DISSERTATION

EXTRACTING COPPER FROM DAIRY FOOTBATHS TO PREVENT HEAVY METAL BIOACCUMULATION IN AGRICULTURAL LAND

Submitted by

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ABSTRACT

EXTRACTING COPPER FROM DAIRY FOOTBATHS TO PREVENT HEAVY METAL BIOACCUMULATION IN AGRICULTURAL LAND

Five experiments were conducted at Colorado State University Department of Animal Sciences to find a way to prevent heavy metal contamination in the soil, due to the use of $CuSO_4$ footbaths in the dairy industry.

These experiments were conducted as proof of concept experiments to determine the feasibility of copper (Cu) extraction from copper sulfate ($CuSO_4$) used in dairy footbaths. In the first experiment, we hypothesized that by collecting the used footbath and implementing chemical and electrochemical procedures, we would be able to extract Cu from dairy footbath solution before the used footbath contents are discharged into the premise lagoon. The first objective was to remove elemental Cu from a 5% $CuSO_4$ solution prepared in our laboratory.

We utilized a platinized titanium inert electrode as the anode and pure Cu electrode as the cathode. Copper was extracted with greater than a 95% efficiency ($P < 0.05$) with a purity of 99.6% Cu in all replicates (n=5). During Cu extraction sulfuric acid was produced. Following Cu removal, 5 g of calcium carbonate ($CaCO_3$) were added to 250 ml of H_2SO_4 solution. This created a reaction where $CaSO_4$ (gypsum) and water were produced ultimately increasing the pH of the solution from 0.7 to 6.5.

The second objective was to determine the feasibility of extracting Cu from a $CuSO_4$ footbath before and after the cows passed through the $CuSO_4$ solution. Footbath samples (from a local dairy in Northern Colorado) were collected from one footbath prior to use and after 600

cows had passed through the footbath. Both pre and post footbath samples were strained through 4 layers of cheesecloth to remove large debris. During electrochemical extraction, foam formed in the footbath sample obtained after 600 cows had passed through the footbath. This was most likely due to protein contaminants in the spent footbath solution denaturing as pH began to decrease during Cu extraction. Foam production was not originally anticipated and significantly reduced the Cu extraction efficiency by more than 23% ($P < 0.05$). Collectively, these data indicate that it is feasible to extract Cu from $CuSO_4$ footbaths and to convert the H_2SO_4 generated in the electrochemical extraction process to $CaSO_4$ and water. Future research examining extraction efficiency and foam production is warranted.

We hypothesized that as cow numbers passing through a $CuSO_4$ footbath increased, that Cu extraction efficiency would decrease and that multiple Cu extractions and $CuSO_4$ regenerations would decrease ultimate Cu recycling efficiency.

The objectives of the second experiment were to determine:

- The effect of the number of cows walking through a footbath on Cu extraction efficiency.
- The impact of multiple Cu extractions, regeneration of $CuSO_4$, and reuse in subsequent footbaths on Cu extraction efficiency.

To accomplish our objectives, footbath samples were obtained from a northern Colorado dairy milking 1,200 Holstein cows two times per day. Since this dairy had one footbath on both sides of the lead up to the 34 head herringbone milking parlor (17 on each side), we obtained samples from one footbath. Footbath samples (1.0 L samples) were collected at time 0 (no cows had passed through the footbath; freshly made footbath), and after 150, 300, 450 and 600 cows passed through the footbath.

Samples were brought back to the laboratory filtered through 4 layers of cheese cloth to remove large debris, electrolysis was started on each sample. We took sub-samples before and after electrolysis and prepared them for ICP analysis to measure Cu concentration. We observed a relationship between the number of the cows that walked through the footbath and the copper concentration. Also, it was noted that the extraction efficiency of Cu decreases as the number of cows walking through the footbath increases. The extraction efficiency was over 95% ($P < 0.01$) for the 150 head samples, while extraction efficiency dropped to 75% for the 600 head group. In previous studies, we have successfully demonstrated that we can regenerate $CuSO_4$ from the used footbath. We hypothesized that laboratory processing techniques would not alter Cu solubility and that the antimicrobial effectiveness of $CuSO_4$ would not be altered by $CuSO_4$ regeneration. Two experiments were conducted to determine:

- The impact of laboratory processing techniques (autoclaving and centrifugation) on the solubility of Cu from dairy footbaths (Experiment 1), and
- Determine the antimicrobial effectiveness of regenerated $CuSO_4$ (Experiment 2).

In experiment 1, $CuSO_4$ footbath samples were obtained from a northern Colorado dairy operation milking 1,200 Holstein cows two times per day. Since this dairy had one footbath on both sides of the lead up to the milking parlor, we obtained samples from one footbath. Footbath samples (1.0 L samples) were collected at time 0 (no cows had passed through the footbath; freshly made footbath) and after 150, 300, 450 and 600 cows passed through the footbath. Samples were brought back to the laboratory and filtered through 4 layers of cheesecloth. After filtration, 12 subsamples (10 ml/subsample) were collected from each collection period and pH determined on each sample. Six samples per collection period were autoclaved while the remaining six subsamples were not autoclaved. After autoclaving, pH was determined on all

samples. Three autoclaved and three non-autoclaved samples were centrifuged at room temperature, while the other autoclaved and non-autoclaved samples were not centrifuged. At the end of these procedures pH was determined on all samples and a subsample obtained from all samples (n=60) and analyzed for Cu concentrations. Data indicated that neither centrifugation nor autoclaving had an impact on Cu solubility (SEM = 2.94; $P < 0.05$). Experiment 2 was designed to determine the antimicrobial effectiveness of regenerated $CuSO_4$. Footbath treatments mixtures were prepared (10 ml) in 20 ml glass tubes and included:

- Conventional 4% $CuSO_4$ footbath solution (made in the lab) for which 4 grams of $CuSO_4.5H_2O$ was added to 100 ml water.
- Regenerated $CuSO_4$ solution made by regenerating $CuSO_4$ for three times after making artificial footbath in the laboratory from the dirty water footbath.
- Footbath at time zero (22.7 kg of $CuSO_4$ in 550 liters of water is ~ 4% $CuSO_4$), which was taken from a fresh footbath with 0 cows passing through the footbath.
- Conventional 4% $CuSO_4$ solution with added sulfuric acid ($4x10^{-3}$/ vol.). Approximately the same amount of acid that the dairy was using in the footbaths (2 liters per 550 liters footbath) was utilized.
- Regenerated $CuSO_4$ used to make a 4% $CuSO_4$ footbath solution with added sulfuric acid ($4x10^{-3}$/ vol.). This is the regenerated $CuSO_4$ solution with the sulfuric acid from the dairy.
- Sulfuric acid alone (from dairy), and
- Deionized water.

Serial 1:2 dilutions were made of each treatment and then all dilutions were autoclaved. The minimum inhibitory concentration (MIC) for each treatment was determined using *E. coli*.

The MIC was at a 1:16 dilution for all the treatments with the exception of the acid treatment (SEM = 3.62) (P = 0.05). For the acid treatment, no bacteria growth was present at our greatest dilution (1:512). To determine the minimum bactericidal concentration (MBC) treatment dilutions of 1:32; 1:16; and 1:8 were plated on Mueller Hinton ready petri dishes and incubated at 37°C temperature for 24 h. Colony counts were then performed on all plates. All treatments showed bacterial growth with the exception of the acid treatments. These data indicate that a dilution of greater than 1:32 is required to determine MBC. In summary, regarding the laboratory techniques, there was no significant difference between the treatments. The MIC was considered at a 1:16 dilution for all the treatments with the exception of the sulfuric acid treatment. Since bacterial growth was present on all plates for all the treatments (with the exception of the sulfuric acid treatment), we need to reanalyze the MBC with less diluted solutions to determine the appropriate dilution for a true bactericidal effect.

Considering this information, we wanted to design an apparatus which can either extract Cu, or by adding some other options, can regenerate $CuSO_4$ from the used footbath. This machine comes with a prefabricated footbath which can be installed on the previously made footbath with some minor modifications to the previous structure. The footbath is long enough to let the cows have at least three full hoof immersions as they pass through the footbath. These footbaths are made of stainless steel and have a rubber padding at the bottom to provide adequate traction for the cows while walking through the footbath. Each unit is going to be on a trailer, and producers can use a tractor to move it to a different locations. This system is a combination of pumps, filtering systems, an electrowinning chamber and other tanks for chemicals.

This machine has an infrared counter that can count the number of the cows that have passed through the footbath and will start to pump out the footbath after 150 head. There is a

second batch of unused $CuSO_4$ that will be pumped back into the footbath while the other one is being regenerating. After each extraction, the operator can turn off the system and scrape the cathode to remove the copper by simply pulling the electrode out, and turning the system back on. Our team tried to design and make an integrated system which is easy to work with, efficient and safe.

Energy consumption of this system is minimal, and the chemicals that are being using for the chemical processing are reasonably cost effective. There is an option of using solar panels to provide the energy for the electrowinning process. The return on investment for this machine is less than four years. We believe that we have a feasible solution to the previously stated problem. The next steps in our research include building a prototype of the system and installing it in a dairy operation in northern Colorado in the near future.

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DEDICATION

I would like to dedicate this research to Mr. William (Bill) Wailes, Jr., who first realized the negative impacts of the disposal of used footbath dairy solutions on the environment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

REVIEW OF THE LITERATURE

1.1. Dairy Industry Yesterday and Today

Domestication of livestock has played a central role in shaping human history. Livestock domestication provided humans with a continuous supply of power and food. During the crop growing months, livestock serving as a source of plowing power, allowed farmers to plant larger crops. The excess crops could be stored as a food supply over the winter months and the crop waste would serve as feed for certain livestock species. Humans that once relied on hunting, fishing, and food gathering as a mean of existence were now able to live in one location and permanently settle. This allowed for trade to become more specialized and for the development of established commerce and governments. Regarding dairy cows, human learned to harvest milk from other mammals more than 12,000 years ago in Mesopotamia, which was located on the Iran-Iraq and Turkish-Syrian borders (Collon, 2011).

Approximately 7,000 BCE in Southwest Asia, domestication of dairy animals reached Europe (Price, 2000). Approximately 2,000 years ago, as the domestication of livestock progressed, nomadic tribes of Europe settled in the area of what is now known as the Netherlands. As domestication proceeded, this population of people began to select livestock for production efficiency traits. It is believed that this group of people began to select black Batavian cattle and white Friesian cows for milk production and feed efficiency traits. Today these animals are known as the Holstein-Friesian breed of dairy cattle (Holstein Association of America, 2017).

European immigrants brought cattle to the United States (US) in the early 1600's to provide dairy and meat products for their families (USDA, 2017a). Even though many different breeds of cattle including Durhams, Ayrshires, Guernseys, Jerseys, and Brown Swiss were brought to the US over the next few centuries, developing a breed specifically for dairy purposes occurred in the late 1800's (USDA, 2017a). From the 1800's through 1950's dairy cow numbers increased to a high of 23 million in the US (Blayney, 2002). Then in the 1960's the demand for milk and milk products began to increase. This demand (coupled with the industrial revolution) began the industrialization of dairy production (Figure 1.1).

Some reports show that Jersey cattle were imported to the US in the early to mid- 1800's (Graves and Fohrman, 1936). As the name indicates, Brown Swiss cattle were developed in Switzerland. There were limited numbers of Brow Swiss imported to the US in 1869 - 1882 (Graves and Fohrman, 1936). Then in the late 1800's the first dairy husbandry school was established at the University of Wisconsin - The Center for Dairy Research at UW-Madison and the nation's first master cheesemaker program (Wisconsin government, 2017).

Dairy producers began selecting for improved genetics and increased overall herd size to gain production efficiencies. In the US, practicing artificial insemination (AI) began in 1936 (Esminger, 1980) and the first dairy cattle AI cooperative was organized in 1938 (Shaffer, 1962). Since the beginning of the industrialization of dairy production, the total population of dairy cows in the US has actually decreased to less than 9 million cows in 2017. However the cow population per dairy has dramatically increased from 6 cows per dairy in 1950 to 187 cows per dairy in 2017.

Total milk production in the US was over 75 million tons in 2000, near 45 percent more than in 1975 (Blayney, 2002). In a review of dairy cow efficiency, Capper et al. (2009) compared

a 1944 Holstein cow to a 2007 Holstein cow. Average milk yield of a 1944 dairy cow was less than a quarter of the yield of a 2007 dairy cow (Capper et al., 2009). Both milk and crop production improvements have reduced the amount of farmland required to support dairy production to 162,000 hectares to produce one billion kg of milk, which is 10% of the land that was needed in 1944 to support dairy cattle production (Capper et al., 2009). Today in the US, dairy farms with more than 100 cows produce 86% of the milk (USDA, 2017b) and the top five dairy producing states are California, Wisconsin, New York, Pennsylvania, and Idaho respectively.

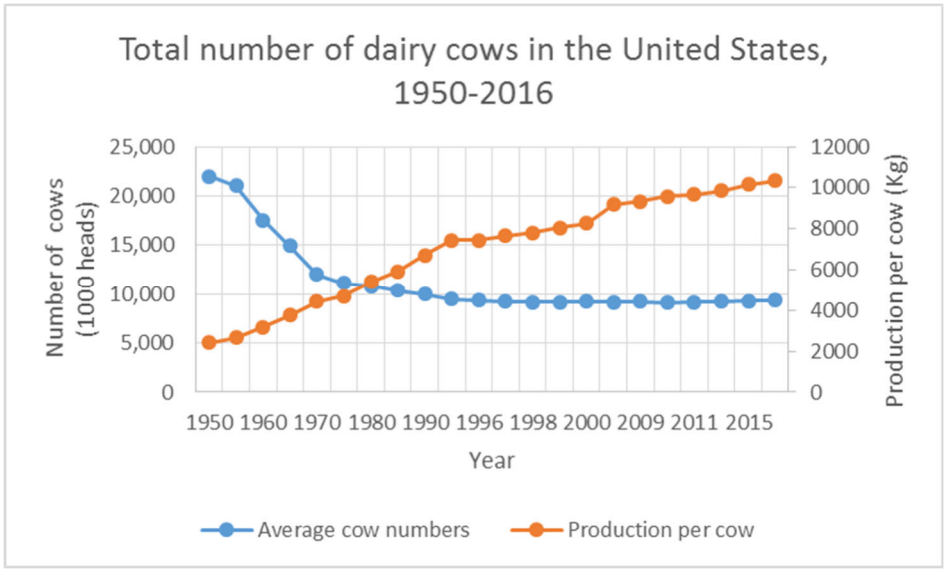

Figure 1.1. United States milk production, 1950-2000 (Blayney, 2002) (USDA, 2017c)

Milk production is a yield trait that has been selected by dairy producers for many years. However, selection of non-yield traits has also played a significant role in dairy herd improvement and longevity of dairy cows (Shook, 2006). One of the most significant advancement in US diary production in past 25 years has been that of preventing instead of treating disease (e.g., focusing on herd health) (LeBlanc et al., 2006). Another significant improvement in non-yield trait selection has been the ability to identify antibodies and DNA in milk to help detect and prevent disease. This is a major health monitoring program currently

being used in Europe (Houe et al., 2006). Furthermore, genetic selection for improved udder health is now available globally (van der Spek, 2015).

Since heifers are more valuable in the dairy industry than male dairy cattle, using sexed semen to produce more female cattle has recently been commercially developed for dairy producers. In the US, five percent of the semen used in artificial insemination (AI) in dairy cows is sexed semen (Seidel, 2014). The slow adoption of sexed semen in dairy production is most likely due to low conception rates in cattle bred with sexed semen.

Technology is another factor that has had a tremendous impact on feed production and dairy management. Using tractors instead of draft horses and sophisticated software to balance rations instead of just using pastures for feeding the animals has increased dairy production efficiency. Dairy farmers are adopting more technology every day, technologies like pedometers and activity monitoring systems for estrus detection (Rutten et al., 2013).

Since the 1980's, world milk production has risen by more than 50 percent, from 500 MMT in 1983 to 769 MMT in 2013 (FAO, 2017). In the 1980's with the introduction of the personal computer, farmers were able to mix more complex rations that could reduce feed costs significantly without the use local of agricultural extension personnel (Esminger, 1980). Even though the increase of milk production has been shown by changing the milking frequency (2X to 3X/day) in the 1930's and 1940's, producers did not adopt this management strategy to improve milk production until the 1980's (Borton et al., 1990). Furthermore, in 1984 the FDA ruled that Recombinant Bovine Somatotropin (rbST) had no risk to human health and is safe for the human consumption (Sechan, 1989).

Globally, India is the leading milk producer in the world with the 18% of the world production. US, China, Pakistan, and Brazil are respectively second, third and fourth in milk

production. In 2016, the US dairy industry produced 96.4 million tons of milk (USDA, 2017). Over the last 40 years, South Asia increased their milk production significantly. (FAO, 2017).

1.2. Dairy Industry Problems and Hurdles

One of the most critical issues to dairy production is animal welfare as evidenced by a survey of Belgian consumers where it was reported that Belgian customers believe that welfare is the second most important priority after food safety (Vanhonacker et al., 2007). The animal welfare includes its physical and mental state, and good animal welfare indicates both health and a sense of well-being. Each animal which is kept by humans, must be protected from preventable suffering. The Farm Animal Welfare Council has an excellent framework for animal welfare which consists of five freedoms (Farm Animal Welfare Council, 1979):

1. **Freedom from hunger and thirst:** Having access to clean water and feed to sustain health and strength.
2. **Freedom from discomfort:** Having a proper shelter and resting area.
3. **Freedom from pain, injury or disease:** Prevention and/or quick diagnosis and treatment.
4. **Freedom to express normal behavior:** Providing adequate space, appropriate facilities, and another animal from the same species as a company.
5. **Freedom from fear and distress:** Providing circumstances and accommodations which avoid mental distress.

Having good animal welfare on a dairy farm is not only socially good, but it will also reduce economic losses for a dairy. The most straightforward example of improving animal welfare is heat stress management. Heat stress not only causes an economic loss, but it is also an animal welfare issue. Significant improvements in environmental management, including fans,

misters, sprinklers, and cooled waterbeds, can reduce the impacts of thermal stress on cow health, production, and reproduction. Rotating cow brushes are sometimes used in some dairies to allow cows to brush and scratch their bodies. It may additionally keep them occupied and, if placed appropriately (e.g., placed in shaded areas) can help keep the cow cooler. Other management strategies such as having a functional disease prevention and management plan in place and making sure animals are supplied with balanced diets and clean drinking water are relatively commonplace today.

A plan for preventing disease is one of the most essential strategies that we should keep in mind in order to sustain a successful dairy system. As we discussed in the previous paragraph, dairy producers are doing these things not only because of the profitability but because it is ethically the right thing to do. There are four major diseases in dairies:

- Displaced abomasum
- Lameness
- Mastitis
- Metritis

As discussed by (Charles Guard, 2009), lameness is one of costliest diseases in dairy production (Table 1.1). Although it is not the most expensive diseases per cow, it is the costliest disease per herd because the frequency of occurrence is much higher than other conditions.

Table 1.1. Costly diseases in dairies (Charles Guard, 2009)

Disease (cost/year)	Cost per cow	Cost per Herd
Displaced abomasum	$489	$2,447
Lameness	$478	$14,330
Mastitis	$262	$10,490
Metritis	$325	$4,874

There are multiple studies regarding frequency of lameness in dairies in Europe and the United States. The average prevalence of lameness across study farms in England and Wales was 36.8% (Barker et al., 2010). In Wisconsin and Minnesota, average lameness prevalence was reported to be approximately 25% (Nigel B. Cook, 2003). Further reports from Northeastern states and California designate that lameness occurrence ranges from 34 to 63% (von Keyserlingk et al., 2012).

There are several ways to prevent lameness. The most important prevention method is keeping the hoofs and alleyways clean and dry. Drier alleys will lead to fewer hoof problems and injuries. Another method to avoid lameness is walking the cows through a footbath that contains an antimicrobial agent, after milking.

Different types of chemical solutions are available for use in dairy footbaths. Two of the most common footbath solutions are copper sulfate ($CuSO_4$) and formaldehyde. The most common concentration for use in dairy footbaths is 37% formaldehyde with the most common doses being 5, 20, 30 and 50 ml per liters respectively (Cook, 2006). Although the efficacy of formaldehyde for preventing lameness is excellent, formaldehyde is a known carcinogen and it is a serious hazard to the workers (Collins and Lineker, 2004) and can alter microbial populations in a premise lagoon.

Copper sulfate is the most popular antibacterial used in the dairy industry today with 63% of herds using it at concentrations ranging from 1 to 10% of the footbath (Cook, 2017). $CuSO_4$ footbaths are typically emptied and refilled three times a day, once after each milking. Used footbath solution is typically discharged into the premise lagoon. Dairy farmers then use the water from the lagoon for irrigation. This management practice transfers copper (Cu) to the farmlands and over time can accumulate in farmland soils.

When Cu is added to soil, it can end up in several forms within the soil:

- In the soil solution
- On exchange sites
- Sorbed
- Occluded in soil oxides
- In the frame structure of primary and secondary minerals

One factor that contributes to Cu accumulation in the soil is the prominent type of silicate existent in the soil. There are six major silicate groups: *Tectosilicates* (Framework), *Phyllosilicates* (Sheet), *Inosilicates* (Chain), *Cyclosilicates* (Ring), *Sorosilicates* and *Nesosilicates*, and Inorganic residues and living organisms (Adriano, 2001). Heavy metal contamination is a prominent matter, especially in agricultural production systems (Jafarian and Alehashem, 2013). Symptoms of plant toxicity associated with Cu are stunted growth, chlorosis, necrosis, and death of the crop (Pierzynski, 1994). Cu less than 250 mg/kg had no effect on plant growth; however, Cu applications of more than 250 mg Cu/kg increased corn Cu concentrations and reduced growth (Ippolito et al., 2010). Cu accumulation can also have negative effects on potato production as well (Moore et al., 2013). Figure 1.2 depicts the difference in root mass between high and low concentrations of Cu treatments. These treatments were applied with six

different rates of $CuSO_4$ solution of 50, 100, 250, 500 and 1000 mg Cu/kg soil (Moore et al., 2013).

Figure 1.2. Root mass is smaller and darker for the 1000 mg Cu/kg treatment (right) in comparison with the 0 mg Cu/kg (left) (Moore et al., 2013)

Dr. Jim Ippolito (Ippolito et al., 2010) has conducted research investigating the impact Cu accumulation due to footbath disposal in lagoons by repeatedly applying the lagoon water to one or two parcels of land under center pivot irrigation in a town called Wendell, Idaho (Figure 1.3). This causes Cu induced iron deficiency in corn and other crops (Figure 1.4). The goals of this research were to recognize Cu application effects on corn growth and Cu concentration, total and diethylenetriaminepentaacetic acid (DTPA)-extractable soil Cu content, and the soil bacterial population. Results revealed that corn growth was unaffected by soil Cu concentrations up to 250 mg/kg; however, soil Cu concentrations greater than 250 mg/kg increased corn Cu concentrations and reduced corn plant growth. In soil, DTPA-extractable Cu content increased as Cu application increased. After 30 days of Cu application, 60% to 75% of the added Cu was still available to plants (Figure 1.3).

