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Coach : JW Spronck
Professor : JW Spronck
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Development of an automated production line for bone processing

With an emphasis on multi-frequency ultrasonic cleaning

J.T.F. Spierings

Supervisor: Ir. J.W. Spronck

Department of Precision and Microsystem Engineering

Master programme of Mechanical Engineering

Technical University Delft

Abstract

In orthopaedic and dental surgery, bone grafts are used by surgeons as bone substitutes. A bone graft provides support to the skeletal structure and stimulates the ingrowth of new bone. There is a growing demand for bone grafts due to an increasing world population and an ageing demographic. Tissue banks produce bone grafts originating from either human or animal bone. Bone grafts have been investigated profoundly. However, there is no literature that provides a standard protocol for the production process of bone grafts. The current production processes for bone grafts are inefficient because of many manual actions by humans in the production line. Many complex steps in the bone cleaning process are divided over several different machines. This thesis aims to provide a bone processing protocol and investigate the first steps to an automated production line for bone processing. The automated production line must transform fresh donor bone to an end-product that is a clean and sterile bone graft consisting mineral and collagen. An important step in the production process is ultrasonic cleaning. There is a hiatus in knowledge in the cleaning process of bone using multiple ultrasonic frequencies. This thesis provides new insights in processing bone with ultrasonic frequencies and solves the research goals through an iterative process based on an extensive literature review, numerical modelling, testing and analysing cleaning procedures for bone samples.

A literature review was executed to research and select the best chemical and mechanical processing steps for an optimal bone processing protocol. This optimized protocol allows for modest production costs, adequate sterility levels and high purity of the bone grafts. The criteria from this processing protocol forms the basis of the design of the automated production line. The processing protocol consists of chemical and mechanical steps. The selected processing protocol is tested on human femoral heads (*caput femoris*). These tests showed that chemical cleaning agents for dissolving lipids such as acetone and isopropanol had little success in dissolving marrow from within the femoral head. Increasing temperature reduced the viscosity of marrow but did not adequately remove the marrow from the bone. The best results were obtained by treating the femoral head with hydrogen peroxide.

Cleaning by ultrasound is selected as the best tool for mechanical cleaning during the chemical baths. Ultrasound produces acoustic cavitation which is used as a mechanical cleaning force. The literature review suggested that combining ultrasound frequencies is beneficial for cleaning. Low ultrasonic frequencies of 35 kHz have a better potential in removing large particles such as blood clots. High frequencies of 130 kHz are better for cleaning small parts such as small lipid and virus particles. Combining both frequencies should lead to optimal cleaning results and a reduction in cleaning time. A reduction in cleaning time can optimize the surface intactness of the cleaned bone grafts by reducing the contact time with abrasive cleaning agents. This thesis started with building a numerical model to predict the effect of cavitation in a chemical bath and continued with testing the effect of multiple ultrasonic frequencies on the efficiency of cleaning for bone.

A numerical model was built in COMSOL to simulate the ultrasonic cleaner. The model gives an pressure distribution in the bath which relates to the pressure threshold for acoustic cavitation. The model uses an improved value for damping compared to previous models in literature. The model is used for placing a bone sample in the bath. Thereafter a parallel experiment has been executed to compare the effect of different ultrasonic frequencies on the efficiency of cleaning. Nine bone samples of human femoral heads coming from three different donors were cleaned at 35 kHz, 130 kHz and a combination of both frequencies alternating. There was no significant difference in cleaning results shown by a residual lipid analysis, a histological analysis and a compression test. The possible explanation for this is that the cleaning agent, hydrogen peroxide was the dominant factor.

The results from the model, tests and analyses were used to design an automated production line. The model indicated that for optimal ultrasonic cleaning the bone have to be placed above the transducer in the bath but not near the surface. The tests and analyses concluded that the ultrasonic frequency used for cleaning is free to choose. Sample size, operation time and chemical cleaner control the cleaning effect. I choose to combine all chemical baths in one machine, while the heating is performed in a separate machine. This shortens the operation time per batch and allows drying through infrared. The automated production line minimizes human interference, is environmentally friendly, secures a safe process, has a fast turnaround with small batch sizes and produces bone grafts of optimal quality. This thesis contributes new knowledge for cleaning bone with ultrasound and generated a new design for an automated production line for cleaning bones.

Content

1. Introduction

Bone grafts are bone substitutes used during surgical procedures to compensate for bone loss in patients. The grafts are implanted during reconstructive surgery in bone cavities to stimulate the ingrowth of new bone and to support the skeletal structure. There is a growing demand for bone grafts due to an increased global population and an ageing demographic. The production of bone grafts is a high-tech industry that is constantly innovating to optimize their products and to keep up with demand. Bone grafts and the production thereof have been investigated profoundly, however there is still no standard protocol for production. The current production processes for bone grafts are inefficient because of the need for extensive human action by manual labour in the production line and the inclusion of many complex steps, which are fragmented over several machines. This requires an improvement in the processing protocol and the production line. There is a need for an fully automated continuously production line that produces clean and sterile bone grafts efficiently. Such an automated production line with a simple and cheap process does not exist today. The production line should minimize human interference to prevent the risk of contamination. The difficulty in building a compact line is the integration of all the individual cleaning steps with a variety of chemicals in one machine while fulfilling all safety requirements. Besides the difficulty of integrating all steps, there is a hiatus in the current knowledge about cleaning bone grafts with ultrasonic frequencies. Cleaning bone with a combination of different ultrasonic frequencies has not been investigated. Previous research suggest that higher ultrasonic frequencies are more effective in cleaning small particles and lower ultrasound frequencies are better in removing bigger structures such as blood clots. A combination of multiple frequencies could enhance the cleaning process and this hypothesis is investigated in this thesis.

This thesis is missioned to research the current bone processing techniques and the possible improvements that can be made regarding efficiency and safety. This knowledge is used to design a new automated production line. This results in the development of a better production line and a new protocol for bone processing. The thesis starts with a literature review of the current production processes and by conversations with experts in the field. The results are used to conclude the optimal production protocol. The production protocol aims to improve accessibility, safety, low cost and efficiency. This protocol sets up the criteria for a compact automated production line which minimizes human interference, reduces costs, has a fast turnaround with small batch sizes and optimal quality of the bone graft. The production line consists preferable of only one machine that continuously processes the donor bone to clean grafts. Designing all steps in one machine is enhanced by avoiding difficult operating settings in the selection of the production protocol. The minimization of human interference by an automated process will limit human errors as well as the risk of contamination by human handling.

A literature review is executed to investigate the chemical and mechanical process steps to prepare sterile bone grafts for implantation. It is investigated which types of bone graft exists and what the advantages and disadvantages of each type are. Next, the processing steps are investigated to conclude a final protocol. The chemical and mechanical process will be used to develop criteria for an automatic compact production line. The criteria that were selected to be executed by the machine are tested and the results are the basis for a future machine.

The literature review revealed out that there is a hiatus in knowledge about cleaning bone with ultrasonic frequencies in chemical baths. Most bone banks use ultrasound to clean bone. However there is no scientific research on the use of multiple ultrasonic frequency for cleaning bone. Literature shows that high ultrasonic frequencies are beneficial for removing small particles while low frequencies are more suited to remove resistive clots. A combination ultrasonic frequencies

could be beneficial for cleaning bone. Multiple frequencies enables better cleaning results and it reduces the contact time between the cleaning agent and the bone scaffold. The contact time with the cleaning chemicals can be detrimental to the bone scaffold. The intactness of the bone scaffold is of major importance because it enhances the osteoconductive properties of the bone. The effect of ultrasonic frequency at 35 kHz and 130 kHz are investigated. For this experiment, a human donor bone is cleaned and analysed for residual fat content and cell residue. The behaviour of the ultrasonic cleaning process is modelled in COMSOL. This model simulates the ultrasonic frequencies and enables the prediction of cavitation zones. Cavitation is a physical phenomenon that is formed by ultrasound waves in liquids. Cavitation causes mechanical forces for cleaning.

Bone grafts are used in orthopaedic and dental surgery for skeletal reconstruction when there is a need for bone reconstruction and bone augmentation. Bone grafts are implanted during reconstructive surgery in iatrogenic bone cavities to accelerate the ingrowth of new bone and to support the skeletal structure. A bone graft can be an autograft, allograft and xenograft. Autografts and allografts originate from human donor bone whereas xenografts have an animal origin. Autografts are harvested from the patient during surgery and are used for that patient. An autograft means that a patient receives their own bone. Harvesting autologous bone requires additional surgery and comes therefore with additional risks. Allografts are made of human bone donated by post-mortem donors or femoral heads which are resected during prosthesis hip implantation surgery. Xenografts are mainly made of bovine bone. Allografts and xenografts are available in larger quantities and are simpler to harvest than autologous bone.

Autologous bone is considered the golden standard (An et al., 1995). However, Foster et al. (2010) indicated in a review study for knee reconstruction that there are no distinctions in results between the usage of autografts or allografts. A risk of allografts and xenografts is cross-contamination of pathogens from donor to recipient. Allogenous and xenogenous bone must be sterilized to prevent cross-contamination. Furthermore, the bone grafts must be biocompatible, osteoconductive and have the right mechanical properties to ensure successful clinical outcomes (Fesseha et al., 2020).

The literature search revealed a number of necessary steps in the cleaning process. Each step can be performed in several ways. Bone will be processed first mechanically to remove soft tissues like muscle, tendons, periost, connective tissue and cartilage. This is followed by several chemical baths which for the removal of fat, pathogens and proteins but with exception of collagen. The mineral structure is preserved during these steps. The final steps are dehydration to improve shelf life and irradiation to secure sterility after packaging. The final product after these steps is an allograft comprising of bone mineral hydroxyapatite and collagen.

The paragraph above provides a summary of the mandatory steps in bone processing by an automatic production line. The necessary steps are based on the literature review. The literature review compares multiple processing techniques. The review of the current processing techniques and the consultation of experts in the field have highlighted the bottlenecks in the current production methods. The shortcomings in the current process are largely dependent on human interference. Human interference causes risks for human operators, is cost inefficient, has the risk of production errors and the risk of contamination of the product. These shortcomings are minimized in the newly developed protocol and production line where the human factor is minimized. This automatic production line serves as an impulse for the tissue industry in their aim to provide the world with excellent bone grafts.

This thesis is commissioned by Spierings Tissue Processing. They are active in the tissue processing industry and strive to improving the current production methods by technical innovations. ETB-Bislife

provided the research with human bone from living donors. This donor bone was rejected for clinical practice. ETB-Bislife is a Dutch bone bank and non-profit organisation. The Netherlands Forensic Institute (NFI) helped analysing the bone product with their expertise in forensic anthropology and chemical identification research. The thesis was executed as master thesis at the Technical University Delft. See figure 1 for the parties involved.

This thesis has the following structure. The first chapter starts after the introduction starts with the purpose and requirements of bone grafts and its biological mechanism are discussed. The third chapter discusses the mechanical cleaning treatments for bone and the process of ultrasonic cleaning in detail. In the fourth chapter, the most common chemical processing techniques for allografts are described and compared to one another. The fifth chapter describes the selected bone processing protocol. It explains the rationale for each selected processing technique. The sixth chapter summons the criteria for the production line. The seventh chapter discusses the implementation of these criteria. The eight chapter provides the model of the ultrasonic cleaner in COMSOL and shows the influence of different ultrasonic frequencies on cavitation. The nineth chapter is describes the setup for the executed tests and analysis. Chapter ten reveals the results of the tests and analysis. The eleventh chapter discusses the implications of the results for the design of an automated production line. Chapter twelve concludes this thesis.

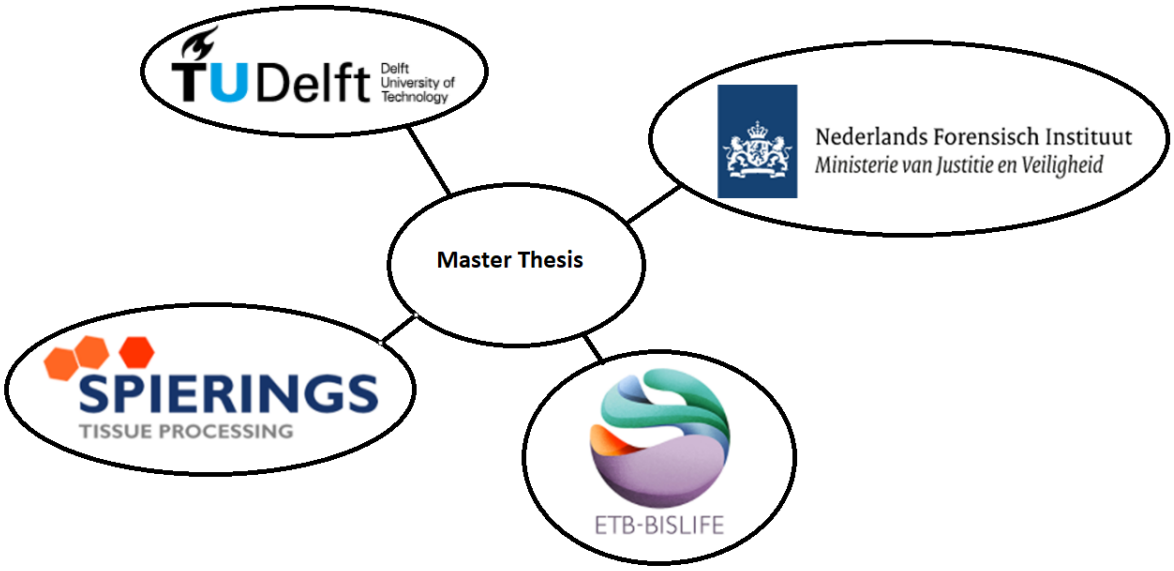

Figure 1: Parties involved in this thesis project. Spierings Tissue Processing commissioned the research, TU Delft provided supervision, the Dutch Forensic Institute helped analysing the bone samples and ETB-Bislife provided the human femoral heads for the tests.

2. Bone grafts

This chapter explains the relevance of producing bone grafts and its implications. The chapter starts with a discussion of the clinical application of bone grafts. This is followed with an overview of the types of bone grafts. Next, the physiology of bone is discussed. This is followed by the biocompatibility and pathogens in bone grafts. This chapter closes with an economic perspective on bone processing and discusses the laws that regulate the processing of bone grafts.

2.1 Clinical application

Bone grafts are used in dental, orthopaedic, trauma and reconstructive surgery. The purpose of using bone grafts includes repairing bone loss, maintaining contour, structural support, filling of dead spaces, to fuse joints to prevent movement, and reducing postoperative infection (Fesseha et al., 2020).

Bone grafts serving as a filler and scaffold are used in dental surgery for new bone formation. The most common dental treatment that requires bone grafts is an alveolar ridge defect (Kumar et al., 2013). An alveolar ridge defect is the result of teeth extraction which results in bone loss. Yet not enough bone exists to support a tooth implant. The bone loss is supplemented by bone grafts often in the form of bone powder.

Bone grafts have gained importance in orthopaedic surgery for joint arthroplasties and spinal surgery. Joint arthroplasty is the surgical procedure to replace or remodel the surface of the musculoskeletal joint, common places are the hip or knee joint. It is executed to restore functions of the joint after damage by arthritis or other trauma. Bone grafts are used to fill up voids and to provide support or to connect ligaments. In spinal surgery blocks of bone grafts are used to provide support to the spinal structure.

2.2 Overview of bone grafts

Bone grafts aren't the only resources available to serve as bone substitutes. Inorganic materials can serve as bone substitute as well. Synthetic bone substitutes are less effective, because its composition, comprising trace element, and its structure are hard to mimic. There is a common preference among surgeons for bone substitutes with an organic origin.

Grafts, that are made from organic bone, exist in a wide range of types. These types will be discussed subsequently based on their donor source, composition, type of bone, part of bone and shape. The mechanisms of action and application varies between those types of grafts. The mechanisms of action comprise osteoinductive, osteoconductive, osteogenitive and structural support. These mechanisms are explained paragraph 2.3 physiology of bone.

The source can be autograft, allograft or xenograft. The type of bone used for the graft differs with availability and minimal invasive surgery. The most common types of bone used are the iliac crest and femoral heads. Femoral heads are widely available from living patients undergoing a total hip replacement and are often used as allograft. The iliac crest is commonly used for autografts. Xenografts are derived from all types of bones, often long bones.

The structure of bone exists of cortical bone that has a dense mineral structure and of cancellous bone that has a high porosity. Cancellous bone is also known by the name trabecular or spongious

bone. Cancellous bone has a Eiffel tower structure to bear stress. The porous structure of cancellous bone gives its spongious appearance and make it easier to clean. Cancellous bone forms the main volume of bone in a femoral head. On the other hand, cortical bone is denser which makes it harder to penetrate with chemicals, but provides more strength.

The composition of bone grafts can exist of pure mineral, mineral plus collagen, demineralized bone matrix (DBM) or peptide enhanced bone grafts. The enhancement with peptides consists of bone morphogenic proteins which induce the growth of bone. Mineral and collagen provide the strength of the bone structure.

Bone grafts are produced in different forms based on their application. The shapes that exist are for example: powder, chips, paste, blocks or massive bone. Powder is used for small cavities where structural support is not necessary. Cylindrical blocks are used to fill up the void created by drilled holes.

2.3 Physiology of bone

The properties of a bone graft depend on its composition and its mechanism of action. First is explained the composition of bone and thereafter the mechanisms of action in bone. The composition is important for the strength of the bone graft. The mechanisms of action are important for the incorporation of bone grafts. Mechanisms of action are osteoinductivity, osteoconductivity and osteogenitivity.

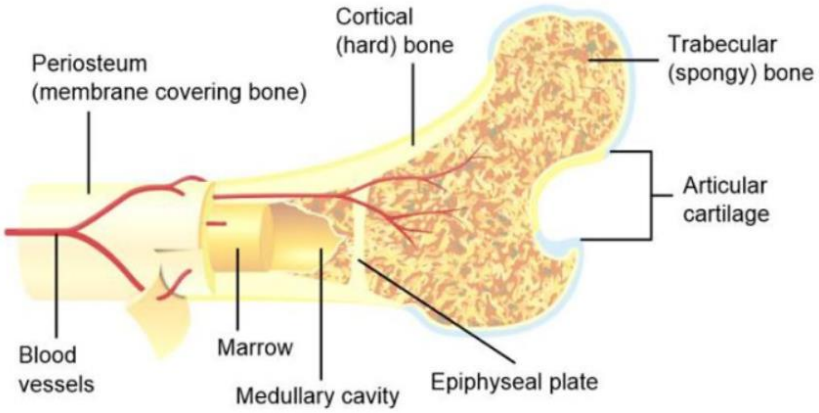

Figure 2 (Biggane et al., 2016): Anatomy of bone.

Bone is a living tissue that comprises fat, bone marrow, blood vessels, cells, proteins, minerals, see figure 2. Roughly 65% of the mass of bone is composed of mineral and 25% is organic and the remaining 10% is water that is bound to the collagen-mineral composite. Collagen comprises around 90% of the organic weight. The strength of bone is determined by its mineral content and collagen fibres. The structure of bone could be compared to reinforced concrete. The mineral content is the concrete for compressional strength. The collagen fibres are the steel cables and provide its tensional strength. The mineral content is composed of a poorly crystalline carbonated apatite.

Bone is constantly adapting itself during a life time to bear changing loads and to restore small ruptures in the bone. The process of adaptation of the bone is called remodelling. Remodelling strengthens parts of the bone that are weak and tears down parts that are unnecessary strong (Burr & Allen, 2019). Remodelling is a constant process in every human body. The breakdown of bone,

which includes the dissolution of the minerals as well as the breakdown of the collagen, is executed by cells named osteoclasts. The generation of new bone is organised by osteoblasts.

An easy conduction of cells through the bone structure is called osteoconductive. The scaffold of bone grafts serves as a framework for osteoblast to spread and promote new bone growth. Osteoinductivity is the generation of bone cells and a catalysator for bone remodelling. Bone morphogenic proteins are the most studied osteoinductive cell promoters. Osteoinductive properties in bone grafts promote the formation of new osteoblasts and faster incorporation of the graft. Osteogenesis is the growth of new bone due to presence of osteoblasts. Autografts have only the property of osteogenesis.

The osteoconductive and osteoinductive properties of bone implants determine its quality for osseointegration in the recipient. The allograft must exist of hydroxyapatite and collagen for its strength. Fat has to be removed from the bone implant, because it decreases the osteoconductive properties of bone (Chappard et al., 1993). The removal of fat residuals improves the properties of bone grafts, because fats are known for causing immune reactions and to disturb bone formation (Aspenberg & Thoren, 1990; Horowitz, 1996).

2.4 Biocompatibility

The biocompatibility depends on the source of the bone graft. Human bone has less implications on biocompatibility compared to animals. Cells in human allografts, as in blood or fat, could produce an autoimmune response and should be removed (Thoren et al., 1993). Bones from animals can cause immune reactions because the presence of fat, cells and proteins.

Cleaned xenografts have risks in biocompatibility due to collagen. Collagen consists of three strings of polypeptides intertwined with each other. A peptide differs from a protein, because of its small number of amino acids in its molecule. There exists around 30 types of collagen. Human bone and skin are mainly composed of type I as well as porcine and bovine bones. The collagen of animals has still three options to cause a possible weak immune reaction in humans (Furthmayr & Timpl, 1976). The first is the helical structure of the collagen which is recognised by antibodies. The second possibility is a different amino acid sequence. The latter are the nonhelical terminal regions of the strings.

Between two and four percent of all people have an allergy to bovine collagen type I (Lynn et al. , 2004). Bovine collagen is often used in as an injectable in dermal treatments and gives even with prior skin tests still in one to two percent of the cases an immune reaction (Keefe et al., 1992; DeLustro et al., 1990). These percentages are in contradiction with the percentage found in a literature survey by Bracey et al. (2020) on bovine bone xenotransplantation's. He found that bovine bone grafts gave in 50 percent a poor bone ingrowth result, which would be hard to allocate only to collagen compared to the dermal results from Keefe et al. (1992).

Another explanation besides collagen for the poor results of bovine bone grafts are, suggested by myself, poor removal of the fat and cells in the grafts. The different composition in mineral composition in bovine bone compared to human bone cannot be the reason. Namely, hydroxyapatite alone as produced by calcination does not cause an autoimmune response. The response to collagen of porcine bones is not completely understood so far, but gives promising results in new studies (Bracey et al., 2020).

2.5 Pathogens

Effective elimination of the possibility of contamination with bacteria, viruses, spores or prion disease is of utmost importance. Human donors should be screened very carefully on viruses such as human immunodeficiency virus (HIV) which were reported to be transmitted in the past by tissue allografts. Prions are proteinaceous infectious agents and examples are Creutzfeldt-Jakob Disease (CJD) and bovine spongiform encephalopathy (BSE). These prion diseases induce no immune response and take years to develop. The Netherlands is free of BSE for the last ten years. Porcine bones have less dramatic viruses and prions so far except for Porcine Endogenous Retroviruses (PERV). Until now there are no known cases of the transmission of PERV to humans from xenotransplants nor to butchers who were exposed heavily to pig tissue (Elliott et al., 2001; Paradis et al, 1999). Some tissue banks have left xenografts due to the fear for transmission of zoonotic diseases to the human population (Dziedzic-Goclawska et al., 2005). All viruses, bacteria, prions and antigens should be eliminated in the process. This is done by removing all cells, proteins, viruses and prions through a validated process that reduces any risk of contamination and assures sterility.

2.6 Economic perspective

Bone is the second most implanted substance after blood transfusion. The number of grafts implanted annually are estimated to be around 600.000 grafts (Fesseha et al., 2020). At present, the tissue business is an costly part of the health care system. Tissues used for spinal surgery in the United States alone are already estimated on $300 million dollars in revenue for tissue banks (Anderson and Bottenfield, 2004). A country has high annual tissue importation cost, when it does not produce its own tissues (Phillips & Morales, 2002). An ageing demographic in Europe and the United States demands higher quantities of bone grafts. A growing demand for cheaper bone grafts is a necessity in Asia, Africa and South America. Those countries have limited resources for costly bone graft production lines while these countries are having a growing demand for bone grafts due to a rapidly growing population.

2.7 Law and regulations

Depending on the raw material for the bone graft, it falls either under the Medical Devices Directive of the European Union (EU) or under the Tissue Legislation of the EU. The first applies if it is of animal origin, the second only on human bone. There is specific legislation for human tissue. The Food and Drug Administration (FDA) of the USA requires for implantable devices a sterility assurance level (SAL) of 10^{-3}. In most tissue banks this SAL is closer to 10^{-6} for processed allografts (Vaishnav & Vangsness, 2009). Those risks are small compared to postoperative orthopaedic infection rates which ranges from 0.6 to 6.6 % (Centers for Disease Control and Prevention, 2000). The European Union advises for the removal of prions a treatment with 1 M sodium hydroxide or chlorine (European Commission, 2011). The European Union has no regulations for processing machines. They recommend that machine parts should be easy to clean and to sterilize.

3. Review of mechanical cleaning

This section discusses the mechanical treatments used to process bone to a clean bone graft. The focus lies on processing of a femoral heads, because they can be obtained from living donors. The mechanical treatment enhances the penetration of the chemical liquids and has the ability to detach particles. Debriding soft tissue, flushing by a water jet gun, fluid mixing and circulation by brushing, washing, agitation, spray and ultrasonic are all options for cleaning. Their function is divided in two phases. The first phase cleans the outside of the bone by removing the soft tissue and the cortical layer. This is called the mechanical pre-treatment. The second phase of the mechanical treatment disturbs the surface and circulates the fluid during the chemical baths. Ultrasound is most suitable for this second phase, because its sound waves are able to penetrate the complex structure of the bone and it creates microstreaming. Spraying or washing by a rotating propeller do not have this ability. This is why only ultrasound is discussed in section 3.2. Ultrasound has a wide range in optional parameters which require an extensive in depth knowledge. This is elaborated profoundly in this literature review.

