethanol 50% and ethanol 100% showed better results after two months rather than after one month.

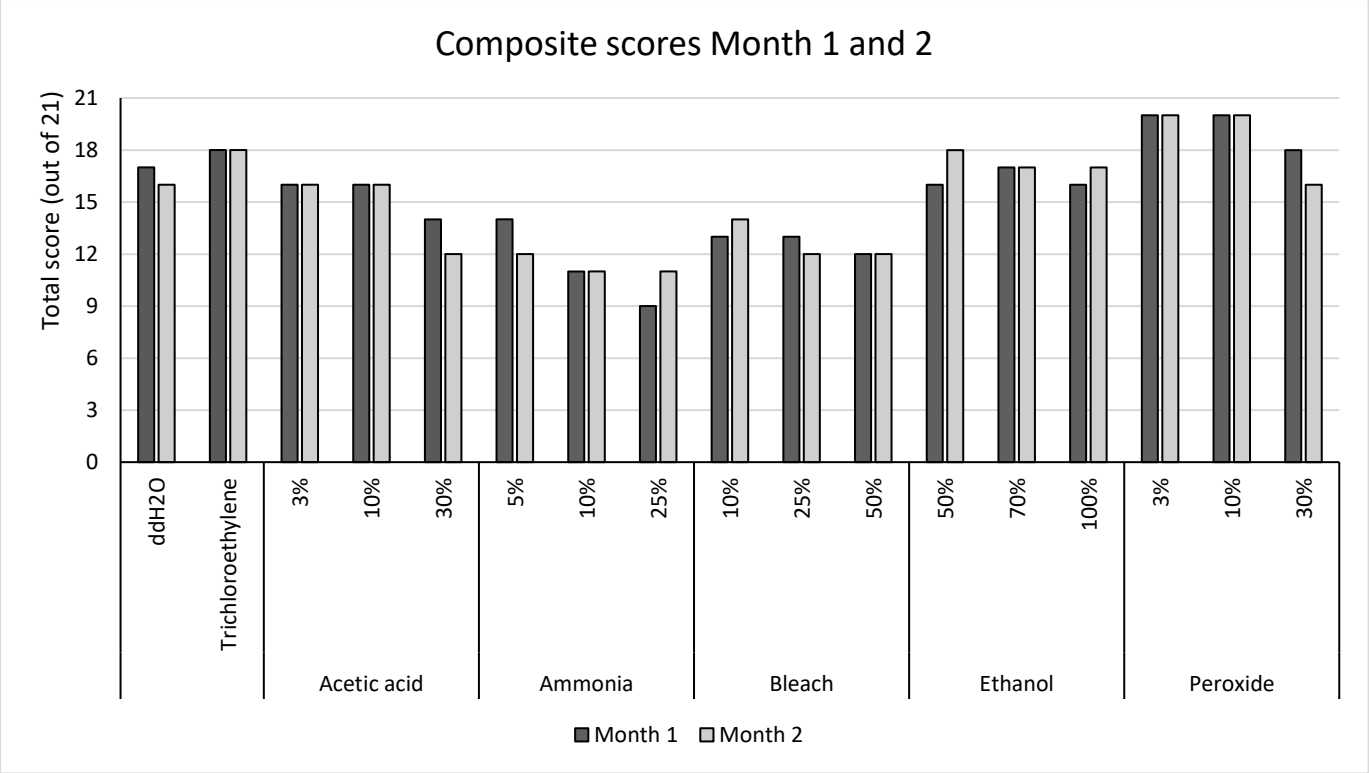


Figure 3: Combined scores of colour difference, bone morphology, odour, sediment layer and fatty layer for Month 1 and Month 2.

4.4. Bone mass

4.4.1 Month 1

Bone mass was weighed directly after the bones were taken out as well as after drying for one week. The percentage weight loss or weight gain is displayed in the table below (Table 2). During month 1, some chemicals caused weight gain in the bones initially and followed by weight loss after 1 week, including acetic acid 3%, acetic acid 30%, all ammonia concentrations, all bleach concentrations, distilled water and ethanol 50%. In contrast, some chemicals caused immediate weight loss and increased weight loss after one week, including acetic acid 10%, ethanol 70%, ethanol 100%, all peroxide concentrations and TCE.