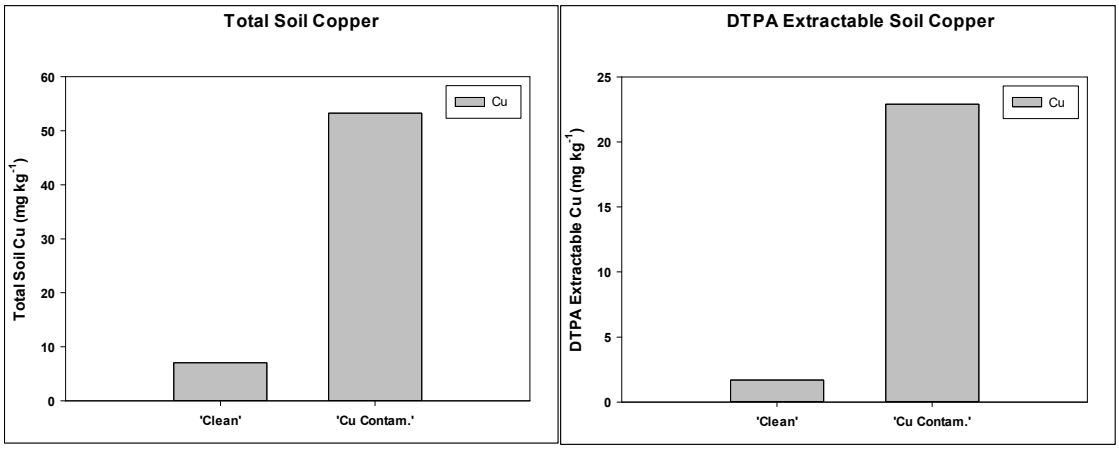

Figure 1.3. Difference in total soil copper content (left) and difference between plant available Cu content (right) (Ippolito, 2010)

Figure 1.4. Copper induced iron deficiency in corn (Ippolito, 2010)

1.3. Hoof Anatomy

A cow's hoof is cloven, divided into two approximately equal parts usually called claws (Shearer et al., 2005). Hoofs are fully evolved on both main digits III and IV and are composed of modified skin with a thick, strongly cornified epidermis (Bragulla et al., 2003). Dewclaws are shortened digits (II and V) that are connected without synovial joints. They do not touch the ground except in cases of foot softening. Softening of the ligaments of the suspensory apparatus will lead to pedal bone rotation and putting pressure on the digital cushion.

Mechanical damage or softening and weakening of the interdigital skin by cut wounds or continuous exposure to muddy conditions are necessary to provide entrance points for infectious agents. At the center of the claw are the support structures consisting of the third phalanx, the distal sesamoid bone, second phalanx, distal interphalangeal joint as well as tendons and ligaments.

The subcutis forms a layer of padding between the corium with horn capsule and support structures also known as the digital cushion. The corium is highly vascularized and innervated and helps to form a secure connection between the horn capsule and the third phalanx. The germinal layer that covers the corium initiates the production of hoof horn.

The capsule has a thickness of 10 mm in the dorsal section and 5 mm at the axial section. The epidermis growth forces the cornified portions distally at the rate of 5 mm per month. On the third trimester of pregnancy and at high producing months, horn development will reduce dramatically. The hoof capsule has five major segments. These segments are:

1- Perioplic segment
2- Coronary segment (Corona)
3- Wall segment (Paries)
4- Sole segment (Solea)
5- Bulbar segment (Torus ungulae)

The outermost perioplic segment comprises of relatively soft horn and is limited to the upper part of the hoof wall. This segment is approximately 1 cm wide. Superficial fascia forms a slightly arched perioplic cushion dorsally and abaxially.

The preoptic dermis protects the subcutis. Horn tubules are formed by periople, covering the dermis. The coronary section is responsible for hoof wall production, which protects the claw

and consists of tough horn material, in addition is distal to the perioplic segment and spreads to another section at the middle of the hoof. It forms the larger portion of the hoof capsule including the weight bearing border of the wall.

A connecting layer of the horn is located between the corium and hoof wall and consists of the laminar horn which interdigitates with the laminae of the corium. This firm digitation belongs to the suspensory apparatus between the third phalanx, soft tissue, and horn capsule and comprises all the tissues that connect to the bone to the horn capsule (Figure 1.5). Its function is to transform the pressure exerted by the body weight within the hoof capsule to a tractional force; the connecting intermediate layer is visible at the sole as the area of the white line. The white line (white zone) has three different parts: external part, middle part and internal part.

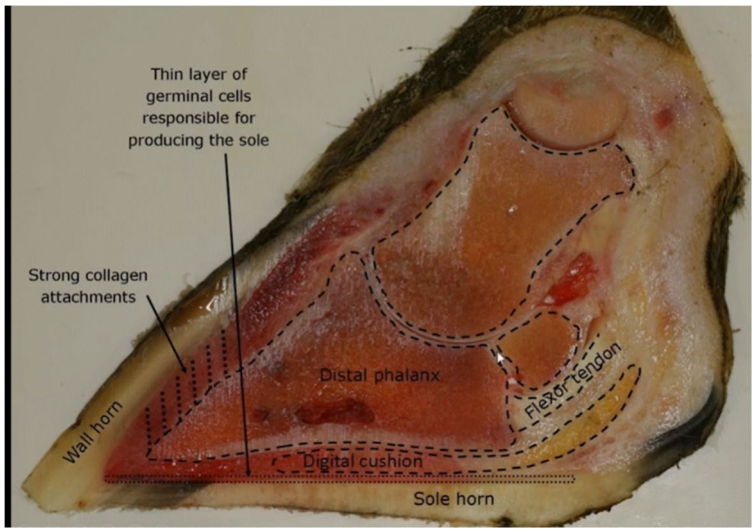

Figure 1.5. Anatomy of the hoof (Huxley, 2015a)

The outer part is a portion which we can see with the naked eye as a bright 1 mm wide stripe. Intermediate sections of the horny lamellae are responsible for the development of the middle parts. The internal portion of the white line entails the crests of the horny lamellae and linking them to the terminal tubular horn. The small sole segment is connected to the heel horn the cranial heel together with the sole section is referred as the sole (Figure 1.6). White line acts

like a cement and will keep the wall and the sole together. Most of the cracks start at the white line and will lead to introduction of the bacteria and other microorganisms to the hoof which will cause lots of complications for the animal.

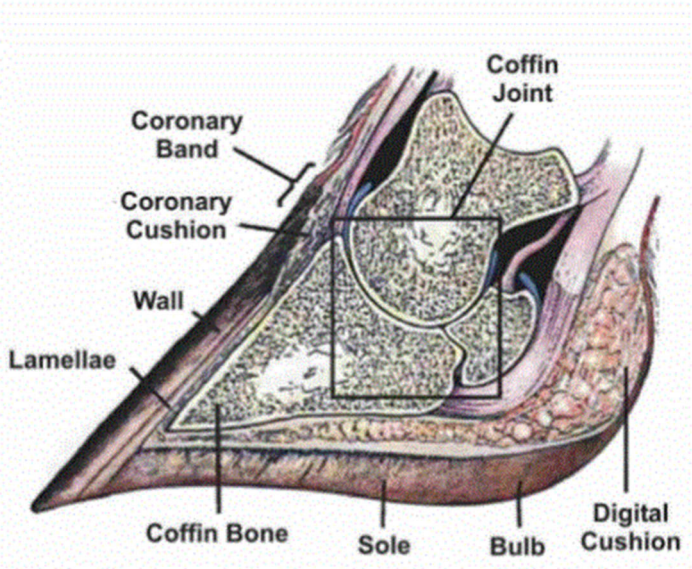

Figure 1.6. Bovine hoof anatomy (Raven, 1985)

The interdigital space separating the main claws (III and IV) of a limb is bridged by the interdigital skin which lacks hair and has a dense cornified layer. This area is dark and moist so it is ideal for bacterial growth and cause lesions and pain for the animal.

1.4. Lameness

One of the most challenging issues facing the dairy industry today is lameness. It has a negative impact on animal welfare and profitability (Bicalho et al., 2009). Lameness is a multifactorial disorder (Figure 1.7) (Sanders et al., 2009). The main factors contributing to lameness are infectious agents (e.g., foot rot), laminitis, conformational or other lesions (leg injury), and claw lesions such as white line disease, thin soles, thin sole-induced toe ulcers, sole ulcers, heel ulcers, toe ulcers, and sole punctures (Sanders et al., 2009).

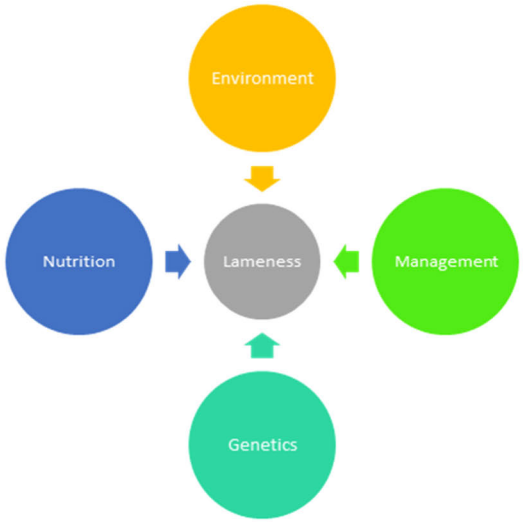

Figure 1.7. Important factors that will cause lameness (Jalali, 2016)

In general, there are four main factors that cause lameness: Environment, Management, Genetics, and Nutrition (Jalali, 2016). Globally, 25 to 40% of dairy cows suffer from some type of lameness (Huxley, 2015b). Environmental factors like housing type, stall surface, and season can have an impact on the occurrence of lameness (Nigel B Cook, 2003). Providing a comfortable environment is not only important to help prevent injury, but it is also crucial after or during the treatment period for laminitis (Whay and Shearer, 2017).

There are several ways that we can identify a lame animal. Stance, posture and weight shifting is one of the common ways to identify a lame animal. A lame animal will modify the posture to alleviate pain. The other valuable way to detect a lame animal is the locomotion scoring system. Locomotion scoring is based on the way cows stand and walk, and back posture also is essential in lameness detection. Lameness will be measured from 1 to 5 (normal to severely lame). Score 1 (Normal) animal has straight leveled back and makes long confident strides. Score 2 (Mildly Lame) stand with a straight back but arches when starts walking, gaits are somewhat shorter than a healthy animal. Score 3 (Moderately Lame) animal has shorter strides, and the back will be arched while standing and also walking. Score 4 (Lame) has arched

back while standing and walking, these animals prefer one or more limbs to the other but still have problems putting weights on them. Score 5 (Severely Lame) has a notably arched back, and they are not able to move. Severely lame animals cannot complete weight transfer off the injured limb (Sprecher et al., 1997).

The treatment period is extremely important because it is at this time that the animal is dealing with a problem and is more prone to other complications. For instance, an animal that has been treated for laminitis has a difficult time laying down and standing up. The stalls must have sufficient room for the cows to lie down and stand up, without difficulty, distress or fear. If this individual is not provided with a comfortable area to rest, this might cause more complications for the animal.

If cows have a comfortable place to rest they will be out of the way of other cows so they can get to their feed faster and with lower stress. Dairy cows prefer to lie down while ruminating. If they are found to stand with their back outside the cubicle or to lie outside the cubicles, this is a sign of discomfort, and probably the stalls are not long enough. Having enough space is essential for the cow's head for lying down and rising. A small stall or a wall in the front of the animal's head is the most popular characteristic of poor designed free stalls. Having at least 0.5 m of extra space in front of an adult cows head is required to allow the animal to stand comfortably. (total length at least 2.70 m) (De Laval, 2007).

Confinement has the luxury of cooling the cow in the hot weather, and the shelter will act as a barrier to protect animals from the wind, snow, and rain (Shearer and Amstel, 2007). One of the main factors contributing to laminitis in modern dairy facilities is flooring. The most popular type of flooring is concrete. It is easy to clean and maintain. However, cows are not designed to spend their life on such a hard surface.

Hard surfaces will cause the overgrowth of hoofs. Drainage is not good on concrete surfaces and will therefore, which keep the pen surface wet and cause the hoof to become soft and become more vulnerable to different complications, e.g., sole lesions and bacteria growth. Slippery concrete and low traction surfaces are another contributor to lameness in cows. Hoof wear will be increased on rough concrete surfaces; this will raise the risk of lameness in dairy cattle (Wells et al., 1993). However, rubberizing concrete surfaces will improve cow comfort and traction (Shearer and Amstel, 2007).

Practices such as making flooring slip resistant should reduce the occurrence of lameness (Solano et al., 2015). Another consideration regarding reducing lameness is to eliminate sharp surfaces like screws and nails of the flooring. Sharp pointy surfaces can penetrate the sole and can cause injury and infection. One of the regularly occurring problems in the interdigital area is Hyperkeratosis, which happens due to mechanical stress. A particular disease of the interdigital area is interdigital hyperplasia (limax). Limax is the out sticking hypertrophy of the scar tissue and is detected mainly in fat cows and breeding bulls.

An infectious disease of the claws of global significance is digital dermatitis (DD; strawberry footrot - Mortellaro's disease (Figure 1.8). DD was discovered in 1974 by two Italian scientists Cheli and Mortellaro (Cheli and Mortellaro, 1974). The standard round reddish and elevated lesions. Digital dermatitis is primarily a skin disease which secondarily affects the interdigital region and may also spread in the claw causing disruption of the horn capsule (Budras and Habel, 2011). Digital dermatitis is one of the major causes of lameness in dairy herds (Laven and Logue, 2006). It is an important problem for the dairy industry in many countries, causing diminished animal welfare and economic loss.

Figure 1.8. Digital dermatitis. Typical acute lesion (strawberry foot rot) in the skin of the pastern region over the coronary band of the hoof. (Jalali and Engle, 2016)

Digital dermatitis lesions can evolve into a chronic stage which is dominated by proliferative as well as hyper and parakeratotic processes that typically transforms into warts. The preliminary lesion or M1 stage is a restricted granulomatous region which is usually small and is generally not painful (Holzhauer et al., 2008). The lesion will progresses into the M2 or standard ulcerative stage. At this stage, lesion is typically more extensive and is tender on palpation. When the lesion starts to heel, scab forms over the ulcer and now the lesion will be described as M3. The M4 or the chronic stage is characterized by surface proliferation. M4 lesions are not painful but are infectious and can become M1 lesions (Berry et al., 2012). The healed, healthy skin will be classified as M5. This infectious disease is a polymicrobial disease, yet the major bacteria that is responsible for DD is *Treponema* (Zinicola et al., 2015). Like other types of microorganisms, *Treponema* has different strains. However, the most famous strains that are available in the literature that causes DD is *Treponema Phagedenis* and *Treponema Denticola*.

Management plays a substantial role in preventing lameness. Keeping animals cool in the summer can help prevent lameness. Heat stress can induce ruminal acidosis in cattle. Lameness

and subacute ruminal acidosis (SARA) both appear to be highly prevalent in the US dairy industry (Stone, 2004). Dairy cows need the energy for milk production, growth and also calf development. These animals also need energy for their daily activities like walking, breathing, eating, etc. That's what is called maintenance energy. The energy requirement of the animal increases with heat stress. The way the animals were grouped in dairies also affects the DD incidence. By giving more time to the heifers to adapt more with the environment DD incidence was reduced significantly. Spending more time with lactating cows before calving increased the risk of DD (Somers et al., 2005).

Dairy cows exposed to heat stress typically produce less milk. Solar radiation, air movement, and relative humidity can cause heat stress. As we know, acidic conditions are not ideal for rumen microbes. Low pH can affect enzyme activity and cellular structure. The pH regulation of blood is as essential. Heat-stressed animal lose potassium and this loss will decrease the pH of the blood. Potassium (K^+) and sodium (Na^+) ions are the main cations associated with maintaining acid-base status, and in alkalosis, K^+ will exchange with H^+ and enter cells to sustain electro-neutrality.

By increasing the dietary cation-anion difference (DCAD), we can raise the blood pH. The dietary cation-anion difference (DCAD) usually consists of two cations [potassium (K) and sodium (Na)] and two anions [chlorine (Cl) and sulfur (S)]. DCAD affects the animal's acid-base balance, Ca levels around calving, and mineral utilization. There is always a balance between anions and cations to maintain the electrochemical neutrality. Animals which are mainly fed diets with high levels in cations will have alkaline urine (pH > 7) though cows which are fed diets that are low in cations will have acidic urine (pH < 7) (Erdman and Iwaniuk, 2017).

Another management strategy that can be implemented to prevent DD is using footbaths. The primary function of a footbath is to improve foot hygiene and reduce bacterial load on the hoof. Keeping the alleys clean and scraping they are essential as well.

The discussed previously, most popular compound used in footbaths is $CuSO_4$ which is being used in different concentrations from 3 to 10%. The ideal dimensions for a dairy footbath are 3.7 m long, 0.6 m wide and 0.28 m deep to have three successful hoof immersions per foot per animal (Cook, 2017). Experiments have been conducted that have reported that well-designed footbaths have significant impacts on reducing cases of DD cases (Solano et al., 2017). Prewashing the hoof prior entering the footbath can help the longevity and effectiveness of the solution (Manning et al., 2017). There are three main benefits of footbaths:

1- To strengthen the hoof

2- To improve the hoof cleanliness, and

3- To control the bacterial population on the hoof (Cook, 2017).

The other element that plays an essential role in lameness occurrence is genetics. Some studies have reported that there is a high correlation between genetics and lameness. A study from Van der Waaij (van der Waaij et al., 2005) indicated that most of the foot disorders in cattle were heritable, especially DD, which had a significant phenotypic and genetic correlation with locomotion. Genetic selection can be an invaluable tool for the producers to improve herd productivity and reduce operation costs (Obike, 2009).

Low body condition score (BCS) is another contributor to lameness. Animals with lower BCS are more susceptible to become lame (Kougioumtzis et al., 2014; Huxley, 2015b). They are more prone to lameness because they have less fat in their body. Animals with negative energy balance will start to mobilize fat from different locations in the body to provide energy for the

vital organs that are essential for the animal to survive. One of the places that fat will be mobilized from is the subcutaneous digital cushion. When this digital pad gets mobilized, it is hard to get replaced moreover the third phalanx of these animals will put more pressure on the sole and will cause sole ulcers and discomfort for the cows.

One of the most critical factors that can cause lameness in livestock is nutrition. The history of lameness and nutrition goes back to ancient Greece when Aristotle associated equine laminitis with indigestion (Bergsten, 2003). There are plenty of reports that fructans and glucose, will escalate lactic acid production and this will lead to laminitis. (Lean et al., 2013). The connections among acidosis and lameness are explained by some studies confirming that fructose and other sugars produce lactic acid and oligofructose in the rumen and this cause laminitis when fed to both cattle and horses. Feeding animals the right diet is also an essential part of good management (Owens et al., 1998).

We already know that by utilizing large quantities of highly fermentable carbohydrates, the rumen pH will drop. A reduction in rumen pH will cause a shift in rumen flora. Certain rumen flora that grows at lower pH can produce endotoxins. These endotoxins with other vaso-active substances like lactic acid can be absorbed into the bloodstream and will impact blood flow in the several tissues of the hoof, particularly the corium. Death of tissues from hypoxia or nutrients resulting from inadequate blood flow is a potent inflammatory stimulus, this prevents differentiation of the cells and redevelopment in the germinal layer and the keratinization of epithelial cells in the spinous layer. (Figure 1.9).

The horn quality is reliant upon keratinization which provides the horn cell skeletal rigidity and health. In situations following a vascular compromise, like laminitis, the keratinocyte can get damaged and inflammation will occur due to insufficient nutrients. Inflamed

corium will start to release some potent metalloproteinase enzymes which will destroy the suspensory apparatus of the third phalanx.

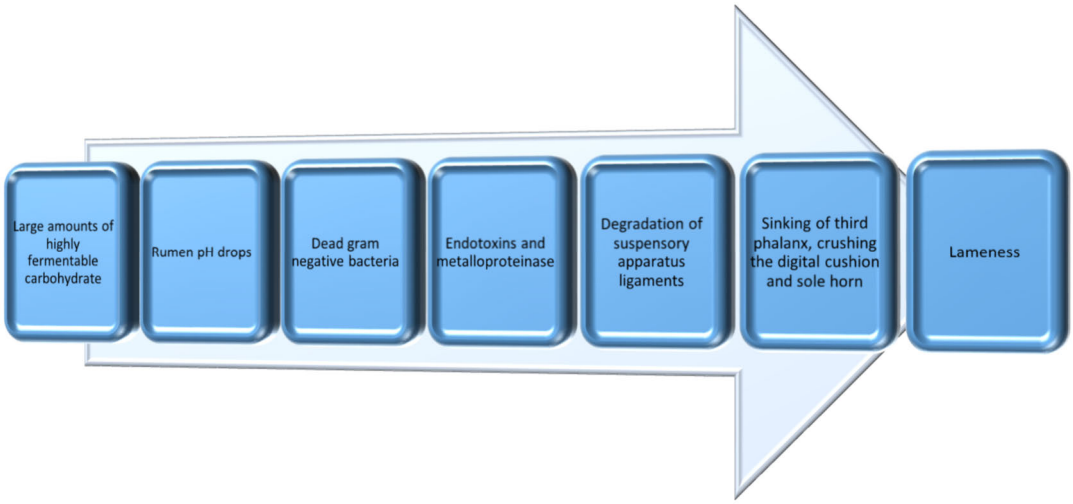

Figure 1.9. From large amount of carbohydrates to lameness (Jalali, 2016)

After the suspensory apparatus being destroyed, the third phalanx will put pressure on the sole and crush the corium which can cause a secondary issues like sole ulcers and white line disease (Figure 1.10). Fat content in the feed has also been reported to influence laminitis. The digital cushion which consists of fat and loose connective tissue, has an important function in the structure of the claw (Shearer, 2010). Digital cushions consist of three parallel cylinders which act as shock absorbers in cattle (Figures 1.11 and 1.12) (Bicalho et al., 2009). Digital cushions are remarkably efficient shock absorbers. In a functional cooperative interaction with the soft elastic horn of the bulb, they absorb forces through the first ground contact during weight bearing, diffusing the force equally inside the hoof. The composition of the fat in these pads changes with the age and physiological state of the animal. The amount of fat in the digital cushion is higher in cows than in heifers. The amount of fat tissue is inferior in animals with sole abscesses compared animals with healthy claws. Shock absorbing capability is different in fat pads with various fat compositions.