3.1 Pre-treatment

The pre-treatment is meant to prepare the bone graft for the chemical baths. This includes two stages. First the bone graft has to be undone from its soft tissue and cortical bone before the bone graft goes through several chemical baths. Secondly part of the fluid volume such as bone marrow and blood should be removed by a simple flushing method without damaging the bone scaffold. The soft tissue and cortical bone is removed by a femoral head reamer (Cuttica &Hyer, 2011) or a Fortios bone debrider from Spierings Tissue Processing. The femoral head reamer is an automatic slicer device with is sold by the company Spierings Tissue Processing, named Noviomagus Femoral Head Reamer. The Fortios bone debrider is a spinning steel brush. Here after only the cancellous part of the femoral head remains. This can be flushed by either an air pressure gun or a water jet. A too high applied water pressure is detrimental to the bone properties (Hua et al., 2020). Hua at al. (2020) found that a water pressure of 6 MPa for 10 seconds at distance of 10 cm from the bone graft resulted in a fat residue of only 1.45%, but for this research were used only very small samples of 5 mm in diameter.

3.2 Ultrasonic cleaning

Ultrasonic cleaning is applied during the chemical baths and is explained in detail in this section. Ultrasonic cleaning is used to enhance the cleaning process by detaching pollutants from surfaces and by improving the chemical reactions. Ultrasonic cleaning uses sound waves above the human hearing which is above 18 kHz. These waves are produced by piezoelectric transducers and travel through a liquid medium. The travelling wave produces pressure oscillations in the liquid. In the rarefaction of this wave is the pressure so low that water can evaporate. This is comparable to the process of boiling. However the pressure is now lowered instead of increasing the temperature, see figure 3. A vapour bubble can grow in the presence of an imperfection in the liquid. This imperfection is a weak spot in the liquid which exists commonly of a particle of a microscopic gas bubble and is named a nucleus. The bubble cannot form without a nucleus because the water surface tension is too high in pure water. Nearby gasses such as dissolved oxygen can diffuse into the bubble. The formed vapour bubble increases and decreases during the oscillating pressure waves. During these waves the bubble grows until it reaches its resonant frequency. At this point the bubble becomes

unstable and implodes, see figure 4 and 5. The process of formation, growth, oscillation and bubble implosion is called cavitation.

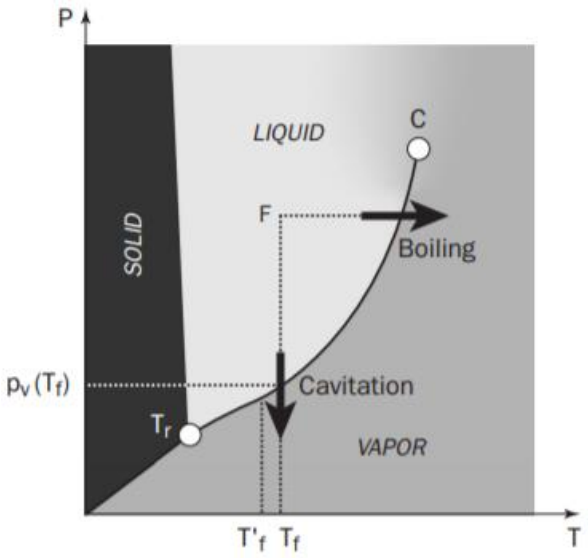

Figure 3 (Franc & Michel, 2007): Phase diagram which shows the transition from liquid to vapour during cavitation.

Bubble implosions release an enormous amount of energy which results in shockwaves, microjets, light-emission and a localized increase in temperature, see figure 6. The compression of gas releases heat which leads to short lived thermal hotspots of 5000 K (Suslick et al., 1999). These local hot spots form highly reactive radicals in water such as hydroxide and hydrogen atoms which degrade organic pollutants (Suslick, 1989). The physical effect can cause shockwaves and microjets, see figure 7. A stable implosion causes a shockwave. The implosion can also happen unstable which produces a microjet. These physical effects are used to detach impurities from nearby surfaces. Micro-agitation caused by implosion causes a better mixing between the solvent and the contaminant. This improves the efficiencies in dissolving and removing contaminants.

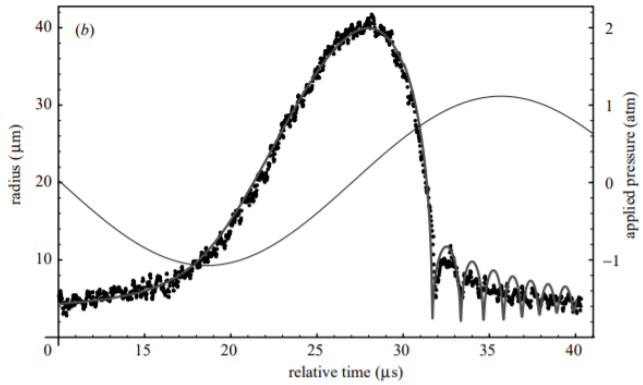

Figure 4 (Matula, 1999): The final oscillation during bubble implosion. The pressure oscillation induces the growth of the bubble which collapses when the pressure changes from negative to positive.

Acoustic cavitation is normally divided in a stable and transient form. Stable cavitation is the oscillating effect in bubble size during the pressure waves. The bubble exists for many pressure cycles. Transient cavitation is named when the bubble collapses violently and exist only for one cycle.

The collapse happens when the bubble reaches its resonant frequency. The collapsed bubble breaks up in multiple smaller bubbles which grow again in size during the pressure oscillation (Laborde et al., 1998). These bubbles can collide together which is called coalescence and rise to surface or implode again. Another option for this multiple bubble nuclei is rectified diffusion which is the growth of single bubble until implosion. The surface size of the bubble is larger during the expansion period as during the contraction. The larger surface during expansion leads to a greater inflow of gas than during the contraction period. This results in a net inflow of gas for the bubble over time which causes the bubble to grow and finally collapse.

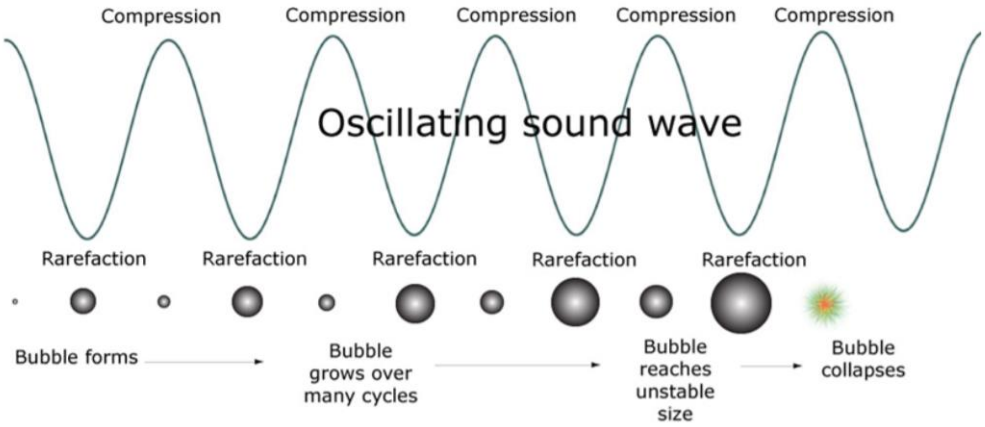

Figure 5 (Leong et al., 2011): The pressure oscillation causes the bubble the oscillate in size during the wave and finally to collapse at resonant size.

The threshold for this violent collapse is called the cavitation threshold. The threshold is dependent on the pressure amplitude during rarefaction. The threshold increases with an increasing frequency, because the bubble has less time to grow due to the shorter wave length. Increasing the applied power to transducer increases the peak amplitude of pressure in the bath. The applied power has to be high enough to reach the cavitation threshold.

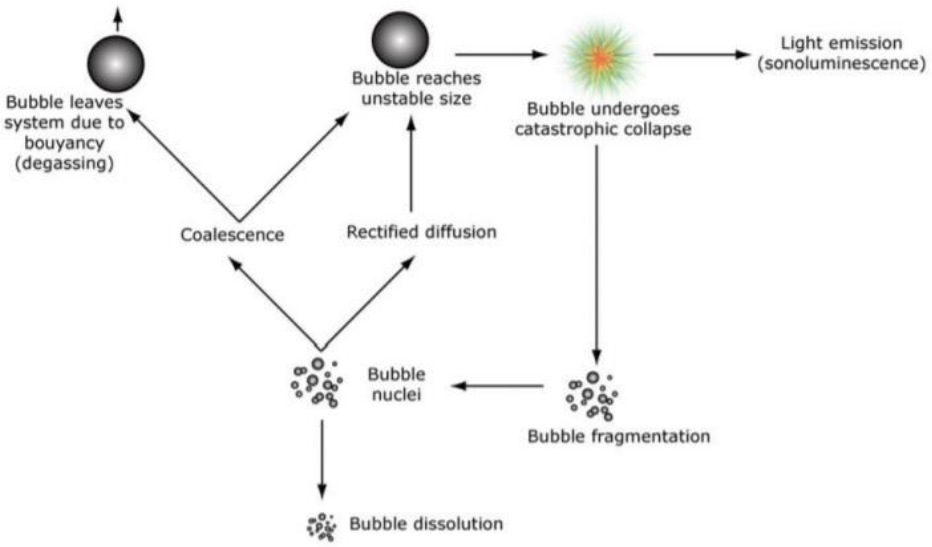

Figure 6 (Leong et al., 2011): The cavitation cycle from bubble nuclei to implosion or degassing at the surface.

The intensity of the of cleaning by cavitation depends on temperature, gas content and liquid height, (Niemczewski, 2007). Non-condensable gas decelerates the collapse. This is why machines are developed with a degassing function prior to the ultrasonic cleaning. The liquid height increases cavitation when the height is equal to half the wave length of the ultrasound. This happens because the wave travelling through the liquid is reflected by the surface. This results in standing waves over the medium and causes resonance (Antony, 1963). The standing waves have pressure nodes and antinodes. Small bubbles gather near anti-pressure nodes, because the primary Bjerknes force is here greater than the buoyancy force. Bubbles which are greater than their resonance size are trapped at the pressure node (Yamashita et al., 2018). Violent bubble implosions happen at the anti-pressure node. This can give a sequence pattern on the cleaned object. The harmonics depend on the velocity of sound in the fluid and the applied frequency. The velocity of sound depends on the density of the liquid which varies per liquid and with temperature. Water at 25℃ has the first standing wave at half the wave length which results in a length of 1.75 cm at an ultrasonic frequency of 40 kHz. The wave length is calculated by: $\lambda = c/f$, λ is de wavelength, c is velocity of the travelling sound wave and f is the frequency. Acetone has its first standing wave under the same conditions at 1.45 cm. Different liquids cavitate with different intensities even when corrected for their column height, due to different parameters such as surface tension and air content. The intensity measured in water is known to cavitate most intensely. Acetone cavitates at 44% of the intensity of cavitation in water (niemczewski, 1980). The cavitation intensity is measured by the following formula:

$$J = \frac{K}{T}\frac{1}{2\rho c}\sum_{k=1}^{M} n_k p_k^2$$

J is the cavitation intensity, K is proportional coefficient, T is the total measurement time, ρ is the density of the fluid, c is the speed of sound, M is the number of pressure intervals, k is the successive number of intervals, n_k is the number of pulses in the interval and p_k is the value of the pressure amplitude in the interval. Beside sound velocity, the type of liquid influences more parameters. The most important ones which improve cavitation intensity are a high surface tension, low vapour pressure and low viscosity (Antony, 1963).

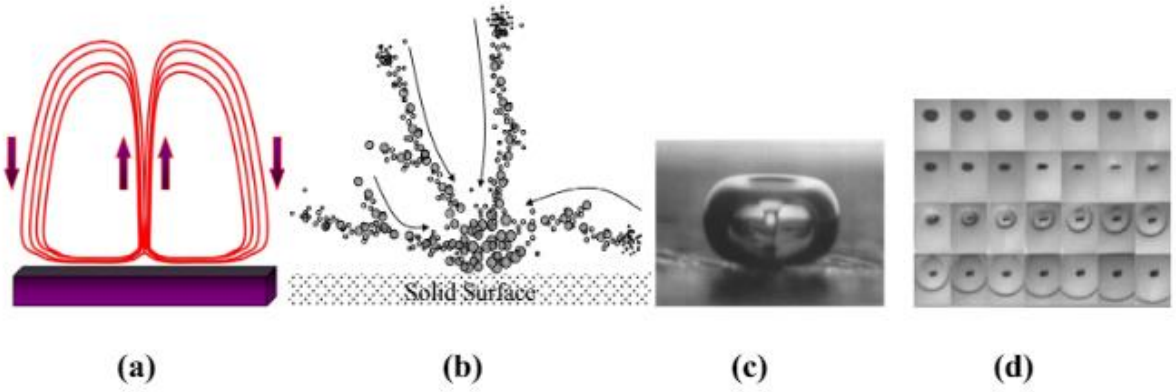

Figure 7 (Yusof et al., 2016): The physical effects of cavitation include the following; a) streaming in the bath, upwards above the transducer, b) the bubble stream towards antinodes due to Bjerknes forces, c) an unstable collapse of the bubble creates a microjet, d) a stable collapse of the bubble creats a shockwave.

Ultrasonic cleaning that is applied to bone cleaning ranges from 28 to 40 kHz as found in literature (Hua et al., 2020; DePaula et al., 2005). The standard frequency for cleaners is between 20 and 45 kHz. The mean size of bubbles depends on the driving ultrasonic frequency and the applied power

(Brotchie et al., 2009). The frequency determines the time that a bubble can grow. The resonant bubble size is inversely proportional to the driving frequency (Laborde et al., 1998; Merouani et al., 2013). Ultrasonic waves cause at higher frequencies smaller bubbles which expel smaller quantities of energy and are better distributed over the bath. High ultrasonic frequencies are more suited for scrubbing tiny features and openings. Lower frequencies are creating bigger bubbles which release more energy when imploding and are less well distributed. This is used to scrub large features heavily. The approximated relation between the resonance frequency of bubbles in relation with frequency is given by (Young, 1999):

$$fR \approx 3$$

In which f is the frequency in Hz and R is the bubble radius in meters. The accurate version of this relation is given by (Young, 1999):

$$R_r = \sqrt{\frac{3\gamma p_\infty}{\rho\omega^2}}$$

R_r is the resonance size, γ is the specific heat ratio, ρ is the liquid density, p_∞ is the ambient liquid pressure and ω is the angular frequency.

It could be advantages to test higher frequencies compared to the standard around 35 kHz, because of three reasons. The first reason is that higher frequencies could work well for cleaning the tiny holes in bones to obtain better results for the removal of fat, because smaller stable bubbles could scrub the surface in tiny pores by their oscillating size which creates micro streaming (Mason, 2016). Cavitation bubbles range in size from 1 µm to 100 µm dependant on frequency and applied power (Brotchie et al, 2009; Brennen, 2011) The pores in bone range from 20 µm to 1500 µm (Murphy & O'Brien, 2010). This means that bubbles of the right size will fit in all the pores of the bone. The second reason is that the number of bubbles increases with higher frequency (Ashokkumar, 2011). This means that bubbles are better divided over the bone graft and the likelihood of reaching all the tiny pores increases. A third advantage of using a higher frequency is that it reduces the viscous boundary layer that forms between the liquid and the part that has to be cleaned (Fuchs, 2015). This viscous boundaries hinders tiny particles to escape from the surface. By increasing the ultrasonic frequency, this boundary layer becomes smaller and small particles can diffuse easier through the layer. The formula for the acoustic boundary thickness is given by:

$$\delta_{ac} = \sqrt{\frac{2v}{\omega}}, where\ \omega = 2\pi f$$

δ_{ac} is the acoustic boundary thickness, v is the viscosity of the medium and f is the acoustic frequency. The hydrodynamic boundary layer which applies to laminar and turbulent flows is given by:

$$\delta = 0.16(\frac{v}{Ux})^{1/7}x$$

δ is the hydrodynamic boundary thickness, v is the fluid viscosity, U is the fluid velocity and x is the distance from the leading edge. The boundary layer for acoustic flow is smaller compared to hydrodynamic boundary layer and allows better exposure to higher velocities (Bakhtari et al, 2006). This streaming inside the boundary layer is discovered by Schlichting and called Schlichting streaming (Schlichting, 2016). These high velocity gradients in the boundary for acoustic boundaries causes steady viscous stresses which contribute to the removal of contaminants (Gale & Busnaina, 1995). A

smaller boundary also allows cavitation bubbles to come closer to the surface. It is questionable if this makes any difference, because the energy released by the imploding bubble reduces with increasing frequency.

The adhesion mechanism that predominates in liquids is the Van der Waals force which is a dipole attraction. The Van de Waals force for a sphere adhering to a flat surface is given by:

$$F_{vdw} = -\frac{Ar}{6z^2}$$

Where F_{dvw} is the Van der Waals force, A is the Hamaker constant which is related to the property of the involved substances and z is the distance between the sphere and the surface. This formula shows that that the adhesion force decreases linearly with the decreasing particle size. The removal forces for mechanisms such as centrifugal or hydrodynamic drag are decreasing by the second or third order with an decreasing particle size and thus are less beneficial to remove tiny particles compared with ultrasonic cleaning (Gale & Busnaina, 1995).

McQueen (1986) investigated the frequency dependence of ultrasonic cleaning. The research showed that fingerprints on a metal surface are better cleaned by a higher frequency of 200 kHz than compared to a frequency of 40 and 100 kHz. A higher power only accelerated the process. Fingerprints exist of fats and proteins which are of sub-microscopic scale and are probably tied to the object by their diffusion rate through the viscous boundary layer. The study of McQueen (1986) showed that larger particles of microscopic scale such as blood cloths were better removed by lower frequencies at 40 kHz. Vetrimurugan (2012) showed that the use of multiple frequencies, namely 40, 58 and 132 kHz resulted in better cleaning results instead of using only one frequency for the cleaning of hard disk drive heads. A study by Naddeo et al. (2015) on reducing membrane fouling in waste waters showed that cleaning those filters with high ultrasonic frequency of 130 kHz improved the removal of organic matter, while lower frequencies of 35 kHz slowed down the process of fouling. This means that investigating the combination of 35 and 130 kHz for cleaning bone could lead to promising results.

Conclusion

Cleaning by ultrasound is selected as the best tool for mechanical cleaning during the chemical baths. Ultrasound produces acoustic cavitation which is used as a mechanical cleaning force. The literature review suggested that combining ultrasound frequencies is beneficial for cleaning. Higher ultrasonic frequencies cause lower resonance sizes of the bubble. This means that bubbles implode at smaller sizes and their impact is less heavy. Bubbles have less time to rupture and grow at a higher frequency due to the shorter wave cycle. This results in a larger cavitation threshold. The cavitation threshold in the bath should be investigated by a numerical model that simulates the pressures in the bath under influence of the driving frequency. A higher ultrasonic frequency causes a smaller viscous boundary layer between the surface and the fluid. This means that it is easier for smaller particles to be removed because they can diffuse easier through the boundary layer. Lower ultrasonic frequencies are beneficial for causing energetic implosions which is used to detach cloths of blood and other large structures. In conclusion, low ultrasonic frequencies of 35 kHz have a better potential in removing large particles such as blood clots. High frequencies of 130 kHz are better for cleaning small parts such as small lipid and virus particles. Combining both frequencies should lead to optimal cleaning results and a reduction in cleaning time. A reduction in cleaning time can optimize the surface intactness of the cleaned bone grafts by reducing the contact time with abrasive cleaning agents.

4. Review of chemical bone processing

This chapter discusses the most common bone processing methods and comes up with the best solution for the processing protocol. Bone can either be processed where only the mineral component remains or to a product where the minerals and collagen remain. First, the process which results in a mineral composition will be discussed. Secondly, the more elaborate process resulting in a mineral composition with collagen will be discussed. A suitable bone graft for implantation has many processing options to become its final composition. There are several steps and each step has multiple optional techniques that lead to the required result. Blood, fat, cells, proteins, spores, prions and viruses should all be removed in chemical and mechanical processing steps to assure sterility of the graft. The first step is the removal of the soft tissue and cortical layer as well as flushing out blood and marrow. Hereafter starts the chemical process which consists of several chemical baths to remove and destroy specific organic components remaining in the bone. This results in the removal of fat, cells, proteins and viruses. The bone is dehydrated, packaged and finally sterilized by irradiation.

4.1 Calcination

Bone powder is not implanted for its strength, but for its osteoconductive properties for bone regeneration. Thus, the preservation for collagen is not required for improved strength. Calcination is a method to produce bone powder in a fast and simple way. During calcination bone is heated stepwise at a rate of 10℃/minute to a temperature in the range of 700 to 900℃ and hold there for 3 to 6 hours (Khoo et al. 2015; Barakat et al, 2009). In this period, all organic compounds including all pathogens are eliminated. Heating to 650℃ does not remove all the organic content (Sobczak et al., 2009). The only remnant is natural hydroxyapatite. Natural hydroxyapatite is a mixture of calcium in combination with phosphate, hydroxyl and carbonate groups. Hydroxyapatite incorporates many trace elements such as Na, Zn, Mg, etcetera. The wide variety in trace elements limits the ease of a synthetical reproduction (Akram et al., 2014). The calcium/phosphate ratio for human and porcine bones is the same, namely 1. 67, but differs from bovine bones that have a higher ratio. The hydroxyapatite alters during calcination at increasing temperatures from 700 to 1100℃. The morphology of the hydroxyapatite changes from amorphous to crystalline during this temperature increase, its particles grow and its carbonate content decreases (Khoo et al. 2015). Hydroxyapatite is thermally stable till 1100℃ (Bano et al., 2017). Other techniques for extracting pure natural hydroxyapatite are a subcritical water treatment or an alkaline hydrothermal bath, but these treatments produce very small nano particles (Bakarat et al, 2009).

4.2 Defatting

The defatting of bone can be performed in many ways. The most common methods are soaking in acetone, chloroform methanol, alcohol, high pressure washing and extraction by supercritical CO2.

Supercritical CO2 is a technique that is used in the food industry for example in extracting caffeine from coffee beans. Supercritical CO2 is claimed to be effective in extracting lipids from cancellous bone, because it diffuses easily into micro holes. After treatment of the bone with supercritical CO2, 1.5 percent of fat resides in the bone (Fages et al., 1994). The method does not affect the strength of the bone (Mitton et al., 2005). The drawback of using supercritical CO2 is that it is applied under high temperature and pressure. This warrants a machine with high purchase price.

Chloroform in combination with methanol is used in a ratio of methanol/chloroform 1:2. This method is the most common one in literature. The choice of the applied procedures with these chemicals depends on the percentage in fat loss required and the accessibility of the solid. Chloroform is a toxic substance that has to be completely removed from the bone graft. Kalus et al. (2005) describes a treatment for cancellous bone by a two hour bath of chloroform/methanol followed by eight rinses with methanol followed by two rinses of purified water. This treatment results in a chloroform concentration less than 25 ppm which is an acceptable concentration. Nevertheless, less toxic defatting methods are preferred. The process with chloroform and methanol does not deteriorate the mechanical properties of bone (Thorén et al, 1995).

The usage of lipase is promising so far on porcine bones with a residual fat percentage of 0.5 (Zhang et al., 2014). Lipase, which is a product of the pancreas, excludes the usage of any chemical reagents. The drawbacks of the use of enzymatic delipidation are the costs involved. The current market price is around 50 euros per litre for an effective concentration of lipase in solution.

Acetone and ethanol are both on their own, or in combination used for defatting. A recent paper by Hua et al., (2020) compares the following defatting methods; acetone, alcohol and high pressure washing at 6 MPa. The tested bone graft samples are blocks of 5 mm in the x, y and z direction. The results for alcohol and ethanol are statistically the same and result in a residual fat percentage of 1.28 and 1.13. High pressure washing with a duration of 10 seconds resulted in a percentage of 1.45%. The maximum compressive stress of the bone is around 10 MPa for the methods with acetone, ethanol and the 10 second pressure washing. The compressive stress is the comparable to fresh bone and bone after treatment by supercritical CO2. Longer pressure washings of 20 and 30 seconds results in significant reduction in compressive strength. Defatting bone with a gradient alcohol is beneficial because it ruptures the fat cell and dissolves the lipids (Gardin et al., 2015). When the alcohol concentration is too high it causes the intracellular proteins to denature too fast which prevents alcohol from entering the cell. Thus one should start with a low alcohol concentration.

4.3 Protein and cell destruction

The defatting stage is followed by the destruction of cells, proteins and viruses in exception of collagen. This step is executed in two phases to secure that all harmful bacteria and viruses including prions are destroyed. The first step is to destroy and remove all cells and proteins. The most investigated sterilizing methods for bone grafts are hydrogen peroxide, moist heat sterilization, dry heat, urea and ethylene oxide.

Moist heat and dry heat sterilization are not recommended, because the strength of bone grafts decreases at temperatures above 70℃ due to collagen shrinkage (Vagnsness et al., 1997). No negative effects were observed on the ingrowth of bone grafts when exposed to temperatures lower than 44 ℃ (Eriksson & Albrektsson, 1984). This is probably due to denaturation of proteins at temperatures above 40℃. Dry heat is performed at a temperature of 250℃ for effective sterilization (Rashed et al., 2020). Moist heat requires a temperature of 135℃, but does not kill all pyrogens (Salama & Mobarez, 2015). Pyrogens are fever causing parts of Gram-negative bacteria such as E. coli. However, the deteriorating effects of moist heat on the bone strength are small (Vastel et al., 2009)

A new disinfection method is high hydrostatic pressure. The volume of fluids changes when very high pressures are applied. The volume of water decreases by 12% under 400 MPa at room temperature.

This is used for the destruction of microorganism by changing the non-covalent bonds of macromolecules and the conformational state of lipids (Knorr, 1999; Diehl et al., 2008). The tests on disinfected bone grafts are promising so far (van de Sande et al., 2017), but it needs further clinical investigation.

Hydrogen peroxide is an often used step. Hydroperoxide can also be destructive when it is too long in contact with bone. DePaula et al., (2005) showed that hydrogen peroxide 3% is detrimental for the osteoinductivity when the cleaning produce lasts for three hours. Hydrogen peroxide is used to destroy pyrogens at low costs, while the mechanism of destruction is unknown (Salama & Mobarez, 2015). Hydrogen peroxide also seems to reduce the fat content. Fages et al. (1994) shows that a hydrogen peroxide 35% rinse after a supercritical CO2 cleanse reduces the fat content from 1.5 to 0.5 percent.