Bleach 10% caused the least weight loss while acetic acid 10% caused the greatest weight loss.

Table 2: Bone weight loss after Month 1

Chemical	Bone Loss after 0 days (%)	Bone Loss after 7 days (%)
Distilled water	2.75	-1.15
TCE	-13.3	-13.76
Acetic acid 3%	2.75	-5.49

Acetic acid 10%	-11.21	-16.67
Acetic acid 30%	0.78	-11.15
Ammonia 5%	15.74	-4.71
Ammonia 10%	26.82	-13.78
Ammonia 25%	26.03	-10.63
Bleach 10%	5.56	-0.72
Bleach 25%	7.07	-1.18
Bleach 50%	2.67	-2.44
Ethanol 50%	6.27	-2.44
Ethanol 70%	-1.55	-5.28
Ethanol 100%	-9.73	-10.62
Peroxide 3%	-2.34	-3.13
Peroxide 10%	-0.52	-4.46
Peroxide 30%	-0.32	-4.9

4.4.2 Month 2

Bone mass was weighed directly after the bones were taken out as well as after drying for one week and two weeks. The percentage weight loss or weight gain is displayed in the table below (Table 3). During month 2, some chemicals caused weight gain in the bones initially and thereafter caused weight loss after 1 week, including acetic acid 3%, all ammonia concentrations, all bleach concentrations, distilled water, ethanol 50% and ethanol 100%. In contrast, some chemicals caused immediate weight loss and increased weight loss after 1 week, including acetic acid 10%, acetic acid 30%, ethanol 70%, all peroxide concentrations and TCE. The bones were weighed again after two weeks and extra weight loss was noted in all concentrations, however not to a great extent.

Distilled water caused the least weight loss while acetic acid 30% caused the greatest weight loss.

Table 3: Bone weight loss after Month 2

Chemical	Bone loss after 0 days (%)	Bone loss after 7 days (%)	Bone loss after 14 days (%)
Distilled water	3.59	-2.11	-2.53
TCE	-9.51	-10.93	-10.93
Acetic acid 3%	2.77	-8.1	-8.74
Acetic acid 10%	-8.19	-19.59	-20.18
Acetic acid 30%	-10.89	-20.04	-21.01
Ammonia 5%	9.39	-15.52	-16.06

Ammonia 10%	24.58	-5.91	-6.28
Ammonia 25%	32.19	-18.43	-18.92
Bleach 10%	3.01	-3.01	-3.41
Bleach 25%	3.45	-3.45	-3.69
Bleach 50%	4.02	-3.41	-3.82
Ethanol 50%	1.98	-3.56	-3.56
Ethanol 70%	-9.06	-11.59	-11.96
Ethanol 100%	3.23	-9.68	-10.08
Peroxide 3%	-3.17	-6.35	-6.35
Peroxide 10%	-1.45	-5.78	-6.07
Peroxide 30%	-11.51	-14.29	-14.29

DISCUSSION

5.1 Colour Difference

The aim of degreasing is to get rid of the dark brown, sticky appearance of bone and produce a light colour which ranges between a light yellow and yellow-white. Peroxide 30% resulted in the whitest colour, while peroxide 3% and peroxide 10% ranged from white to light yellow, making peroxide the overall most successful chemical concerning colour, as the TCE positive control also resulted in a light yellow colour.

All concentrations of acetic acid resulted in the same dark brown colour and showed almost no colour change from the original colour, making acetic acid the least successful chemical concerning colour changes. All ethanol and ammonia concentrations resulted in a dark yellow to brown and sticky colour, making them unsuccessful in colour change as well. While bleach coloured the bone shafts white, the distal and proximal ends of the bones remained dark brown and sticky, making it rather unsuccessful regarding colour change.