Figure 1.10. Initial phase of the sole lesion and cracks on the white line on a dairy cow's sole (Jalali and Engle, 2016)

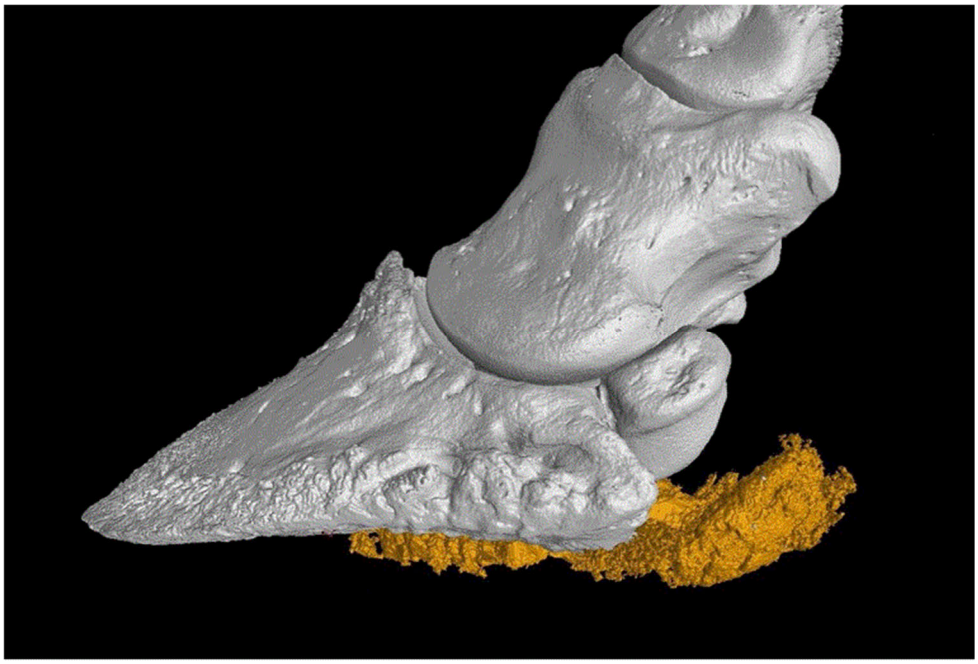

Figure 1.11. Hoof skeleton and digital cushion from a dairy cow hind limb (3D reconstruction generated by X-ray computed tomography) – lateral view (Huxley, 2015b)

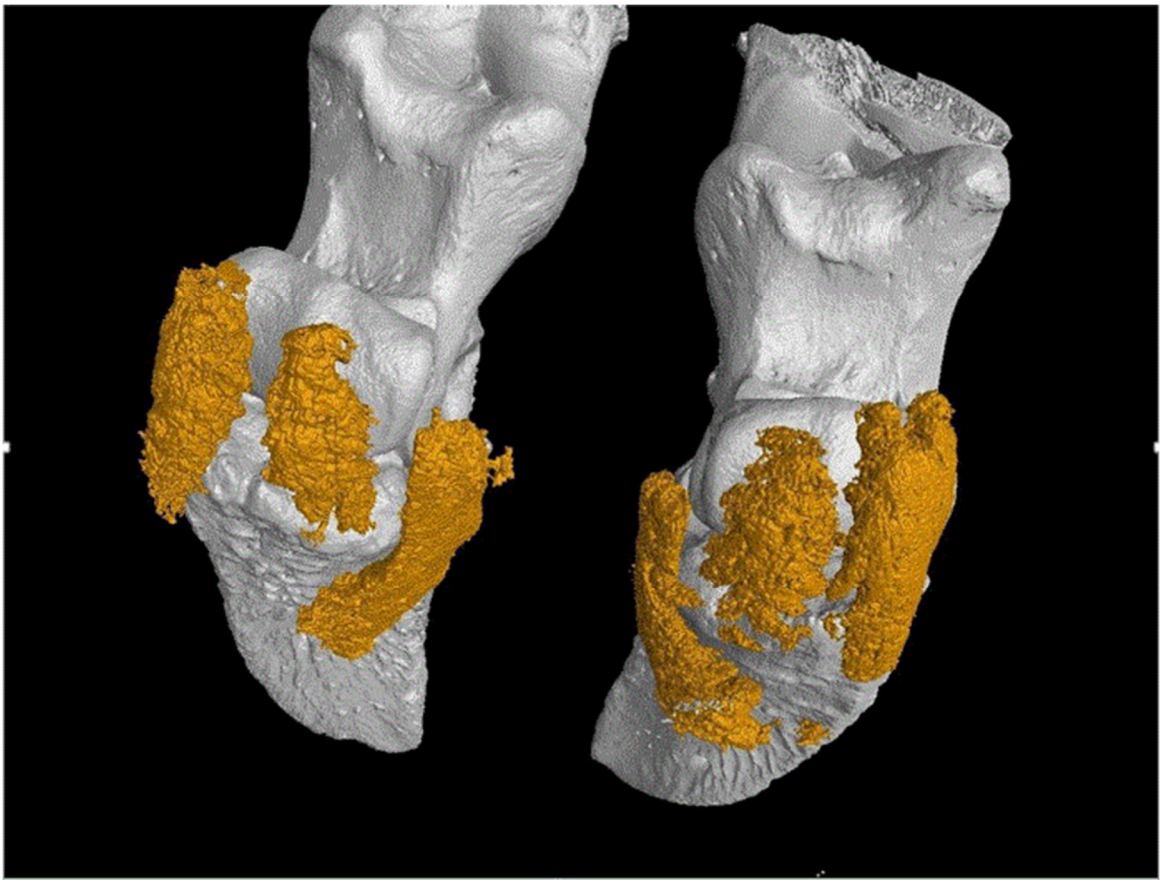

Figure 1.12. Hoof skeleton and digital cushion from a dairy cow hind limb (3D reconstruction generated by X-ray computed tomography) (Huxley, 2015b)

1.5. Lameness Prevention Methods

Lameness can be categorized into two different types: infectious and non-infectious. There are various types of prevention for these types of lameness. For the contagious diseases, focus should be placed on hygiene and cleanliness of the hoofs by keeping the alleys clean and implementation of footbaths. Footbaths are important tools to prevent infectious hoof disease in dairy herds (Cook, 2017). By using the hoof and leg, hygiene scoring chart producer can determine whether or not to use footbaths.

As mentioned previously, $CuSO_4$ or formaldehyde are the most common additives to footbaths. Producers use $CuSO_4$ in various concentrations. The two most commonly used $CuSO_4$ concentrations are 5 and 10%. For a 300 liter footbath, 14.1 kg and 28.2 kg of $CuSO_4$ should be

added to target 5 and 10% concentration, respectively. The rule of thumb for the footbaths is the footbath should be changed after 300 cows pass through it (Cook, 2006).

Overall, additives to footbaths are categorized as bactericidal (which destroy the bacteria) or bacteriostatic (which reduces the speed of growth in the bacteria) (Hajipour et al., 2012). Copper particles can attach to the membrane of bacteria via electrostatic interaction and disrupt the bacterial membrane's integrity (Thill et al., 2006). Furthermore, these metal ions will produce free radicals resulting in initiation of oxidative stress and the production of reactive oxygen species (ROS). Reactive oxygen species will permanently damage the bacteria (Figure 1.13). Ionic and metallic types of Cu can damage essential proteins and DNA in the bacteria by producing hydroxyl radicals (Wang et al., 2011).

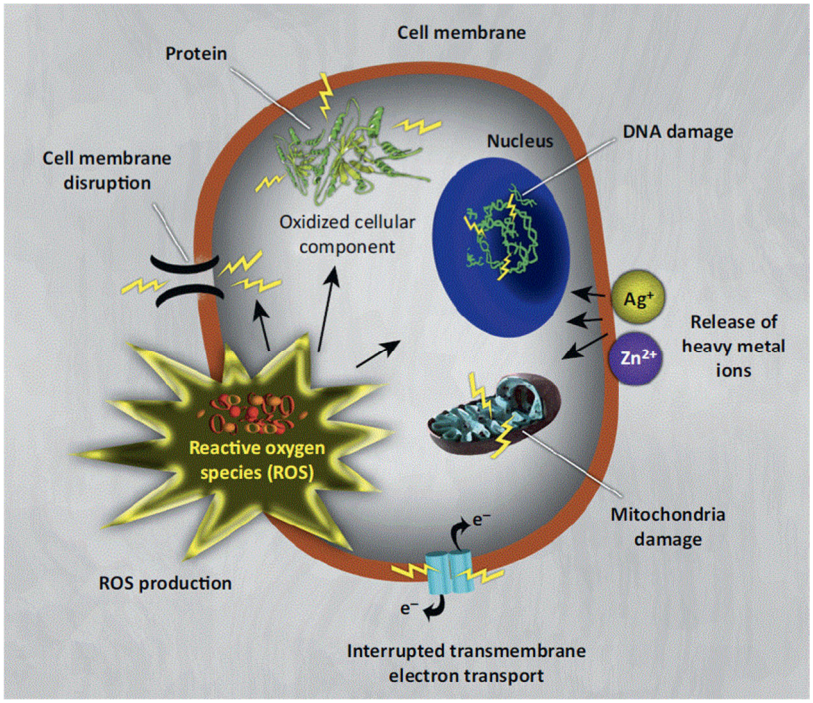

Figure 1.13. Toxicity of nanoparticles toward bacteria (Hajipour et al., 2012)

Several factors can affect Cu nano-particles toxicity in a solution. These factors are as follows:

- Cu concentration in the solution,

- Temperature,
- pH levels, and
- Bacteria concentration itself.

Agglomeration is another factor that can affect the toxicity of the nanoparticles. Agglomeration of nanoparticles happens when you have a small particle with a large surface area compared to their size. This phenomenon usually occurs in the particles sized between 1 and 100 nm. Particle sizes will change with the $CuSO_4$ concentration when it increases from 0.05 mol/L to 0.5 mol/L the average size increased from 14 nm to 50 nm which is precisely in the range of agglomeration (Zhou et al., 2015).

Lower pH will cause lower levels of agglomeration and therefore more surface area for the particles to interact with bacterial membranes and solubility of the Cu ions in the solution (Pramanik et al., 2012). As pH decreases Cu becomes more soluble. Therefore, some of the dairies add acid as to footbaths to increase Cu solubility.

For the non-infectious prevention can be the right design of the stalls to let the animals get in and out without injuring themselves. Use anti-slip material like rubber in the alleys to prevent slip injuries in the herd. As discussed before, concrete is not a perfect surface for the animals to walk on. Cows are designed to walk on surfaces like soil. Hoof trimming is another critical thing that should be keep in mind as a strategy to prevent and also treat lameness. Since cows are spending lots of time on concrete which is abrasive to the hoofs, this will cause the overgrowth of the foot.

Another important area in prevention laminitis is to have a reliable and safe hoof trimming chute. In large operations, it is better to have either a portable hoof trimming station or multiple trimming stations in a strategic way. Having several locations around the dairy farm

where hoof trimming can be performed can significantly reduce the distance the animal must walk on the concrete.

Dairy cows should have their hooves trimmed at least two times per year. In the bigger dairies having an onsite hoof trimmer is highly recommended. Having a skilled hoof trimmer with excellent observational skills is a great asset for an operation. A skilled hoof trimmer can find a lame cow by locomotion score and use low-stress handling techniques to take the animal to the hoof trimming station. The hoof trimmer will keep the lameness records for each animal and will also follow up in the future. It should be kept in mind that welfare aspect of this problem has the highest level of importance compared to other issues.

1.6. Copper Sulfate in Agriculture

Copper is naturally present in the Earth's crust, and it is available in two different states: Cu^{+} and Cu^{2+}. Copper belongs to the group 11, and its atomic number of 29. The *s* shell is filled with one electron ($3d^{10}4s^{1}$) and can quickly gain and lose that electron and turn in to a positive ion.

Copper sulfate is an inorganic chemical that combines sulfur with Cu (Cornell University, 2008). The salt has multiple degrees of hydration. Copper sulfate has different names such as cupric sulfate and blue vitriol, bluestone, the vitriol of Cu, Salzburg vitriol and Roman vitriol. Copper has a molar mass of 249.685 g/mol and has an electric blue color. Copper sulfate pentahydrate ($CuSO_4.5H_2O$) is highly soluble in water (1.502 molal at 30°C).

$CuSO_4$ is used widely used in different industries especially in agriculture. The most frequent uses of $CuSO_4$ are:

- Bordeaux and Burgundy mixtures which are used as fungicides. Bordeaux mixture is a blend of $CuSO_4$ and calcium hydroxide. This mix was first invented in Bordeaux region

of France more than two hundred years ago. Burgundy mixture is a mixture of $CuSO_4$ and sodium carbonate.

- As a feed additive in swine feed and broiler chickens at pharmacological concentrations improve growth efficiency (Hill et al., 2000).
- correction of the Cu deficiency in the soil (Grundon, 1980).

Since the late 1970's and the early 1980's dairies have been using $CuSO_4$ as an antibacterial product to prevent infectious hoof problems that affect skin next to the claw horn of dairy cattle (Digital dermatitis, hairy heel warts). Copper sulfate is popular in the industry because of its low cost and effectiveness on the lesions.

1.7. Environmental Bioaccumulation

Accumulation of heavy metal in the soils has become a serious problem due to the unfavorable effects on food safety, crop growth, and environmental health (Nagajyoti et al., 2010). Copper is one of the most extensively used metals in the world**.** Environmental bioaccumulation of Cu in the soil has become a crucial problem (Ahmadpour et al., 2014). Soil's Cu contamination is not only critical because of the danger of leaching into the water resources but also because of the high availability to the plants. Excess Cu can prevent plants from absorbing essential minerals like iron. This will cause iron deficiency in the plants and decrease the growth rate and in certain cases can inhibit plant growth.

There are multiple sources of heavy metal contamination in the environment:

- Natural sources
- Agricultural sources
- Industrial sources
- Domestic sewage

- Atmospheric sources, and
- Other sources (Nagajyoti et al., 2010).

Heavy metals are defined as metals having a specific density of more than 5 g/cm^3 (Järup, 2003). Some organic pollutants can be degraded by microorganisms; however, they are not able to degrade heavy metals like Cu (Ma et al., 2009). One of the heavy metals that can often be found in the wastewater is Cu. (Lambert and Leven, 2000). Agriculture production in general is a significant contributor to heavy metal contamination in the soil. There are numerous ways that agriculture is contributing to the heavy metal bioaccumulation in the soil.

The primary use of heavy metals like arsenic, cadmium, lead, and mercury in agricultural land, arises from the use of fertilizers, organic wastes, and industrial byproduct wastes. Some farming methods, like irrigation, can increase the levels of selenium (Se) in soil, which can cause selenium toxicity in downstream water reservoirs (Wu, 2004).

Agriculture is not the only cause of heavy metal contamination. Industrial sources like metal processing in refineries, coal burning in power plants, combustion engines, nuclear power plants and high tension lines, plastics, textiles, microelectronics, wood preservation, and paper processing plants (Arruti et al., 2010). Cigarette filters are also another major contributor to heavy metal contamination especially Cadmium (Cd).

Interaction of Cu with the environment is complex; however, reports show that most Cu is introduced to the environment rapidly becomes stable which is not a risk to the habitat. As a matter of fact, Cu is not expanded or bioaccumulated in the food chain, unlike the synthetic materials. (Wuana and Okieimen, 2011).

There are different ways to remediate the toxic soil condition. Physical approaches like scavenging and burial of the contaminated soil or removing Cu from the soil via electrical

dialysis, which is too expensive in large scales of remediation (Zhenli He et al., 2010). The way burial works is only removing the soil from the topsoil to the lower layers of the earth. This will prevent phytotoxicity and heavy metal contamination in our food chain. However, depending on the location and situation, Cu may leach into the water sources and cause water pollution.

The most cost-effective way is phytoremediation, which requires a lot of time. According to United Nations Environment Programme (UNEP), Phytoremediation is the direct use of living plants for elimination, degradation, in soils, sludges, sediments, surface water, and groundwater (UNEP (United Nations Environment Programme), 1998). When the soil becomes contaminated by heavy metals, there is no easy way to fix the issue. Soil with the pH< 6.5 has higher Cu availability status than a soil with pH>6.5 (Zhenli He et al., 2010).

Plants like other living organisms can be affected by both deficiencies and also excesses of heavy metal ions. Copper toxicity is a vital obstacle to food crop growth. Higher Cu concentration is toxic to plants creating significant adverse effects varying from morphological and physiological/molecular alterations. Several publications report a significant reduction in productivity and plant growth because of high levels of Cu in the soil. The abundance of Cu in soil is toxic to the cells, which provokes stress and causes damage to plants (Lewis et al., 2001).

Copper toxicity can influence plant growth by altering the following:

1. Seed germination
2. Growth and morphology
3. Biomass and grain yield
4. Mineral composition
5. Photosynthetic apparatus and pigments
6. Antioxidant function

7. Genotoxic effects (Adrees et al., 2015)

Typically with Cu toxicities, as with most toxicities, growth stunting of plants is observed. Plants can lose turgor, or turn white due to too much Cu-based fungicide application, but more often than not in the early stages of Cu toxicity, plants look Fe deficient. Retention of Cu happens in the plant root to xylem, and after being absorbed by the roots, Cu will translocate to shoots within xylem and phloem (Ando et al., 2013). Highest Cu concentration is at the epidermis of the root.

Copper is one of the main cofactors for many enzymes; therefore, it is expected that Cu toxicity enhances "Reactive Oxygen Species" (ROS) production (Küpper and Andresen, 2016). To prohibit ROS production in plants, antioxidant systems involving enzymatic antioxidants like catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR) and also non-enzymatic molecular antioxidants like ascorbic acid (ASC) are present to prevent oxidative damage (Ivanova et al., 2010).

1.8. Conclusion

Copper sulfate is a functional, relatively cheap and easy to use chemical that can help to prevent DD and lameness. However, the bioaccumulation of Cu in areas of high use is becoming an environmental issue. Copper has the potential of contaminating water and soil in farmlands, which are the primary resource for our food production. Having less agricultural land and clean water will be a significant issue in the future as the global population increases. Remediating areas that are contaminated with Cu is time consuming and expensive. Therefore, it is imperative that environmental bioaccumulation of Cu used in agriculture production be prevented.

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CHAPTER 2

EXTRACTING COPPER FROM COPPER SULFATE FOOTBATHS IN DAIRIES – PROOF OF CONCEPT

2.1. Summary

The experiments presented in this chapter are conducted as proof of concept experiments to determine the feasibility of copper (Cu) extraction from dairy copper sulfate ($CuSO_4$) footbaths. We hypothesized that by collecting the used footbath and implementing chemical and electrochemical procedures, we would be able to extract Cu from dairy footbaths prior to the used footbath contents being discharged into the premise lagoon. Our first objective was to remove elemental Cu from a 5% $CuSO_4$ solution prepared in our laboratory. We used a platinized titanium inert electrode as the anode and pure Cu electrode as the cathode. Copper was extracted with greater than a 95% efficiency ($P < 0.05$) with a purity of 99.6% Cu in all replicates (n=5). During Cu extraction sulfuric acid was produced. Following Cu removal, 5 grams calcium carbonate ($CaCO_3$) of was added to 250 ml of H_2SO_4 solution. This created a reaction where $CaSO_4$ (gypsum) and water were produced ultimately increasing the pH of the solution from 0.7 to 6.5. Our second objective was to determine the feasibility of extracting Cu from a $CuSO_4$ footbath before and after the cows passed through the $CuSO_4$ solution. Footbath samples (from a local dairy) were collected from one footbath prior to use and after 600 cows had passed through the footbath. Both pre and post footbath samples were strained through 4 layers of cheesecloth to remove large debris. During electrochemical extraction, foam was formed in the footbath sample obtained after 600 cows had passed through the footbath. It is possible that this is due to protein contaminants in the spent footbath solution denaturing as pH

began to decrease during Cu extraction. Foam production was not originally anticipated and significantly reduced the Cu extraction efficiency by more than 23% ($P < 0.05$). Collectively, these data points indicate that it is feasible to extract Cu from $CuSO_4$ footbaths and to convert the H_2SO_4 generated in the electrochemical extraction process to $CaSO_4$ and water. Future research examining extraction efficiency and foam production is warranted.

2.2. Introduction

Digital dermatitis (DD) is a bacterial infection that primarily affects the skin on the heels of cattle. Infection provokes inflammation and skin destruction, leading to distress and pain (Laven and Proven, 2000). Digital dermatitis (DD), also identified as *papillomatous digital dermatitis*, is an extremely contagious, proliferative skin disease of the foot in cattle, sheep, and goats induced by primary or auxiliary spirochete infection (Shearer, 2009). Digital dermatitis is one of the principal causes of lameness in the dairy industry, and it causes reduced animal welfare and economic loss. Digital dermatitis has been reported globally from all over Europe to the Middle East, East Asia, and the United States (Read and Walker, 1998).

Various species of bacteria have been cultured from DD lesions, including *Fusobacterium spp., Bacteroides spp., Campylobacter spp.* and *Peptococcus spp* (Ohya et al., 1999). However, researchers believe that the leading cause of the Digital dermatitis is different strains of *Treponema* which belongs to Spirochetes family (Choi et al., 1997) (Hartshorn et al., 2013)(Walker et al., 1995). Since digital dermatitis is a painful disease for the animal, it is a severe welfare problem, which should immediately be treated. In the United Kingdom, the most common method to treat digital dermatitis is specific topical medication per animal mainly with antibiotic compounds. However, preventing DD with the help of footbathing is the a common practice in the US and is becoming more accepted in Europe (Laven and Logue, 2006).

Several types of solutions are used in footbaths. Copper sulfate ($CuSO_4$) seems to be the most effective and safe antibacterial agent, although there are concerns about disposal also accumulation of Cu in the environment (Cook, 2017). The most common concentrations of $CuSO_4$ are 3, 5, and 10% (Cook, 2006).

After cows walk through the footbath, the used footbath solution is typically discharged into the premise lagoon. The disposal of used footbaths containing $CuSO_4$ has unintended outcomes on the environment. Agricultural soils undergo Cu accumulation when footbaths are disposed of in wastewater lagoons used for irrigation. By using the water in these lagoons for irrigation, Cu is then transferred from the lagoon to the farmland (Ippolito, 2010).

From a cost standpoint, producers are spending approximately $42 USD per cow per year on $CuSO_4$ footbaths. This cost estimation was based on replacing footbaths four times per week (Cook, 2017). However, the cost per cow would be greater for dairies that replace footbath solution after every milking. For example, a 1,200 head dairy with two 550 liter footbaths adding 22.7 kg of $CuSO_4$ to each footbath twice a day would require 90.8 kg of $CuSO_4$ every day, which translates to 33,142 kg per year.

With the assumption of the price of $90 US per bag (22.7 kg) of $CuSO_4$, this dairy would spend approximately $131,400 US annually just to purchase $CuSO_4$ for their footbaths. Consequently, a method that would be allow for removal of Cu from used footbath solutions prior to being discharged into the premise lagoon would help to prevent Cu accumulation in farmland where the lagoon effluent is applied. Furthermore, it would allow for Cu to be recycled which could help offset the cost of purchasing $CuSO_4$ for footbaths. Therefore, this experiment was conducted as a proof of concept to determine the feasibility of Cu extraction from dairy $CuSO_4$ footbaths. Our overarching hypothesis was that by collecting the used footbath and

implementing chemical and electrochemical procedures, we would be able to extract Cu from dairy footbaths prior to the used footbath contents being discharged into the premise lagoon.