4.4 Prion elimination

A second chemical is added to improve the reduction factor of the remaining prions, namely sodium hydroxide. Sodium hydroxide (>1M/L) and chlorine (>1000 ppm) are known as most effective in destroying present prions, but their corrosiveness should be taken into account (Rutala et al., 2010). Sodium hydroxide is most promising for bone grafts and reduces by >5.6 $\log^{10}$ the prion load (Fichet et al., 2004). Hereafter, the sodium hydroxide is flushed away, the residues of sodium hydroxide are neutralized by the addition of sodium dihydrogen phosphate 1.2% (w/w). Sodium dihydrogen phosphate reacts with sodium hydroxide by the following reactions:

$NaOH\ (s) \rightarrow Na^+\ (aq) + OH^-\ (aq)$

$NaH_2PO_4\ (s) \rightarrow Na^+\ (aq) + H_2PO_4^-\ (aq)$

$OH^-\ (aq) + H_2PO_4^-\ (aq) \rightarrow H_2O\ (l) + HPO_4^{2-}$

Chlorine is applied as a gas by chlorine dioxide for 6 hours at 30℃ for sterilization of medical equipment (Govindaraj & Muthuraman, 2015). Vaporized hydrogen peroxide gives also good results in sterilization of prions (Rogez-Kreuz et al., 2009). Vaporized hydrogen peroxide is dangerous for humans when inhaled.

Urea in a concentration of 6 mole/L could do the same, but is more destructive to bone and reduces its mechanical strength by 30% (Vastel et al., 2004). Attention should be paid that urea 6 mole/L deteriorates the collagen which decreases the mechanical strength. Urea 4 Mol/L is also enough to destroy prions and probably less destructive to collagen (Rutula & Weber, 2010).

4.5 Dehydration

The bone graft is now assessed as clean. The water content should be less than 6 percent to preserve bone grafts at room temperature instead of frozen. Dehydrated bone grafts need to be hydrated before surgery to obtain better ductile properties (Erivan et al., 2017). There are two general options for this steps, freeze drying and dehydration by washing with high concentrations of ethanol. Freeze drying is disputed in the literature because of detrimental effects to the properties of bone grafts (Conrad et al, 1993).

Ethanol is a relative simple procedure and has no known negative effects. First is added 95 percent of ethanol for 3 hours which dilutes the remaining water in the bone. Five percent of water remains in

the bone graft. A second solution of 99 percent of ethanol is added for two hours. A concentration of 0.95 times 0.99 results in less than one percent of water remaining in the bone graft. The remaining ethanol in the bone graft has to be evaporated and is dried for 12 hours in an airflow at 40℃.

4.6 Final sterilization

The dehydrated bone grafts are kept in the existing form or are grinded to powder. Those products are vacuum packaged and irradiated. The irradiation is a final sterilization step to secure that any contamination is excluded. The ionising irradiation exists of gamma irradiation or electron beam irradiation. Gamma irradiation has the benefit of penetrating easily through objects. However it is harder to control its exact dose and has the problem of scattering of rays “bremsstrahlung”. This requires a long irradiation time and as consequence higher temperatures. Electron beam irradiation can be applied very precise and has short operation times (Yusof, 2018). The disadvantage of electron beam irradiation is a low penetration depth and cannot be applied to large batches.

The sterilization works by the collision of gamma rays or electrons with atoms or molecules whose chemical bonds are disrupted. This can be directly on the targeted molecules or indirectly by the production of free radicals such as hydroxyl from the present water which reacts with pathogens. The effectivity of sterilization by ionizing radiation depends on multiple factors. The sensitivity of the pathogen for irradiation. The most resistant pathogen *Bacillus* and *Bacillus pumillus* needs 1.75 kGy to destroy 90% of its pathogens (Nguyen et al, 2007). The pathogen load that is present determines the irradiation dose. According to Hilmy et al. (2000), the most radiation resistant pathogens in combination with an average bioburden should result in 14.4 kGy for a sterility assurance level of 10^{-6}. The quantity of the irradiation energy that is absorbed is dependent on the materials and packaging. Lower temperatures make pathogens more irradiation resistant and reduces the effectiveness of irradiation at a certain dose. The water content can increase the sterilization effectiveness due to the formation of hydroxyl radicals. Pathogens in dehydrated bone are more resistant to irradiation. This all makes that the irradiation quantity is specific for the bone process that preceded. The presence of oxygen can form, when irradiated, hydrogen peroxide with the hydroxyl radicals which improves the effectiveness of the irradiation for sterilization. Radiation protective substances such as ethanol and glycerol reduce the effectiveness. Defatting is beneficial for the performance of the bone graft, because irradiation could alter lipids that become toxic (Dziedzic-Goclawska et al., 2005)

Irradiation can be detrimental to collagen under high doses (Rasmussen et al., 1994). The irradiation is assumed to be responsible for cleavage of the polypeptide chains in the collagen. Present moisture could reduce the cleavage of bonds by restoring crosslinks in the collagen (Yang et al., 2019). The damage can be reduced significantly by using electron beam irradiation which has a small effect on the biomechanical properties compared to gamma irradiation (Hoburg et al., 2015). Free radical scavengers such as mannitol, acorbic acid or vitamin C could be a solution, because they remove moisture and oxygen which prevents the formation of free radicals. Those free radicals destroy covalent bonds in the collagen (Yang et al., 2019). The best solution would be the addition of a cross-linking agent such as 1-(3-dimethylaminopropyl-3-carbodiimide) in combination with free radical scavengers according to Seto et al. (2009).

At present the sterilization dose ranges between 10 and 35 kGy for tissue banks (Vangsness et al., 1996). Gamma irradiation is most common now adays and the advised standard is 25 kGy to obtain a sterility assurance level of 10^{-6}. The amount of 25 kGy is based on previous studies who predict that this amount is high enough to kill all viruses and bacteria as validated by ISO 13409 for bone

allografts (IAEA Radiation Sterilisation of Tissue Allografts, 2007). However, Dziedzic-Goclawska et al., (2005) recommend a dose of 35 kGy by stating that 25 kGy is not high enough for unprocessed infected bone, stating that viruses and prions are very resistant to ionising radiation. It should be noticed that the bone graft is already sterilized by hydrogen peroxide and sodium hydroxide and needs less irradiation to be effective. Besides, a dose higher than 20 kGy could be detrimental to the properties of the bone (Jinno et al., 2000). This is why the quantity of irradiation should be determined specifically for the preceding bone process (Nguyen et al, 2007). Most beneficial is when this load stays under 20 kGy for its properties and most likely this is possible due to previous sterilization baths.

Ethylene oxide is a common option for sterilization, but it is not possible to do this after the product is packaged which includes an extra contamination risk. Ethylene oxide is toxic and can reside in the bone when it bonds to water or fat. There is a procedure to sterilize without ethylene oxide residuals, but this consists of three days of evaporation (Kakiuchi et al., 1996). A benefit of ethylene oxide is that it can be used to sterilize large batches to reduce costs, but in the case of bone graft production, it is not beneficial enough.

Another possibility is microwave irradiation. Microwave radiation is quite new as sterilization technique and the effects on bone are not known yet. It does kill however all bacteria in 60 seconds at 2450 MHz and 900 W (Singh & Singh, 2012). The use of a microwave would be a very cheap solution.

5. Final bone processing protocol

This chapter discusses the selection of the final processing protocol consisting of six main steps. The final protocol will be discussed in detail step by step. The six mayor processing steps are mechanical pre-treatment, defatting, protein and cell destruction, prion annulation, dehydration and final sterilization. The selected protocol can be seen in table 1.

5.1 Selection of bone processing techniques

Mechanical treatment

The selection for the pre-treatment is a debridement of the soft tissue and cortical bone by a Fortios bone debrider. This is followed by a water jet to flush out the bone and marrow. Hereafter ultrasonic cleaning is used during the chemical baths. The applied ultrasonic vibration is 35 kHz followed by 130 kHz. As explained in chapter 3.7, lower frequencies produce bigger cavitation bubbles with more energy release but less well distributed. Higher frequencies are more gentle, have less energy release but are better distributed and better in cleaning sub-microscopic particles. Bubbles at higher frequencies fit better in tiny pores where they can scrub the walls by micro streaming. Starting at 35 kHz will detach the bigger impurities on the surface and the 130 kHz vibration will clean the smaller pores in bone. These specific two frequencies depended on the available piezoelectric transducer by manufactures. The piezoelectric transducer has to resonate at two frequencies. The advised cleaning time per frequency is 25% of the time at 35 kHz, followed by 25% at 130 kHz, then 25% again at 35 kHz and finally 25% at 130 kHz. The precise time division should be fine-tuned by experiments. Stirring by a rotor instead of ultrasonic cleaning is not advised, because it generates large turbulent flows which inhibit the low pressures for cavitation initiation.

Defatting

Acetone 100% in alternation with ethanol 50% has been chosen for the defatting step. Acetone and ethanol are cheap and easy to use at room temperature. The only disadvantage is that acetone is toxic, so the produced gassed should by extracted safely and secure. Components cleaned in a mixture of ethanol and water are significant cleaner than in pure water (Antony, 1963). Super critical CO2 has complicated machine requirements due to the high temperature and pressures applied, which makes it unsuitable for a simple and efficient machine. Lipase is a good solution for defatting. The only disadvantage of lipase is that it is very expensive. It costs around 50 euros to create one litre of solvent. The chloroform methanol mixture has not been selected because it is toxic and requires too long evaporation times to dissolve from the bone graft.

Protein destruction

The protein and cell destruction is done by a hydrogen peroxide 35% (w/v). This method is cheap, well known and gives good results. Vaporized hydrogen peroxide is too aggressive and makes the machine requirements complicated. Moist heat and dry heat are not opted for because they destroy the collagen content due too high temperatures. Hydrostatic pressure is not chosen, because it is a relative new technique without much publications concerning bone grafts. The machine requirements become more difficult when high pressures need to be applied. Urea is not selected, because it is too aggressive and reduces the bone graft properties.

Prion elimination

Sodium hydroxide is selected for the prion elimination. Sodium hydroxide is recommended by the European Union for sterilizing prions. Chlorine and vaporized hydrogen peroxide are not selected

because they have to be applied as vapor which increases the difficulty of the machine specification. They are also too aggressive for the bone grafts. Chlorine and hydrogen peroxide are very toxic, even when inhaled in small quantities, which increase the level of safety measures that have to be applied. This makes them unsuited.

Dehydration
The dehydration has two options, freeze drying or ethanol. Freeze drying deteriorates the bone graft properties. That is why ethanol is selected for this step. Ethanol is also easier to apply as a step in the production line because it does not require cooling as for freeze drying.

Sterilization
The final sterilization could be done by irradiation or ethylene oxide. Ethylene oxide is not selected because it is toxic and needs long evaporation times. Irradiation has as advantage that it can be performed after packaging, which increase the certainty of a sterile end-product. Gamma or beta irradiation can be selected as irradiation and the dose can be varied. Beta irradiation is selected because it produces less heat in the bone graft at the same dose. Gamma irradiation can be detrimental to the bone graft above 20 kGy. The applied dose is 25 kGy with Beta irradiation which is recommend by the European Union for sterilization.

Table 1: The final bone processing protocol

Bone processing protocol					
Step	*Process*	*Additive*	*Time*	*Temperature*	*Agitation*
1	Mechanical debridement	-	-	room temperature	-
2	Rinse	Purified water	10 min	room temperature	ultrasonic
3	Soak	Acetone 100%	3 hours	room temperature	ultrasonic
4	Soak	Ethanol 50%	1 hour	room temperature	ultrasonic
5	Soak	Acetone 100%	3 hours	room temperature	ultrasonic
6	Soak	Ethanol 50%	1 hour	room temperature	ultrasonic
7	Rinse	Purified water	10 min	room temperature	ultrasonic
8	Soak	Hydrogen peroxide 35%	2 hours	40 °C	ultrasonic
9	Rinse	Purified water	10 min	room temperature	ultrasonic
10	Soak	Sodium hydroxide 4%	1 hour	room temperature	ultrasonic
11	Rinse	Sodium dihydrogen phospate 1.2%	30 min	room temperature	ultrasonic
12	Soak	Ethanol 96%	3 hours	room temperature	ultrasonic
13	Soak	Ethanol 99%	2 hours	room temperature	ultrasonic
14	Drying	Air flow	12 hours	40 °C	-
15	Irradation	Beta irradiation 25 kGy	-	-	-

5.2 Processing steps

Step 1

The 15 steps of the process are elaborated per step and recommendations will be given for optimization per step. The first step is to remove the remnants of soft tissue on the bone. These remnants can be cleaned easily and quickly by a high speed rotating clean and sterile metal brush. In most companies this step is still done by hand with a scalpel. Using the brush gives a time benefit.

The bone grafts are placed in a stainless steel basket after debridement. Stainless steel is often used because it does not corrode and is easy to sterilize after use in an autoclave. The size of the basket and processing machine should match well to prevent useless space. All the space up to several

centimetres above the bone grafts have to be filled with fluid. A ratio of 1:5 is recommend between the bone volume and the added liquid. An excess of liquid would be a costly waste. A too small volume of liquid would endanger the process in its capacity to dissolve substances.

Step 2

The second step is a rinse with purified water. This first washing is done to remove blood and cells that are easy to wash away. The rinsing is done by ultrasonic agitation for a good diffusion through the porous bone. Prior to this step could be added a manually operated water jet to remove these substances as well. This gives good results in removing fat as well according to Hua et al., (2020).

Step 3-6

The third till the seventh step is the defatting procedure. First acetone is added to the bone grafts. This solution is agitated by ultrasound. The solution resides for 3 hours at room temperature. Here after, the solution is washed away at an overflow. The fat floats on top of the acetone and is drained at the top overflow. This is done to prevent contamination of the bone grafts by draining it at the bottom. The fifth step is washing the bone grafts with a 50 percent ethanol 50 percent water solution. The purpose of this step is to remove the residing remnants of acetone with fat from the porous bone and removing as well some fats for which acetone is less suited. This steps takes 1 hour. The fifth step is again acetone which remains for 3 hours again with ultrasound. Step six is again a rinsing with 50 percent ethanol solution.

Step 7

Step seven is a rinse with purified water for 10 minutes with ultrasound. The purpose is to remove all the remaining liquids and clots from the defatting procedure.

Step 8-9

Step eight is removing cells, bacteria, proteins and viruses. Hydrogen peroxide is added for 2 hours at 40℃. A shorter time will not reduce the viral load as effective. During the two hours, the machine will be agitated by ultrasound. A rinse with purified water is executed to remove the remnant from step eight. This purified water takes 10 minutes under ultrasonic vibrations.

Step 10-11

The tenth step is a bath in sodium hydroxide. The sodium hydroxide bath is to make sure that any remaining prions are killed. Even though hydrogen peroxide does this as well, repeating it with sodium hydroxide makes the reducing factor even larger (Fages et al., 1998). The sodium hydroxide is neutralized in step eleven by replacing the sodium hydroxide with sodium dihydrogen phosphate 1.2% for 30 minutes at room temperature under ultrasonic vibrations.

Step 12-14

The twelfth step is dehydrating the bone grafts for storage at room temperature. The grafts have to have a water content less than 6 percent. First is ethanol 95% added for 3 hours and then drained. This is followed by a second bath at ethanol 99% for 2 hours. The water content should be less than 1 percent after these two steps. The step is followed by airflow drying at 40℃ to evaporate all the remaining ethanol in the porous bone structure.

Step 15

The final step is packaging the bone grafts in their current shape or optionally grinded to powder. Grinding to powder should be done after the cleaning process otherwise the powder will be lost in the washing process. These are vacuum packed and sterilized by beta irradiation at 25 kGy.

The expected viral load in the end product is less than 10^{-6} due to the four sterilizations steps, namely the degreasing and washing, the hydrogen peroxide, sodium hydroxide and the irradiation. This is comparable to studies as Fages et al. (1998), which investigated the viral load.

6. Production line requirements

The requirements for the production line are listed in this chapter. The requirements for the production line are based on the bone processing protocol as selected in chapter five. The functional requirements are directly related to the processing protocol. Several factors have an allowable range and will be optimized in the design. These variable criteria are safety, efficiency, cleanability, manufacturability and human interference.

Functional requirements:

- Minimal human interference
- Ultrasonic agitation 35 & 130 kHz separately
- Heating bath till 60 ̊C
- Inflow different types of chemicals fluids without backflow
- An overflow for floating lipids
- Drainage at the bottom
- No corrosion (pH 14)
- Airflow drying
- Venting
- Machine parts easy to clean and sterilize
- A safe production line
- Automatic operation
- Minimal cross-contamination
- No overheating transducers
- Uniform cleaning results from ultrasound
- Compatibility with all chemical agents

Detailed design specifications

There are eight distinct fluids that are added to the bath. Those come from eight different storage tanks. Which results in eight distinct inlets.

- Chemical liquids do not mix before inlet
- Drainage of liquids according to environmental standards
- Down sloping floor towards the drain
- Transducers mounted on a flat floor for balance
- Heating panels providing uniform temperature over the volume

7. Conceptual design production line

This chapter discusses the implementation of the design criteria to create an automated production line. It implements the chosen proposal and describes the design choices in detail. The design describes the production line and elucidates decisions as the production size and the number of separate machines used. The detailed production line functions are elaborated such as in and outflow of liquids, heating and vibration. The design includes further safety precautions, endurance regarding corrosion and proper cleaning to prevent contamination between batches. These design choices are explained as follow by this chapter: production size, multiple versus one bath, material choices, functionality, fluid transport system and safety.

7.1 Production size

The production size is determined by the batch dimension. The production line for bone grafts is a first design. A first design and practical trial are bringing unforeseen problems along and require new inventions along the way to work out all the kinks. This implies that it would not be wise to start with an investment in a very large volume. A processing goal of 3200 femoral heads a year is a reasonable one. The processing time of the machine is 29 hours. The machine will not operate all the time uninterrupted because the batch has to be replaced with a new one after processing, the machine will undergo maintenance and will be out of production on national holidays. Thus the average time per batch size is estimated on 36 hours. This results in 243 batches a year. 3200 divided by 243 is 13.17 femoral heads per batch. A machine with a rectangular size is better for its functional requirements such as balance and heating. A batch size of 15 is chosen. This results in processing roughly 3645 femoral heads a year. A femoral head ranges in the size on average from 40 to 54 mm in diameter and is spherical in shape (Affatato, 2014). To have some tolerance, a cube with sizes of 70 mm in all directions is preserved per femoral head. This results in a basket of 210 mm width, 350 mm length and 70 mm height. This basket has to fit in the liquid baths. The liquid baths should be kept as small as possible to prevent wasting chemicals and to reduce costs. The total volume of the liquids baths will be 5.145 litre. This leads to a minimal volume of 1250 litres a year per chemical liquid.

7.2 Multiple versus one bath

The production line can be made by separate baths which all execute a processing step with an automatic transport system in between. Another option is to combine all those steps into one bath in one machine. Both options have advantages and disadvantages. A production line with several baths will result in a maximum of five baths. This number of five baths depends on the bone processing protocol which has six main processing operations. The sixth processing operation is not executed by this production line because the gamma irradiation will be outsourced to a licenced facility after the product is packaged. The five processing operations for the baths are defatting with ethanol and acetone, protein destruction by hydrogen peroxide, prions elimination with sodium hydroxide, dehydration with ethanol and evaporation of ethanol by air drying. The first benefit of keeping these steps in multiple baths is that the production time is reduced to the longest step. The longest step is drying which takes 12 hours. The disadvantage of multiple baths is that every bath requires a sterilization of the bath after one batch to prevent possible cross-contamination between the batches. All steps combined in one machine with one bath does not require a cleaning step at the end of the processing protocol, because the processing protocol is already a sterilization procedure.

This procedure is not complete in one bath when the process exists of multiple baths with in each bath only one chemical process step. Multiple baths have as disadvantage that there has to be a transport system for the basket between the baths. Designing all steps in one machine gives a compacter production line and it needs less repetition in machine parts which saves costs. The challenge in designing all steps in one machine is to satisfy all machine requirements and to assure a safe process while using flammable liquids such as acetone. Based on the previous arguments is selected a combination of all processing steps in one machine.

7.3 Material choices

Chemical fluids are deteriorating the machine, storage tanks and pipes when the material properties are not chosen well. They can react with the materials or stimulate corrosion. The material used for the machine and fluid transport system should be selected carefully, not only for degradation of its part but also to prevent contamination of the fluids. First, the behaviour of corrosion that could occur in the production line are described and the design choices therefrom derived. Hereafter are discussed the degradational behaviour and the properties of every chemical and as conclusion is given a recommendation for the compatible materials.

Corrosion

Corrosion is the main process in deteriorating metals. Two types of corrosion should be taken into account for the machine and storage tanks. Those types of corrosion are named uniform corrosion and galvanic corrosion. Uniform corrosion takes place over the whole surface and is the most common type. The rusting of metal is the result of an oxidation reduction reaction. An oxidation reaction occurs when three components are present, a metal, a corrodent and water. A metal in water ionizes and will oxidize in the solution. The result is a precipitate called rust. Stainless steel is recommended to prevent uniform corrosion.

Galvanic corrosion occurs where two different metals are in contact with each other in a humid condition (Oldfield, 1988).The two metals have a difference in electrical potential which creates an electrical current of electrons flowing from the anode to the cathode. The anodic metal is less noble than the cathodic metal. This is called a galvanic couple between two different metals, it accelerates the corrosion of the anode which corrodes faster compared to when it was alone. On the other hand, the corrosion of cathodic metal slows down compared to when it was alone. The galvanic area ratio between the two metals is important for the corrosion rate. If the anodic area is way larger than the cathode it reduces the reaction significantly or almost completely. This is happening because the potential difference between the two metals is now divided for the anode over a larger area resulting in a lower potential difference. This means that the type of metal for small parts in the machine, which are in contact with the fluids, need to be carefully selected. An even better solution would be that the surface area in contact between parts exists of only one material.

Cavitation erosion

Materials are sensitive to cavitation. The implosions caused by cavitation stresses the wall of the bath and repetition overtime can cause pitting. The implosion produce violent shock waves and microjets. Especially these microjets stresses the surface at a very small area. The impact on the surface causes elastic and plastic deformation. Overtime this can result in metal fatigue and results in an accumulation of small cracks. Zakrzewska & Krella (2019) showed that metals performed best against cavitation erosion with a homogenous fine grained structure with high endurance and high

corrosion resistance. This surface of the material needs to be smooth and plate structures are more resilient.

Hydrogen peroxide

Hydrogen peroxide is non-flammable but it can accelerate the combustion of organic matter. Organic matter can be oxidized by hydrogen peroxide when it is present in high concentrations. The concentration of 35% is not high enough for hydrogen peroxide to intensify a fire. It is still recommendable that hydrogen peroxide should be kept away from flammable substances and not stored in wood for example. Hydrogen peroxide is decomposing slowly and thereby releases oxygen. A release valve has to be installed to prevent a pressure built up which could rupture the storage tank. When the storage tank is made of metal, it has to be grounded. Suitable material to store or transport hydrogen peroxide are stainless steel 304 or 316, PVC 100, very pure aluminium (99.5%), aluminium magnesium alloys, polyethylene, polypropylene and polytetrafluoretheen (teflon).

Sodium hydroxide

Sodium hydroxide 4% has a pH of 14. It attacks chemically the protective coating in galvanized steel due to presence of zinc. Sodium hydroxide is stable for a long time. Concentrated sodium hydroxide has to be stored at temperatures above 16℃ otherwise it crystallizes which causing damage to pumps for example. This applies only for concentrations higher than 30%. The temperature should be kept lower than 50℃ for carbon steel tanks and lower than 38℃ for polyethylene tanks. So indoor storage is preferred. Sodium hydroxide cannot be discharged in water or acid directly, because it reacts violently exothermic inducing a rapid increase in temperature. Sodium hydroxide can be stored or transported in stainless steel, carbon steel, PVC, polypropylene and polyethylene. The corrosion of stainless steel 316L is 0.4 mm/year for concentrations of 30-50% sodium hydroxide (Monteiro et al., 2019).

Acetone

Acetone is highly flammable and can form explosive mixtures when mixed with high oxygen concentrations. The flashpoint of acetone is -20℃. Fires occur only when oxygen is present so acetone should be kept away from an oxygen supply. Acetone is compatible with stainless steel 316, carbon steel and polypropylene. The storage tanks should be properly grounded when they are metallic. Acetone is not compatible with PVC.

Ethanol

Pure ethanol evaporates easily and has a flashpoint at 13℃. Excess evaporation of ethanol should be prevented, because it creates a flammable environment and reduces the stored quantity. It should be stored in a closed container that is grounded in case it is metallic. Ethanol has to be stored dry, because it absorbs water from its surrounding. Ethanol is compatible with stainless steel and polypropylene and high density polyethylene (HDPE).

Sodium dihydrogen phosphate

Sodium dihydrogen phosphate is an acid with a pH around 4. It can be stored like most acids in a material of stainless steel, polypropylene or polyethylene.

Conclusion

The recommended material for the machine is stainless steel 316L, because it is protects against all chemicals that are used, it provides strength for the construction of the machine and it is easy to

clean. The storages tanks and pipe network can be made of polyethylene or polypropylene. These plastics storage tanks and pipes are cheaper than the stainless steel variant. The rubbers that can be used to seal the machine by its opening can be made of Ethylene-Propylene-Diene-Monomer (EPDM), Butyl or Kalrez which are compatible with all the chemical baths.

7.4 Functionality

Inlet

The processing protocol exists of eight different liquids namely, purified water, acetone, ethanol 50%, ethanol 96%, ethanol 99%, hydrogen peroxide, sodium hydroxide and sodium dihydrogen phosphate. All these liquids will flow in from distinct reservoirs and are used in different stages of the process. This results in 8 distinct inlets. These inlets need to be designed properly so that they don't obstruct the inlet of the basket with the bones. The inlets do not have a closure mechanism because the inflow of liquid will be managed by the fluid transport system between the machine and the reservoir tanks.

Drainage

The machine has to drain eight different liquids that are contaminated by substances derived from the femoral heads. These liquids cannot be reused because cross-contamination could occur between the batches. The liquids have to be disposed in line with environmental requirements. Different chemicals have different disposal systems. The liquids will be separately drained form the machine and captured in storage tanks. The ethanol in different percentages will be combined in one storage tank and requires therefore only one drain. This results in a total of six drains at the bottom. The floor at the bottom has to be tapered so that all the liquid will flow in the direction of the drain. The drains will be operated by an automatic gate that opens and closes them. The drainage of the fats will be done by an overflow. Fat floats on water and therefore will contaminate the bones if it is drained only at the bottom. This overflow can surround the basket at all sides or be a single slit. This depends on the amount of fat that is left on the wall at the top of the liquid volume.