5.2 Bone Morphology

Degreasing should not affect the morphology of the bone and keep the bone intact without making it brittle or flakey. While peroxide showed success in colour difference, it was less successful regarding bone morphology. Peroxide 3% showed no change in bone morphology, while peroxide 10% and peroxide 30% resulted in slightly brittle bone. However, TCE caused approximately the same amount of changed morphology than the peroxide concentrations.

All acetic acid concentrations as well as bleach 10%, distilled water and all ethanol concentrations showed the least change in bone morphology. However, acetic acid 30% caused white crystals to form on the distal end of the bone, which is an unwanted side-effect. Ammonia 25%, bleach 25% and bleach 50% caused the most change in bone morphology, making these concentrations the least successful concerning bone morphology preservation.

5.3 Odour

Odourless degreasing would be the desired outcome, yet it is not always possible. However, a slightly noticeable odour will be preferred over a lasting, unpleasant smell. Most chemicals resulted in either no odour or only a slightly irritating odour, which makes all of them quite successful regarding odour. However, ammonia and bleach concentrations left a lasting odour on the bones. Distilled water left an unpleasant smell on the bone.

5.4 Sediment layer

Degreasing should occur without a sediment layer forming, as a sediment layer indicates that breakdown of bone occurred. Neither TCE, distilled water, all ethanol concentrations, all acetic acid concentrations (except for acetic acid 30% in month 2) or all peroxide concentrations caused a sediment layer to form in their respective test tubes, making them the most successful regarding sediment formation.

Both bleach and ammonia caused sediment formation in all concentrations, making these chemicals the least successful in this aspect.

5.5 Fatty layer

The presence of a fatty layer during the degreasing process is not always disadvantageous, however it adds an extra step to the process as this fatty layer needs to be removed somehow, making it rather unideal. Neither TCE, distilled water, all peroxide concentrations, all ethanol concentrations, acetic acid 3% and acetic acid 10% caused a fatty layer to form in the test tube, making them the most successful regarding this aspect.

Acetic acid 30%, peroxide 10% and peroxide 30% caused a vague fatty layer to form. Both ammonia and bleach in all concentrations caused the test tube to become cloudy. Bleach causing a white cloudiness and ammonia causing a yellow, murky cloudiness. These two

chemicals are then least successful regarding fatty layer formation.

5.6 Bone mass

Loss of bone mass is not ideal yet unpreventable. This loss of bone mass could indicate anything from dehydration of fats and moisture inside the bone by the chemical, to breakdown and damage to the inner part of the bone. Further studies should be done to investigate the reason behind this loss of weight. However, in this study, loss of bone mass was regarded as disadvantageous.

All chemicals caused a loss in bone mass over the time period, some to a greater extent than others. After 1 month, bleach 10% caused the least weight loss (0.72%) while after 2 months, distilled water caused the least weight loss (2.53%). After 1 month, acetic acid caused the greatest weight loss (16.67%) while after 2 months, acetic acid 30% caused the greatest weight loss (21.01%).

5.7 Cost-effectivity

Degreasing in a bone lab or research unit, is a process that will take place often, therefore cost is an important factor to consider. In terms of buying costs, ammonia>ethanol>TCE>hydrogen peroxide>acetic acid>bleach>distilled water. However, one has to also take into account disposing costs where TCE>ammonia>all other chemicals. Hydrogen peroxide again seems to be the most affordable as it is not the most expensive chemical to buy and is easily disposable.

5.8 Safety

TCE is a carcinogenic substance and not ideal for use. Furthermore, various unwanted health effects such as dizziness, slowed reaction time, eye irritation, headache and drowsiness can be caused by exposure to the vapour of TCE (Cadet & Bolla 2007; van der Spuy 2008; Agency for Toxic Substances and Disease Registry 2016). During this experiment, laboratory personnel trained in working with TCE had to handle the chemical during each part of the experiment as it is unsafe for an untrained person to use.