2.3. Materials, Methods, Results, and Revisions

2.3.1. Objective 1: Removal of Elemental Cu from a 5% $CuSO_4$ Solution Prepared in our Laboratory

To determine the feasibility of Cu extraction from dairy $CuSO_4$ footbaths five, 250 ml 5% $CuSO_4$ solutions were prepared in our laboratory to determine Cu extraction efficiency. The 5% $CuSO_4$ solution was prepared using $CuSO_4.5H_2O$ (Product Number: 209198, Sigma-Aldrich, Saint Louis, MO, USA) by adding 50 grams of $CuSO_4.5H_2O$ to one liter of deionized water. The mixture was then mixed with a glass stir rod by hand for 2 to 3 minutes, until all $CuSO_4.5H_2O$ was dissolved. After making the 5% $CuSO_4$ solution, the one liter solution was divided into 250 ml aliquots in beakers that were modified to hold an electrolysis apparatus. For electrolysis, a pure Cu cathode (Frey Scientific Cu electrode strip, 12.7 cm long, 1.9 cm wide, and with a thickness of 0.11 cm) and platinized titanium (2.54 cm x 10.16 cm) anode (SRA Soldering Products, Walpole, Massachusetts, United States) were inserted the 250 ml of $CuSO_4$ solution. The dimensions of the 250 ml beaker were 7 cm x 9 cm. Prior to using the electrodes, both of the electrodes (cathode and anode) were weighed. The initial weight of the Cu electrode was 25.81 grams, and the platinized titanium was 10.89 grams (Table 2.1). This table shows the weight of the electrodes before and after the electrolysis, as well as the calculated Cu extraction efficiency.

Table 2.1. Weight of the electrodes before and after the electrolysis

	Initial Weight[a]	Final Weight	Delta Weight	Total Weight[b]	Efficiency[c]
Cathode	25.81	27.11	1.302	2.403	76.8%
Anode	10.89	10.89	-	-	-

[a] All weights are in grams.
[b] Total weight has been calculated by weight of the electrode plus the weight of precipitated Cu.
[c] Efficiency has been calculated by the total weight of extracted copper divided by total weight of the available copper in the solution of 3.125 grams.

Each electrode was connected to a DC power supply (Tekpower TP3005T Variable Linear DC Power Supply; 0 - 30V at 0 - 5A) with 16 gauge insulated wire; the platinized titanium was connected to the positive charge (anode) and the Cu electrode was connected to the negative charge (cathode). The DC power supply was set at 5A and 10V then turned on and electrolysis initiated. Briefly, the negative electrode cathode becomes more negatively charged with excess electrons from the circuit. The positive electrode or anode becomes more positively charged because the circuit removes the electrons. The positive ions (cations) migrate towards the cathode, and negative ions migrated towards the anode as described below:

The ions present in copper (II) sulfate solution are:

- $CuSO_4$ (aq): Cu^{2+}, SO_4^{2-} ions.
- H_2O (l): $H^{+,}$ OH^- ions.

At cathode:

- The Cu and hydrogen ions are attracted to the cathode.
- $Cu^{2+} + 2e \rightarrow Cu$
- Copper is deposited at cathode.

At anode:

- The sulfate and hydroxide ions are attracted to the anode.
- OH^- - e → OH
- OH + OH → H_2O + O
- Oxygen is produced at anode.

Using the formula $P = V. I$, we calculated the power used in the process of electrolysis. P is power, measured in Watts, V is the voltage, measured in Volts and I is the electric current measured in Amps. Multiplying the power by time yields the kilowatt hour (Watt hour) used. The standard reduction potential for the reactions will determine which of H^+ or Cu^{2+} and O^{2-} and SO_4 will be deposited at the cathode and the anode respectively.

The reduction potentials for all the elements and compounds in the associated reactions are:

- Cu = 0.340 V
- H = 0 V
- SO_4^{2-} = -0.94 V
- OH^- =1.23 V

To determine the quantity of time required to produce a known quantity of a substance given the amount of current flow, we calculated the amount of material generated/consumed in moles. The concentration of elemental Cu was 0.198 mol/L and the balanced half-reaction involved was:

$Cu \rightarrow Cu^{2+} + 2e^-$

Next, the number of moles of electrons required was calculated.

$2 \times (0.198) = 0.396$ mol

In order to calculate the appropriate electron flow, in Faraday, electrons are converted to coulombs. One mole of electron equals one Faraday, and one Faraday equals 96,485 coulombs. By multiplying the required number of moles by the number of coulombs in one Faraday, the total number of coulombs needed for the electrolysis was determined.

$96485 \times 0.396 = 38208.06$ Coulombs

To calculate the total time the following equation was used: $Q = I.\ t$, where Q is the charge measured in coulombs (C), I is current measured in amperes (A) and t is time measured in seconds.

$5A \times t = 38208.06$

$t = 38208.06/\ 5 = 7641.61s = 2.12\ h \approx 2h: 07m: 12s$

The electrolysis was operated for 2h: 07m: 12s with 6V and 5A, electrolysis was terminated, electrodes were removed, air dried and weighed (Cathode weight after electrolysis increased to 27.56 g; anode weight increase; negligible and the precipitated Cu was 0.6 grams) Prior to and post electrolysis, temperature and pH of the solution were also determined using Fluke VT04 IR thermometer (Fluke Corporation, Everett, Washington, USA) and benchtop pH meter (VWR Symphony SB70P, Pennsylvania, USA). The pH after electrolysis decreased from 3.6 to 1.1 ± 0.2. After extraction, the solution was filtered through a dried, reweighed Whatman filter paper (Grade 541 Circles Particle Retention >20 to 25μmm) using a Buchner porcelain funnel and flask fitted with a vacuum pump. Table 2.2 shows the initial and final weights for the electrodes, as well as the pH measured at the beginning and the end of the electrolysis.

Table 2.2. Initial and final weights for the electrodes and pH measured at the beginning and the end of electrolysis

	Initial Weight[a]	Final Weight	Delta Weight	Total Weight[b]	Efficiency[c]	Initial pH	Final pH
Cathode	25.81	27.56	1.75	2.35	75.2%	-	-
Anode	10.89	10.90	0.01	-	-	-	-
Electrolyte	-	-	-	-	-	3.6	1.1

[a] All weights are in grams.
[b] Total weight is the weight of the electrode plus the weight of precipitated Cu.
[c]Efficiency is the total weight of extracted copper divided by total weight of the available copper in the solution of 3.125 grams.

Since the extracted Cu had a porous and spongey structure it was left to dry under a fume hood for approximately 24 h. Total weights of the electrode and the residual Cu were determined after air drying. Based on air dried weights, the extraction efficiency of Cu from the $CuSO_4$ was 76% ± 2. This indicated that not all Cu was extracted (Figure 2.1).

Figure 2.1. One day old extracted copper (left) and freshly extracted copper (right)

After further investigation it was determined that the DC power supply was faulty, because it did not deliver the proper current and voltage. Therefore the power supply was

replaced by a BK Precision 1901 (Yorba Linda, California) DC power supply (Figure 2.2). All procedures previously discussed, were followed and extraction efficiency of greater than 95% (SEM 0.06) was achieved over 5 replicate samples.

Figure 2.2. Electrolysis setup with the BK Precision DC power supply

Table 2.3 shows the initial and final weights of both anode and cathode and also the weight difference. Total weight is the total weight of the extracted Cu (on the cathode plus the precipitated). pH measurements before and after the electrolysis are also shown in this table.

Table 2.3. Initial and final weights for the electrodes, pH measured at the beginning and the end of electrolysis and SEM

	Initial Weight[a]	**Final Weight**	**Delta Weight**	**Total Weight[b]**	**Efficiency[c]**	**Initial pH**	**Final pH**	**SEM**
Cathode	25.81	28.03	2.22	2.97	95.0%	-	-	0.06
Anode	10.89	10.90	0.01	-	-	-	-	-
Electrolyte	-	-	-	-	-	3.6	0.9	-

[a] Average of weights (n=5)
[b] Total weight is the weight of the electrode plus the weight of precipitated Cu.
[c]Efficiency is the total weight of extracted copper divided by total weight of the available copper in the solution of 3.125 grams.

In an attempt to reduce the extraction time, the current and voltage were increased from 5A to 15A and from 6V to 20V, respectively which reduced the electrolysis time to approximately 25 minutes. With 5 A and 6 V, we have been able to calculate the time needed for the extraction, which was 127 minutes. To increase extraction rate the current was increased to 20 A, which theoretically should reduce the extraction time to approximately 30 minutes.

Visual appearance of the solution was also evaluated. The electrolysis process was terminated when there was no observable blue from the $CuSO_4$. As expected, as Cu was removed from the solution resistance was decreased and voltage and amperage increased until they reached the maximum parameters. Once this maximum electrical flow was achieved, the electrolysis procedure was terminated. The time for full electrolysis completion was 27 ± 3 minutes. As anticipated the temperature of the extraction also increased due to the elevated A and V applied. The ideal temperature for electrowinning is 60°C (Schlesinger et al., 2011). In some of our attempts, the temperature increased to approximately 90°C. After Cu extraction, the remaining sulfuric acid solution was analyzed for Cu concentration via Inductively Coupled Plasma Mass Spectrometry (ICP) as described by Ahola (Ahola et al., 2005). Samples were analyzed for Cu concentration before and after the electrolysis. Samples taken before electrolysis were diluted (1:50) prior to Cu analysis to ensure that they were in the analytical range of the ICP. To achieve this dilution, 100 µl of the solution was diluted with 4.9 ml of 1.2 M HCl (total volume of 5 ml).

Figure 2.3. Is taken with Fluke IR thermometer. This figure shows the temperature of the electrolysis chamber during electrolysis. The thermometer reads 188 ° F/ 86.7° C.

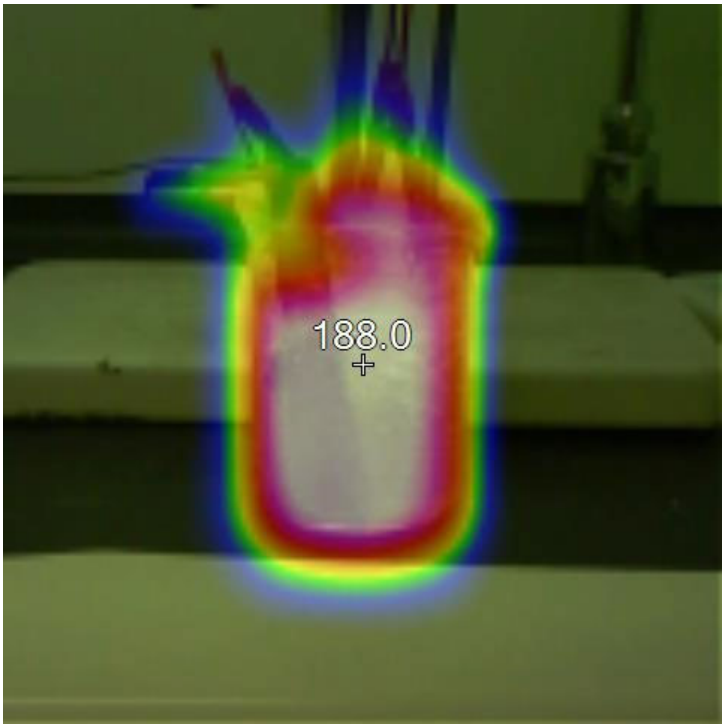

Figure 2.3. Temperature of the electrolysis chamber during electrolysis

2.3.2. Objective 2: Determine the Feasibility of Extracting Cu from a $CuSO_4$ Footbath Before and After the Cows Passed Through the $CuSO_4$ Solution

Footbath samples (from a local dairy) were collected from one footbath prior to use and after 600 cows had passed through the footbath (Figure 2.4). This dairy was using a premade $CuSO_4$ footbath solution (Figure 2.5).

Figure 2.4. Two 40 gallons footbaths in a dairy in northern Colorado (Jalali and Engle, 2016)

Figure 2.5. Premade copper sulfate solution in a dairy in northern Colorado (Jalali and Engle, 2016)

Both pre and post footbath samples (1.0 L) were obtained at the appropriate times and were strained through 4 layers of cheese cloth to remove large debris. All samples were subjected to electrolysis for 20 mins at 15 A and 20 V as described above in 5 replicates. A 95% Cu extraction efficiency was obtained from the initial footbath solution (Figure 2.6). However, during electrochemical extraction foam was formed in the footbath sample obtained after 600 cows had passed through the footbath (Figure 2.7). This was most likely due to protein contaminants in the spent footbath solution denaturing as pH began to decrease during Cu extraction. Foam production was not originally anticipated and significantly reduced the Cu extraction efficiency. The Cu extraction efficiency was reduced to 40% (SEM = 0.58) when this foaming event was noted. By recycling the foam from the first extraction into a subsequent extraction (e.g. capturing and re-extracting the Cu form the foam), extraction efficiency was increased to 80% (SEM = 1). The challenge with this approach is that electrolysis time and energy is doubled.

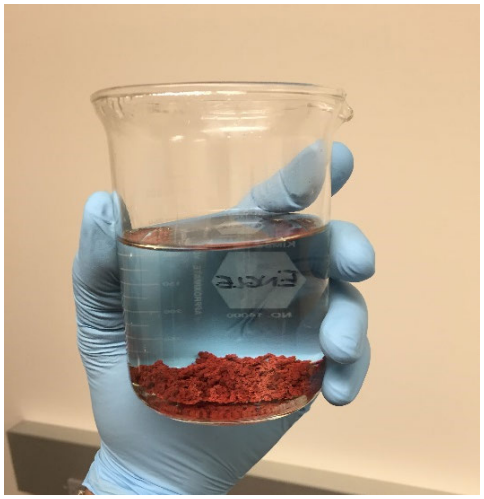

Figure 2.6. Extracted copper in the sulfuric acid (Jalali and Engle, 2016)

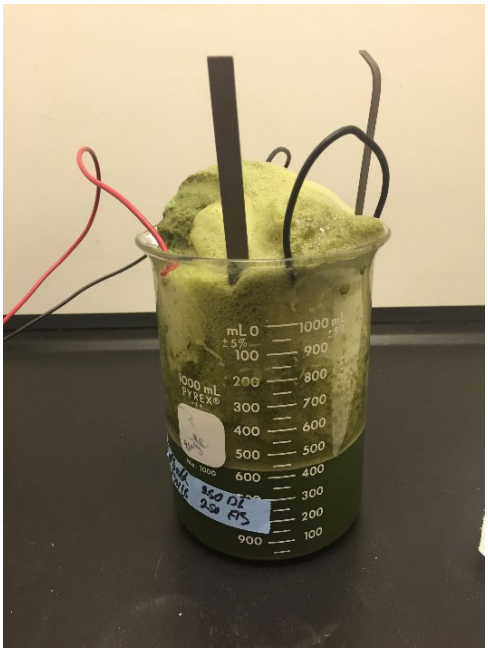

Figure 2.7. Foam build up in electrolysis (Jalali and Engle, 2016)

After Cu extraction, the remaining solution has a low pH (pH < 0.5) and is comprised primarily of sulfuric acid and water. Due to the low pH and sulfuric acid content this solution cannot be discharged into the environment. Therefore, a method of neutralizing the pH of solution was investigated. Following Cu removal via electrolysis for our CuSO4 solution generated by the electrolysis procedure, 1 g of calcium carbonate ($CaCO_3$) was added to 100 ml

of H_2SO_4 solution and mixed with a glass stir rod for approximately one minute. Once $CaCO_3$ was visibility mixed into solution, pH was measured.

2.4. Results and discussion

To reach a pH of 6.5 a total of 5 grams of $CaCO_3$ needed to be added to 100 ml of the solution. Adding $CaCO_3$ to sulfuric acid generated $CaSO_4$ (gypsum), water, and CO_2 and ultimately increased the pH of the solution from 0.7 to 6.5 (Figure 2.8). Gypsum can be used in agriculture as fertilizer (Hamza and Anderson, 2003) and in manufacturing building materials and the water can be discharged into the environment (Figure 2.9). As previously described, the chemical reactions predicted were:

$CaCO_3 + H_2SO_4$

$CaCO_3 + 2H^+ + SO_4{}^{2-} \rightleftharpoons Ca2^+ + SO_4^{2-} + H_2O + CO_2$

$Ca^{2+} + SO_4^{2-} \rightleftharpoons CaSO_4$

Figure 2.8. Generated gypsum after adding calcium carbonate to the sulfuric acid (pH of the solution is 6.5) (Jalali and Engle, 2016)

Figure 2.9. Generated gypsum after filtration with Whatman 541 filters (Jalali and Engle, 2016)

The aforementioned procedure was repeated with the solution remaining after Cu extraction via electrolysis form used dairy $CuSO_4$ footbath. The initial pH of the sulfuric acid solution generated after Cu extraction from the used dairy footbath solutions was 0.4. By adding the first gram of $CaCO_3$, it increased to 0.5. However, by adding another gram of $CaCO_3$, the pH increased to 4.7. For the third, fourth and fifth one gram additions the pH increased to 5.5, 5.8 and 6.4, respectively (Figure 2.9).

2.5. Ancillary Discussion

Electrolysis and electro-winning have been commercially available for more than 120 years. Concentration/smelting/refining of sulfide ores produces about 80% of the world's Cu from ore. The other 20% is generated by heap leaching/solvent extraction-electrowinning of 'oxide' and chalcocite ores (Schlesinger et al., 2011).

Electrowinning requires:

a) Sinking metal cathodes and inert (but conductive) anodes in $CuS0_4$-H_2S0_4-H_20 electrolyte
b) Utilizing an electrical potential within the anodes and cathodes
c) Plating pure metallic Cu from the electrolyte onto the cathodes (Schlesinger et al., 2011).

In other words, the items that we need for the electrowinning procedure are: a rectifier or DC power source, an anode and a cathode and of course an electrolyte to extract the non-ferrous metal from the electrolyte. The electrowinning products are:

1. Pure Cu metal at the cathode
2. Oxygen gas at the anode, and
3. The regenerated sulfuric acid in the solution.

In the anode, platinum is doing the primary work while titanium is just the structure of the electrode. There is only a thin layer (1 micron) of platinum on top of the titanium. There are a lot of advantages to the platinized titanium; these electrodes have a long life expectancy and they are easy to maintain. They have excellent corrosion resistance and the essential characteristic is that they have a perfect current and thermal distribution.

In 2010, approximately 4.5 million tons of pure Cu were electrowon (Schlesinger et al., 2011). With the help of electrowinning, we have been able to extract Cu from the used footbaths with more than 95% ($P < 0.05$) efficiency and with a purity of more than 99%. We also have demonstrated the economic feasibility of the extraction process. Cathodes weight after electrolysis was 27.56 grams and the anodes weight did not increase in weight. We have also measured the pH after electrolysis. The initial pH of the $CuSO_4$ solution is 3.6 and it decreased to 0.7. The amount of calcium carbonate required to increase the pH of 100 ml of the solution to 6.5 was 5.0 grams.

At the beginning we had low efficiency and after some investigation we were able to determine that the low extraction efficiency was due to a malfunctioning DC power supply. Our current was supposed to be maintained and 5A, but was unable to go above 3.5A. The weight of the submerged Cu was 3.73 g. After letting it to dry the amount of Cu extracted was 2.94 g of Cu with an extraction efficiency of 94% and in some cases, it was even more than the actual amount in the prepared Cu sulfate solution, because we have been able to extract the Cu in manure and other external sources. In one of our experiments, we have been able to remove 1.87 g of Cu which the total amount of Cu in the footbath was 1.85 g.

We have been able to demonstrate that it is possible to extract Cu from the used footbaths successfully. Foam build up is an issue which can be solved by changing the footbaths and using electrolysis more frequently. By using 20V and 15A in approximately 15 minutes, we can successfully extract the Cu with purity as high as 99.6%. This grade is one of the purest grades of Cu available on the world Cu market. We also successfully demonstrated that we can increase the pH and generate another useful byproduct like gypsum by adding an abundantly accessible cheap source of $CaCO_3$. After generating gypsum, we can simply filter the water and inject it into the irrigation system to irrigate the farmland while the gypsum can be sold to fertilizer companies or building material manufacturing companies.

Electricity is reasonably priced in the United States and developed countries, for example in Colorado which is not the cheapest state in the country, electricity price is less than 10 cents (U.S. Energy Information Administration, 2018) per kilowatt-hour. Let's keep that in mind that we used only 15 minutes which is just a quarter of an hour, so the electricity costs are less than 2.5 cents for 250 ml of extracted footbath solution. For the footbath which is approximately 300 liters, we have larger electrolysis chamber and also larger electrodes. The electricity that we will

use is going to be minimal too. Even if we use 1 kW for one hour, this will cost us around 0.12 dollars.

2.6. Conclusion

From the results of these experiments we are confident that it is possible to extract Cu from dairy footbaths in an efficient and productive way. We have been able to successfully demonstrate that it's possible to remove Cu from used footbaths to prevent heavy metal contamination in the soil. We can extract the Cu and sell the extracted Cu to cover the costs for the Cu removal. Future studies investigating extraction efficiency and foam production are warranted as well as Cu extraction and regeneration of $CuSO_4$ to be reused in subsequent footbaths.

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CHAPTER 3

OPTIMUM COW THROUGHPUT FOR OPTIMIZING COPPER EXTRACTION AND COPPER SULFATE REGENERATION FROM COPPER SULFATE FOOTBATHS

3.1. Summary

The objectives of this experiment were to determine:

- How many cows can walk through a footbath before copper (Cu) extraction efficiency is reduced
- The impact of multiple Cu extractions, regeneration of copper sulfate $CuSO_4$, and reuse in subsequent footbaths on Cu extraction efficiency.

We hypothesized that as cow numbers passing through a $CuSO_4$ footbath increased, that Cu extraction efficiency would decrease and that multiple Cu extractions and $CuSO_4$ regenerations would decrease ultimate Cu recycling efficiency. To accomplish our objectives, footbath samples were obtained from a northern Colorado dairy milking 1,200 Holstein cows two times per day. Since this dairy had one footbath on both sides of the lead up to the 34 heads herringbone milking parlor (17 on each side), we obtained samples from one footbath. Footbath samples (1.0 L samples) were collected at time 0 (no cows had passed through the footbath; freshly made footbath) and after 150, 300, 450 and 600 cows passed through the footbath.

Samples were brought back to the laboratory and after filtration to remove organic matter, and then subjected to electrolysis procedure. Samples were taken before and after the electrolysis and prepared for ICP to measure the Cu concentration. There is a correlation between the number of cows that walk through the footbath and the Cu concentration within the footbath. Also, the extraction efficiency decreases as the number of cows that walks through the

footbath increases. The extraction efficiency was greater than 95% ($P < 0.05$) for footbath that had 150 heads through it, and the efficiency decreased to 75% for footbath that had 600 heads through it.

3.2. Introduction

Copper sulfate ($CuSO_4$) is one of the most popular chemicals used in dairy footbaths to help prevent lameness (Cook, 2017). The most common concentrations of $CuSO_4$ in dairy footbaths range from 3-10%. After cows walk through a footbath, the used footbath solution is typically discharged into the premise lagoon. By using the water in these lagoons for irrigation, Copper (Cu) is transferred from the lagoon to the cropland (Moore et al., 2013).

In some locations within the US, dairy farmers are experiencing iron deficiencies in their corn fields that have been irrigated with lagoon effluent. However, this deficiency is not due to reduced soil iron concentrations, but rather due to elevated Cu concentrations inducing an iron deficiency in the plant (Ippolito, 2010). It is advisable to stop Cu addition to soils that contain greater than 50 ppm extractable Cu (Ippolito and Moore, 2013).