Closing mechanism

The basket with the femoral heads is replaced during each batch. This is possible by an opening in the roof of the machine or by a side door. The benefit of a roof opening is that is does not get into contact with the fluids and it is easier to handle the basket. The side opening has the benefit that a venting system and fluid inlets can be made at the top. An top opening can have a lid that is removable or connected by a hinge. The benefit of a hinge system is that is does not have to be laid down somewhere which can get contaminated. The side opening can be a door or a cylindrical screwable lid. A door on top of the machine is preferred. The doors can be sealed by rubbers that are resistant to all chemicals.

Heating

The heating of the bath depends on the volume, the specific heat capacity of the liquid and the change in temperature. The volume of the bath is 5.145 litres. Hydrogen peroxide 35% is the only liquid that has to be heated to 40℃. Hydrogen peroxide enters the bath at room temperature, because it is stored at this condition. So it needs a temperature increase of 20℃. The specific heat is 3.5 J/g/K at 25℃. One litre of 35% (w/v) hydrogen peroxide weighs 1124 grams at 30℃. So the total heat that has to be added is about 78.7 KJ. The machine has to heat the volume in about 5 minutes.

Shorter would be better for the accuracy of the process, but 5 min is assumed as reasonable. This results in a heating element that produces at least 263 watt. More watts are required because of heat loss to the environment. The heating element can be mounted on the sides of the bath. The sheet metal of the bath will conduct the heat quickly and does not have to be considered in detail. The recommend solution will be heating plates at the side of the bath that produce together at least 300 watt. Known ultrasonic cleaners that are for sale can reach with 300 watt up to temperatures of 80℃. A temperature sensor should be added to the bath to control the heating. Convection will transport the heat through the bath. Ideally, the heating element would be placed under the bath. This is however not possible because the ultrasonic sound producers are mounted here. An better solution is preheating the volume size needed for one batch. This makes that the liquid enters the bath at the right temperature and no time is lost with heating. Heating only one batch size prevent high heating costs for heating the whole storage tank.

Ultrasonic vibration

Ultrasonic vibration is applied to produce acoustic cavitation which is used for cleaning. The ultrasonic waves are produced by piezoelectric transducers which are deformed by the applied voltage or by magneto-strictive transducer which produces an expanding and contracting material under the influence of an alternating magnetic field. There is chosen for an piezoelectric transducer, because it is more efficient than a magneto-strictive transducer which has more power losses and operates worse at frequencies above 20 kHz. The magneto-strictive transducer has high power losses because it has to convert electrical energy first into magnetic energy before turning it into mechanical energy which includes eddy currents and hysteresis.

The piezoelectric transducers are mounted on to the bottom. Their power is based on the weight of the volume. If the applied power is not high enough cavitation will not occur and the fluid will only oscillate in form and size. A power supply of 130 watt is needed for a volume of 5 litres. The power cannot be too high, because this hinders the process of cavitation. When the power is too high, the bottom plate detaches from the liquid and the acoustic energy is lost. This is called decoupling (Mason, 2016).

Dissolved gas also hinders the violent implosion during cavitation. Degassing reduces the cavitation threshold and makes the machine more efficient. Degassing is executed by pulsing the solution the first 5 minutes of operation. The ultrasonic vibration concentrates the dissolved gas into a larger gas bubble. The pauses in applying ultrasonic vibration gives air bubbles the time to rise and thus leaves the liquid. Complete degassing is impossible as partly regassing occurs as stated by Niemczewski (2014). Degassing happens also automatically during ultrasonic cleaning but takes approximately 20 minutes when used without degassing function.

Cavitation is more intense due to resonance when the liquid height has the length of a multiple of the standing waves. These harmonics depend on the velocity of sound in the fluid and the applied frequency. The velocity of sound depends on the density of the liquid which varies per liquid and with temperature. The multiple liquids that are used differ in height for their harmonics which makes it impossible to design a length at which all liquids resonate. However, the total amount of liquid should be controlled at the inlet. This can be used to vary the total height of the column per liquid bath. The liquid height for water at 20℃ is 4 times 1.75 which is 7 cm, resulting in its 4^{th} harmonic. The liquid height for acetone is 1.4625 times 5 which is 7.3125 cm, resulting in its 5^{th} harmonic. See table 2 for the liquid heights of all the chemical agents.

Table 2: The speed of sound of each liquid and its column length at the 4th or 5th harmonic. The table shows the range in optimal column height of each liquids for a frequency of 40 kHz.

Liquid	Speed of sound (m/s)	1st harmonic at 40 kHz (cm)	Column height (cm)
Acetone (20 °C)	1192	1.490	7.45
Ethanol 50% (20 °C)	1500	1.875	7.50
Ethanol 99% (20 °C)	1159	1.449	7.24
Water (20 °C)	1482	1.853	7.41
Hydrogen peroxide 35% (40 °C)	1560	1.950	7.80
Sodium hydroxide 4% (20 °C)	1600	2.000	8.00

The specification for the driving frequency are 35 kHz and 130 kHz. A frequency generator will be installed to convert the incoming frequency of 50 Hz of the power supply to the demanded frequency for the transducer. The frequency has to vary the frequency in small deviations around 35 kHz and 130 kHz to create a sweep. This sweep prevents hotspots of cavitation in the bath at the length of the standing waves. Undulation, which is the movement of parts during cleaning, moves the part as well and should together with a sweep function prevent dead spots which have no cavitation.

The frequency generator regulates the transducer. A fan has to be installed to cool the generator, because it can overheat in the housing. Thickness of the wall is stressed by the ultrasound and cannot be too thin. A thickness of 2-3 mm is normally used for the vibrating bottom plate of the stainless steel container.

Ultrasonic cleaning cannot be used in combination with flow generators like a propeller for example. The created flow disturbs the low pressures created by ultrasonic sound and hinders the formation of cavitation.

Drying

The ethanol has to be evaporated from the bone during the last step. This is accomplished by blowing hot air over the bones. The air has to be heated to assure that there is no water added to the bones, because this is performed after the dehydration step. Heated air adsorbs more water than dry air. The air will be heated inside the machine to 40℃ by the heating panels on the side of the walls. Those can be the heating panels that are also used for heating the baths. However there is also a need for air flow in the bath. Acetone is not compatible with an airflow and requires the bath to be completely sealed off from the surrounding. A better solution is a separated machine that performs the drying. The drying can be through infrared drying or hot air drying. Infrared drying has better drying times compared to hot air (Nozad et al., 2016).

Operating system

The system will be automatic to prevent human interference which could cause contamination and reduces costs as well. The system will be manged by a microcomputer that is programmed to perform all tasks at the right times for one batch loop. The computer has to regulate the temperature, opening of valves, running of the motors, operating the fans and control the ultrasonic vibration. This will be done by a PLC control.

7.5 Fluid transport system

The fluids have to be transported from the reservoir tanks to the machine, see figure 8. The driving force can be gravity or a pump. The benefit of pumps is that they can control the total amount of liquid to flow. Valves can be used when gravity is the driving factor. The benefit of using valves is that they are cheaper. The whole piping system has to be designed tapered to secure that fluids will never flow back in the system. Otherwise check valves have to be installed to prevent back flow. The required pumps need to full fill two important tasks. The first one is to deliver a flow rate of at least 5 litres per minute which assures that the bone processing does not delay. The second is that it can regulate precisely the total amount of inflow. This does not have to be accurate to the mm^3, but should be in the range of 10 cm^3. The main two types of pumps that exist on the market are centrifugal pumps and positive displacement pumps. Positive displacement pumps are more suited because they can regulated accurately the total pumped volume. Centrifugal pumps could be used, but a flow rate sensor should be installed to control the inlet volume. The flow rate of centrifugal pumps depends on the pressure difference between inlet and outlet. The pressure at the inlet side is variable because it depends on liquid height. This means that a standard operating power is not possible and sensors should be installed or a pressure control valve. Installing a positive displacement pump is a simpler choice for the system, because its flow rate does not depend on the suction pressures. Every liquid supply line should have its own pump because the chemicals should not mix. This results in eight pumps, which makes the selection of the pump more dependable on price. The supply line does not need automatic valves because the eight distinct pumps will work as valves for each liquid inflow. The outlet needs an automatic valve. The storage tank should have an air inlet valve to compensate for the loss in volume when they are drained. A transient tank has to be placed in line between the pump and the machine to preheat the liquids at the desired temperature, so the temperature only has to be maintained in the machine.

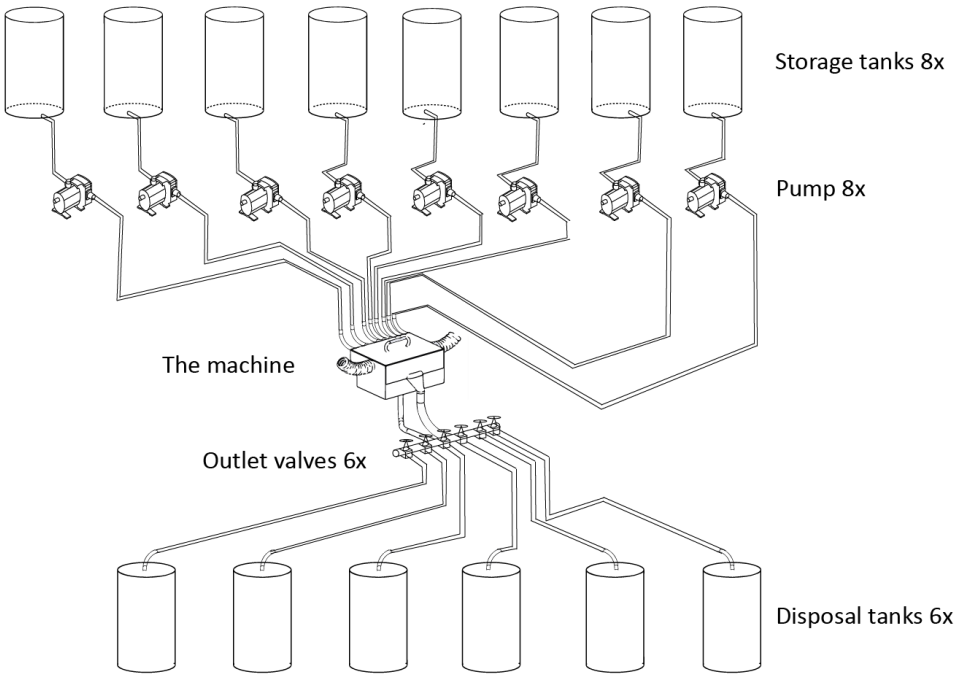

Figure 8: Schematic representation of the fluid transport system.

7.6 Safety

Safety is the most important part of the whole design. People are endangered by the process if it is not understood well. Safety risks are explosion, releases of toxic chemicals, ignition and electrocution. This could be due to the usage of chemicals such as acetone, ethanol, hydrogen peroxide 35% and sodium hydroxide and to the use of mechanical and electrical systems. One must be aware of the risks when designing storage, transport, usage and disposal. These risks s will be discussed subsequently, first, the general risks by using toxic chemicals and secondly the mechanical and electrical risks

Chemical risks

According to European regulation no. 1272/2008 are acetone, ethanol, hydrogen peroxide 35% and sodium hydroxide harmful chemicals with each its own safety risks. Hydrogen peroxide 35% is harmful when swallowed, causes skin and respiratory irritation and could cause serious eye damage. Sodium hydroxide 4% may cause severe skin and eye burns. One should avoid contact with sodium hydroxide. Acetone is dangerous because its highly flammable and has a flash point at minus 20℃. Acetone causes eye irritation and may cause dizziness. Ethanol is dangerous for is highly flammable liquid and vapour.

All these chemicals should be stored in a cool, dry and well-ventilated buildings with low fire hazards. The temperature has to be controlled in summer to prevent heating which could lead to dangerous situations with the flammable liquids. The tank containing hydrogen peroxide should have a pressure valve for overpressure, because hydrogen peroxide slowly decomposes and the released oxygen builds up pressure. The flammable liquids in the machine are a risk for fire in mixture with air. There are two solutions, namely the prevention of a mixture with air by preventing air to enter the machine or the complete replacement all the air by a another gas which prevents acetone from reacting with oxygen. This noble gas could be argon gas which dissipates oxygen. However argon dissolves in water and it is a costlier application. Hence, the prevention of oxygen entering the machine is the best solution.

The air that is let into the drying machine should be filtered to prevent contamination. The air has to be clean as in clean room conditions class 7 as described by ISO 14644-1:1999, which is the same class as for packaging medical devices. This is obtained by operating the machine in a clean room facility that is already in use by Spierings Tissue Processing. The machine should never be opened during processing to prevent the release of chemical fumes and to prevent any contact with the chemicals.

Mechanical and electrical risks

There are fluid stresses which creates risks for overpressures and electrical circuits which cause risks on electrocution. The mechanical risk is the pressure build up by using positive discharge pumps. The pipe in and outlet connected to the pump should always be open when the pump is in operation. If one end is closed by a valve, the pump can build up pressure and the pipe can break. The housing of the machine should always be grounded to prevent electrocution in case of a short circuit. The grounding prevents also an electric pulse to the liquid in the bath which could be a risk when this liquid is flammable such as acetone. An emergency break should be added to shut everything down in case of an unforeseen situation. This break should be between the hot wire of the wall outlet and the machine. The emergency brake should never be installed between the machine and the neutral wire, because there current is only supplied through the hot wire in common wall outlets in the Netherlands.

8. Modelling of the ultrasonic cleaner

Modelling an ultrasonic cleaner shows the spatial distribution of the standing waves in the bath. Cavitation is dependent on pressure fluctuations and is most prevalent at the anti-pressure nodes. This provides insight in the pressure distribution and therefrom can be derived the cleaning effect in space on bone. This enables an estimate on how bones should be places in a ultrasonic cleaner for optimal cleaning results. The modelling focuses on the effect of different ultrasonic frequencies. The frequency affects the spatial pressure distribution in the bath and the cavitation threshold helps to predicts the locations undergoing cavitation. A certain negative pressure has to be obtained for a given driving frequency to result in cavitation.

This chapter consist of six parts. The first part discusses previous studies with the limitations in their simulations. The second chapter discusses the physics of acoustic waves in a liquid bath. Thirdly, the implications for the cavitation threshold are discussed. Hereafter is the setup of the model explained. This is followed by the results. At last, the model is validated and its application is discussed.

8.1 Previous modelling studies

Multiple studies attempted to model the pressure distribution in an ultrasonic cleaner, but all fail in their assumptions and oversimplify the physics. The models are representative for the spatial distribution of the standing waves, but are lacking a correct damping or excluding damping at all. This is why a new model is necessary to predict the pressure distribution more correctly in the bath to make correct assumptions for the cavitation distribution which depends on the cavitation threshold. I have found eight articles that tried to model ultrasonic cleaning, most of these used Comsol and a minority Ansys. These articles are Li et al. (2013), Lais et al. (2018), Wei et al. (2016), Rokad and Pandya (2020), Lewis (2009), Niazi et al. (2014), Zong et al., (2014), Tangsopa, (2019). Most of these models are orders off in magnitude for the acoustic pressure due to neglecting at all such as in Li et al. (2013), Lais et al. (2018), Rokad and Pandya (2020), Niazi et al. (2014) and Zong et al., (2014), see figure 9.

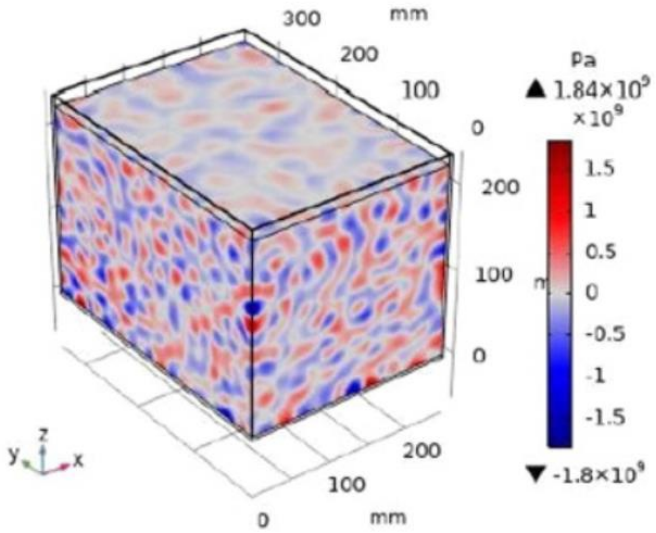

Figure 9 (Rokad and Pandya, 2020): The model is several orders in magnitude too high for the pressure, because the impact of damping is neglected. The model uses a driving frequency of 40 kHz.

Some ignore damping by cavitation which results in pressures that only depend on the standing wave pattern or on the spatial spreading of the wave such as in Tangsopa, (2019) and Wei et al. (2016), se figure 10. One paper (Lais et al., 2014) estimates the cavitation threshold on a value of 500 kPa for a frequency of 40 kHz at a 40 Watt input. This value is 10 times too large and the referenced paper for this value is misunderstood and actually suggest a cavitation threshold of 40 kPa.

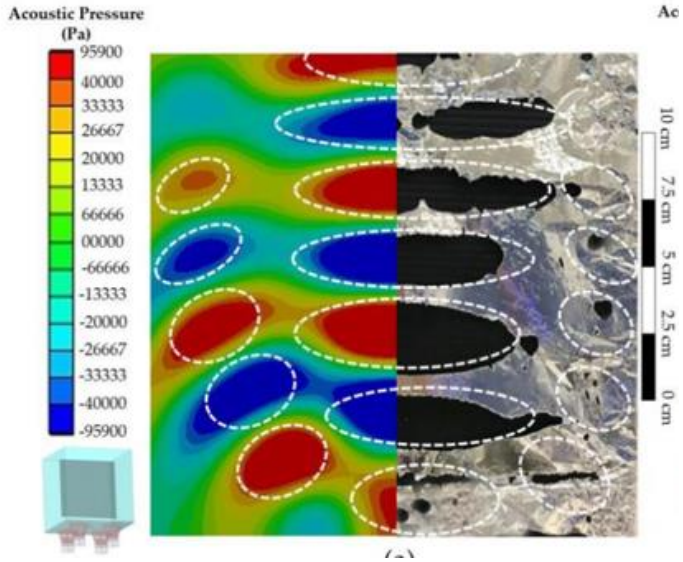

Figure 10 (Tangsopa, 2019): The model above ignores damping by cavitation which results in over estimation of the pressure near the surface. There should be a descending gradient in pressure from the bottom to the top of the bath.

The only articles that makes a realistic model for ultrasonic cleaning is the one of Lewis (2009). The only problem in Lewis (2009) is that the value for the damping by cavitation is estimated too low when you compare the dominant nuclei size to the cavitation threshold. This is further explained in the next two sections. The goal of the model in this research is to make a better prediction of the pressures in the bath formed by the standing waves. This helps to eliminate differences in the cleaning process due to difference in cavitation fields to enables a better comparison of the effect of the cleaning frequency.

8.2 Physics of acoustic waves

The physics of an ultrasonic cleaner depend mainly on the behaviour of acoustic waves in a liquid bath. The acoustic wave is provoked by a deformed piezoelectric transducer that is bounded to the flat bottom plate of the bath. The piezoelectric in the transducer is sinusoidally deformed by an applied voltage. This creates a sinusoidal vibration at the bottom of the metal container. This metal is slightly deformed in the order of micrometres and creates sinusoidal pressure waves. The pressure of this wave is dependent on the applied power. The instantaneous power of a sound wave is given by

$$P_{inst} = \frac{F\dot{u}}{A} = p\dot{u}$$

P_{inst} is the instantaneous power, F is the force, A is the area, p is the pressure and $\dot{u}$ is the acoustic particle velocity. The impedance is related to the pressure and the velocity by:

$$Z = \frac{p}{\dot{u}}$$

While the impedance is also given by:

$$Z = \rho c$$

This gives for the velocity the following relation:

$$\dot{u} = \frac{p}{\rho c}$$

This is recombined to the following formula for the instantaneous power:

$$P_{inst} = \frac{p^2}{\rho c}$$

The pressure input at the bottom of the bath is sinusoidal and varies in time and space by the following formula,

$$p(t) = P_{max}\exp\,(i\omega t)$$

in which p is the pressure, P_{max} is the maximum pressure, ω the radial frequency and t is time.

This sinusoidal power input means that to obtain the average power input the instantaneous power has to be divided by two. The combination of all the formulas gives the boundary pressure at the at bottom of the tank:

$$p = (\frac{2\rho_f C_f P_{mean}}{A})^{1/2}$$

p is the applied pressure, ρ_f is the density of the fluid, C_f is the speed of sound of the fluid, P_e is the applied power and A is the area. This formula relates the applied power of the transducer to the pressure produced by the transducer. However there is a small deviation is real value due to power losses by heat generation in the transducer and deformation in the metal container.

The produced sound travels with a speed that is dependent on the bulk modulus and the density of the medium for fluids. The formula for the speed of sound is given by:

$$c = \sqrt{\frac{K}{\rho}}$$

c is the speed of sound, K is the bulk modulus and ρ is the density. Solid mediums have to add the shear modulus. The frequency and the speed of sound are related to each other by the wave length. The formula is:

$$\lambda = c/f$$

λ is the wave length, c is the speed of sound and f is the frequency.

The pressure oscillation at the bottom of the tank generates an acoustic wave travelling through the fluid medium. The acoustic wave equation is based on the assumption of maintaining motion and preservation of mass. The wave equation describes the changes in pressure induced by the sound wave in time and space and is as follows:

$$\nabla^2 \boldsymbol{p}(t) - \frac{1}{c^2}(\frac{\partial^2 \boldsymbol{p}(t)}{\partial t^2} = 0$$

p is the pressure, c is sound velocity, t is time, ρ is the density. This acoustic wave equation is valid for inviscid flow. Including viscosity results in the following formula:

$$\nabla^2 \boldsymbol{p}(t) - \frac{1}{c^2}\left(\frac{\partial^2 \boldsymbol{p}(t)}{\partial t^2} + \frac{3\mu}{3\rho}\frac{\nabla^2 \boldsymbol{p}(t)}{\partial t}\right) = 0$$

p is the pressure, c is sound velocity, t is time, ρ is the density and μ is the viscosity.

The viscoelastic behaviour causes an energy loss which results in an attenuation of amplitude of the acoustic wave. The attenuation coefficient for the viscoelastic effect is given by:

$$\alpha = \frac{2\omega^2 \mu}{3\rho c^3}$$

There is a second damping effect due to cavitation which results in energy losses. This effect will be discussed in 8.3.

The wave is a self-sustaining propagation at the same velocity. However the particles hit by this wave have a changing displacement in time due to the changing pressure gradient produced by the wave. This makes the pressure 90 degrees out of phase with the displacement caused by the travelling wave, see figure 11.

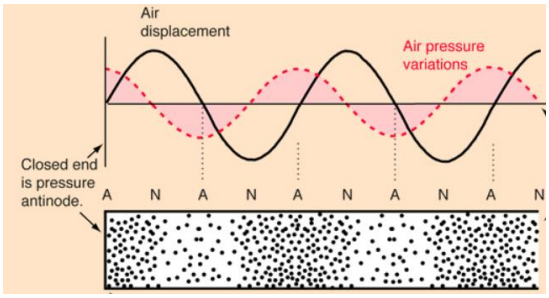

Figure 11: The relation in phase between pressure and particle displacement caused by a longitudinal wave.

The wave reaches the liquid air interface at the top of the bath. The difference in mass between the liquid and air is so big that most of the incident wave is reflected. Only a very small part of the wave is absorbed or transmitted. The reflection coefficient for a plane incoming wave is given by:

$$R = \frac{\rho_1 C_1 - \rho_2 C_2}{\rho_1 C_1 + \rho_2 C_2}$$

R is the reflection coefficient, ρ_1 and C_1 are the density and speed of sound of the incident wave and ρ_2 and C_2 are the density and speed of sound of the reflected. This means for an air water interface that 99,99% is reflected. The reflection coefficient is even more for incoming waves under an angle. This reflection coefficient makes that the boundary condition approaches a solid boundary where the $\frac{dp}{dx} = 0$, where p is the pressure and x the distance. This high reflection coefficient causes standing waves to arise in the bath. The standing waves amplify when the liquid height of the bath approaches the resonating length. Therefore modelling the harmonic response of the acoustic waves in a finite element approach gives a clear image of the pressure distribution in the bath which is related to the cavitation effect.

8.3 Cavitation threshold

Cavitation is produced by the change in pressure in a longitudinal wave. Microscopic bubbles grow during the rarefaction of the pressure wave and implode when they reach resonance size. The pressure changes are the biggest at the anti-pressure nodes. Bubbles flow from pressure nodes to the anti-pressure nodes. These anti-pressure nodes are of most interest because they obtain the maximum pressures and are most likely to reach the cavitation threshold.

The cavitation threshold is the minimum pressure to produce cavitation. Cavitation depends on a lot of parameters such as temperature, gas content, nuclei, nuclei size, column length, lithostatic pressure, acoustic power, bubble size and the liquid medium. The liquid medium influences cavitation by its vapour pressure, viscosity and surface tension. It is worth investigating the effect of different liquids on the pressure distribution in an finite element analyses because different liquids are used for cleaning bone.

The cavitation threshold is approached by the following formula (Crum, 1982):

$$R_t = \frac{0.13}{f}\left(\frac{P_\infty}{\rho}\right)^{\frac{1}{2}}\left\{\frac{P-1}{\sqrt{P}}\left[1+\frac{2}{3}(P-1)\right]^{\frac{1}{3}}\right\} for\ P \leq 11$$

$$R_t = \frac{0.3}{f}\left(\frac{P_\infty}{\rho}\right)^{\frac{1}{2}}\left[\frac{2}{3}(P-1)\right]^{\frac{1}{3}} for\ P \geq 11$$

R_t is the radius of the free bubble acting as nucleus, ρ is the density of the liquid, f is the driving frequency, P∞ is the ambient pressure and P=Pt/P∞ where Pt is the minimum acoustic pressure amplitude to initiate cavitation.