Peroxide, ethanol, bleach and acetic acid are considered relatively safe chemicals and can be poured down the drain after use. Although the above-mentioned compounds are generally safe to use, accidental ingestion, exposure and inhalation can cause health issues. Inhaling large

amounts of these compounds can cause vomiting, nausea, mucous membrane and throat irritation as well as breathing difficulties (Meadows 2016). Working with these chemicals during the experiment were easy as latex gloves were worn and no health issues were encountered. Hydrogen peroxide is highly destructive and can not only cause damage to the structural integrity of the bones but can also be harmful to human skin (Ososky n.d.; Rowley 2015). Nevertheless, even though hydrogen peroxide is deemed destructive and dangerous, contrary to other chemical substances, hydrogen peroxide does not produce residues or gasses thereby decreasing the health risks accompanying inhalation (Solver Chem Publications 2016).

Ammonia is a bit more difficult to use as it can be hazardous to health when not handled correctly. Inhalation of lower concentrations of ammonia may cause coughing and throat irritation (Agency for Toxic Substances and Disease Registry 2004; New York State Department of Health 2004; Canadian Centre for Occupational Health and Safety 2017). Ammonia presents with a strong odour and can cause olfactory fatigue, making it dangerous to work with over a prolonged period of time (New York State Department of Health 2004; Canadian Centre for Occupational Health and Safety 2017). Furthermore, rapid skin or eye irritation may also occur due to exposure to ammonia. Higher concentrations of ammonia, for example before diluted for degreasing, may cause severe injury such as skin burns, permanent eye damage and even blindness (Agency for Toxic Substances and Disease Registry 2004; New York State Department of Health 2004). Ammonia caused respiratory issues during making up of the concentrations as well as discarding the solutions after experimentation took place as it caused severe coughing and bronchoconstriction. Eye irritation also occurred, and a strong smell lingered in the laboratory afterwards, making it difficult to work in that environment. Ammonia also needs to be discarded of using laboratory protocols and cannot be poured down the drain.

CONCLUSION

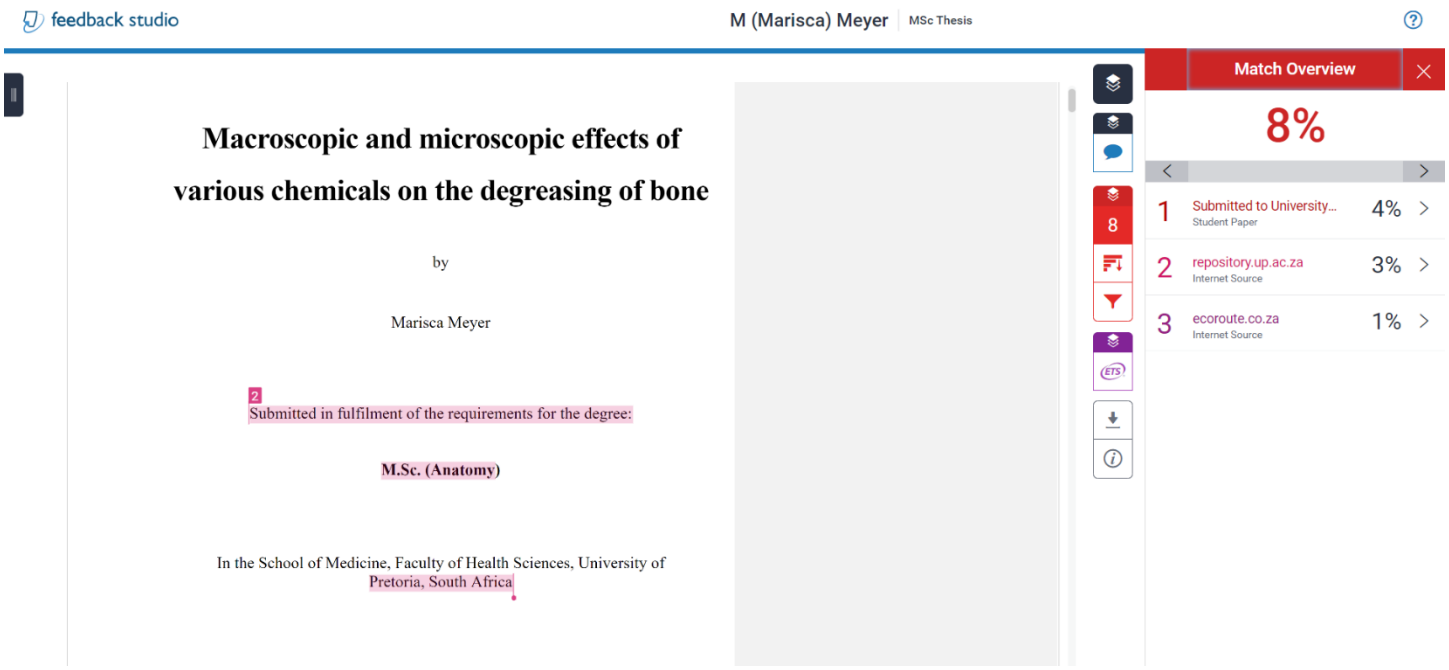
Taking all factors into account, peroxide 3% is the most successful degreasing chemical tested in this study. The bone is whitened without breaking down bone morphology, no residing odour is produced, the bone is successfully degreased and no sediment layer or fatty layer forms with this concentration. No health or safety problems occurred using this chemical and minimal loss of bone mass occurred. However, further testing needs to be done to confirm its degreasing success. Ammonia resulted in being the worst chemical during this study. The bone remained