Once Cu has accumulated in the soil at concentration in excess of 50 ppm extractable Cu, iron deficiency can be produced in plants grown in this soil ultimately making it difficult to remove Cu form soil. Recent research from our laboratory has shown that Cu from a $CuSO_4$ solution can be extracted with greater than 95% efficiency and 99% purity (Jalali et al., 2018, unpublished data) with H_2SO_4 formed as a bi-product. We have also determined how to regenerate $CuSO_4$ from extracted Cu (Jalali et al., 2018, unpublished data) using a modification of the Bordeaux system (Bordeaux, 1972) with the addition of a strong oxidizing agent (H_2O_2) to convert elemental Cu and H_2SO_4 to $CuSO_4$ and water ($Cu + H_2SO_4 + H_2O_2 \rightarrow CuSO_4 + 2\ H_2O$). However, extracting Cu from used footbath solutions collected on dairy farms was problematic

because of foam buildup in the electrolysis phase of Cu extraction. Therefore the objectives of the current series of experiments were to determine:

- How many cows can walk through a footbath before Cu extraction efficiency is reduced
- The impact of multiple Cu extractions, regeneration of $CuSO_4$, and reuse in subsequent footbaths on Cu extraction efficiency.

3.3. Materials and Methods

3.3.1. Determining the Number of Cows that Can Walk Through a Footbath Before Cu Extraction Efficiency Is Reduced

Footbath samples for this experiment were collected from a dairy in the northern Colorado that was milking 1200 Holstein cows two times per day. This dairy had two footbaths, one on each side of the lead-up to the herringbone milking parlor. Each footbath was approximately 550 liters, and per dairy protocol, 22.6 kg of $CuSO_4$ and approximately 2.0 L of a commercial sulfuric acid (H_2SO_4; Advantage™) was added to each of the footbaths (Figure 3.1 and 3.2 respectively). Footbaths were emptied after the first milking and a fresh footbath was made prior to the second milking.

Figure 3.1. A bag of copper sulfate (~22.6 kilograms) dumped in an empty footbath (~550 liters) ready to be filled with water.

Figure 3.2. Sulfuric acid (pH= -0.8) with the commercial name of Advantage™ added to each footbath (~ 2 liters) after adding water.

Footbath samples were obtained at five different time points. Since this dairy had one footbath on both sides of the lead-up to the herringbone milking parlor milking 34 cows at a time (17 on each side). Samples were obtained from one footbath. Footbath samples (1.0 L samples) were collected into Pyrex™ reusable media storage bottles (Fisher brand) at time 0 (no cows had passed through the footbath; freshly made footbath) (Figure 3.3) and after 150, 300, 450 and 600 cows passed through the footbath (Figure 3.4). Prior to collection the footbath samples, the footbath was thoroughly mixed by hand for approximately 1 min and pH determined using a handheld pH meter (Cole-Parmer WD-35462-10 Vernon Hills, IL 60061 United States). After all samples were collected, samples were transported back to our laboratory, agitated for 30 s by hand and pH determined again using a benchtop pH meter (VWR Symphony SB70P, Pennsylvania, USA).

Figure 3.3. 1 liter sample bottles

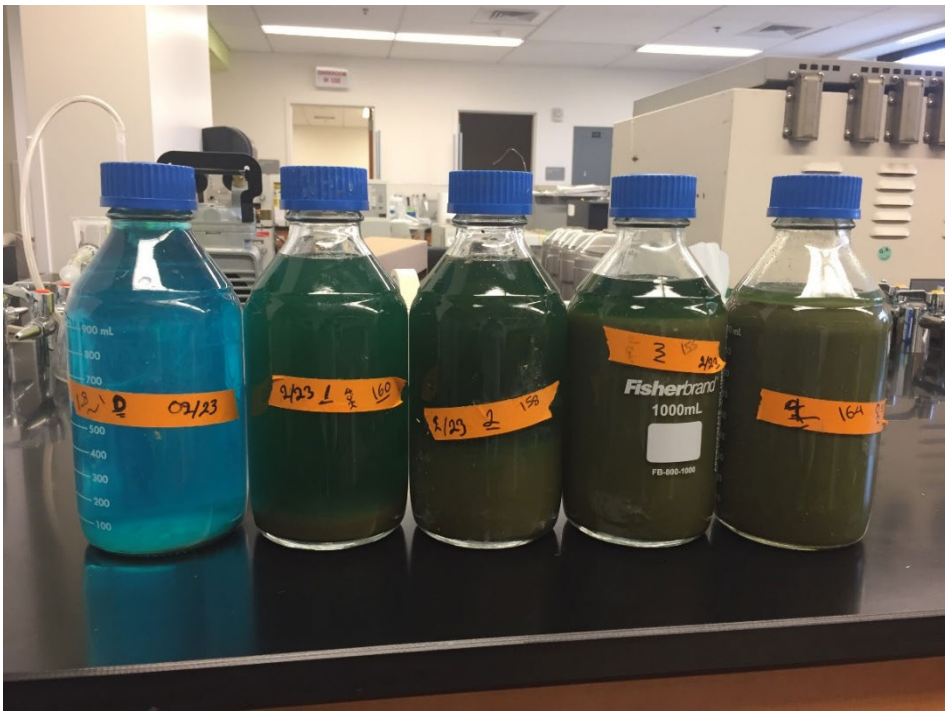


Figure 3.4. Collected footbath samples at different time points from left to right

After pH determination, samples were agitated for 30 seconds by hand and a 5 ml an aliquot was obtained for each sample for Cu analysis via ICP-MS (Inductively coupled plasma

mass spectrometry), Cu concentrations were read at 324.7 nm using a flame atomic absorption spectrophotometer (model 1275, Varian, Walnut Creek, CA) explained by Ahola (Ahola et al., 2005). Briefly, 4.9 ml of 1.2 molar hydrochloric acid and added 100 µl of each sample aliquot in 9 ml test tubes in triplicate (Figure 3.5).

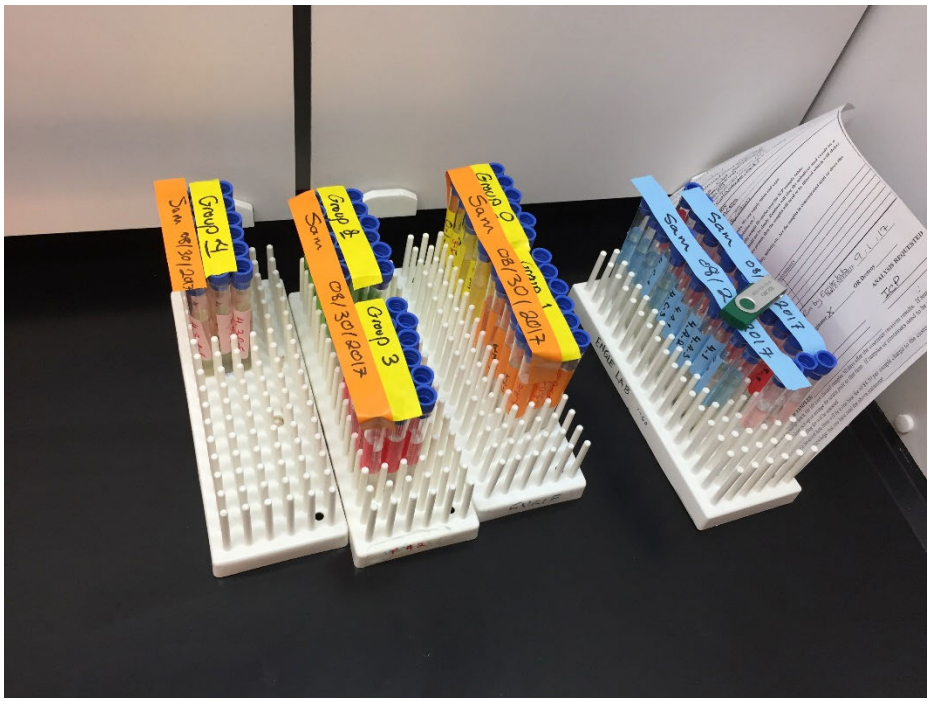


Figure 3.5. 0.1 ml samples diluted with 4.9 ml 1.2 molar HCl in 9 ml test tubes are ready for the ICP to measure the soluble Cu concentrations

3.3.2. Electrolysis

Electrolysis was performed in triplicate for each sampling time. For electrolysis, each sample was agitated by hand for 30 seconds and 100 ml of each sample was transferred to a 150 ml beaker where electrolysis was performed. The electrolytic cells were composed of a cathode (pure Cu; Frey Scientific Cu electrode strip, 12.7 cm long, 1.9 cm wide, and with a thickness of 0.11 cm) and anode (platinized titanium; 2.54 cm x 10.16 cm). Electrolysis was initiated for 10 min at 20 Volts and 15 Amps. Prior to and immediately following electrolysis, pH of the solution

was determined and 100 µl was removed from each flask for Cu analysis. After electrolysis the electrode was removed and the Cu was scraped into a weighing dish (Figure 3.6).

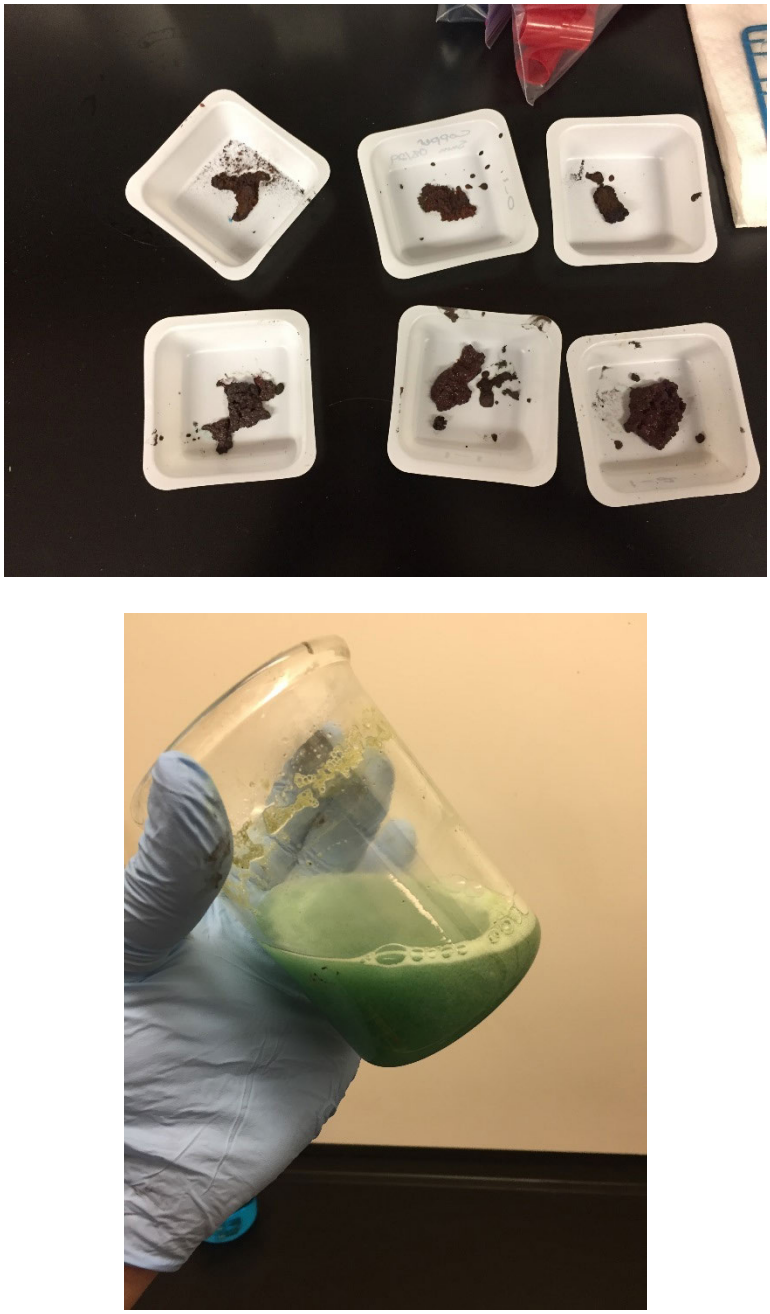


Figure 3.6. Dried extracted Cu and being scraped and transferred to the weigh plates (top). Regenerated $CuSO_4$ solution from the used footbath (bottom) (Jalali and Engle, 2016).

The weight of the cathode was measured to determine if there an increase in weight due to electrolysis. The H_2SO_4 was then filtered to separate the precipitated Cu using Whatman filter

paper (Grade 541 Circles Particle Retention >20 to 25µmm, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA)

3.3.3. Determining the Impact of Multiple Cu Extractions, Regeneration of $CuSO_4$, and Reuse in Subsequent Footbaths on Cu Extraction Efficiency

Footbath samples for this experiment were collected at a different time from the aforementioned dairy. Prior to milking, a 200 L rubber footbath was placed in front of the $CuSO_4$ footbath and filled with the same water used to fill the $CuSO_4$ footbath (Figure 3.7). The only difference from the footbath that was placed in front of the original dairy $CuSO_4$ footbath was that this footbath did not contain any $CuSO_4$ (water only footbath).

Figure 3.7. Empty 200-liter plastic footbath right before the empty copper sulfate footbath (left). Plastic 200-liter footbath filled with water and ready to use copper sulfate footbath (right).

The purpose of this footbath was to collect the debris that normally is found in the $CuSO_4$ footbath after 150 cows have passed through the footbath but not have Cu in the solution in order to simulate a used footbath in the laboratory. The previous experiment indicated that Cu extraction from the samples where 150 cows passed through the $CuSO_4$ footbath gave the

greatest Cu extraction efficiency. Therefore, 2.0 L of sample was obtained from the water only footbath after 150 cows had passed through the footbath and 2.0 L of the $CuSO_4$ footbath was obtained prior to any cows walking through the footbath.

The 2.0 L samples was transported back to our laboratory, agitated by hand for 30 s and the pH determined. The sample containing the original $CuSO_4$ footbath was agitated by hand for 30 s and 100 ml was transferred to a 150 ml beaker and subjected to electrolysis as describe above. One hundred µl of the sample was obtained prior to and immediately after electrolysis for Cu analysis as describe previously.

After electrolysis, Cu was scraped from the anode back into the H_2SO_4 solution that was generated during the electrolysis procedure and 10 ml of 30% H_2O_2 (Fisher Scientific, Janssen Pharmaceutical, Geel, Belgium) added to the mixture. Due to the exothermic nature of this reaction, H_2O_2 was added in 5 ml increments separated by 15 min. At the end of this procedure $CuSO_4$ and water were produced. Once the reaction was complete the water was evaporated by placing the beaker on a hot plate for 20 min (Figure 3.8). After all of the water was evaporated, 100 ml of our water-only footbath solution was added that contained debris from 150 cows.

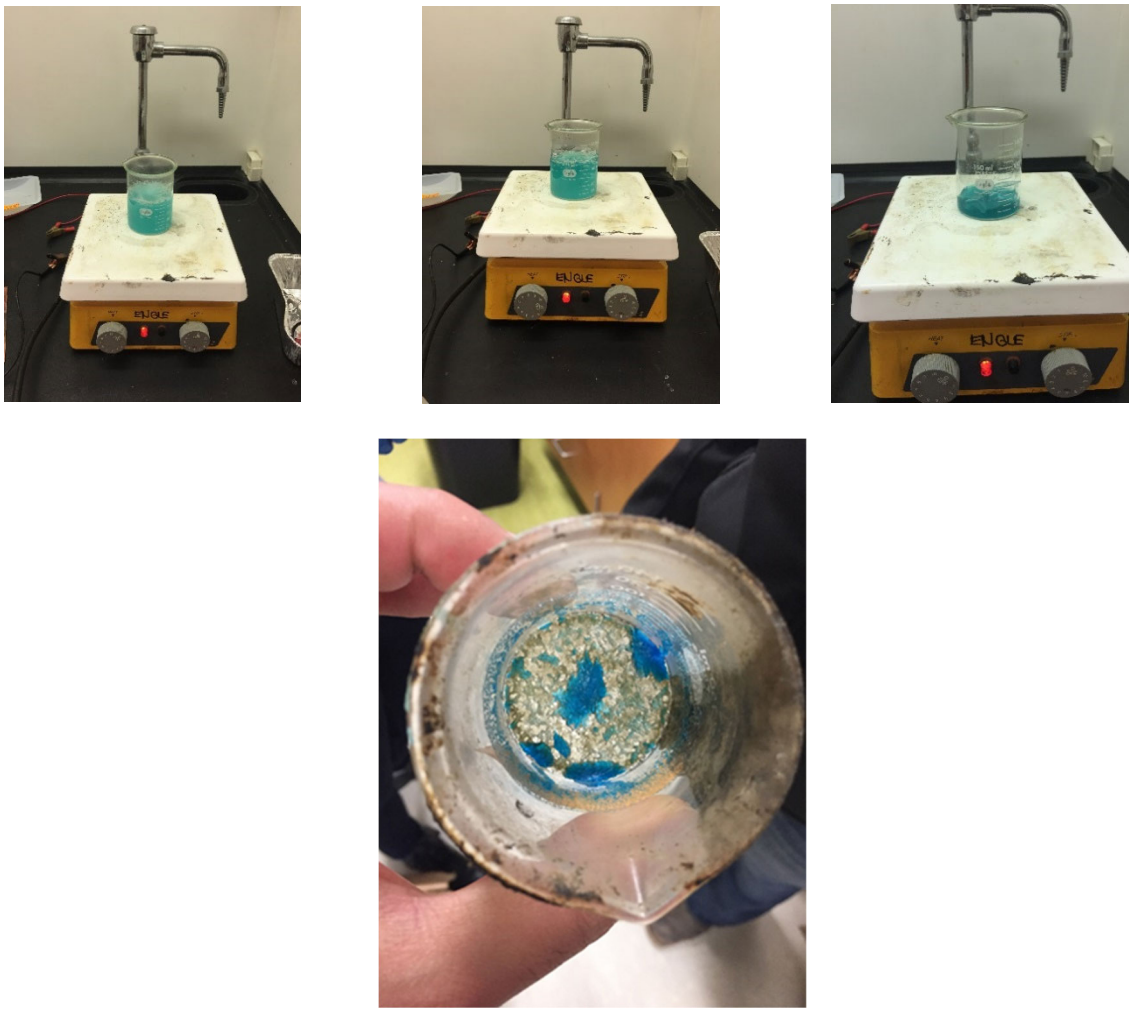


Figure 3.8. Water evaporation process with the help of hotplate from left to right. Blue copper sulfate crystals can be seen in the bottom picture.

A clean glass stirrer bar was used to solubilize $CuSO_4$ in the solution. After visually confirming that no $CuSO_4$ crystals were present at the bottom of the beaker another round of electrolysis as described previously was initiated. Prior to and after electrolysis, pH was determined and a 1 µl sample of the solutions was obtained for Cu analysis. To determine the extraction efficiency, the following formula was used:

Extraction efficiency = 100-(([Cu] after electrolysis*100) / [Cu] before the electrolysis)

3.4. Results and Discussion

3.4.1. Determining the Number of Cows That Can Walk Through a Footbath Before Cu Extraction Efficiency Is Reduced

Figure 3.9 describes the extraction efficiency over time for a $CuSO_4$ footbath on a commercial dairy. Extraction efficiency was 95.7%, 98.4%, 83.7%, 89.6% and 74.8% for T_0-T_4, respectively. It is interesting to note that the Cu extraction efficiency goes down. However, at T_1 (150 head) more Cu was extracted, even more than T_0. This is likely because of the soil and other minerals that cows brought with themselves to the footbath. We speculate Cu extraction efficiency for T_0 compared to T_1 are similar and the difference was due to soil and minerals contamination.

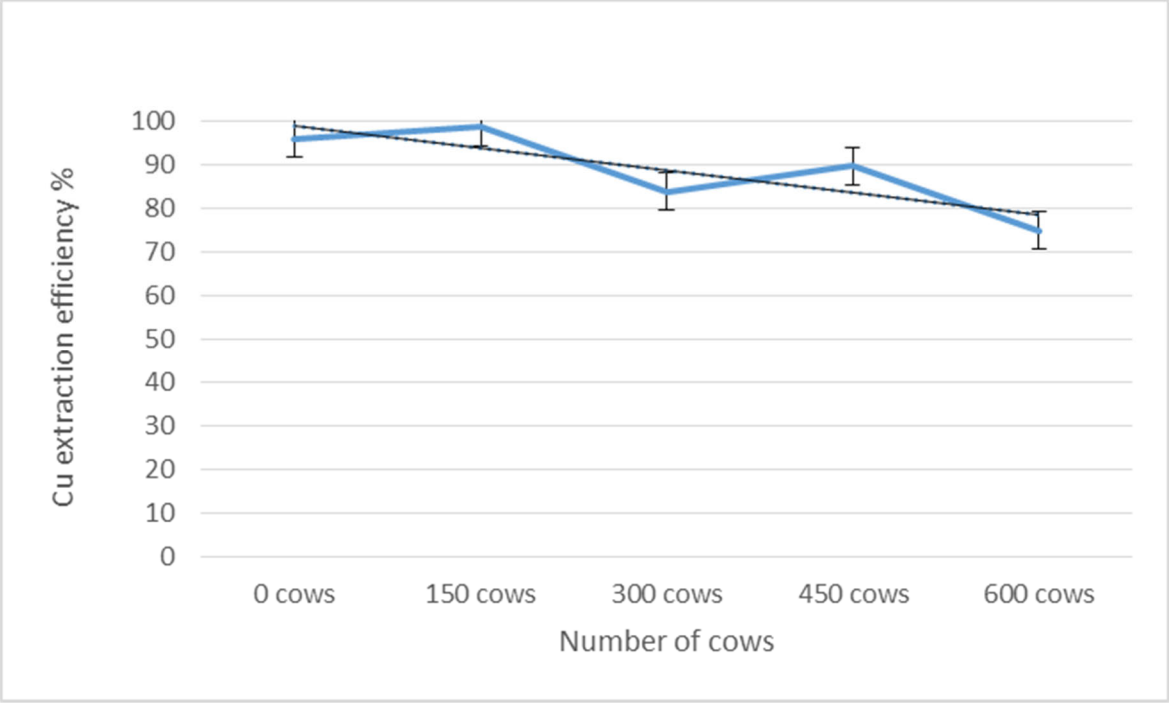


Figure 3.9. The influence of the number of cows passing through a copper sulfate footbath on Cu extraction efficiency.

A shown in the plot below (Figure 3.10), there is, an effect on the number of cows passing through the footbath.