This threshold is only an approach, because there is no uniform bubble size in a liquid. It is necessarily to know the bubble size distribution to come up with a reasonable approach for the average nucleus size. There is however hardly any data on the nucleus sizes in liquids. There is data for the cavitation threshold from empirical research. Nguyen et al. (2017) shows a logarithmic relation between the cavitation threshold and frequency, see figure 12. Using the data for the cavitation threshold allows to calculate the dominating nucleus size. However this data of Nguyen is only valid for water. Extrapolating this threshold and nucleus size to other liquids is not valid.

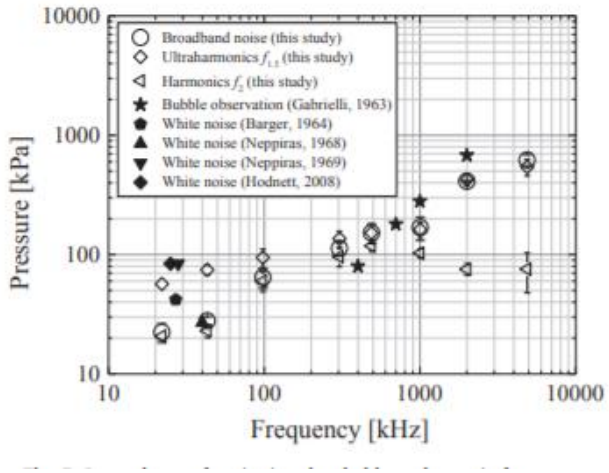

Figure 12 (Nguyen et al., 2017): The logarithmic relationship between frequency and pressure for the cavitation threshold.

Empirical data shows a logarithmic relation between threshold pressure and frequency see figure 12. According to figure 12, the cavitation threshold for a frequency of 35 kHz is around 30 kPa and the threshold is at an frequency of 130 kHz around 70 kPa at an temperature of 25℃.

Gates (1977) measured that most nuclei have an size between 5 and 10 micron, see figure 13. The total range is nuclei in water lies between 5 and 500 micron. However larger bubbles are lost faster from the liquid as small bubbles. This can be calculated by the formula

$$V_b = \frac{gD^2}{18v}$$

in which Vb is the velocity, g is the gravitational force, D is the diameter and v is the dynamic viscosity.

This formula shows that bubbles of 10 micron take 100 times longer to rise out of the liquid as bubbles of 100 micron for a column of 1 meter, so a few minutes compared to a few hours.

Even though a nucleus size for water can be obtained from empirical data and the cavitation threshold as well, it is too straight forward to link the nuclei size too other types of liquid. Nuclei size depends on liquid specific properties and circumstances such as temperature. It is therefore not possible to come up with an estimate for a cavitation threshold independent of the liquid. This makes it impossible to model in a finite element analysis (FEA) a reasonable pressure value yet for various liquids such acetone or isopropanol.

A last factor that should be included is the damping effect of cavitation. The resonating and subsequently collapsing bubble cause an energy loss in the propagating ultrasonic wave and heat up the bath. A formula is given by Qi et al., (1995) for the effect of damping by cavitation:

$$D = 8.69\left(\frac{8.84 * 10^{-4}}{3(2\pi)^{\frac{1}{2}}} \frac{\omega_D^{\frac{3}{2}}}{c^3} (R_0\omega_b)^2\right)$$

D is the attenuation coefficient per unit length, ω_b is the natural frequency of a single bubble, ω_0 is the driving frequency of the source and R_0 is the equilibrium bubble radius.

The attenuation by cavitation depends as well as the cavitation threshold on the values for the dominant bubble size in the liquid. There is a big uncertainty in estimating the cavitation attenuation factor and the cavitation threshold for various liquids other than water. FEA models for cavitation in water have been approached by Lewis (2007) and Tangsopa et al., (2019). It is possible to model the spatial distribution of pressure in an ultrasonic bath for various liquids. There is no known value for the cavitation threshold necessary, because the spatial pressure distribution is not dependent on the cavitation. The cavitation attenuation factor is of importance for the real pressure value but less to the relative pressure, because the bath has a small liquid height and standing waves form. The FEA model in thesis will focus on the spatial pressure distribution for various liquids at various frequencies as well as on the cavitation threshold. This leads to new knowledge about the cavitation distribution zones in the bath which enables an improved placement of bones in an ultrasonic cleaner.

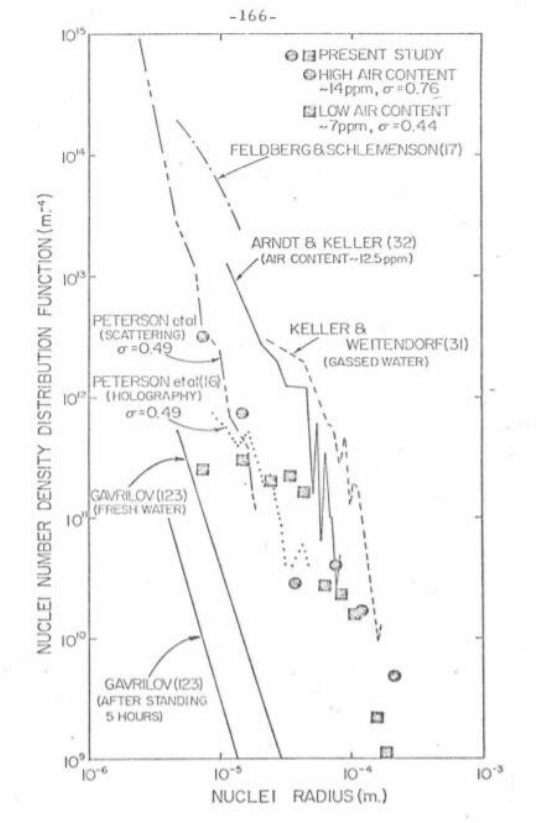

Figure 13 (Gates, 1977): The nuclei radius versus the nuclei number density distribution.

8.4 Model set-up

The 3D numerical model is based on the Elmasonic Ti-H 10 MF3 which is the ultrasonic bath used for the experiments. The model is solved in Comsol Multiphysics 5.6 in the frequency domain for 2D at 35 kHz and 130 kHz and in 3D only at 35 kHz. The frequency domain is chosen because the time domain requires many computational steps and the steady state result is of most importance. The 3D model at 130 kHz is not solved, because it requires too many elements to solve, namely 16 million and is out of reach for the processor. While in 2D, the model has to solve for 130 kHz only 38619 elements.

The model solves the pressure for an acoustic wave in water with an acoustic structural boundary interface between the metal casing and the water. A linear elastic equation is solved for the metal casing which calculates its displacement. The input excitation for the model is a sinusoidal pressure at the base. This sinusoidal boundary load is 276395 Pa. This excitation pressure is calculated from the total energy input and there is assumed that there are no losses in the transducer. Transducers in eigenmode are very energy efficient.

The value for damping is 5 Np/m, which is deducted from Qi et al. (1995). The damping depends on many factors including frequency and nuclei radius. The nuclei radius can be calculated from the cavitation threshold. A nuclei radius of 20 µm for 35 kHz and 3,4 µm for 130 kHz is calculated based on the cavitation threshold. This would give a damping coefficient of around 8 Np/m. It is likely that the cavitation threshold gives the dominant nuclei size but it does not exclude other radii as well. Therefore empirical data suits better and is used from Qi et al (1995) that measured at a frequency of 30 kHz. This value is used for both 35 and 130 kHz, because better data is not available. A higher

frequency should cause more damping according to formula .. , but a higher frequency has also a smaller bubble radius. More empirical data is needed to specify the damping factor for cavitation. The damping by viscous properties of water and heat conductivity are neglected because they are multiple orders lower than damping by cavitation.

8.5 Modelling results

The modelling shows a standing wave pattern that shifts according to the phase of the pressure excitation at the base of the bath. The 3D pattern is obtained for 35 kHz in figure 14.The 130 kHz variant needed too many computational elements which made the model insolvable. The 130 kHz needed many elements because of the shorter wave length to obtain a good representation of the wave. In 2D are all frequencies solvable. The 3D model is reasonably comparable to the 2D model when looking at figure 14 and figure 17 The 3D model seems a bit more damped. The model of 35 kHz in 3D shows that the highest pressures are reached near the bottom and damp out near the top of the bath, because of damping due to cavitation. The shift in phase during one pressure cycle makes that the standing wave pattern moves up and down a bit. The sweep frequency as shown in figure 19 and 20 causes a vertical shift for standing wave pattern at 33 and 37 kHz. The combination of the pressure cycle and the sweep frequency make that there are no dead spots due to pressure nodes for cleaning. The same shift in standing wave pattern is observable for the frequency of 130 kHz. However the shift due to the sweep is smaller at this frequency, because the wave length is smaller.

The cavitation threshold of 30 kPa is easily obtained for the frequencies around 35 kHz as can be seen in figure 17. The cavitation threshold of 70 kPa for 130 kHz is just reached in some areas of the bath and less distributed to the side of the bath as compared to 35 kHz, see figure 21.

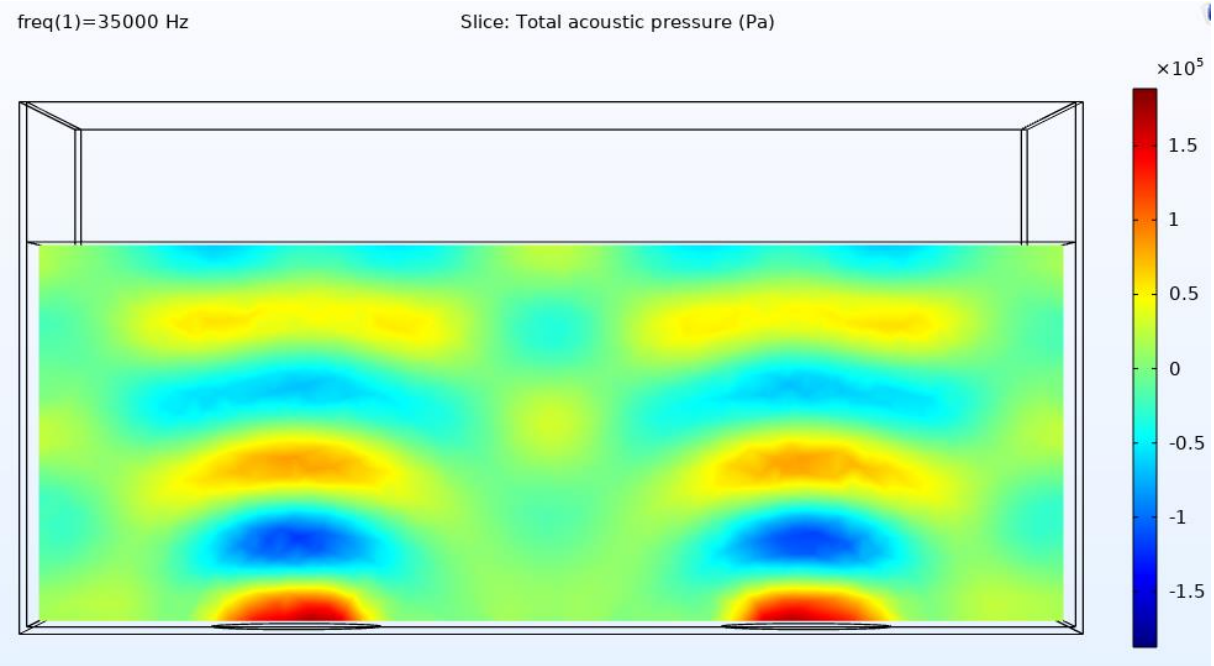

Figure 14: The pressure distribution in a ultrasonic bath for a 35 kHz excitation frequency in 3D at maximum pressure. There are two transducer at the bottom where the pressure is at maximum. The bath is surrounded by a stainless steel tank of 2 mm. The top of the bath is open.

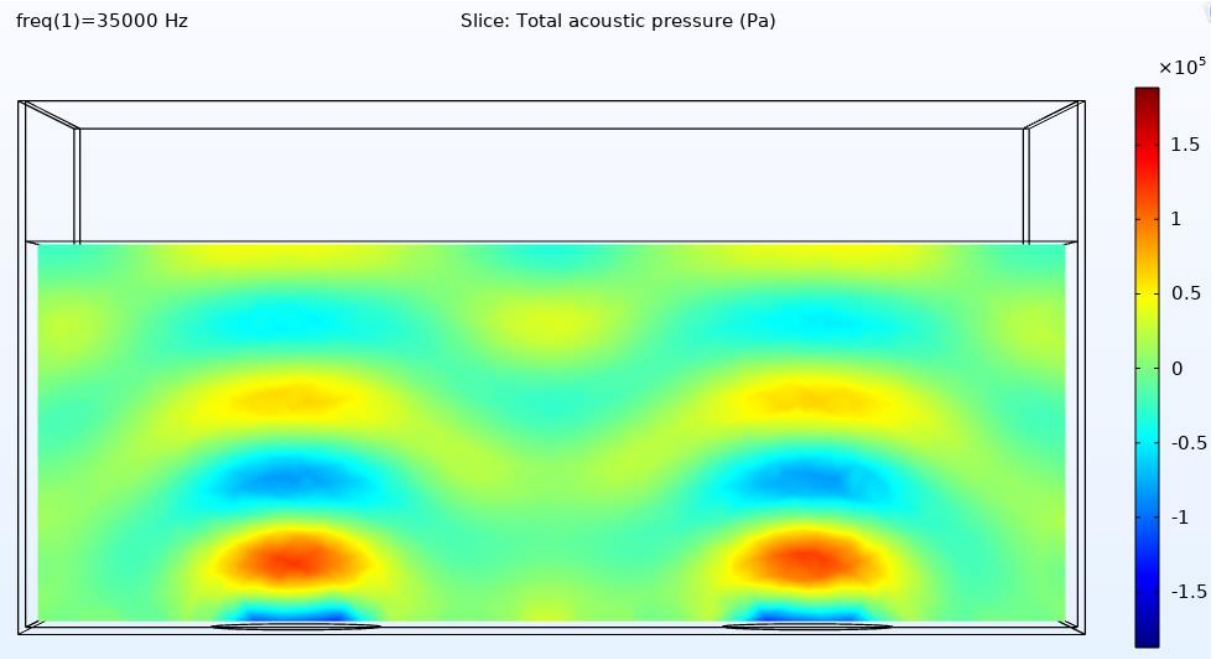

Figure 15: The pressure distribution for 35 kHz excitation in 3D. It is 120 degrees out of phase with figure 14.

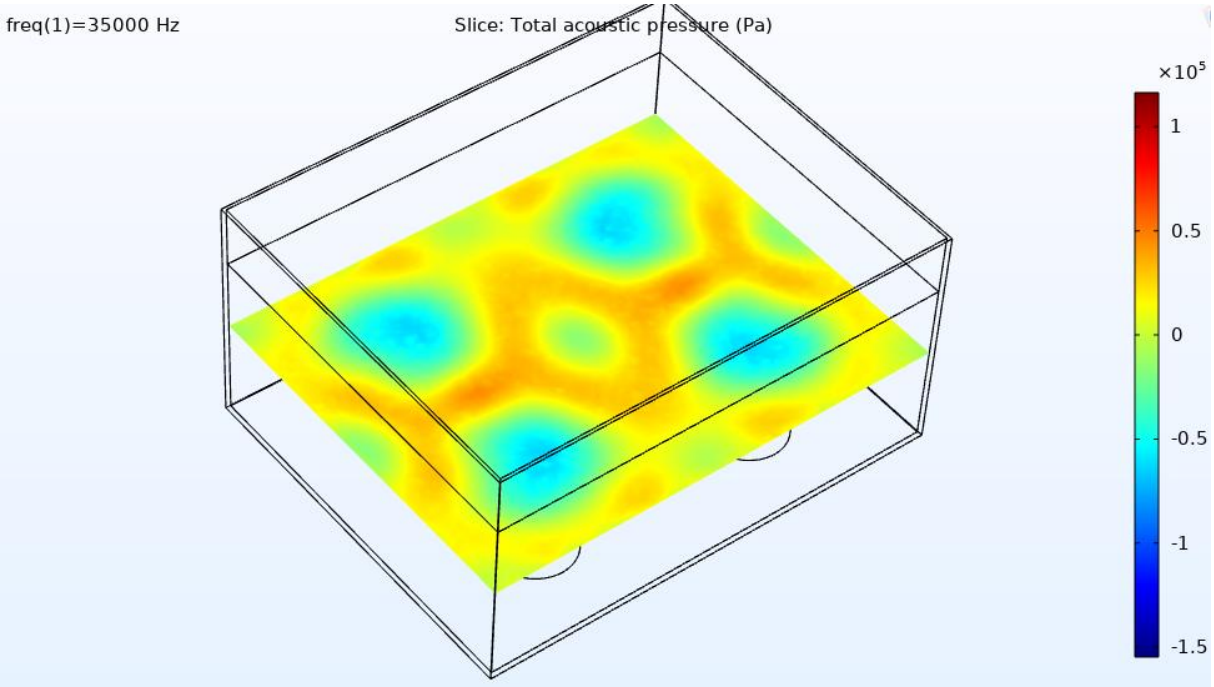

Figure 16:The pressure distribution for 35 kHz in 3D seen from above at a liquid height of 7 cm.

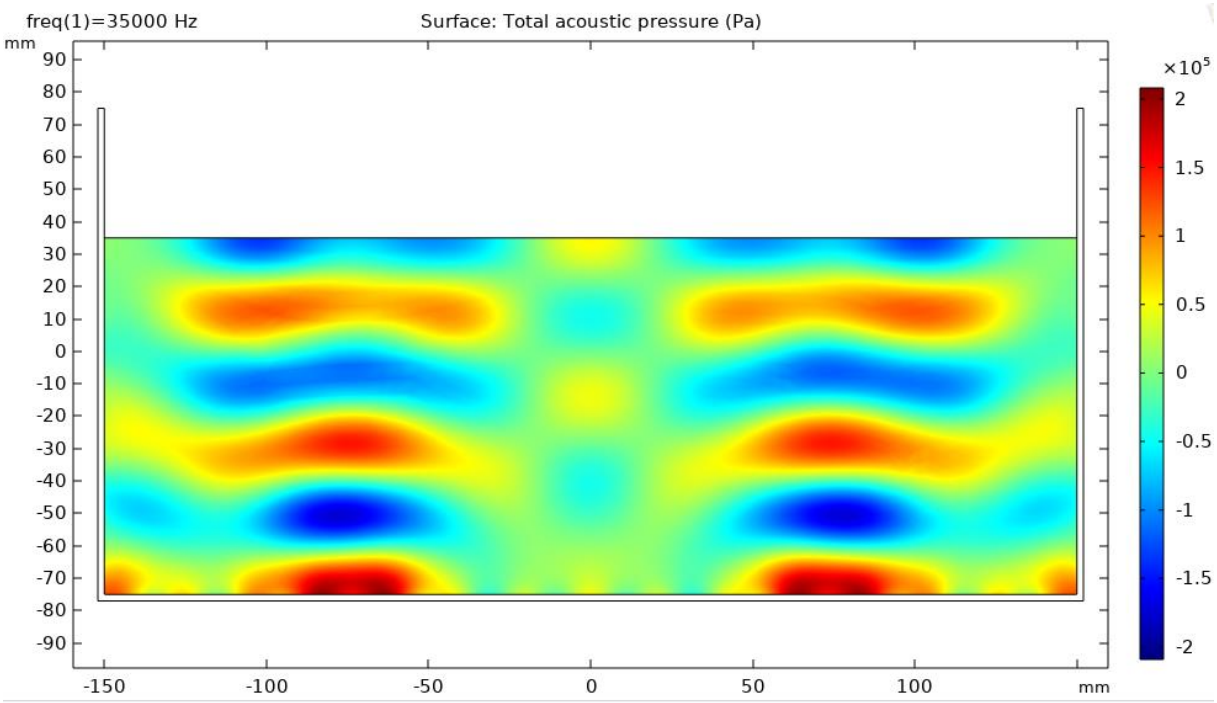

Figure 17: The pressure distribution for 35 kHz excitation in 2D at maximum pressure.

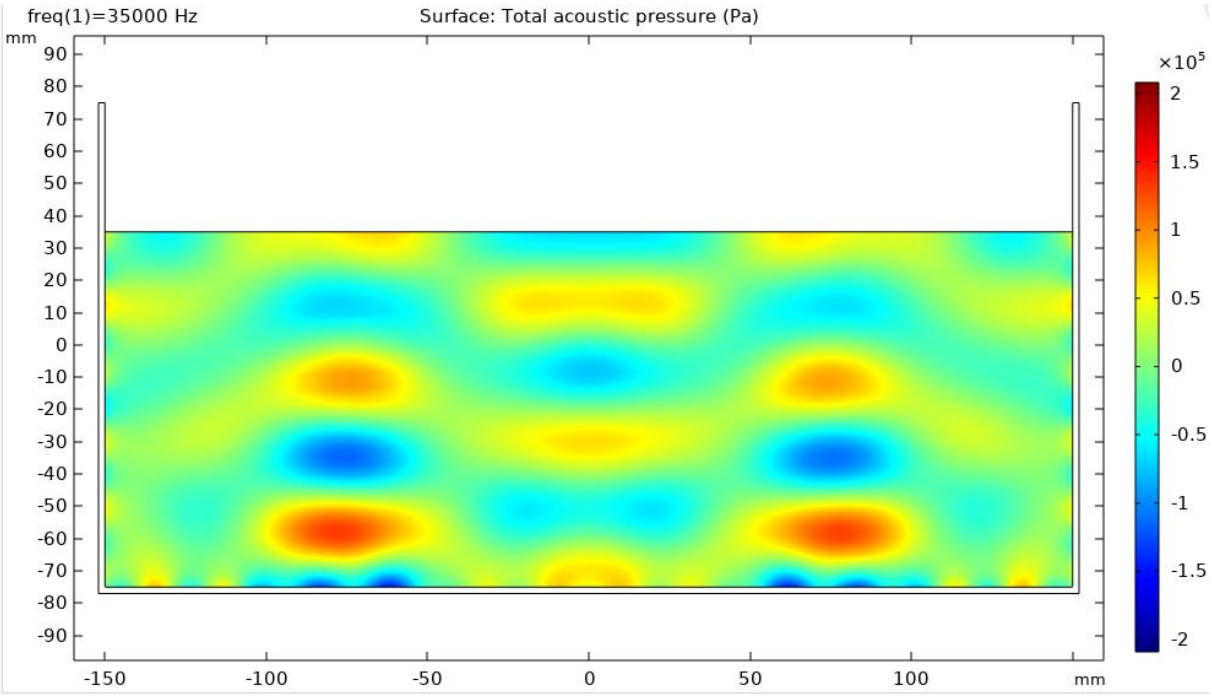

Figure 18: The pressure distribution for 35 kHz excitation in 2D. It is 120 degrees out of phase with figure 17.

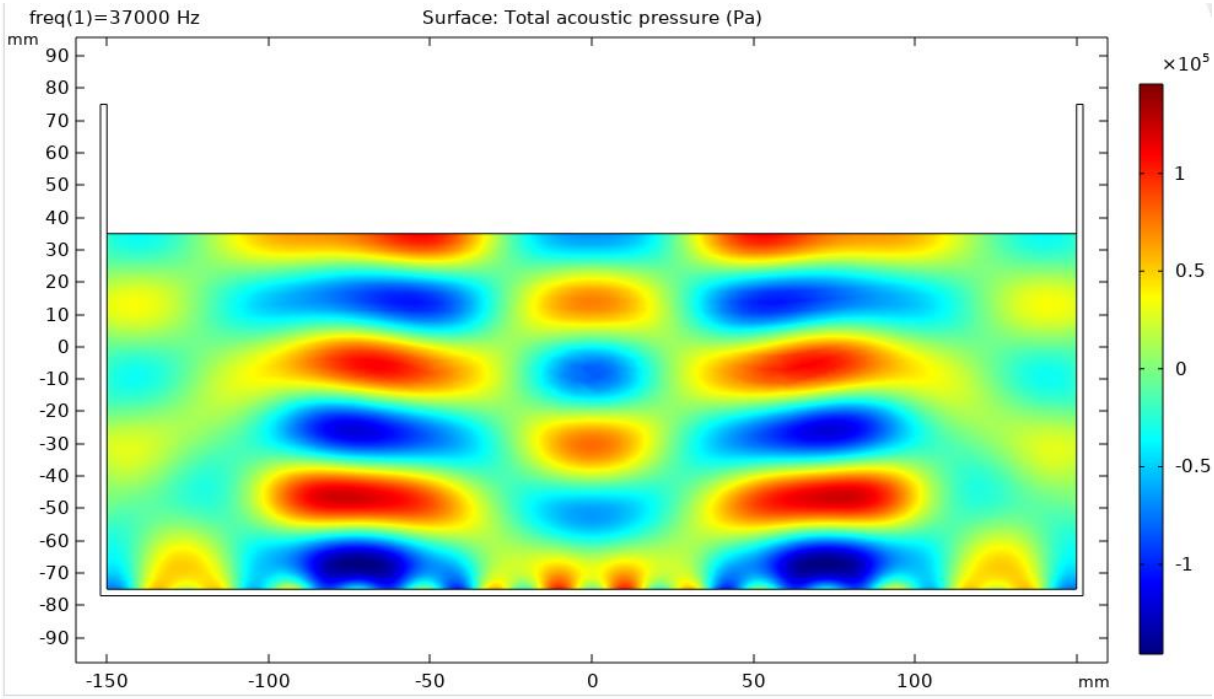

Figure 19: The pressure distribution for 37 kHz excitation in 2D.