dark and sticky, bone morphology changes could be noted, a sediment layer formed, and the solution became murky and slimy. A strong odour was produced using ammonia and some health issues including eye and throat irritation occurred. Ammonia also caused substantial loss of bone mass after each month.

Limitations

A few limitations of this study include that only metacarpals were used and the effect of these chemicals on larger bones are still unknown. During the scoring process of month 1 week 1, the principal investigator (MM) could not score the bones and consequently the supervisor (JM) had to do the scoring for that week, creating an opportunity for interobserver error. Hydrogen peroxide has been said to work better in a dark environment away from UV sunlight, yet the experiment was carried out in a laboratory with natural light, which could have influenced the outcome of the hydrogen peroxide results. The effects of inter and intra-observer error was not included in the study and future research is required to determine whether scoring is accurate. The effect of these chemicals on internal bone characteristics as well as combining chemicals to observe their combined effect is beyond the scope of this research project yet is a promising field for future research.

APPENDIX 4: Turnitin report



APPENDIX 5: Ethics approval letters

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 26, 2022 and Expires: 03/04/2023.

11 June 2020

Approval Certificate
New Application

Ethics Reference No.: 318/2020
Title: Macroscopic and microscopic effects of various chemicals on the degreasing of bone.

Dear Miss M Meyer

The **New Application** as supported by documents received between 2020-05-22 and 2020-06-10 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2020-06-10.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year and needs to be renewed annually by 2021-06-11.
- Please remember to use your protocol number (318/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
Room 4-60, Level 4, Tswelopele Building
University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

Faculty of Health Sciences **Research Ethics Committee**

13 May 2021

Approval Certificate
Annual Renewal

Dear Miss M Meyer

Ethics Reference No.: 318/2020
Title: Macroscopic and microscopic effects of various chemicals on the degreasing of bone.

The **Annual Renewal** as supported by documents received between 2021-04-14 and 2021-05-12 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2021-05-12 as resolved by its quorate meeting.

Please note the following about your ethics approval:
- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2022-05-13.
- Please remember to use your protocol number (318/2020) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
***Deputy Chairperson** of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 46 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2016 (Department of Health)

Research Ethics Committee
Room 4-60, Level 4, Tswelopele Building
University of Pretoria, Private Bag x323
Gezina 0031, South Africa
Tel +27 (0)12 356 3084
Email: deepeka.behari@up.ac.za
www.up.ac.za

Fakulteit Gesondheidswetenskappe
Lefapha la Dikgwerekwa tša Maphelo