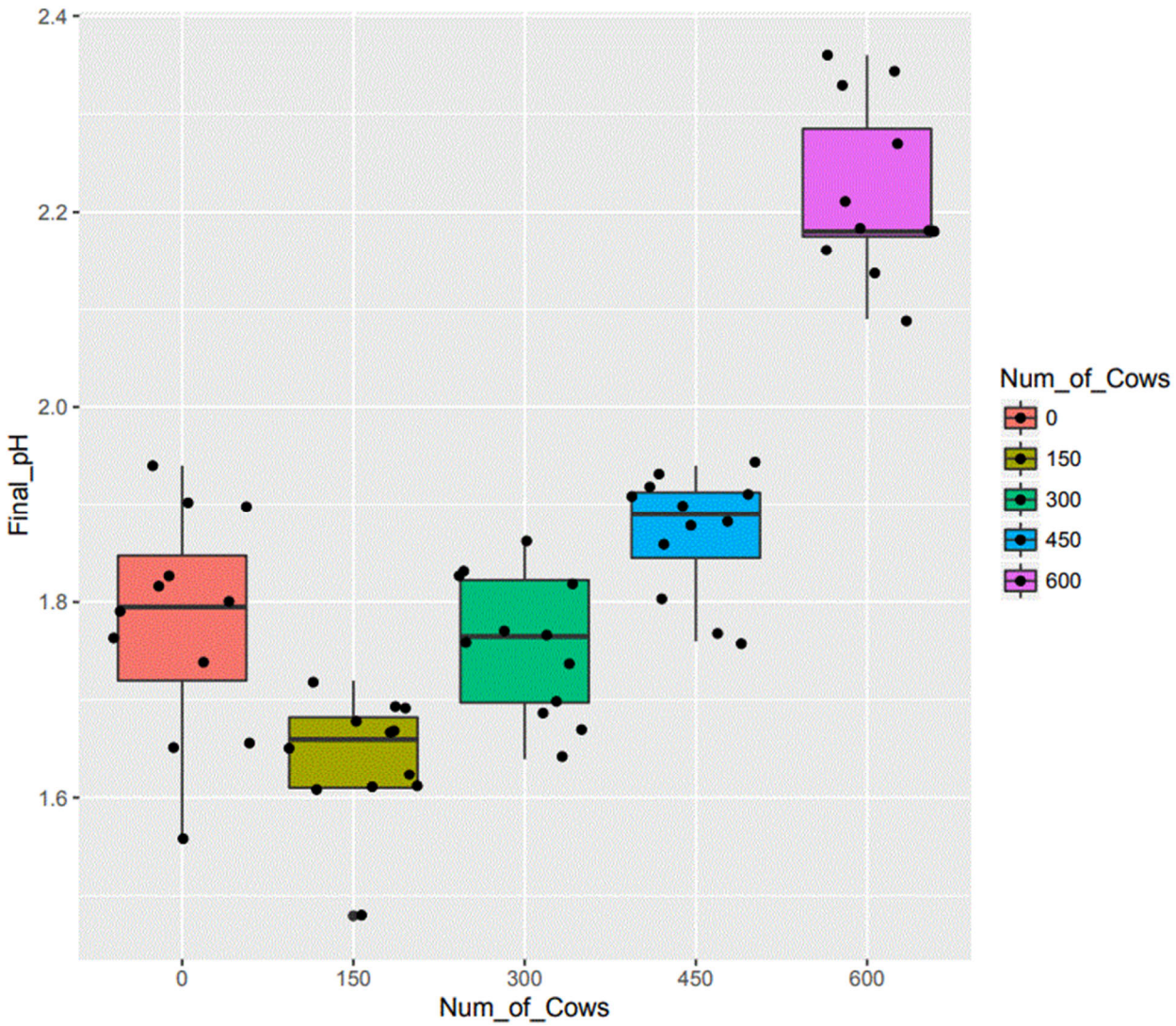
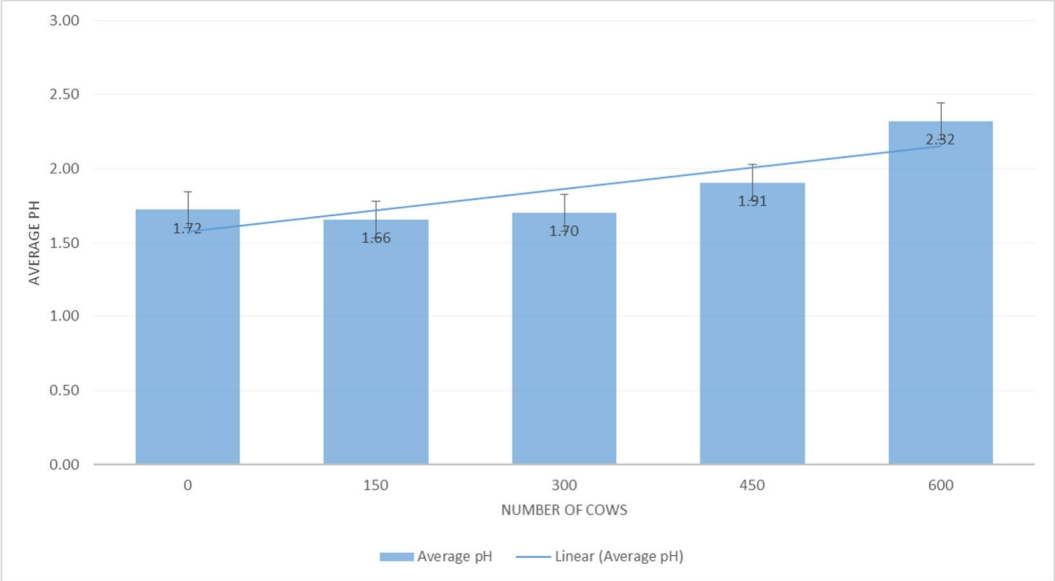


Figure 3.10. The influence of the number of cows passing through a $CuSO_4$ footbath on footbath pH level.

The graph depicted in Figure 3.11 shows the relation between the increase in pH and the number of the cows passed through the footbath. At the bottom of the graph, the table shows one way ANOVA for the number of the cows and pH levels.



	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Num_of_Cows	4	4027.65	1006.91	23.36	0.0000
Residuals	58	2500.07	43.10		

Figure 3.11. Relation between the increase in pH and the number of the cows passed through the footbath. Bottom table shows one way ANOVA for the number of the cows and pH levels.

The reason for the greater pH in T_0 compared to T_1 is most likely due to improper agitation of the footbath when the acid was added. After the first set of 150 cows passed through the footbath the acid added was most likely agitated enough to be homogenous within the footbath. Analyzing the data with Tukey's Honest Significant Difference (HSD) test to determine which groups were statistically different from one another indicate that there are significant differences between the groups T0 and T1 ($P < 0.05$).

It appears that the acid adgitation influence occurred between T_0 and T_1. T_0 and T_2 weren't significantly different ($P = 0.96$). T_1 and T_2 were significantly different ($P < 0.01$) from eachother. There was also a significant diffrenece between T_1 and T_3, T1 and T_4 ($P < 0.05$). There was significant difference between all other groups.

Based on these data, our greatest Cu extraction efficiency was after 150 cows had passed through the $CuSO_4$ footbath. Although we did not determine extraction efficient for any less than 150 cows, from a practical standpoint, 150 cows would be the minimum number of cows that could pass through the footbath prior to Cu extraction while maintain 90% (SEM = 0.57) or greater extraction efficiency of Cu.

3.4.2. Determining the Impact of Multiple Cu Extractions, Regeneration of Cuso4, and Reuse in Subsequent Footbaths on Cu Extraction Efficiency

Table 3.1 describes the influence of multiple Cu extractions, regenerations, and simulated debris accumulation of 150 cows passing through a footbath on pH and Cu extraction efficiency. The first Cu extraction with a 150 cow simulated debris addition to the extracted Cu (R_0) regeneration efficiency was 85% (R_1). A subsequent extraction and regeneration (R_2) produce a regeneration efficiency of 78%.

Table 3.1. Influence of extraction and regeneration of a copper sulfate footbath on pH and copper extraction efficiency.

Samples	Initial pH	Cu mg/L	SD	Regeneration Efficiency (%)
R_0	2.07	146.12	5.38	-
R_1	2.6	112.86	3.65	85
R_2	1.54	98.39	4.69	78

3.5. Conclusion

The most appropriate time to extract and regenerate $CuSO_4$ from a $CuSO_4$ footbath where 90% or greater of the Cu can be extracted is after 150 head of cattle has passed through the footbath. Data from our simulated used footbath experiment, $CuSO_4$ regeneration efficiency decreases by approximately 15% for each regeneration performed. We speculate that the

regeneration efficiency can be greater in real world applications. A large portion of the $CuSO_4$ solution was lost due to evaporating the solution and drops of the solution jumped out of the beaker due to boiling of the solution. Future research comparing the bactericidal effects of the regenerated $CuSO_4$ is warranted.

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CHAPTER 4

EFFECTS OF LABORATORY TECHNIQUES ON SOLUBLE COPPER CONCENTRATIONS AND ANTIBACTERIAL EFFICACY OF REGENERATED COPPER SULFATE FROM DAIRY FOOTBATHS

4.1. Summary

Two experiments were conducted to determine:

- The impact of laboratory processing techniques (autoclaving and centrifugation) on the solubility of copper (Cu) from dairy footbaths (Experiment 1), and
- The antimicrobial effectiveness of regenerated copper sulfate $CuSO_4$ (Experiment 2).

We hypothesized that laboratory processing techniques would not alter Cu solubility and that the antimicrobial effectiveness of $CuSO_4$ would not be altered by $CuSO_4$ regeneration.

In experiment 1, dairy $CuSO_4$ footbath samples were obtained from a northern Colorado dairy milking 1,200 Holstein cows two times per day. Since this dairy had one footbath on both sides of the lead up to the milking parlor, we obtained samples from one footbath. Footbath samples (1.0 L samples) were collected at time 0 (no cows had passed through the footbath; freshly made footbath) and after 150, 300, 450 and 600 cows passed through the footbath. Samples were brought back to the laboratory and filtered through 4 layers of cheesecloth. After filtration, 12 subsamples (10 ml/subsample) were collected from each collection period and pH determined on each sample. Six samples per collection period were autoclaved while the remaining six subsamples were not autoclaved. After autoclaving, pH was determined on all samples. Three autoclaved and three non-autoclaved samples were centrifuged at room temperature, while the other autoclaved and non-autoclaved samples were not centrifuged. At the

end of these procedures pH was determined on all samples and a subsample obtained from all samples (n=60) and analyzed for Cu concentrations. Data indicate that neither centrifugation nor autoclaving had an impact on Cu solubility (SEM = 2.94) ($P < 0.05$).

Experiment 2 was designed to determine the antimicrobial effectiveness of regenerated $CuSO_4$. Footbath treatments mixtures were prepared (10 ml) in 20 ml glass tubes and included:

- Conventional 4% $CuSO_4$ footbath solution (made in the lab) for this we added 4 grams of $CuSO_4.5H_2O$ to 100 ml water
- Regenerated $CuSO_4$ solution made by regenerating $CuSO_4$ for three times after making artificial footbath in the laboratory from the dirty water footbath
- Footbath at time zero (22.7 kg of $CuSO_4$ in 550 liters of water is ~ 4% $CuSO_4$) which was took from a fresh footbath with no cows pass through it
- Conventional 4% $CuSO_4$ solution with added sulfuric acid ($4x10^{-3}$/ vol.) we used approximately the same amount of acid that the dairy was using in the footbaths (2 liters per 550 liters footbath)
- Regenerated $CuSO_4$ used to make a 4% $CuSO_4$ footbath solution with added sulfuric acid ($4x10^{-3}$/ vol.) this is the regenerated $CuSO_4$ solution with the sulfuric acid from dairy
- Sulfuric acid alone (from dairy)
- Deionized water.

Serial 1:2 dilutions were made of each treatment and then all dilutions were autoclaved. The minimum inhibitory concentration (MIC) for each treatment was determined using *E. coli*. The MIC was at a 1:16 dilution for all the treatments with the exception of the acid treatment (SEM = 3.62) ($P = 0.05$). For the acid treatment, no bacteria growth was present at our greatest dilution (1:512). To determine the minimum bactericidal concentration (MBC) treatment

dilutions of 1:32; 1:16; and 1:8 were plated oh Mueller Hinton ready petri dish and incubated at 37°C temperature for 24 h. Colony counts were then performed on all plates. All treatments showed bacterial growth with the exception of the acid treatments these data indicate that a dilution of greater than 1:32 is required to determine MBC. In summary, regarding the laboratory techniques, there was no significant difference between the treatments. The MIC was considered at 1:16 dilution for all the treatments but the sulfuric acid. Since we had growth on all of our plates for all the treatment but sulfuric acid, we need to redo the MBC with less diluted solutions to figure out the dilution for the bactericidal effect.

4.2. Introduction

Copper sulfate ($CuSO_4$) footbaths are the most widely used footbaths in the dairy industry (Cook, 2017). In previous experiments we have shown that Cu from $CuSO_4$ footbaths can be extracted with 95% efficiency and that we are capable of regenerating $CuSO_4$ for reuse in subsequent footbaths. However, we are uncertain if analytical processing procedures such as autoclaving and centrifugation used in the aforementioned experiments to determine extraction efficiency of Cu impact Cu solubility. We already know that Cu is more soluble in lower pH (Mulligan et al., 2001) (Sauve et al., 1997). However, we do not know if sterilization affects the pH of the footbath or not. Some experiments has been done on measuring the pH of the medium before and after autoclaving, and they have reported that there are significant differences between initial pH and pH levels after autoclaving (Skirvin et al., 1986).

Centrifugation is one of the most valuable and extensively utilized research techniques in biochemistry, cellular and molecular biology, evaluation of suspensions and emulsions in pharmacy and medicine (Majekodunmi, 2015). It may be possible that lots of micro-particles remain in the final discarded supernatant (Jy et al., 2004). Sometimes pretreatment of soil

specimens has been reported to affect the chemical formation of soil solutions after centrifugation (Giesler and Lundstrom, 1993). That is the basis of why we centrifuged all the footbath samples with the different number of cows passed through and took samples before and after centrifugation, to figure out if there is any difference between those samples regarding Cu concentration.

The bactericidal efficacy of regenerated $CuSO_4$ has not been determined. Therefore, the objectives of the present experiment were to determine:

- The impact of laboratory processing techniques (autoclaving and centrifugation) on the solubility of Cu from dairy footbaths, and
- Determine the antimicrobial effectiveness of regenerated $CuSO_4$.

We hypothesized that laboratory processing techniques would not alter Cu solubility and that the antimicrobial effectiveness of $CuSO_4$ would not be altered by $CuSO_4$ regeneration.

4.3. Materials and Methods

Experiment 1: Footbath samples have already been obtained and were collected from one dairy in northern Colorado over the course of the first milking. This was a 1,200 head dairy with a 2 x 17 herringbone milking parlor and had two 550 liters footbaths on both sides. A representative sample was obtained from the footbath before use, and then after, 0, 150, 300, 450 and 600 cows walked through the footbath. Before beginning this experiment, each subsample was filtered through 4 layers of cheesecloth to remove large feed particles. After straining through cheesecloth, twelve, 10 ml subsamples were obtained from each collection period (n=5) and pH determined on each sample. Six 10 ml samples per collection period were autoclaved at 1.51 Bar and 121°C for 18 minutes, while the remaining four subsamples were not autoclaved.

After autoclaving, pH was determined on all samples. Three autoclaved and three non-autoclaved samples were centrifuged (Eppendorf Centrifuge 5810) at 1000 x *g* at room temperature for 25 minutes, while the other six autoclaved, and six non-autoclaved samples were not centrifuged. Following centrifugation, pH was determined on all samples, and then 1.0 ml subsample was obtained from all samples and added to 4 ml of 12M HCl for Cu analysis via ICP as described by Ahola (Ahola et al., 2005).

Experiment 2: Footbath treatments mixtures were prepared and included:

- Conventional 4% $CuSO_4$ footbath solution. To prepare this solution, we used 4 gr of $CuSO_4.5H_2O$ (Product Number: 209198, Sigma-Aldrich, Saint Louis, MO, USA) to 100 ml water, and stirred with glass stirrer for until there were no $CuSO_4$ particles in the solution;
- Regenerated $CuSO_4$ used to make a 4% $CuSO_4$ footbath solution. We used the artificially made dirty footbath and regenerated $CuSO_4$ for three times, we did the regeneration process by electrolysis and scraping the extracted Cu into the produced H_2SO_4 and adding 30% H_2O_2 with the ratio of 3:1. We have repeated this process for three times to regenerate $CuSO_4$ three times from dirty footbaths.
- Footbath at time zero (from dairy's footbath). This dairy uses 22.7 kg of $CuSO_4$ per footbath (550 liters). Since 25% of $CuSO_4$ is Cu, this will be 0.1 gr of pure Cu per 10 ml of the solution
- Conventional 4% $CuSO_4$ solution with added sulfuric acid ($4x10^{-3}$/ vol.). We used the same procedure to make the 4% $CuSO_4$ as the first treatment, the only difference is the sulfuric acid added to the solution. Since the dairy added 2 liters of sulfuric acid to 550

liters footbath we added 0.04 ml of the sulfuric acid from the same batch that they were using in the dairy

- Regenerated $CuSO_4$ used to make a 4% $CuSO_4$ footbath solution with added sulfuric acid ($4x10^{-3}$/ vol.). This solution was made exactly like treatment number two but the only difference was the added sulfuric acid
- Sulfuric acid alone (from dairy), and
- Deionized water. Serial 1:2 dilutions were made of each treatment and then all samples were autoclaved. The minimum inhibitory concentration (MIC) for each treatment was determined using inoculation technique with *E. coli*. *E. coli* was purchased from ATCC (ATCC® 25922). This process required streaking Mueller Hinton plates and incubating the plates at 37°C for 24 h (Figure 4.1). Upon removal, tubes containing Mueller Hinton broth were inoculated with bacteria isolated from the plates and incubated to 37°C temperature for 24 hours to reach a turbidity of 0.5 McFarland standards equating to 5 x 10^5 CFU/ml.

Figure 4.1. Streaked plate with E.coli after 24 hours growth in the incubator (37°C) ready to inoculate the individual colonies

To test the antimicrobial effectiveness of $CuSO_4$ footbath components footbath mixtures (seven treatments) were prepared as described above. Serial 1:2 dilutions were made (n=10 1:2 dilutions ranging from 1:2 to 1:512; Figure 4.2) form each treatment and then all diluted treatments were autoclaved at 121°C and 1.51 Bar for 18 minutes.

Figure 4.2. Dilluted treatments

After autoclaving, 100 µl of Mueller Hinton broth bacterial suspension was added to each well of multiple 96-well plates (Costar 3595 Corning Incorporated, Corning, NY, United States; Figure 4.3) using an 8-channel micropipetter. After adding the Mueller Hinton broth bacterial suspension 100 µl of each diluted antimicrobial treatment was added to the appropriate wells. Each diluent was run in triplicate. Plates were then incubated for 24 h at 37°C. After incubation, all plates were read in a plate reader (Synergy HT, Biotek Instruments Inc., Winooski, VT, United States) 600 nm to measure turbidity which is a proxy indicator for bacterial growth, and we checked them for growth (Figure 4.4).

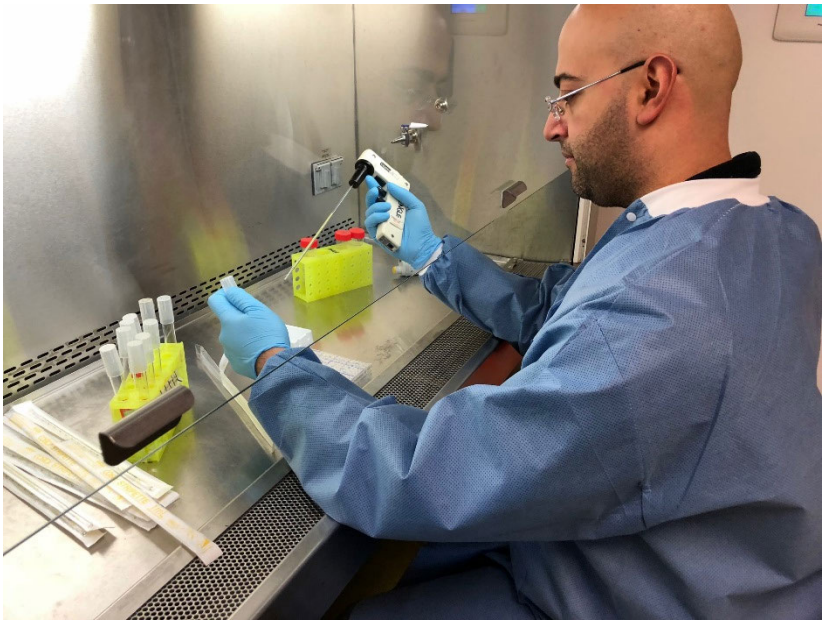


Figure 4.3. Delivering each sterilized treatment to appropriate well in 96 well plates

Figure 4.4. Placing 96 well plates in the plate reader (Synergy HT, Biotek Instruments) to measure the turbidity at 600 nm

Since we had some inconsistency in our readings due to the color of the $CuSO_4$ solution, another plate was used to determine the correction factor for the absorbency of the $CuSO_4$ solution without Mueller Hinton broth bacterial suspension, this correction factor was subtracted from the original readings to give a more accurate reading of turbidity due to bacterial growth.

To determine the minimum bactericidal concentration (MBC) for each treatment following the (Hartshorn et al., 2013) technique for E. coli. Based on the results from the MIC assay describe above, the 1:16 dilution was determined as the MIC. Therefore the 1:8, 1:16, and 1:32 dilutions for each treatment chosen to determine the MBC. Briefly, the sample procedure as describe for MIC was conducted with these three dilutions. After incubation 3 µL of each solution within each well was plated into the appropriate location (Figure 4.5) on a pre-prepared Mueller-Hinton petridish plate. Plates were then incubated at 37°C for 24 hours.