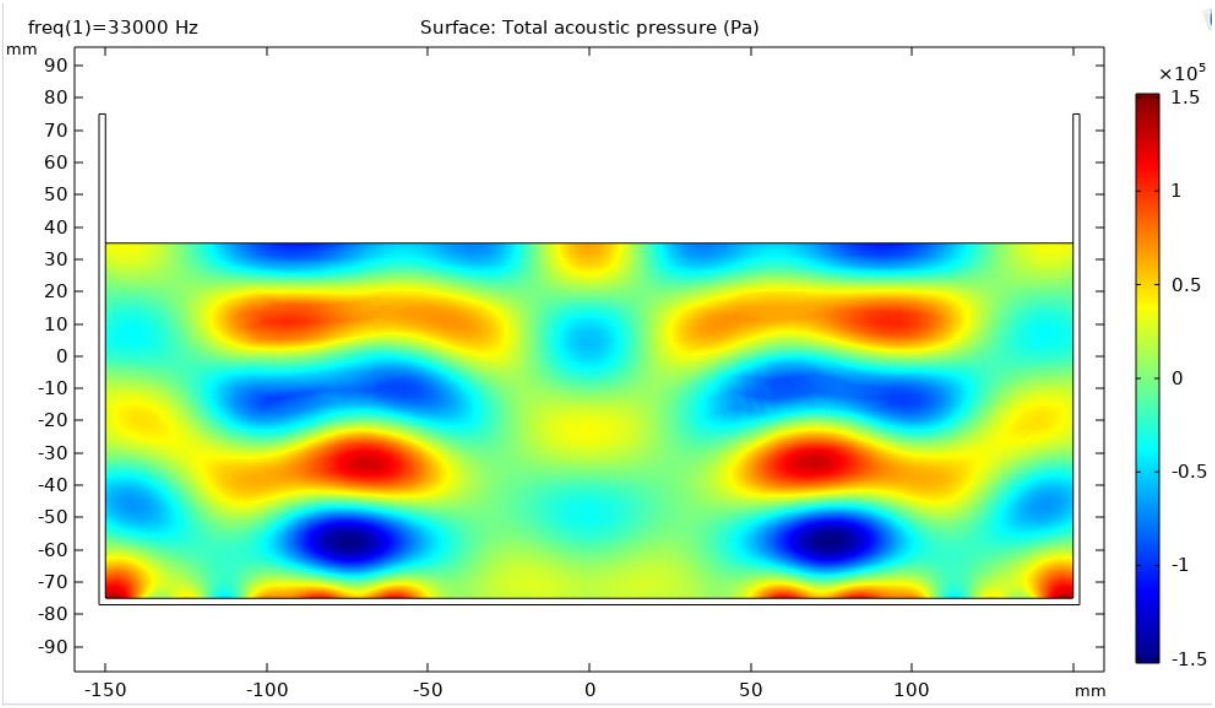

Figure 20: The pressure distribution for 33 kHz excitation in 2D.

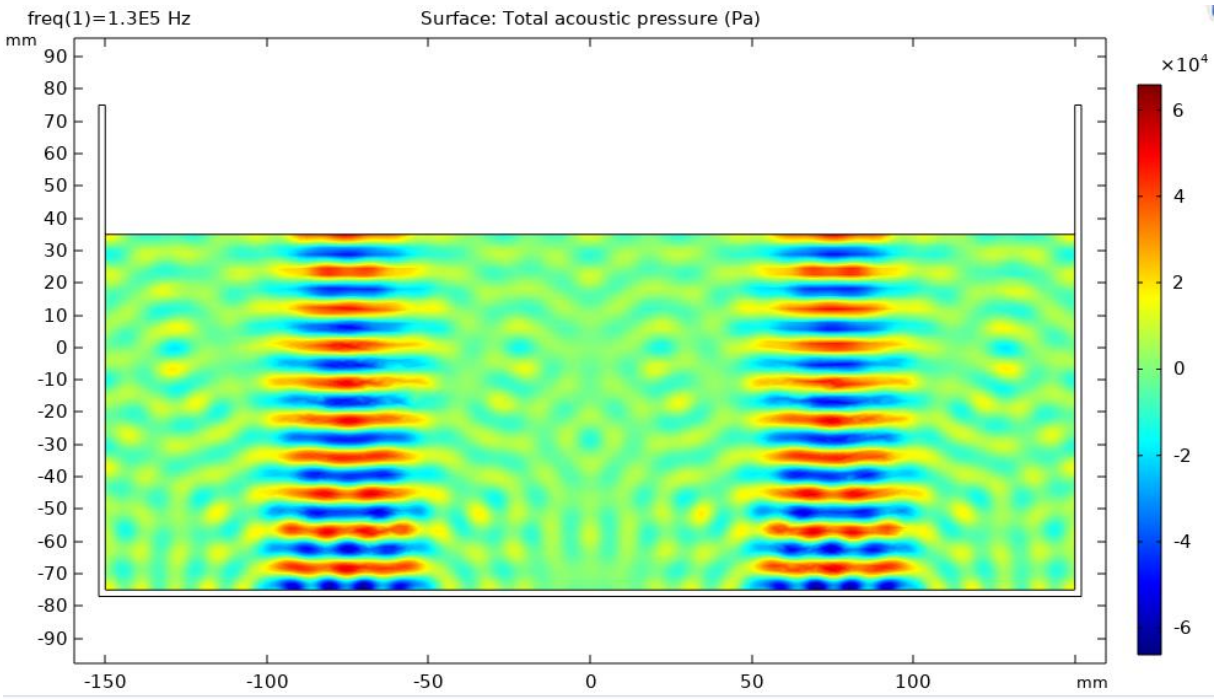

Figure 21: The pressure distribution for 130 kHz excitation in 2D.

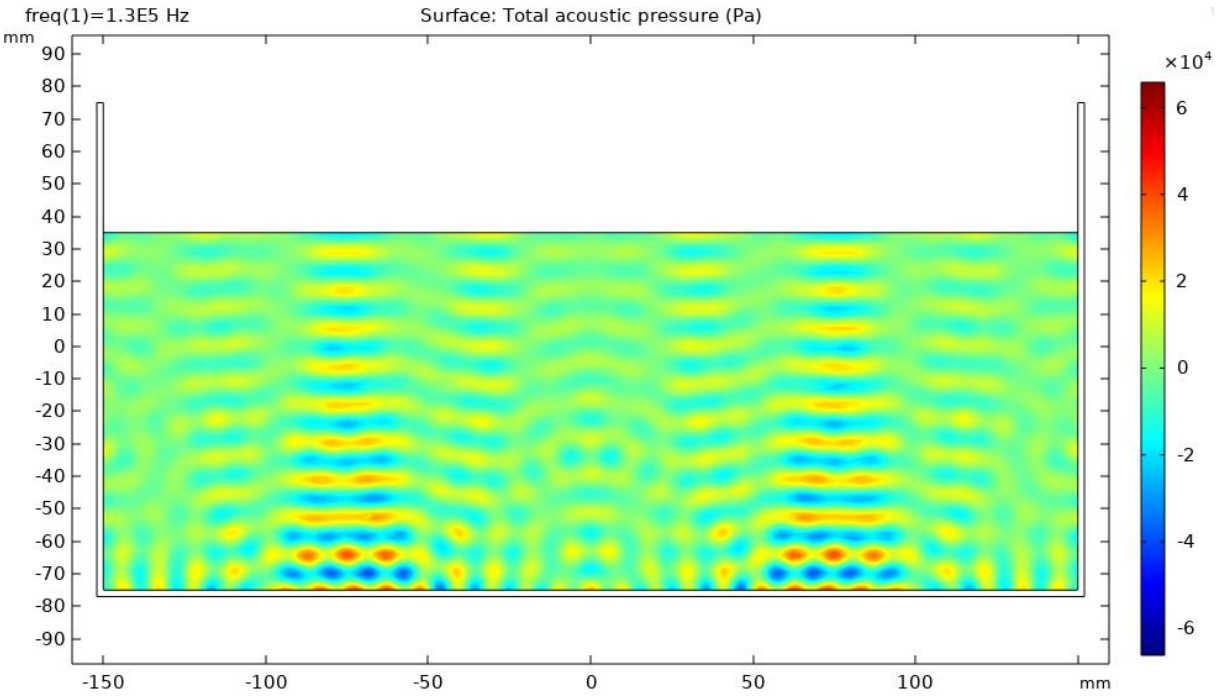

Figure 22: The pressure distribution for 130 kHz excitation in 2D. It is 120 degrees out of phase with figure 21.

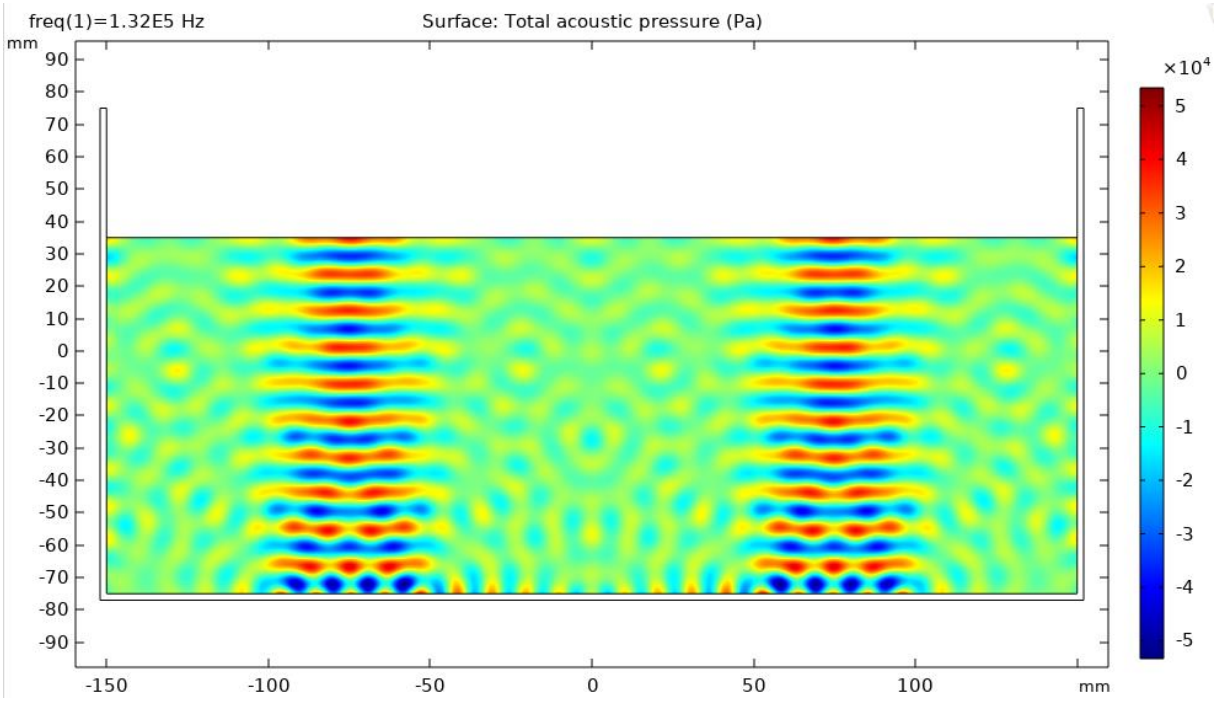

Figure 23: The pressure distribution for 132 kHz excitation in 2D.

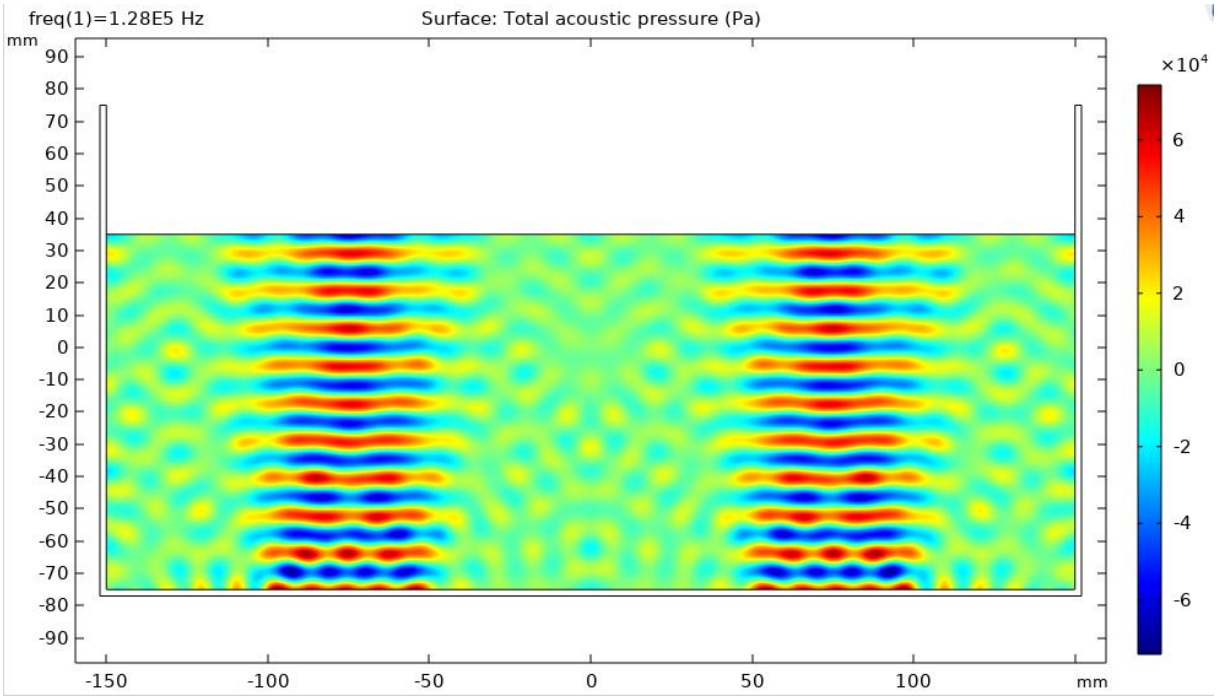

Figure 24: The pressure distribution for 128 kHz excitation in 2D.

8.6 Model validation and application

The model is not validated by direct measurements in the bath, but through a comparison with previous studies. These previous studies have shown the relation between cavitation in the bath and their spatial pressure distribution through aluminium foil tests and probe measurements. These tests do not show the exact pressure value but do show the relative pressure distribution in the bath. Lewis (2009) numerical model for an ultrasonic cleaner approaches reality as best and shows the relation between cavitation and the model in figure 25 and figure 26. The model of Lewis (2009) is only missing the right damping factor but this does not matter in this case because the cavitation threshold is easily reached at the applied frequency.

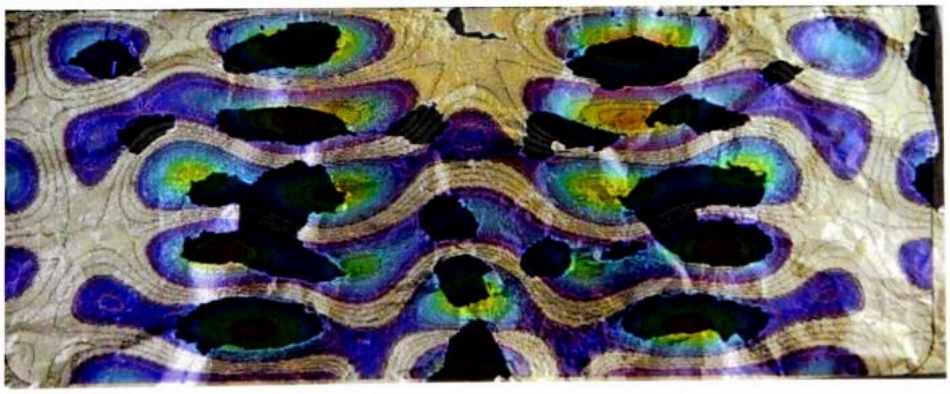

Figure 25: A 2D simulated model of Lewis (2009) which shows the aluminium foil test overlay. The aluminium foil tears down due to cavitation and leaves erosion holes. The aluminium was submerged in the ultrasonic bath for three minutes. The setup has placed two transducers at the bottom of the bath operating at 40 kHz.

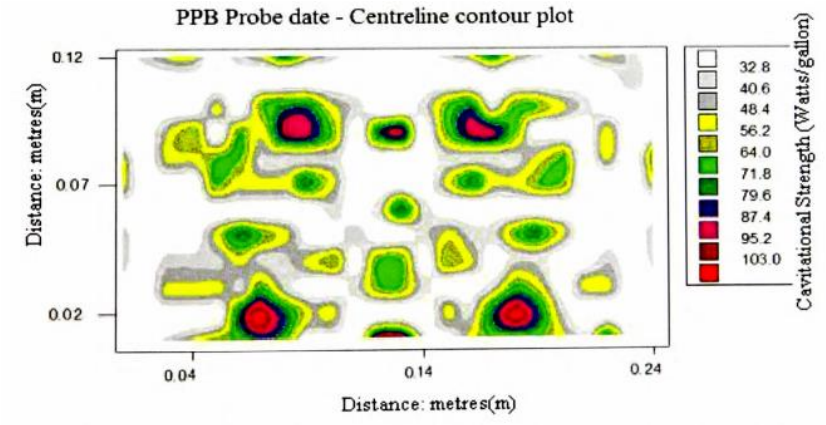

Figure 26: The graph shows the probe data obtained by Lewis (2009) for the same model as in figure 25. The applied ultrasonic frequency is 40 kHz. The strongest cavitation field occurs above the transducer.

The probe data is a bit capricious but shows that the cavitation zones are above the transducers. The aluminium test shows a good synchronisation between the model used by Lewis (2009) and the aluminium erosion regions. Lewis (2009) results are comparable to the modelling results in this thesis. The modelling results in this thesis show a concentration of the pressure regions above the cavitation threshold directly above the transducers. The model of 130 kHz has the strongest limitation in the distribution of the cavitation regions and is limited to only above the transducer. The waves are more likely to spread out at 35 kHz. The frequency of 35 kHz is comparable to the

frequency of 40 kHz used by Lewis (2009). Both the model of Lewis (2009) and the model generated by this thesis show a zone of caviation in the middle between the transducers. These cavitation regions can be found in the aluminium foil test as well as in the probe date. Concluded can be that based on the results of Lewis (2009) the model results in this thesis are valid. The model results shows that the cavitation regions are limited to directly above the transducer, especially for higher frequencies. This should be taken into care by placing the bones in the bath. The probe data of Lewis (2009) shows a lack of cavitation regions near the surface of the bath. This data matches the modelling results in this paper due to an improved estimated value for damping which is 0.435 dB/cm. The numerical model in Lewis (2009) does not match this probe data, because there is a damping of 0.02 dB/cm for cavitation. The lower cavitation values near the surface imply for bone cleaning bone that the sample has to be submerged to secure the effect of cavitation.

8.7 Conclusion

A model for ultrasonic cleaning was generated successfully. The model is a steady state solution that simulates pressure waves in a bath caused by ultrasound at different driving frequencies. The spatial distribution of pressure in the bath is related to cavitation zones by the cavitation threshold. The numerical model of a ultrasonic cleaner is an improvement of previous models in the literature, because a better estimate for the damping is used. The damping is significant increased because cavitation causes high energy losses for the wave propagation. The model is validated by a comparison with probe data and foil tests from previous research. The model can be improved by a better estimate of the pressure input delivered by the piezoelectric transducers. The model should be fine-tuned for more detail by damping only the regions that reach the cavitation threshold. In general, the model is very useful and leads to an improved placement of the bone samples in the ultrasonic cleaner. The bone should not be placed near the surface, because the cavitation threshold is not reached at this location for 130 kHz. The bone should be placed in a vertical column above the transducers for optimal cleaning. The bone should not be placed at the bottom of the bath, because the vibrating bottom plate is harmed by the contact over time.

9. Method

The aim of the tests is to analyse the effect of the chemical cleaning agents and to compare the effect of different ultrasonic frequencies on the cleaning of bones. This is tested in two separate tests. First several consecutive tests are executed on a femoral head to observe the effectiveness of the chosen processing protocol from chapter 5. The effectiveness is examined visually and through cross sections in the bone. Secondly, a cleaning test is executed to compare the ultrasonic frequency of 35 kHz, 130 kHz and the combination of consecutive 35 kHz and 130 kHz. This test used a shorter chemical cleaning protocol as compared to the processing protocol. A shorter version was used to make a better comparison of the effect of the ultrasound frequency on cleaning. The frequency test is executed on small identical cylinders from human femoral heads. The cleaning process has to remove fat, cells, viruses and bacteria. The test was setup for controlling the variables in ultrasonic cleaning and the cleaning process of the bone is described in 9.1. The effectiveness of cleaning was validated by four tests on the cleaned bone samples. The first test measured the residual fat in the bone. This compared the effectives of cleaning fat particles. The second test measured the residual content of DNA. This demonstrated the effective removal of cells in bone. The third test was a histological test. This is an optical validation of cleanliness by examining a thin cross-section of the bone sample. The last test was a compression test to validate if the bone has remained its strength during the processing. Intensive cavitation and chemicals could cause damage to the bone.

9.1 Sample preparation

The samples are derived from human femoral heads. These femoral heads were collected by ETB-Bislife, a bone bank in the Netherlands. The femoral heads were frozen at -80 and -10℃ and different moments in time. They were unfrozen and debrided by the Fortios bone debrider from Spierings Tissue Processing, see figure 27. The debriding step removed soft tissue, cartilage and the outer cortical bone layer. The chemical comparison test used large pieces of human and porcine femoral heads. Three cylindrical cores were drilled for the ultrasonic frequency comparison test from the cancellous part of a human femoral head. The cores have a diameter thickness of one centimetre and are in length one centimetre as well. The cores are all drilled in the same direction from the top of the femoral head down in the direction of the metaphysis. The Fortios drill press from Spierings Tissue Processing is used for drilling the cores. The drilling has to be slowly otherwise it heats the bone too much. See figure 28 for the samples.

Figure 27: Bone debrider from Spierings Tissue Processing

Three femoral heads are used which provides 9 samples per head. The 9 samples per head are divided in pairs of three per cleaning frequency. 9 samples are used for the fat analysis, 9 samples for the DNA analysis and 9 for the strength analysis. The three femoral heads are derived donors which are specified in table 3.

Table 3: Specification of the donated femoral heads.

Donor nr.	Seks	Birth date	Operation date	Age	Weigth Femoral Head
55569	Female	14-6-1954	19-2-2021	66	64
56088	Female	17-6-1955	22-2-2021	65	65
56575	Female	10-3-1951	9-3-2021	69	61

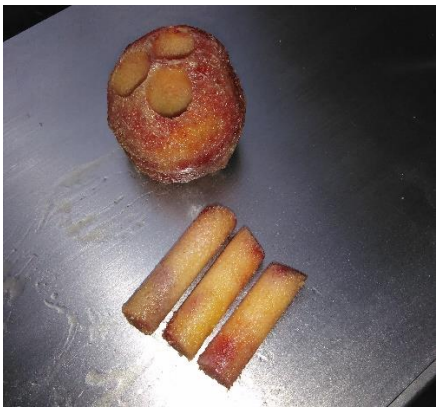

Figure 28: The photo shows a femoral head after debridement of soft tissue and extraction of three columns.

9.2 Chemical cleaning

The chemical comparison tests are executed to observe the effect of the selected chemical agents in the processing protocol of chapter 5. The samples were cleaned in a ultrasonic cleaner Ti-H-3 MF3 from Elmasonic at 35 kHz and observed during the cleaning. The samples were placed in a glass jar in the bath. Ultrasonic waves can travel through a glass jar. Glass jars are commonly used to operate with flammable liquids. A test was executed with increased temperature around between 50 and 60 ℃ for each step. After the cleaning, the samples were cut in half to inspect to core of the bone.

9.3 Comparison ultrasonic frequency

The samples are cleaned in an ultrasonic cleaner by three chemical steps which are selected as the crucial steps for cleaning a bone graft. The samples are placed in a closed basket with a grid size of 5 mm. This grid size does not influence the ultrasound. The bones are separated in the basket over 9 compartments. See figure 29 for the setup. The basket is 2 cm above the bottom. This centres the bones in the middle of the bath to provide uniform impact of the ultrasonic waves. Far away from the bottom is not efficient, because the wave loses energy near the top and bone can float which reduces the penetration of the chemical fluid. The height for the liquid is 11 cm. This height is chosen, because it is near the harmonic frequency for 130 kHz, which is effected less by the frequency sweep function.

Figure 29: The ultrasonic tank with basket and bone samples prior to cleaning.

The ultrasonic cleaner Ti-H-3 MF3 from Elmasonic was used. The piezoelectric transducers are located at the bottom of the bath with four transducer in total. Each transducer delivers 50 Watt and operates with a frequency sweep of plus and minus two kHz. Transducers have a specific resonant frequency but the sweep function allows them to operate outside the resonant frequency. They operate in a plus and minus 2 kHz sweep which is still at least 75 percent of their maximum pressure output.

Three test are performed with the same chemical procedure as shown in table 4. The test at 35 and 130 kHz are constantly vibrated at the given frequency. The combination test of 35 and 130 kHz is done consecutive by 15 minutes of 35 kHz followed by 15 min of 130 kHz. The power is at maximum at 200 Watt. The liquids are heated up in the first 30 minutes from 20 to 50 ℃ and then maintained at 50 ℃.

Table 4: The processing protocol for the bone samples.

Donor nr.	Sex	Birth date	Operation date	Age	Weigth Femoral Head
55569	Female	14-6-1954	19-2-2021	66	64
56088	Female	17-6-1955	22-2-2021	65	65
56575	Female	10-3-1951	9-3-2021	69	61

9.4 Histological analysis

The samples are samples are cast in epoxy. Then they are cut into thin sections. These cross-sections are placed under the microscope. The microscope was a Carl Zeiss Axioscope 20. Figure shows a picture of the cross section of the bone casted in epoxy.

Figure 30: Cutting plate of bone sample 56575 clean at 130 kHz.

9.5 Lipid analysis

The bone samples are crushed by a milling machine that first freezes the bone by liquid nitrogen, see figure 31. The crushed bone powder is extracted with petroleum ether at a shaker at 170 rpm. Here after, the petroleum ether extract is transferred into an evaporating dish. The petroleum ether is evaporated. The residue is weighed and expressed as an percentage of the weight of the sample in mass percentage.

Figure 31: The milled bone sample.

9.7 Mechanical test

The samples are tested under compression at a ZwickRoell Z150 with a 50 kN loadcell. The samples are compressed by 1 mm/minute in axial loading. The test setup is shown in figure 32. The start load is 10 newton. The test were run until 4 mm of total displacement.

Figure 32: The bone sample before (left) and after (right) the compression test. The samples are compressed by a rate of 1mm/minute in axial loading.

10. Results

The results comprise two parts. One part is focussed on the effect of chemicals, temperature, surface area and operation time. The second part is focussed on the effect of ultrasonic frequency. The method and details of the tests of the first part can be find in the Appendix. The results on the effect of ultrasound are described by a histological analysis and a mechanical test.

10.1 The effect of chemicals

The bones that were cleaned following the processing protocol from chapter five were visually inspected during the cleaning. The bones showed less progress in cleaning results during the acetone and isopropanol cleaning step. The surface became grey during these step and penetration of the bone was limited to several millimetres. Figure 33 shows a porcine femoral head after ultrasonic cleaning with acetone and consecutive isopropanol. These chemicals are strong enough to remove the marrow. The marrow is very viscous and contained by the bone scaffold. Placing the bone too long in sodium peroxide is deteriorating the collagen. After two hours in sodium peroxide at 60 ℃ is most of its collagen broken down and remains a pudding of bone minerals.

Figure 33: A porcine femoral head after treatment with acetone and isopropanol according to the processing protocol in chapter five. Only the surface of the bone is partly cleaned by these chemicals.

10.2 The effect of temperature

The second experiment was performed with an increased temperature between 50 and 60℃. The higher temperature caused the marrow to be less viscous, but the overall results in cleaning remained the same. Figure 34 shows a human femoral head cut in half after the test with the increased temperature. The cleaned white parts of the bone were the results of the treatment with hydrogen peroxide. In the centre of the bone remains a yellow stain of bone marrow. The hydrogen peroxide did not penetrate the bone completely.

Figure 34: A human femoral head cut in half after undergoing the processing protocol of chapter five at an elevated temperature between 50 and 60℃. A yellow stain of remnant marrow is visible in the centre of the bone.

10.3 The effect of surface area

Penetration of the chemical solvent depends on time by its effectivity in solving the marrow and by the in and out flow of fluids. The centre of the femoral head is the hardest part to reach. Cutting the femoral in smaller pieces enlarges is surface area. This enables more contact area between the chemical solvent and the bone marrow. It also reduces the penetration depth, which improves its cleaning results. Figure 34 shows that the outer areas of a femoral head cut in half are well cleaned.

10.4 The effect of operation time

Hydrogen peroxide gave so far the best cleaning results. Prolonging the operation time with hydrogen peroxide should ameliorate the results. A previous cleaned bone by the whole process has a marrow stain left in its centre and used again for a test with only four hours of cleaning by hydrogen peroxide, see figure 35 and 36. This resulted in a clean graft without any marrow visible. This means that prolonged steps with hydrogen peroxide can clean the bone completely. A visual inspection of the bone showed some white stains left on the bone which could be calcium phosphate deposits.

Figure 34: The human femoral head was cleaned before in test 01 and is used again for test 02. The photo is taken prior to test 02.

Figure 36: The human femoral head after cleaning for four hours with hydrogen peroxide. The bone was cut in half and showed no visible marrow anymore.

10.5 Histologic analysis

The histologic analysis shows the sample 56575 cleaned at an ultrasonic frequency of 130 kHz. The samples shows an intact bone surface. The bone comprises osteocytes. All the bone samples appear to be intact under the microscope, see figure 38 till figure 44. Figure 42 shows some residues that are possible fat. This possible because the samples used for the histologic analysis were bigger than the samples used for the compression test and lipid analysis. Bigger samples require more time to be cleaned in the interior, so fat residue are possible.

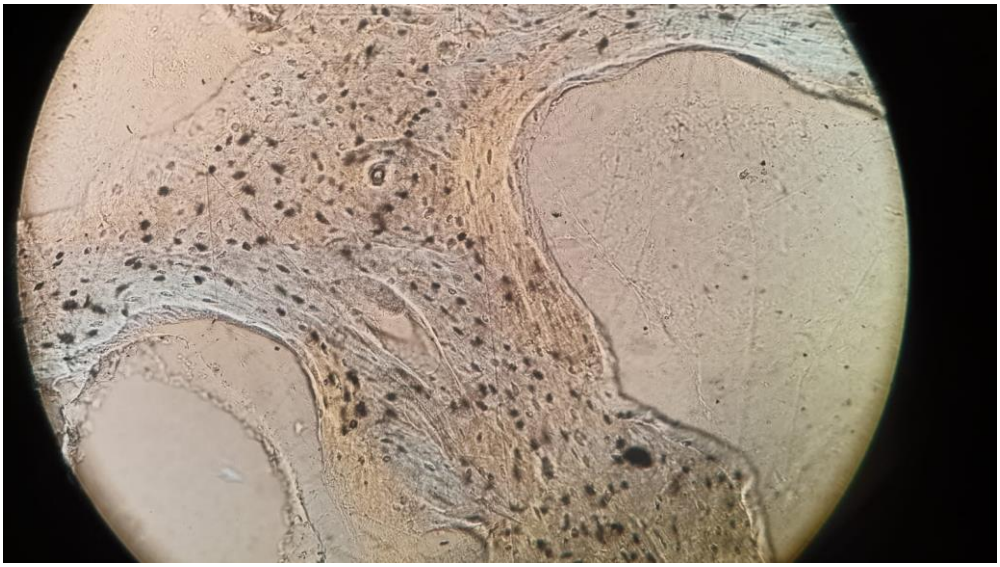

Figure 38: Cross section of bone sample 56575 clean at 130 kHz under the microscope, magnification 40x/0.95.

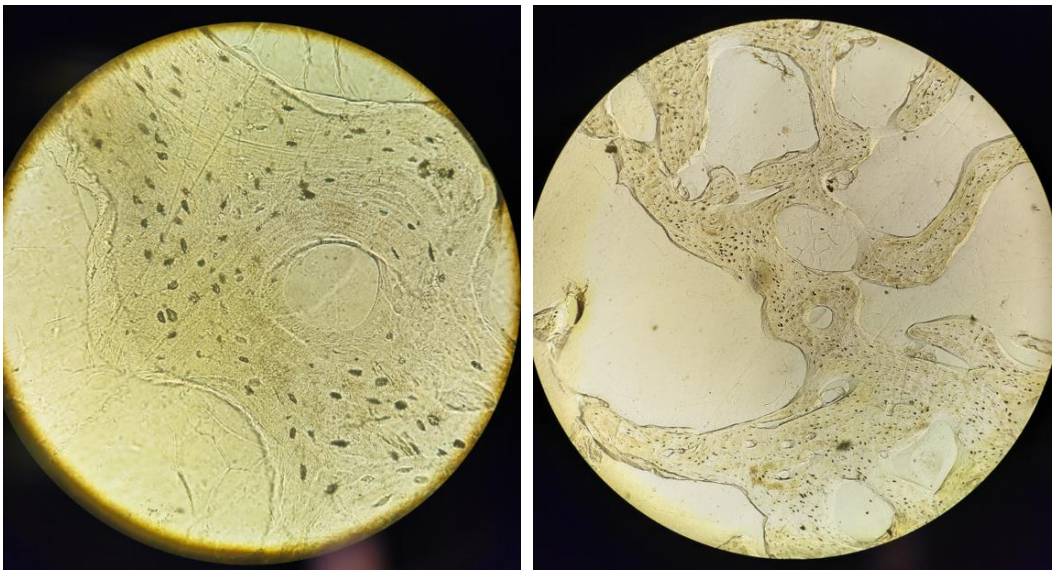

Figure 39: 130 kHz sample 56575, magnification 40x/0.95 (left) and 10x/0.3 (right).

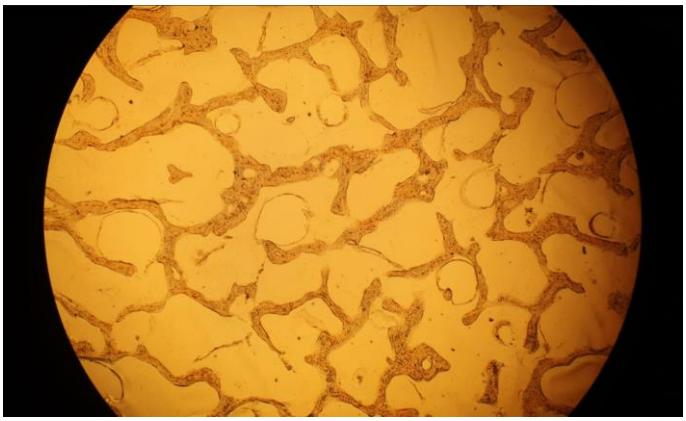

Figure 40: Cross section of bone sample 56575 clean at 130 kHz under the microscope, magnification is 2.5x/0.075.

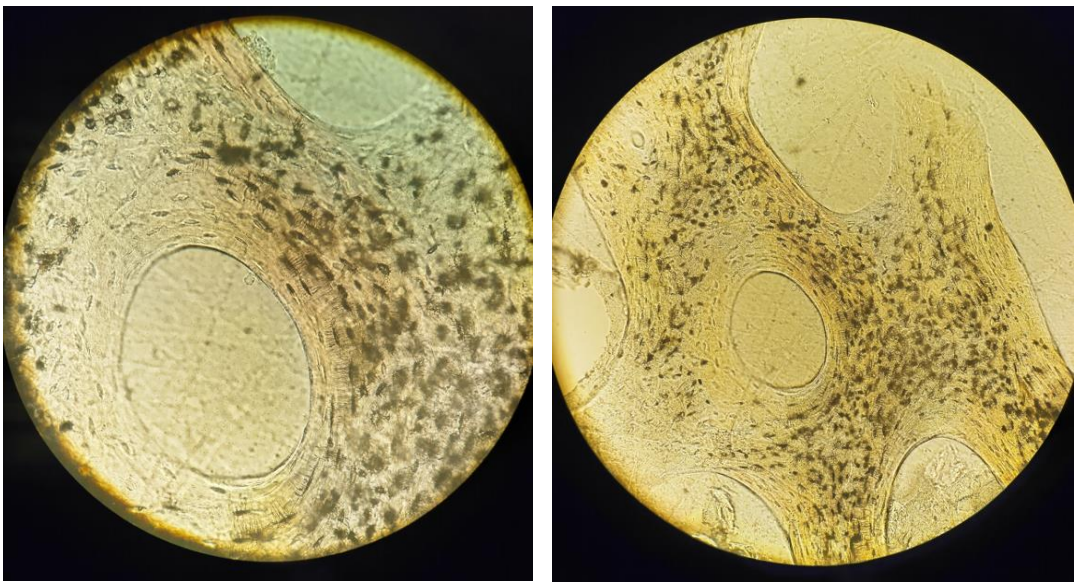

Figure 41: Bone sample 56757 cleaned at 35 kHz. Bone samples is intact and an osteon is visible in the picture. Magnification is 40x/0.95 (left) and 20x/0.8 (right).

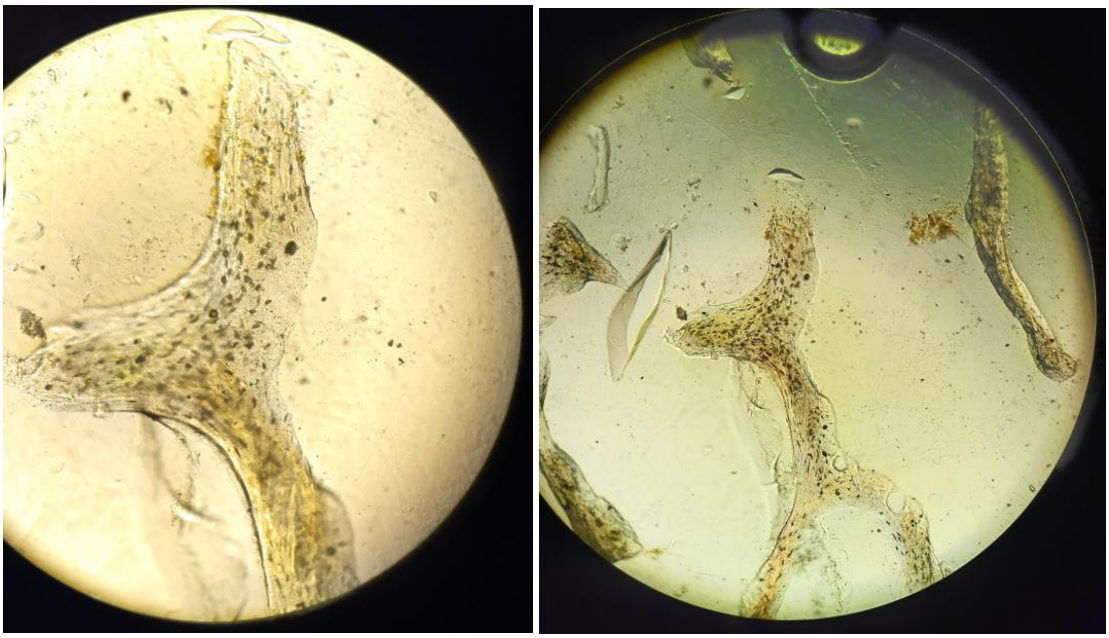

Figure 42: Bone sample 56757 cleaned at 35 and 130 kHz. Bone samples is intact and an osteon is visible in the picture. Magnification is 20x/0.8 (left) and 10x/0.3 (right).

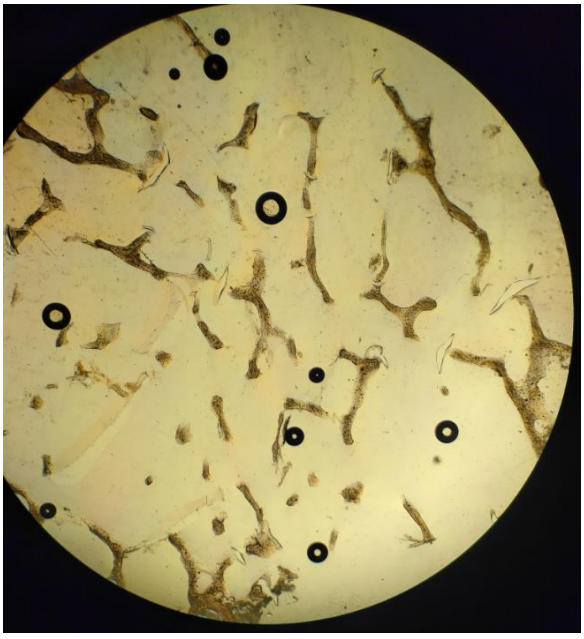

Figure 43: Bone sample 56757 cleaned at 35 and 130 kHz. Bone samples is intact and an osteon is visible in the picture. Magnification is 2.5x/0.075.

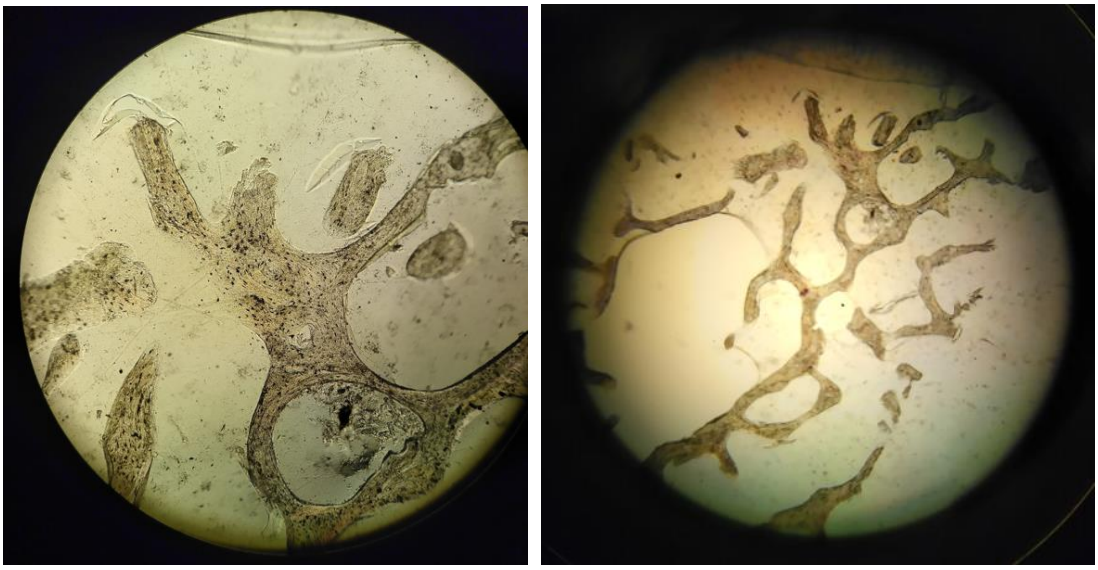

Figure 44: Cross section of a sample that has undergone a 7 hours bath in hydrogen peroxide. The bone appears to be intact. The magnification is 10x/0.3 (left) and 2.5x/0.075 (right).

10.6 Lipid analysis

The lipid analysis has been performed for the three different ultrasonic frequency combinations, 35, 130 and a combination of 35 and 130 kHz. The residual lipid percentage measured varied between 0.02 and 0.95% for all the samples, see table 5. The bone samples weighted on average around 0.4 grams, while the residue weighted on average 0.0020 grams. The samples are all very clean. This makes the error in exact lipid residue higher. The large error makes that there is no distinction possible between the samples in cleaning results based on the ultrasonic frequency. Concluded can be that all samples have hardly any fat residue independent of the ultrasonic frequency used for cleaning.

Table 5: The residual lipid percentage for cleaning with 35, 130 or a combination.

Ultrasonic Frequency (kHz)	Sample	Lipid residue (%)
35 & 130	56088	0.16
35 & 130	56575	0.26
130	56088	0.42
130	55569	0.13
130	56575	0.08
35	56088	0.52
35	55569	0.95
35	56575	0.02

10.7 Mechanical properties

The samples were compressed at an rate of 1 mm/minute. The surface of a sample is 0.785 cm^2. The compressional force is given in newton in figure 45, 46 and 47 per ultrasound frequency.

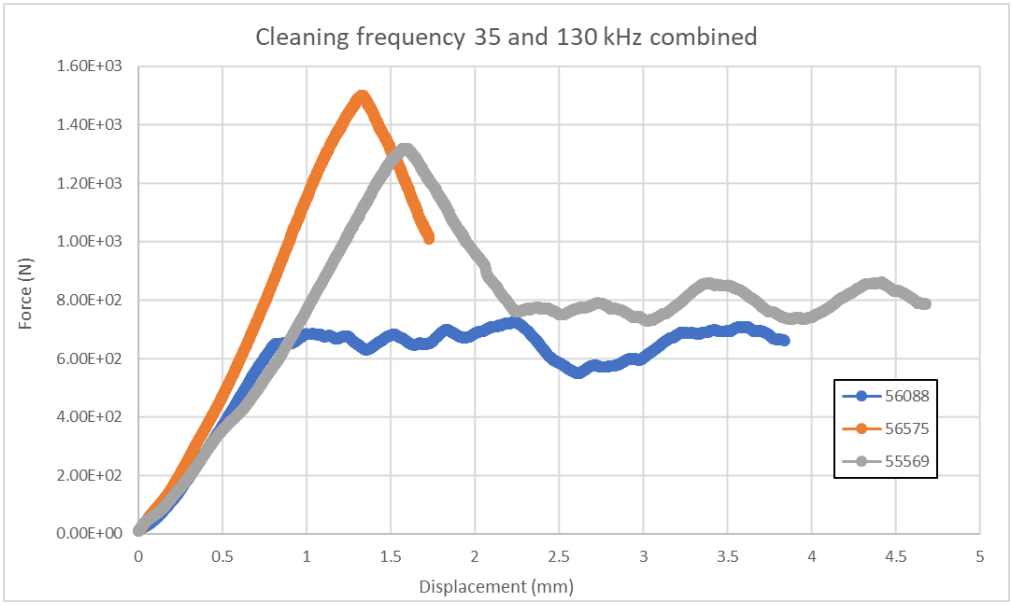

Figure 45: The force versus the displacement for the samples that were cleaned at 35 and 130 kHz combined.

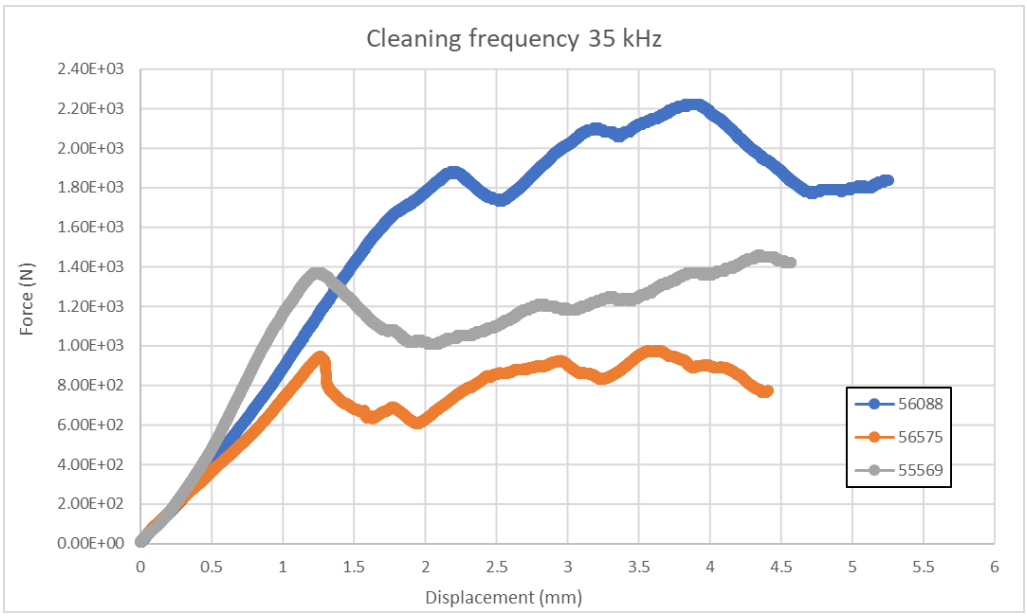

Figure 46: The force versus the displacement for the samples that were cleaned at 35 kHz.

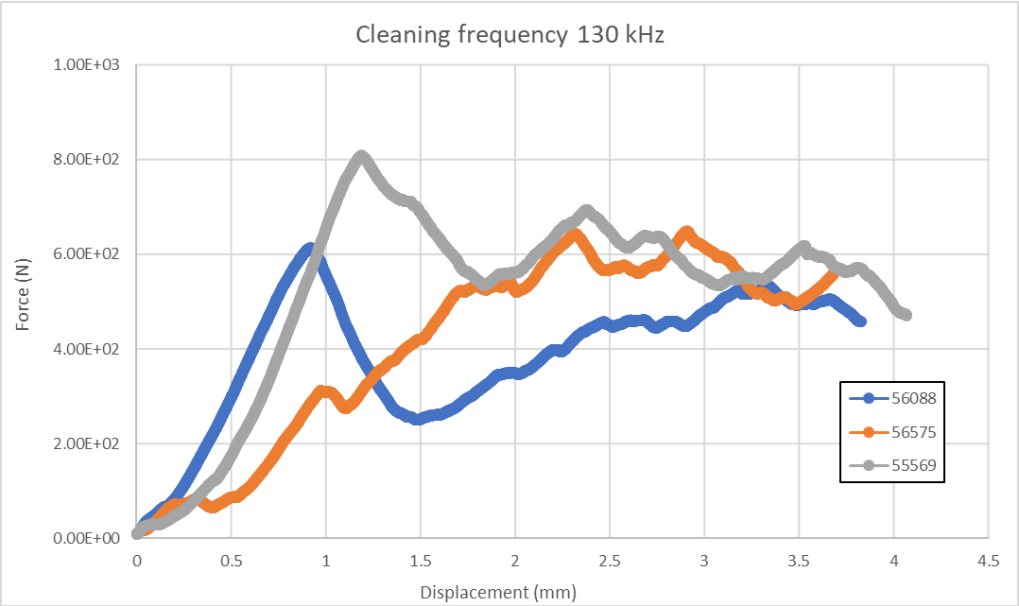

Figure 47: The force versus the displacement for the samples that were cleaned 130 kHz.

The stress to failure values for each frequency and sample are given in table 6.

Table 6: The maximum compressional strength of the bone samples.

Maximum compressional strength		
Sample Nr.	Frequency (kHz)	Compressive Strength (MPa)
56088	35	23.94
56575	35	11.89
55569	35	17.44
56088	130	7.77
56575	130	3.93
55569	130	10.13
56088	35 and 130	8.10
56575	35 and 130	18.97
55569	35 and 130	16.68

11. Discussion

The discussion is divided in four parts. First the outcome of the numerical model on ultrasonic cleaning is discussed. The second part is a discussion of the parameters that influence the chemical cleaning, such as surface area and type of chemical used. Thirdly, the results of the lipid, DNA and histological analyses are evaluated and what these results mean for cleaning with multi-frequency ultrasound are discussed. Finally, an advice is given for the design of the automated production line based on the previous results. It ends with a statement of possible uncertainties.

11.1 Numerical simulation of an ultrasonic cleaner

The modelled pressure distribution in chapter eight is used to provide estimates on the effect of cleaning. Remarkable for the cleaning effect is that for high frequencies the location of the femoral heads in the bath is important. The cleaning effect caused by cavitation is nihil at the sides of the bath for high frequencies of 130 kHz and most strong vertically above the transducer. The location of the femoral heads is less important for lower frequencies of 35 kHz. Placing the bones at lower depths in the bath results in a higher pressures. The cavitation threshold is easier reached under high pressure for high frequencies. The cavitation threshold is in range for the low frequencies. The effect of a pressure node which results in non-cleaned areas is circumvented by using a frequency sweep and there is a shift in pattern during the change in phase of the exiting pressure wave. Undulation counteracts dead spots in the cleaning of bone as well.

The threshold pressure of 30 kPa is easily reached at 35 kHz. However, at 130 kHz, the obtained pressure in the bath is lower while the threshold pressure has gone up to 70 kPa. This threshold pressure is only reached in some areas above the transducer. The threshold is higher for higher frequencies because the bubble has less time to expand during the rarefaction of the wave. Higher frequencies have more waves per time interval which give bubbles an equal amount of time to expand. However, the expansion of the bubble depends on its surface area and time because it is controlled by diffusivity. Less time to expand during one wave cycle means a smaller maximum surface and thus less diffusion of gas into the bubble. Another control factor is the surface tension. Smaller bubbles have a higher surface tension than larger bubbles. High ultrasonic frequencies are related to on average smaller bubbles sizes in the bath which also explains this higher threshold.

The energy of the wave must be released and the damping coefficient increases with an increase in frequency according to Qi et al., (1995). The energy is released by many cavitation bubbles. Previous studies have excluded the effect of damping by cavitation or estimated a limited effect as explained in section 8.1. A high damping factor was selected based on empirical measurements for cavitation. This results in a better estimate of the pressure in the bath. The pressure is lower near the surface of the bath and won't reach the cavitation threshold for high ultrasonic frequencies, see figure 48. Bone in hydrogen peroxide will float to the surface. The vertical movement of the bone must be controlled to prevent it from floating and to optimize its cleaning by cavitation. This is ensured by placing the bone in a metal cage which allows ultrasound to pass but prevents bone from floating to the surface.