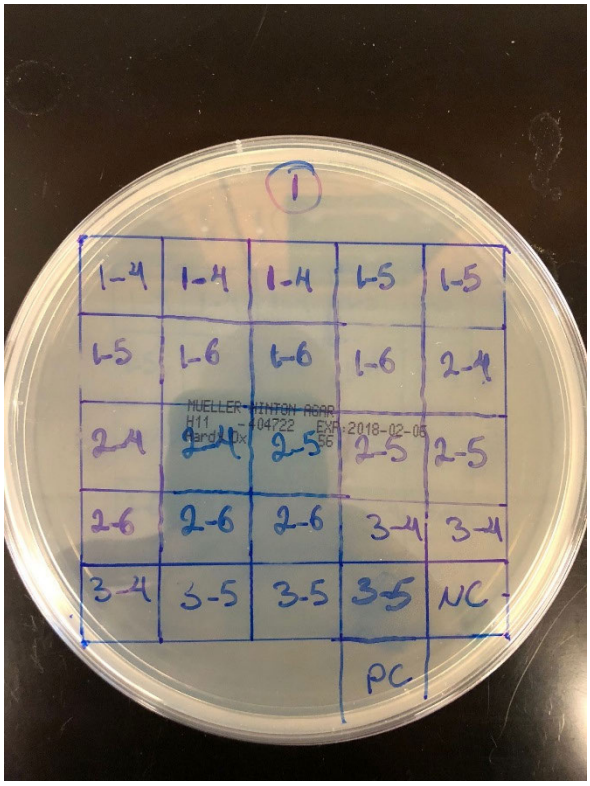
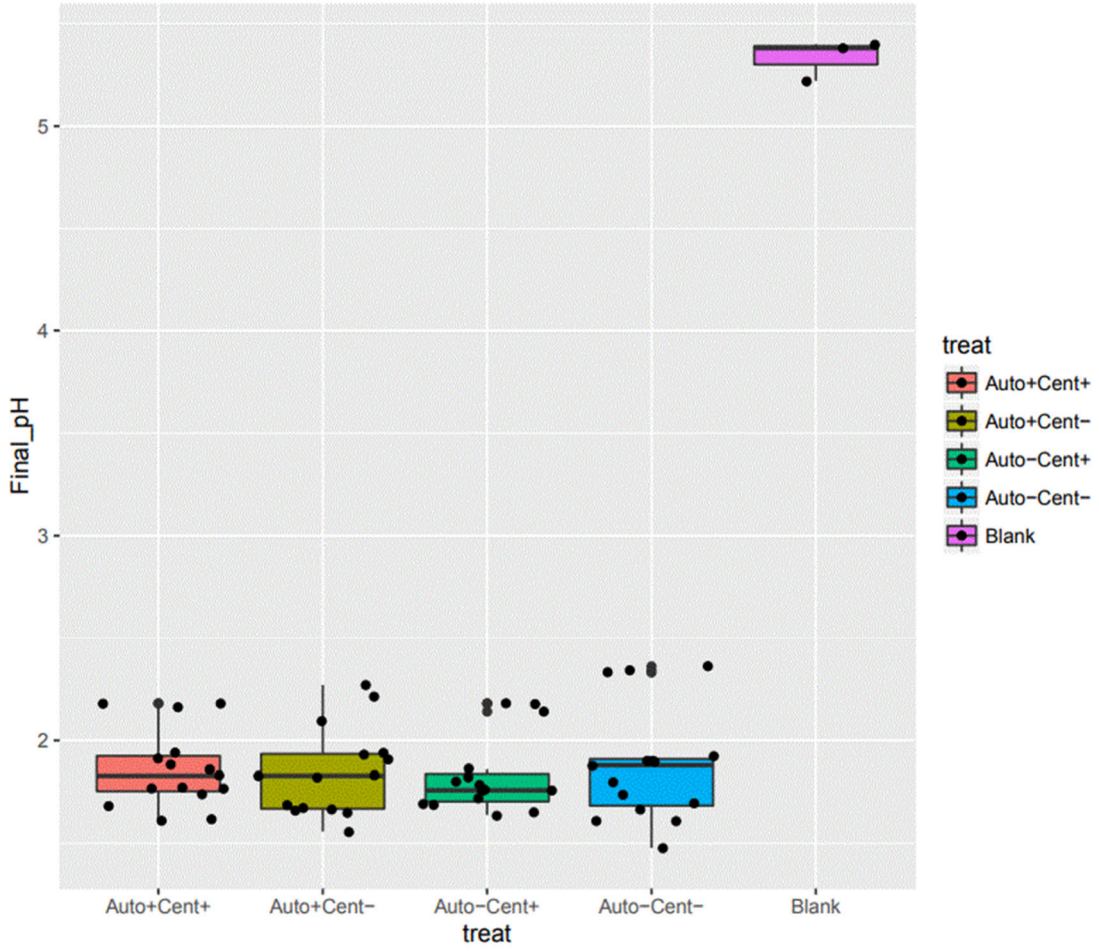


Figure 4.5. Pre-prepared Mueller-Hinton plate for the Minimum Bactericidal Concentration (MBC) assay with 25 squares each representing a treatment and specific dilluton (1:8; 1:16, or 1:32).

4.4. Results and Discussion

Experiment 1: Figure 4.6 describes the effects of centrifugation and autoclaving on pH of each antimicrobial treatmetn. These data indicate that centrifugation and autoclaving had a significant ($P < 0.02$) effect on pH. Therefore, means were seperated using Tukey HSD (Table

4.1). Tukey HSD revealed that the Blank (water) treatment was the only treatment where pH was influenced by autoclaving and centrifugation. These data indicate that laboratory autoclaving and centrifugation do not influence pH of antimicrobial treatmsnt used in Experiment 2 (SEM = 0.04). There was also no significant diffrenece between initial Cu concentration and the final ($P < 0.05$). Table 4.1 also shows that the high variations are only with the blank and not between other treatments.



	Df	Sum Sq	Mean Sq	F value	Pr(>F)
treat	4	1194.11	298.53	3.25	0.0180
Residuals	58	5333.60	91.96		

Figure 4.6. Box plot showing the influence of centrifugation and autoclaving on pH of different antimicrobial dissenfectants used in $CuSO_4$ footbaths. ANOVA table for difference between the treatment groups.

Table 4.1. Tukey's honest significance test to examine the difference between the treatments.

	diff	lwr	upr	p adj
Auto+Cent–Auto+Cent+	-0.73	-10.59	9.13	1.00
Auto-Cent+-Auto+Cent+	-2.80	-12.66	7.06	0.93
Auto-Cent–Auto+Cent+	-0.20	-10.06	9.66	1.00
Blank-Auto+Cent+	18.87	1.79	35.94	0.02
Auto-Cent+-Auto+Cent-	-2.07	-11.93	7.79	0.98
Auto-Cent–Auto+Cent-	0.53	-9.33	10.39	1.00
Blank-Auto+Cent-	19.60	2.52	36.68	0.02
Auto-Cent–Auto-Cent+	2.60	-7.26	12.46	0.95
Blank-Auto-Cent+	21.67	4.59	38.74	0.01
Blank-Auto-Cent-	19.07	1.99	36.14	0.02

Therefore, we find that there is no difference between the various combinations of centrifuge and autoclave procedures. As we demonstrated in the studies, we can easily see the increasing trend of final pH levels as the number of cows passing through the solution increase, for every treatment group, yet we can also see that the treatment trends differ depending on which postprocessing procedure the solution undergoes (Autoclave/Centrifuge), this can be observed in Table 4.2 below.

There is also no difference in the Cu concentration with either centrifuging or autoclaving. However there is a significant cow effect on the Cu concentration ($P < 2e^{-16}$) (Figure 4.7).

Table 4.2. Influence of the number of cows walking through a common 5% copper sulfate solution footbath during the first milking.

Item	Number of cows passing through a common footbath					SEM	P <			
	0	150	300	450	600		Cow	Autoclave	Centrifuge	Autoclave x centrifuge
pH	1.71[a]	1.66[b]	1.76[a]	1.90[a,d]	2.31[c]	0.03	0.0001	0.82	0.92	0.69
Autoclave										
No	1.83	1.63	1.68	1.86	2.25	0.1				
Yes	1.73	1.66	1.81	1.89	2.19	0.09				1.00
Centrifuge										
No	1.74	1.62	1.76	1.91	2.26	0.11		1.00		
Yes	1.81	1.66	1.73	1.83	2.17	0.08			0.99	0.98
Copper, mg/l										
Initial	296.7	402.82	384.7	367.93	294.57	1.74	$2e^{-16}$	0.26	0.94	0.063
Final	307.35	419.22	378.44	364.4	311.32	21.13				
Autoclave										
No	296.7	402.82	384.7	367.93	294.57	1.74			0.92	
Yes	301.01	412.96	412.96	370.1	305.7	24.63		0.88		1.00
Centrifuge										
No	296.7	402.82	384.7	367.93	294.57	1.74				
Yes	302.04	393.42	377.39	344.5	328.32	16.48		0.75		

[1]Means within a row with different superscripts differ P < 0.05.

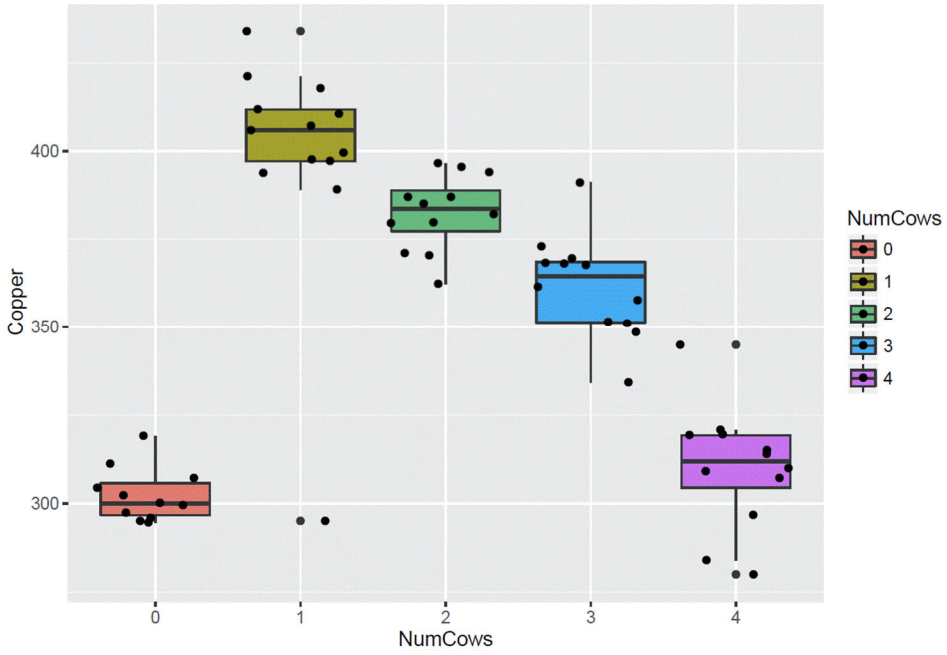


Figure 4.7. Cow effect on the Cu concentration in the footbath (mg/l)

Figure 4.8 shows that there is a positive correlation between number of the cows passed through the footbath and the pH of the footbath, from 0 to 600 heads. It also demonstrates that there is no significant difference between the treatments regarding the pH.

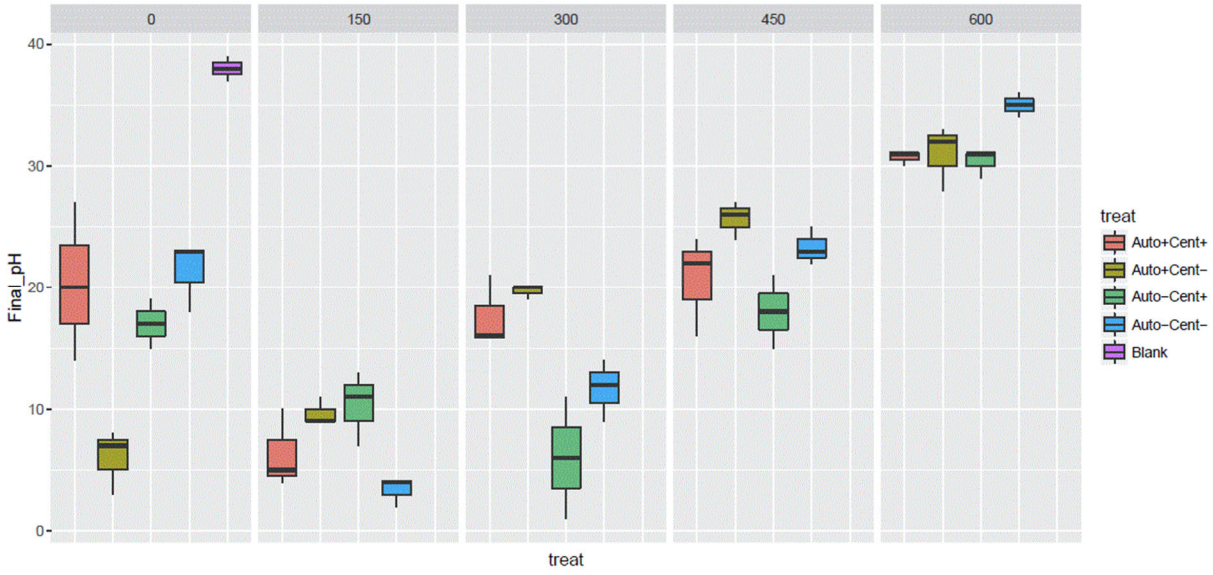


Figure 4.8. Cow, treatment effect for pH levels

Experiment 2: Figure 4.9 describes the influence of all of the six treatments on the bactericidal effects of each treatment measured via MIC. Data were analyzed as a two-way ANOVA model, with treatment and dose (dillution) as factors. Since the design was completely randomized balanced design all factors were orthogonal and that Type II and Type III sums of squares were equivalent to the Type I sums of squares. Therefore Type I sums of squares were used to test for differences between the treatment groups and dose levels. In all cases, extremely large F values wer enoted which correspond to p-values less than $2e^{-16}$ in each case. This means that there is a difference in effect between the treatments and also a difference in effect between the dose levels. On top of that, we also know that the effects of Dose depend on the treatment (the positive interaction effect). We can see from the diagnostics plots that our assumptions for our linear model are reasonably met (Appendix C).

Data indicate that we can easily see that when we do not control for dose, that there is not much difference between the first five treatments (whether in percent inhibition or final reading). We have calculated the percent inhibition by dividing the final readings from the plate reader by the initial readings and multiply that by 100 and deduct that total amount from 100.

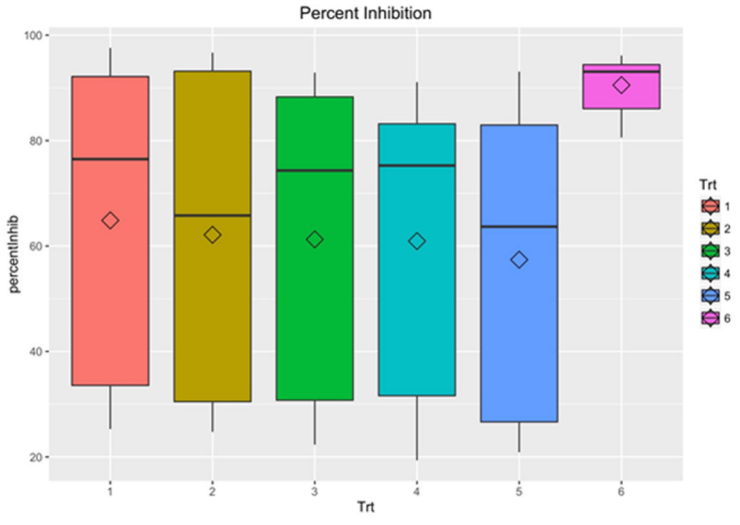


Figure 4.9. The percent inhibition of the treatments

However, we do not even need to control for Dose to quickly see that Treatment 6 (acid) is obviously different. We have looked for dose and find that similar patterns are occurring across the different treatment groups (Figure 4.10).

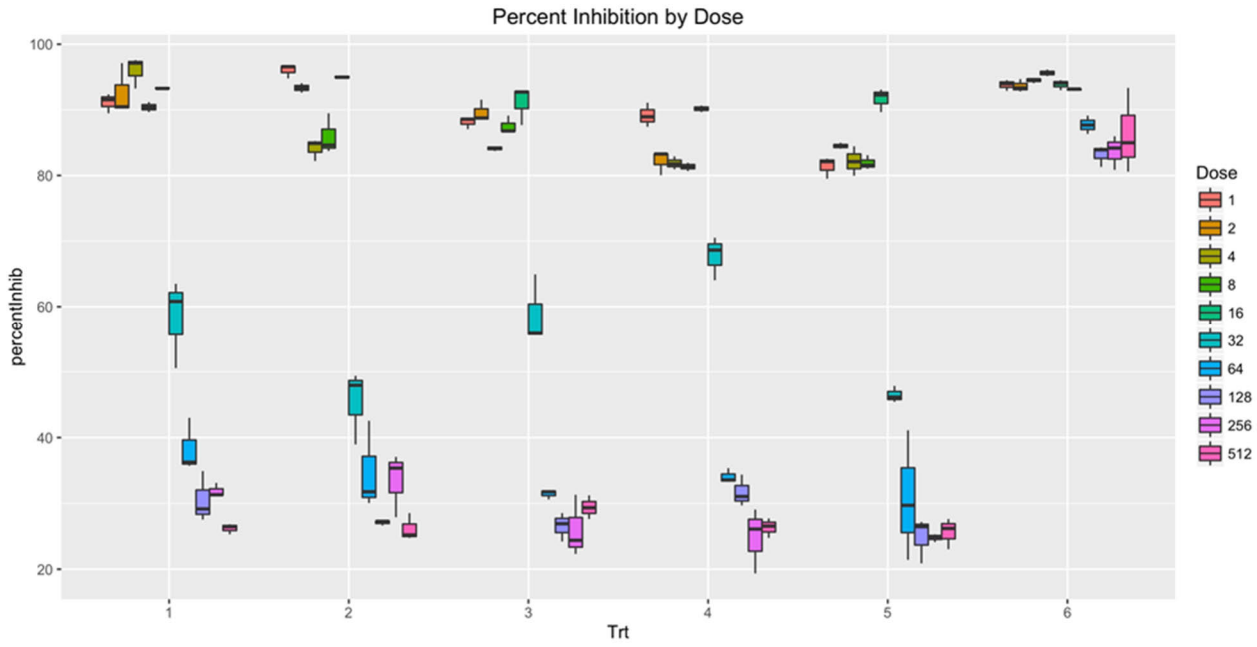


Figure 4.10. The plot is showing different doses (dilution) effects on percent inhibition and perfectly presents a pattern for each treatment

Two-way ANOVA analysis estimates the effects of treatment while marginally controlling for the effects of dose. Means were then separate using Tukey HSD to determine which treatment groups differ from one another. From the Tukey HSD test, we find that Treatments 2, 3, 4 are similar in all dilutions, these treatments are regenerated $CuSO_4$, footbath at time zero and conventional copper sulfate with acid, while the others differ from all the rest.

For the MBC or the microbial bactericidal concentration, we had growth in all the treatment except for acid which had no growth on any of the dilutions or exposure time (Figure 4.11) and (Figure 4.12).

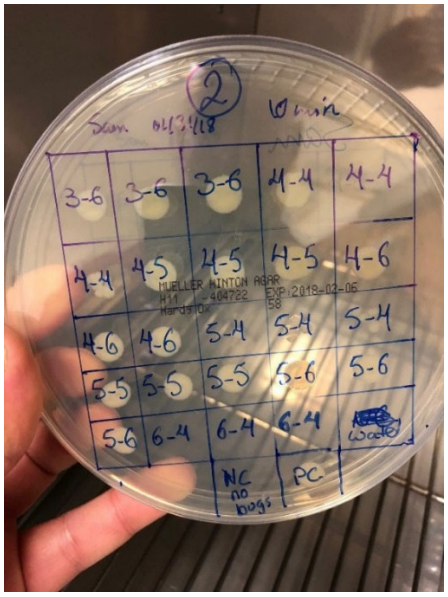


Figure 4.11. E-Coli growth on Mueller-Hinton plate after 10 minutes of exposure to the antibacterial agents and 24 hours of incubation.

Treatment 6 was acid, and even on highest dilution, we did not get any growth. Since we had growth on all other treatments, statistical analyses were performed on MBC.

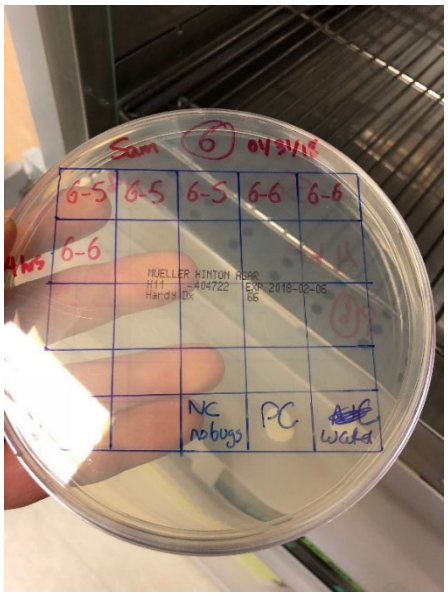


Figure 4.12. Mueller-Hinton agar plate after 4 hours of exposure to the antibacterial agents and 24 hours of incubation.

4.5. Conclusion

Since there was no difference in the Cu concentrations and pH after centrifugation and sterilizing (autoclaving), we can confidently say that there is no problem using these techniques for the regeneration and the recycling process of the footbath.

We have shown that there is no difference between the regenerated $CuSO_4$ and the conventional $CuSO_4$ in regards to efficacy and bacteriostatic effects. Since *E. coli* is not the principal cause of Digital Dermatitis, it would be beneficial to conduct a follow-up experiment using bacteria that are the main pathogens for this disease (e.g. Treponema).

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CHAPTER 5

USED FOOTBATH COPPER EXTRACTION, AND $CuSO_4$ RECYCLING APPARATUS DESIGN AND FEASIBILITY STUDY

5.1. Summary

The objective of this chapter is to design and determine the feasibility of an on-farm copper (Cu) extraction and copper sulfate ($CuSO_4$) regeneration system to prevent Cu from bioaccumulating on dairy farms that use $CuSO_4$ footbaths. Previous research from our laboratory has indicated that we can extract the Cu from the footbath with the efficiency of more than 90 % and regenerate $CuSO_4$ from the used footbath. We also figured out the optimum number of cows pass through the footbath to have the best extraction rate, which was 150 to 200 heads. By using all those information, we have been able to design an integrated system which can extract the Cu or regenerate the $CuSO_4$ from the used footbath. This machine comes with its own prefabricated footbath which can be installed on the previously made footbath with some minor modification to the previous structure. The footbath will be long enough to allow the cows to have at least three full hoof immersions and are constructed from stainless steel with a rubber pad on the bottom of the footbaths to provide adequate traction for the cows while walking through the footbath. The footbath will also contain an infrared counter that will count the number of animals that pass through the footbath.

Once 150 animals have passed through he footbath a pump will drain the footbath and a secondary pump will flush the footbath with warm water that was generated from the regeneration of $CuSO_4$. The used footbath will be pumped through a series of screens to remove large debris. The effluent will then enter an electrolysis tank where elemental Cu will be

extracted. This process will also produce H_2SO_4. The elemental Cu and H_2SO_4 will be transferred to another tank where H_2O_2 will be added to generate $CuSO_4$ and water. This process is exothermic and will generate heat. A cooling water jacket surrounding the tank will transfer the heat to the water in the water jacket which will create hot water.

The hot water will be used to flush the previously emptied footbath. After flushing the footbath with hot water the regenerated $CuSO_4$ solution will be pumped back into the footbath to create a clean footbath $CuSO_4$ solution ready for use. All pumps, tanks, and computers will be housed on one trailer that can be moved with a tractor. Energy consumption is minimal, and the chemicals that we are using for are reasonably cost effective. There is an option of using solar panels to provide the energy for the electrowinning process. The return on investment for this machine is calculated to be less than four years.

5.2. Introduction

Footbaths have been used for several decades to manage and prevent digital dermatitis (Thomsen, 2015). Digital dermatitis is the most critical infectious claw disorder in the modern dairy (Holzhauer et al., 2012). Producers use different types of disinfectants in footbaths for treating and preventing digital dermatitis; Copper (Cu) and zinc (Zn) sulfate (SO_4), formalin, soap and antibiotics (Cook, 2006) (Shearer and Amstel, 2007) (Solano et al., 2017). However, the most predominant disinfectant used in dairy footbaths is $CuSO_4$. After use, footbath solution typically is contaminated with cattle manure, soil, and other debris and is typically released into the premise lagoon. The lagoon effluent is then applied to crops adjacent to the dairy operation (Epperson and Midla, 2007; Ippolito et al., 2010). Copper is a crucial elements for plants; however, excessive amount of Cu in the soil will cause heavy metal contamination which will

reduce the productivity of the crops (Nagajyoti et al., 2010; Ippolito and Moore, 2013; Adrees et al., 2015; Küpper and Andresen, 2016).