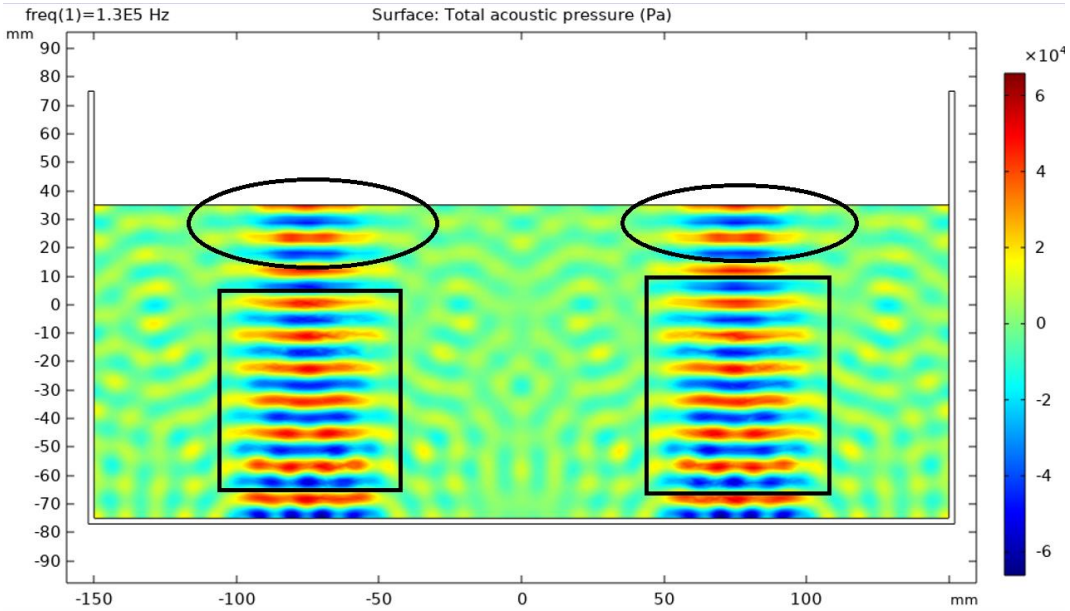

Figure 48: The circled region is located above the transducer and without cavitation due to damping. The squared regions are ideal for the placement of bone. The ultrasonic frequency is 130 kHz.

The absorption coefficient in bone of ultrasound is high and the incoming waves are scattered by the geometry of the bone. This affects the back and forth travelling wave which results in a standing wave pattern. The absorption and reflection impact the pressure of the wave inside the bone and depends on the fat distribution opposite to the cleaned area filled with cleaning fluid. Future research could increase the understanding of the pressure distribution by including a bone with both filled pores and cleaned pores.

The modelling is executed for water, because the bubble size and the cavitation threshold are known for water. Currently, it is not possible to replace water by other cleaning fluids, namely isopropanol or hydrogen peroxide. The properties of these fluids are not sufficiently known enough to design a reasonable model. The bubble size distribution and cavitation threshold should be known for these liquids.

11.2 Cleaning parameters

The cleaning of bone is influenced by the chemicals used for cleaning, surface area, contact time and temperature. The results show that the chosen chemicals have mayor effects on the cleaning results. Fat solvents such as alcohol or acetone have little effect. The contact between the solvent and bone marrow is probably to stagnant and the solving speed to slow. The marrow is very viscous and stubborn in the scaffold of the bone. The only chemical that properly dissolves the marrow is hydrogen peroxide. Hydrogen peroxide produces radicals which are very reactive molecules. The reaction speed of hydrogen is accelerated when it comes in contact with Fe^{2+}. This reaction is the Fenton's reagent:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH\cdot$

$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\cdot + H^+$

Hydrogen peroxide is unstable and reacts spontaneously by itself to hydroxide radicals as well.

$H_2O_2 \rightarrow 2OH\cdot$

These radicals can break up other molecules very easily, especially fats. They oxidize the marrow by cutting the CH_2 of the fat chain. This process produces gas which is visible because the bone in hydrogen peroxide behaves like a shower ball. The disadvantage of hydrogen peroxide is that it could damage the collagen in bone. Tooth bleaching with hydrogen peroxide is known for removing the collagen from the enamel. Shortening the contact between hydrogen peroxide and the bone should decrease its damaging effect on the collagen. Hydrogen peroxide needs time to penetrate the collagen hydroxyapatite structure. It is easier hydrogen peroxide reach the fat in the pores than to penetrate the mineral collagen structure and to damage the collagen

Even when using hydrogen peroxide, it is a challenge to clean the bone located at the centre of a femoral head. Cutting the femoral head in half reduces the penetration depth significantly and enables faster operation times. A femoral head cut in half is therefor much easier to clean. It is not mandated for medical applications to have a complete femoral head. The most wanted grafts are small bone particles. The demand for complete femoral heads is not significant enough to justify the effort.

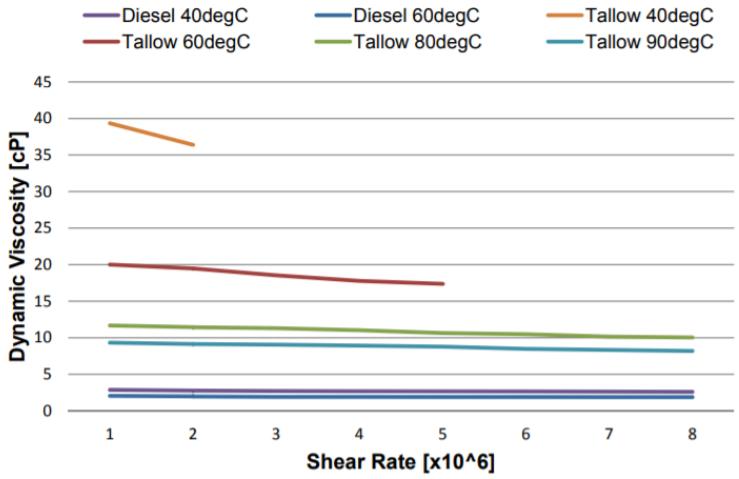

Figure 49 (Piaszyk, 2012): The dynamic viscosity versus the shear rate for tallow and diesel at various temperatures. Tallow is an animal-derived fat. An increase in temperature reduces the viscosity significantly.

Temperature is important for chemical reactions. Hydrogen peroxide is significant more reactive at a temperature over 40℃. Bone marrow is more viscous at lower temperature. Marrow is solid at room temperature. Figure 49 shows the viscosity of animal fat plotted against temperature. Marrow is at body temperature around 40 cP (Gurkan & Akkus, 2008) while water is 1 cP. Increasing the temperature did not significantly improve cleaning results as shown by the results in chapter 10. Tests on the rheology by Bryant et al., (1989) on bovine marrow showed that bovine marrow solidifies at 30℃ with an increasing viscosity. Increasing the temperature did reduce the viscosity but only till 42℃ which is in contradiction to the results in figure 39 A possible explanation is that the adipocyte cell membrane influences the viscosity. The fat in marrow is stored in adipocyte cells. The cell membrane could prevent the fat from dissolving. Destruction of the cell membrane could release the fat and enables it to flow away at higher temperatures. Adipocyte cells can be destructed by changing the osmotic values. The bone is soaked in purified water which will blow up the cells. Purified water is not able to penetrate the bone sample properly when the bone is filled completely with marrow, which was also blocking other solvents such as alcohol. Soaking the bone first in a salt bath could shrink the cells in the pores of the bone scaffold and should create space for the purified water to penetrate the bone deeply. Swelling the cells can break them and releases the fat which is

then susceptible for higher temperature to reduce the viscosity. Swelling is used during liposuction and make removal of adipocyte cells easier. This is worth investigating for bone in the future research.

11.3 The relation between ultrasonic frequency and cleaning bone

The goal was to evaluate and compare the effect of ultrasonic frequency on cleaning bone. The evaluation of the effect of frequency on the strength of the bone sample and the intactness was performed by a compression test and a histologic analysis. A lipid analysis was performed to conclude which ultrasonic frequency had the best cleaning results.

The results of the lipid analysis give a residual lipid percentage between 0.02% and 0.95%. This are a very promising results. In literature, the best value were between 0.5% and 1.5% (Fages et al., 1994;Hua et al., 2020). The obtained value are very low which means that the error in percentage can be big, because a lipid analysis by petroleum extraction has its limitations in preciseness. A DNA analysis to measure the cell residue or a liquid chromatography mass spectrometry for analysis of the residual fat would be a good addition to precisely determine the difference in purity of the samples. The results show that the bone samples are very clean and this is confirmed by visual inspection under the microscope. The difference between the ultrasonic frequencies for the lipid analysis are not significant and range for each frequency in an interval between zero and one percent. Concluded can be that all the samples are extremely free from fat, independent of the ultrasonic frequency used for cleaning.

The cross-sections of the bone samples, used to compare the effect of ultrasonic frequency, were inspected under the microscope, see figure 50. The samples did not show any marrow residues, except one. Sample 56757, cleaned at a combination of 35 and 130 kHz, showed some yellow blobs left of the sample. These blobs are probably fat. The samples used for the cross section were large than the samples for the fat analysis and compression test. The large sample for the cross section requires more time for the cleaning fluid to penetrate its centre. This could lead to same fat residue in the centre of the samples compared to the smaller samples. All sample appeared intact and showed not damage by hydrogen peroxide or ultrasound under the microscope. The intactness of the samples is in agreement with the results of the compression test. The cross-section does show osteocytes residing in the trabeculae. They are hard to reach with chemical cleaning, because they are protected and encapsulated by the mineral collagen structure. Osteocytes do not perform mitosis and are common in allografts and xenografts. The cell membrane could be a cause for a residual fat percentage in the lipid analysis. A cell membrane is made of fat chains.

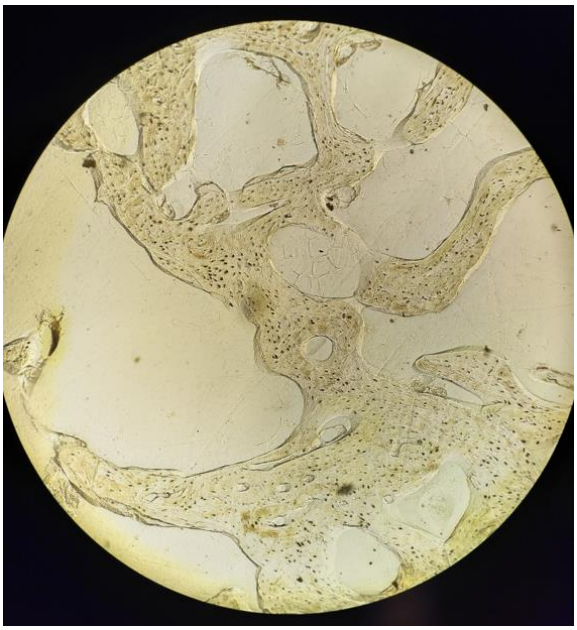

Figure 50: 130 kHz sample 56575, magnification 10x/0.3 (right). Stripes and cracks in the picture are due to cutting the cross section in epoxy. Bone sample appears healthy and intact.

The compression tests showed that the bone has remained its strength after cleaning. The ultrasonic frequency did not affect the strength of the bone. In theory, a lower frequency of 35 kHz could be damaging for the bone structure by the impact of the microjets created by cavitation. Cavitation is more intense at 35 kHz compared to 130 kHz. The bone samples had a maximum compressional strength between 4 and 24 MPa. This is in agreement with compressional strength values in the literature. The average compressional strength for spongious bone is around 10 MPa in the literature (Martens et al., 1983). The strength of bone can vary much due to the anisotropic character of bone and the differences between donors. A highly active person with a high weight produces much denser bone than a small not-active individual.

The comparison of the bone samples under the microscope showed that there was no significant difference between the ultrasonic frequency and cleanness of the sample. All samples appeared to be intact and no marrow was visible. This leads to the preliminary conclusion that ultrasonic frequency is of minor importance for the effect of cleaning. Most likely, hydrogen peroxide is the dominant factor for cleaning. Changing the operation time of hydrogen peroxide changed the percentage of residing marrow significant by visual inspection.

11.4 Design of the automated production line

The automated production line will exist of two parts, one performs the ultrasonic cleaning with the chemical baths and the other part dries the bone grafts. A drawing of the design is shown in figure 51 Separating the drying from the cleaning reduces the production time and enables the cleaning of two batches of bones before they need to be replaced by an operator. The cleaning is performed by an ultrasonic cleaner at 35 kHz. The ultrasonic frequency is of minor importance. 35 kHz is chosen, because it is the most standard cleaner and lowest in cost. The piezoelectric transducer is mounted at the bottom of the bath. There is a preference for as many transducers as possible, because the cleaning effect is the strongest above the transducer. The sides of the bath will be heated by a

heating panel. The material for the bath is 2 to 3 mm thick to withstand vibration for many years. The material used for the bath is of stainless steel 316L, which can withstand all used chemicals. The bath has an overflow at the liquid level for fat to float away. The disposal of fat by an overflow prevents recontamination of the bones with the fat when the liquid is drained at bottom of the bath.

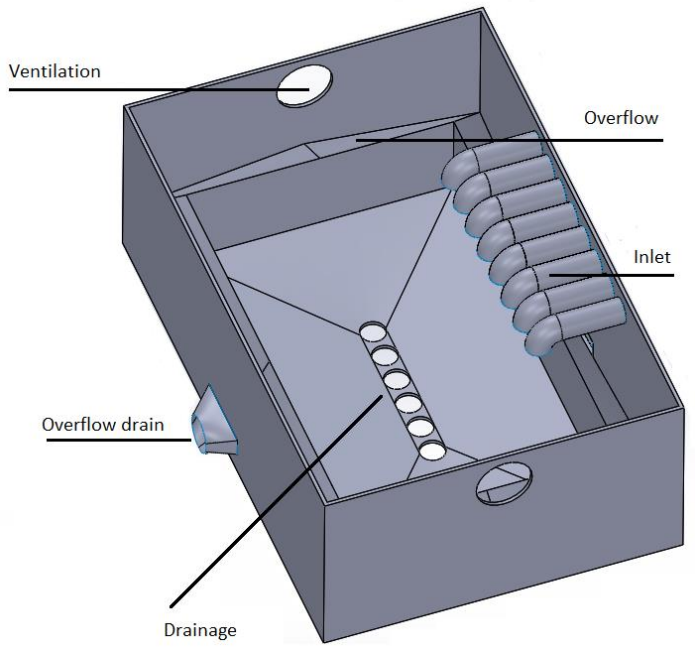

Figure 51: A conceptual design of the ultrasonic cleaner with overflow. The overflow is situated around the bath to prevent the fat from contaminating the bone when drained.

The bones are placed in a metal basket in the bath. This metal has a grid of 5 mm width. The grid size does not influence the behaviour of the ultrasound because the grid spacing is wide enough. The basket is placed one centimetre above the bottom. The basket stops at 4 cm beneath the fluid surface. This prevents the bone from floating to the top during the hydrogen peroxide step. The basket is made of compartments to separate the bone specimens. Each compartment in the basket is located above one transducer, see figure 52. The space vertically above the transducer is optimal for cleaning because the cavitation threshold e is reached best in this zone as is deducted from the modelling.

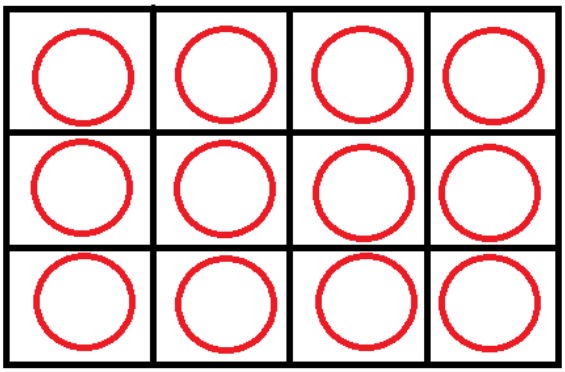

Figure 52: The placement of the transducers at the bottom of the bath in figure 40. The transducer are the red circles. The basket with bones is placed in the bath. The basket has 12 separate compartments for each bone samples. The compartment are located directly above the transducers for optimal cleaning. The compartments of the basket are the black squares.

The fluids used for the bath are hydrogen peroxide, demi water and isopropanol. They are stored in large tanks and pumped to the bath with a positive displacement pump. Between the bath and the storage tank is a transit tank that preheats the fluid. This transit tank is necessarily because heating fluid in the bath takes too much time.

A fan is placed on top of the bath to prevent overheating. Cavitation produces a lot of heat and the temperature with 10℃ per hour on average. This excess heat is discharged by the ventilation. The ventilation is no risk for contamination because the bone is located in the fluid which prevents contamination. The venting is connected to the air circulation of the cleanroom and is therefore extremely clean. Acetone is commonly used in bone processing but would not be compatible with the use of a fan. Acetone is a very flammable substance so the oxygen supply would need to be prevented. This research has found a way of excluding acetone from the bone graft production process. The exclusion of acetone is advantageous for designing a compact automated production line.

After the chemical baths, the bone is cleaned by infrared irradiation. Bone is likely to dry faster in infrared than by a hot air-drying system. A hot air-drying system needs a fresh stream of air that is directly in contact with the cleaned bone, which is a risk for contamination. Infrared drying is not tested at this moment and needs to be examined in further research. A separate machine dries the bone with infrared. An automatic hoist system transports the basket with the bone to the infrared dryer, see figure 53. The machine for chemical cleaning and the machine for infrared drying can be placed next to each other.

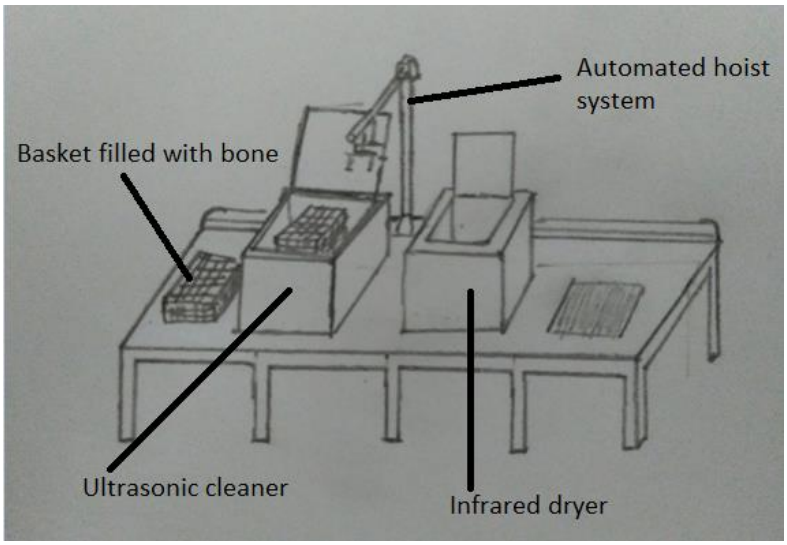

Figure 53: A automatic hoist system transports the basket with the bones from the ultrasonic cleaner to the infrared drying machines. The ultrasonic cleaner refers to the design in figure 40.

The exact operation times for the selected chemical protocol needs further fine tuning. The chemicals are environmentally friendly. Hydrogen peroxide is broken down by to water and oxygen by sunlight. The only waste product is isopropanol which is burned by waste plants for energy regeneration. Isopropanol can possibly be excluded from the process if the infrared drying is effective enough to evaporate all the water. Further research should reveal this.

All steps of the machine can be controlled by a programmable logic controller. A timer, positive displacement pump and valve will regulate the fluid flows. A hoist system controls the position of the basket with the bones. This should be easy to implement and minimize humans interference during the processing. A future prototype should test this.

The chemicals that are used can be deposed environmentally friendly. The chemical used in the highest quantity is hydrogen peroxide. Hydrogen peroxide can be decomposed into water and oxygen. Isopropanol is disposed to a waste plant where it is incinerated as energy for the regeneration of electricity. Isopropanol can maybe be exclude of the production protocol if dehydration of the bone sample by infrared is effective enough. Future tests should prove this.

13. Conclusion

The development of an automated production line is an iterative process with many design choices and changes along the way. Understanding the fundamental mechanisms in cleaning bone allows simpler processing which results in an bone graft of higher quality. This thesis aimed to develop a protocol for bone processing that is implementable for automatic production. The thesis provided a solution for an automatic system for bone processing and verified the design choices by several tests. These tests led to new knowledge in the field of cleaning with ultrasound and gave an improved numerical model for ultrasonic cleaning.

An extensive literature review has selected the optimal chemical and mechanical processing steps. Those are combined in a production protocol which was tested on human femoral heads. These tests showed that chemical agents for dissolving lipids such as acetone and isopropanol had little success in dissolving marrow from the femoral head. Increasing temperature reduced the viscosity of marrow but did not enhance the cleaning results significant. The best results were obtained by treating the femoral head with hydrogen peroxide.

Cleaning by ultrasound was selected as the best tool for mechanical cleaning during the chemical baths. Ultrasound produces acoustic cavitation which serves as mechanical cleaning force. The literature indicated that combining different ultrasonic frequencies is beneficial for cleaning. Low frequencies of 35 kHz should be superior in removing large particles such as blood clots. High frequencies of 130 kHz should be best suited to remove small particles such as small lipids and virus particles. This thesis investigated the effectiveness of different ultrasonic frequencies regarding the cleaning of bone. This was executed through several steps. First a numerical model was created to predict the spatial effect of cavitation in the bath. Secondly the effectiveness of multiple ultrasonic frequencies on the cleaning of bone was tested with experiments.

The effect of ultrasound was investigated by building a numerical model in COMSOL. This model shows the pressure distribution in the bath which enables for allocation of the cavitation regions. Cavitation is the dominant mechanism for cleaning bone by ultrasound. The model is an improved version compared to previous models in the literature. The model is improved with a higher estimate for damping. Previous numerical models neglected or underestimated the impact of damping. The results from the numerical model suggested that the bone samples should be placed in the middle of the vertical axis in the liquid bath. The bone were placed in the ultrasonic bath accordingly.

An experiment was set up to verify the effect of ultrasonic frequency on cleaning bone with real bone samples. The bone samples were taken from the femoral heads of three different donors. The bone samples were cleaned and subsequently analysed by three tests, a residual lipid analysis, a histological analysis and a mechanical compression test. The results of the indicate that the frequency is of minor importance for the cleaning effect. The lipid analysis does not distinguish for a comparison in permille. Further testing by a DNA comparison and a liquid chromatography mass spectrometer could substantiate the cleaning effect. The compression tests showed that the bone retains its strength during cleaning independent of the ultrasonic frequency used. The cleaning result is most likely determined by its chemical agent, hydrogen peroxide. Ultrasound contributes to the overall cleaning, but the frequency of ultrasound is not determining its effectiveness.

Hydrogen peroxide cleans bone through the production of radicals which are highly reactive atoms molecules or ions with an unpaired valence electron. The radicals damage the mineralized collagen fibres minimally which results in a strong bone scaffold of the bone grafts. These radicals clean very efficiently. The exact penetration time for hydrogen peroxide to the core are unknown yet. Further

research should fine tune this. Hydrogen peroxide is environmentally friendly and decays to water and oxygen.

This thesis provided a solution for an automatic system for bone processing and verified the design choices by several tests, see figure 54. The design choices are motivated by minimizing human interference, minimizing risk of contamination, minimizing parts and minimizing costs. The design excludes flammable substances such as acetone which results in a compacter design. The design consists of a bath for ultrasonic cleaning with a compartment to hold the bones in the centre of the liquid column. The ultrasonic frequency is free to choose as validated by experiments. The finite element model in COMSOL has proven that the resonance length for the water column can be ignored, because a sweep function and undulation avert standing pressure nodes to reside at the same location. This prevents dead spots in cleaning. Ultrasound causes cavitation which increases the temperature of the bath. A smart solution for overheating is created by venting to guarantee a constant temperature. This would not have been possible with flammable cleaning agents. The fluid that is supplied to the bath preheated in a transit tank between the bath and the storage tank. The removed lipid residues from the bone marrow will float on the surface and are drained away by an overflow. The bones are dried after cleaning by infrared radiation from above which has a faster drying time than heated air flow and no contamination risks. All these steps fit in one automated production line which excludes errors by human interference and reduces contamination by humans.

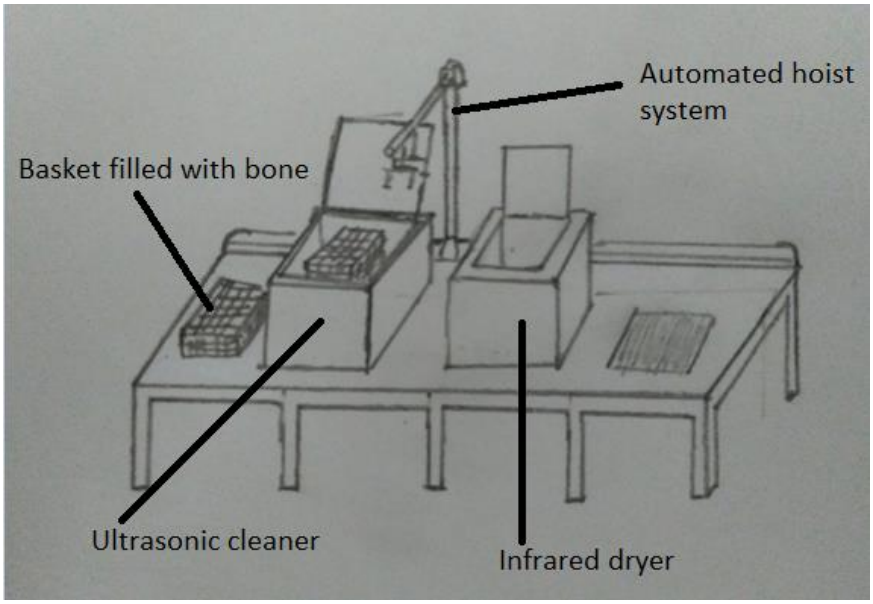

Figure 54: The design of the automated production line to process dirty contaminated bone into clean sterile bone grafts. The ultrasonic cleaner performs several chemical cleaning steps while agitated by ultrasound.

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