Different researchers and organizations have tried to develop alternatives to $CuSO_4$ use in footbaths (Cook, 2006; Kulow et al., 2015). However, to date, alternative solutions that have been investigated, are more detrimental to the environment and not as effective at preventing digital dermatitis as $CuSO_4$ (Speijers et al., 2010; Fiedler et al., 2013; Smith et al., 2014). Numerous research has been conducted to develop technologies to remove heavy metals ions from waste streams (Fu and Wang, 2011). Yet, to our knowledge, no technologies have been developed to remove Cu from $CuSO_4$ footbath solution in an efficient, economical, and practical manner.

In the late 1990's Mr. William Wailes Jr., Department Head and Dairy Extension Specialist, Department of Animal Sciences, Colorado State University recognized that Cu bioaccumulation in soil and plants irrigated with lagoon effluent where $CuSO_4$ footbaths solution were being used could potentially become a problem. Close to 20 years later, our research team has attempted to develop a high efficiency, low cost system to remove Cu form used footbath solution and reuse the extracted Cu in subsequent footbaths.

5.3. General Electrowinning Background

Copper electrowinning is the extraction of Cu metal onto a cathode from the electrolyte. Usually, electrowinning is the final step in the elemental Cu production process from the oxide ores which have been treated with sulfuric acid. This technology is available and has been used as an accepted way to purify Cu since the beginning of 1900's (Kafumbila, 2017). Currently, most of the electrowinning tank houses utilize reusable 316L stainless steel blank cathode. This technique is called permanent cathode technology (Schlesinger et al., 2011). The 316L stainless

steel is a specific type of alloy and may contain up to 0.08% carbon by weight and is resistant to corrosion (Winters, Gary L. and Nutt, 2003). Modern Cu electrowinning anodes are cold-rolled lead (Pb) alloy with 1.35% Sn (Tin) and 0.07 to 0.08% Ca. The Sn provides strength, corrosion resistance, and corrosion layer conductivity. Calcium enhances the mechanical properties and decreases the anode potential (Robinson et al., 2013).

In Cu extraction facilities, electrowinning cells are made of a particular type of concrete, and they are 6.5 to 8 m long, 1.2 m wide and 1.5 m deep. These cells are approximately 12 m^3 in volume (Schlesinger et al., 2011). Stainless steel cathodes are 1.2 m long, 1.0 m wide and has a thickness of 0.003 m. The Pb-Sn-Ca alloy anodes are a slightly smaller than the cathodes to prevent, to prevent Cu plating around the sides of the cathode. The electrowinning operation in the industry is an open system, and the electrolyte will be added to the cell with a constant flow of ~15 m^3 /hour. There is a continuous flow of the electrolyte from a manifold at the bottom of the electrowinning cell (Figure 5.1). This figure shows a schematic view of an electrowinning cell. Anodes and cathodes are designed and installed alternately. Busbars are responsible for the electric contact with a manifold that distributes the electrolyte towards the electrodes (Schlesinger et al., 2011).

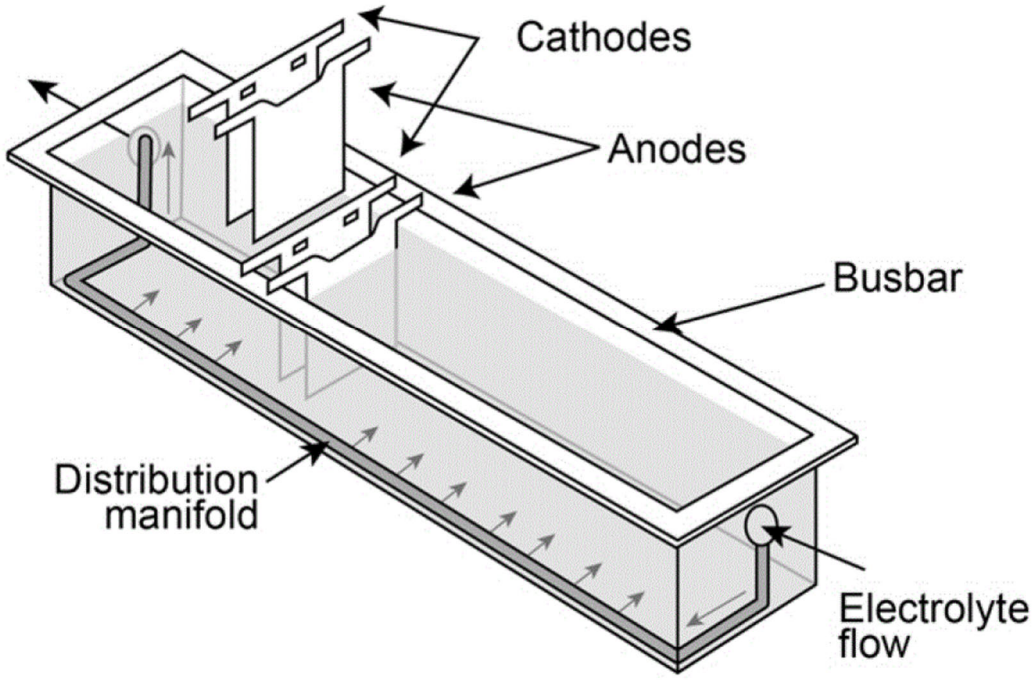

Figure 5.1. Schematic view of an electrowinning cell (Schlesinger et al., 2011)

In the industry, Cu harvesting from the cathode occurs after 4 to 7 days, and typically 50 to 55 kg of Cu will be plated on each side of the cathode (Schlesinger et al., 2011). The cathode will be taken out of the cell with an overhead crane. In the Cu extraction plants, the electrolyte from the solvent extraction has approximately 45 g/Liter of Cu^{2+}, and the spent electrolyte has 5 g/Liter of Cu^{2+}, this is almost 89% extraction efficiency. Having a better conductivity in the electrolyte will reduce the energy and electricity consumption of operation. Two factors affect the electrolyte conductivity in the electrowinning process:

- Electrolyte temperature and
- pH of the electrolyte solution.

The optimum temperature for conductivity is 50 to 55°C (Schlesinger et al., 2011). A modern electrowinning plant produces high purity Cu, using high current, at the same time try to minimize power consumption (Marsden, 2008).

The current efficiency can be measured by the Faraday law:

$m = MIt\varepsilon/nF$

Where m is the mass of Cu plated (g); M is the molar mass of Cu (63.55 g/mol); I is the current passed (A); t is the time for which the current is passed (s); ε is the current efficiency (i.e., the fraction of the total current used in producing Cu; n is the number of electrons involved in the plating of Cu (equal to 2 from; and F is the Faraday constant (96,485 C/mol of charge = 96,485 A/mol).

Using the Faraday law, we can calculate the current efficiency (ε):

ε = 100 (measured mass of Cu plated/theoretical mass of Cu plated)

Current efficiencies in modern Cu electrowinning plants are from 85 to 95% (Robinson et al., 2010).

Some factors can significantly affect the cathode purity, such as positioning the cathodes vertical to the extraction solution which maximized the surface area and prevents the cathode and anode from making contact with each other (Maki, 1999). Further improvements in electrowinning can be achieved by: 1) enhancing energy efficiency and 2) generating high purity Cu at higher current densities (Blackett and Nicol, 2010).

Using anodes with precious metal coating instead of Pb will not only prevent Pb contamination but will also lower the energy consumption by 15% (Schlesinger et al., 2011). However, there are some downsides to these anodes:

- Higher initial investments and
- They need to be handled gently to avoid damage to the precious metal coating.

By using the aforementioned information, we have designed a system which is capable of extracting the Cu efficiently and economically from dairy $CuSO_4$ footbaths. Overall two strategies were developed:

- The first approach is Cu extraction only. The premise of this approach is to sell the purified Cu and to help recover the extraction costs. The other byproduct generated in this process is H_2SO_4, which is a hazardous waste. By adding $CaCO_3$ to the acid, we are capable of producing gypsum. Gypsum can be used in different ways such as fertilizer or in construction building materials.
- The second approach is to regenerate $CuSO_4$ from the used footbath. In this method, the Cu is extracted using electrowinning. This will generate pure Cu and H_2SO_4. Copper and H_2SO_4 would then be placed in a separate container and a strong oxidizing agent (H_2O_2) would be added to the solution to generate $CuSO_4$ and H_2O. In the next section, we will look at the details of the design of the system and how it works.

5.4. Design

When designing the system, compatibility to current dairy footbaths and ease of use were taken into consideration. Our system is categorized into two main components of indoor and outdoor components.

The indoor components are as follows:

Stainless steel footbath: 2.5 m in length, 0.9 m wide and 0.15 m deep with a volume of approximately 340 liters (Figure 5.2). The stainless-steel sheet that we would use for this footbath would be 316L with the thickness of 3 mm. This sheet can tolerate more than 1500 kg/m^2 of compression force. The footbath would also have a 0.5 m stainless steel splash guard extension on both sides of the footbath. As shown in Figure 5.2, there are four nozzles on each

side. These nozzles are connected to a booster pump which is attached to the warm water line. After 150 cows pass through the footbath, an electric valve (76.2 mm) will open and all the used footbath solution will be pumped to the filtration tank. After the removeal of the used footbath a second pump will be activated to pressure wash the footbath for 30 seconds. The water used for the footbath washing prcedure will be warm water generated from the heat produced when regenerating $CuSO_4$. The black covering at the bottom of the footbath demonstrates the antislip flooring (Industrial Anti-Slip Mats, Safeguard, Streetsboro, Ohio). The footbath is designed to gradually slope for better hoof immersion and draining purposes.

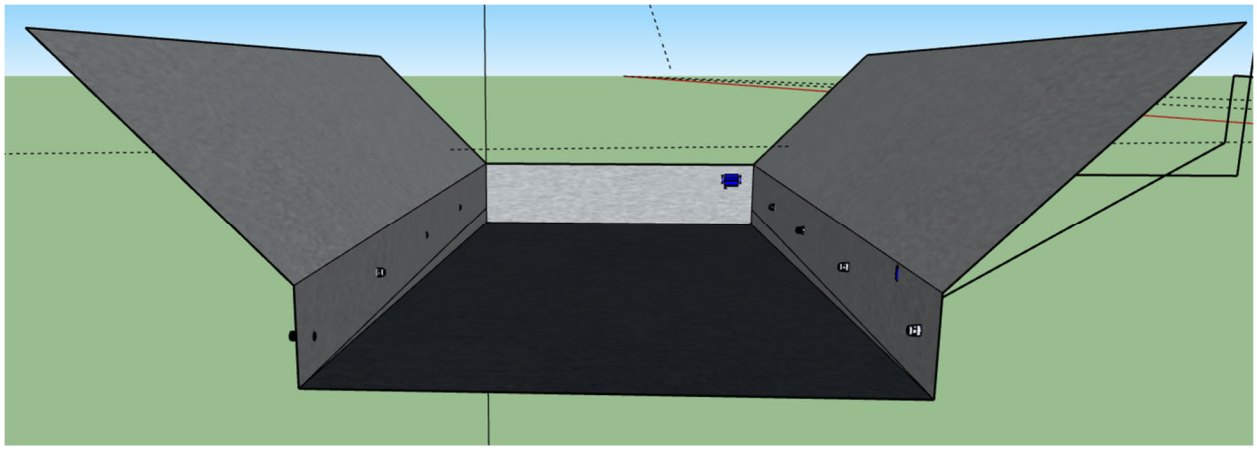

Figure 5.2. Stainless steel footbath design

Figure 5.3 shows the right view of the footbath with an electric valve. This electric valve is responsible for the flow of new (regenerated) $CuSO_4$ solution back into the footbath.

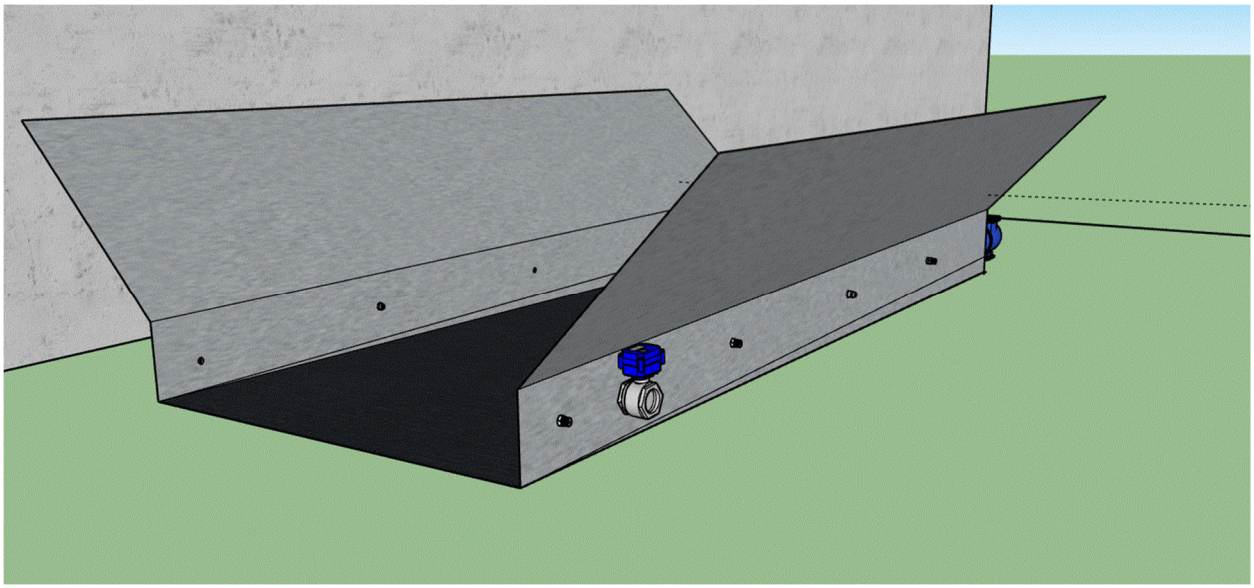

Figure 5.3. Right view of stainless steel footbath with electric pump

Figure 5.4 depicts left view of the footbath demonstrating water nozzles and the slight slope of the footbath for better immersion of the hooves in the footbath solution.

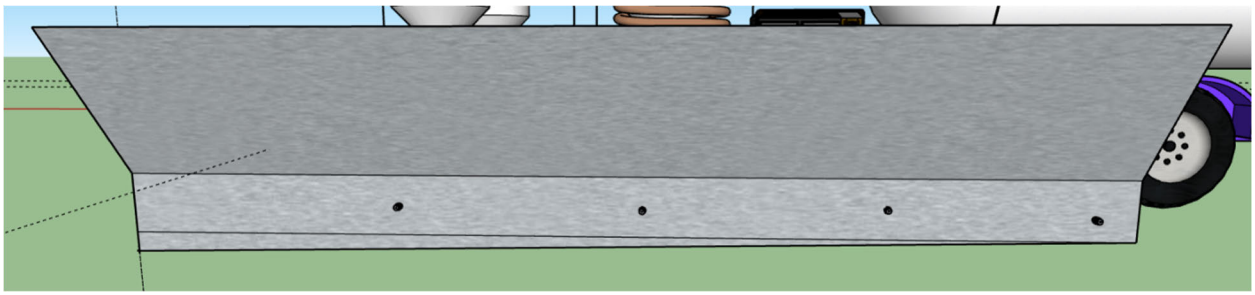

Figure 5.4. Left view of stainless steel footbath with water nozzles

Figure 5.5 shows the back side of the footbath featuring a 400 liter/min pump which is responsible for emptying the footbath and transfering the used solution to the filtration tank. The pump is attached to an electronic valve which will get open after 150 cows pass through the footbath.

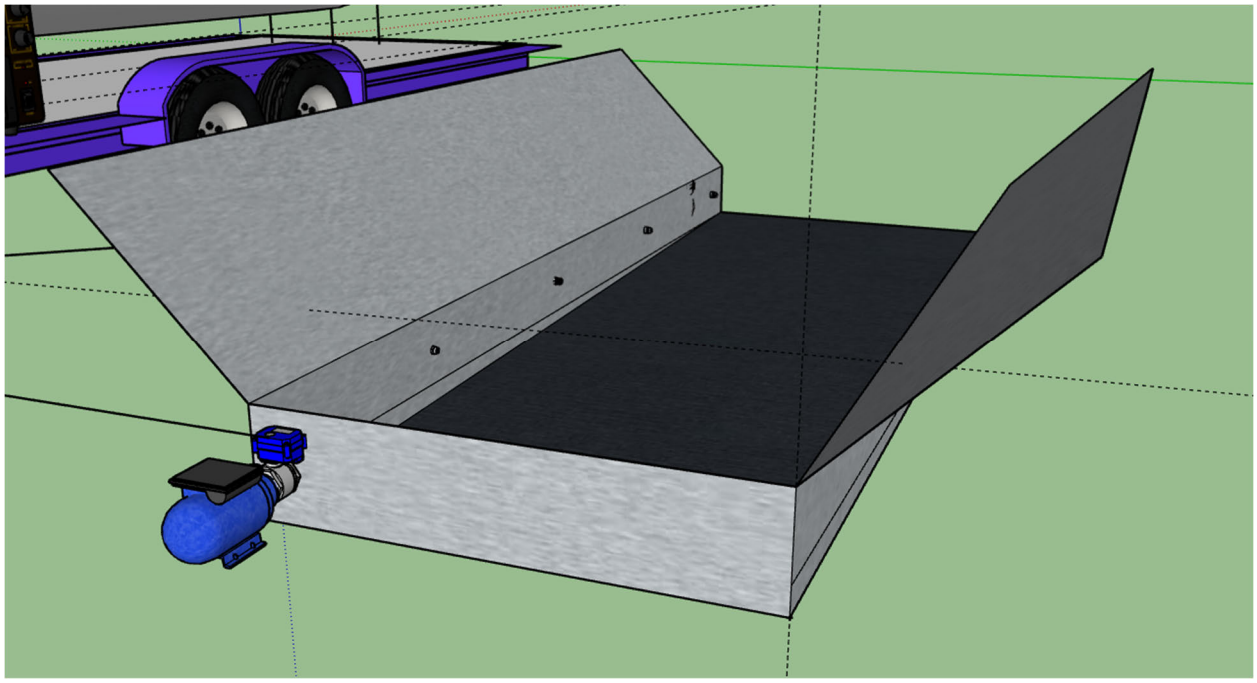

Figure 5.5. Back view of stainless steel footbath with pump for emptying used solution

An infrared counter (TrailMaster's TM300, Goodson & Associates, Lenexa Kansas, United States) will be installed at the exit of the footbath, and it will be connected to the valve and also a 400 L/min pump (AMT Self-Priming Solids Handling Pump - 110 GPM, AMT Pump Company, Royersford, Philadelphia, United States) which will be installed below the footbath (Figure 5.5).

The outdoor components are as follows:

The used footbath solution will be transferred to a 400 L filtration tank with two sets of 1 mm (Mesh 18) and 0.105 (Mesh 140) basket on top of it to capture the large debris. At the bottom of the tank, there will be a large replaceable S7M120 cartridge filter with 584.2 x 482.6 x 482.6 mm dimensions (STA-Rite, Pentair, Delavan, WI, United States). After filtration, the filtered footbath will be pumped out of the filtering tank to a 400 L electrolysis chamber (r = 0.3 m, h = 1.4 m) containing three electrodes: one cathode, and two anodes. The cathode will be a

16-gauge Cu sheet (thickness of 1.58 mm), 1.0 m long and 0.4 m wide, and will be positioned at the center of the tank.

Figure 5.6 shows all of the inside and outside components of the system together. There are three tanks on the left side which are 400 L each. The first one to the left is the filtration tank, the other tank with the Cu coil around it is the electrolysis tank, where the Cu coil will serve to cool the system and provide a source of warm water to clean the footbath. The third tank which is located at back of the picture is the $CuSO_4$ tank. The $CuSO_4$ tank contains the final product, and it is connected to the footbath.

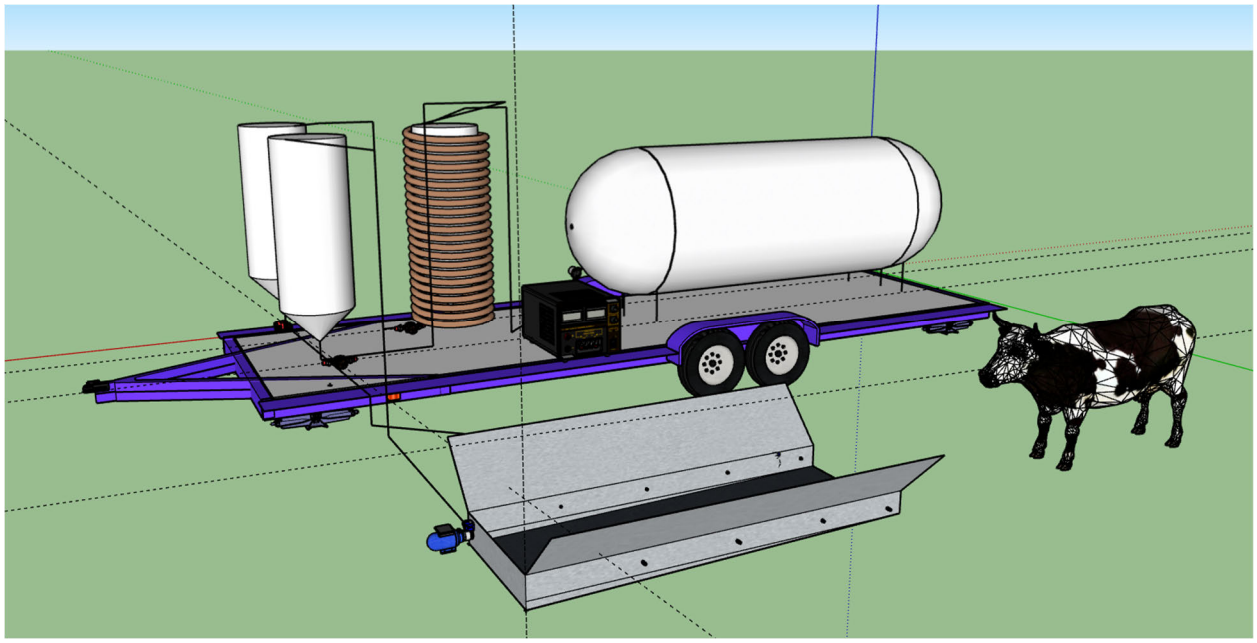

Figure 5.6. Holistic view of the system with both indoor and outdoor components

The anodes are two platinized titanium mesh anodes (type A, ital.V, Metakem, Usingen, Germany; Figure 5.7), coated with 50 g Pt/m^2 nominally 2.5 µm, 0.8 m long and 0.3 m wide. The anodes will be on both sides of the cathode, and 0.1 m apart from the cathode. The lid of the electrowinning tank has a wedge shape scraper which will scrape the Cu off of the cathode when

it is pulled out by a winch. The scraper's opening will be 1.6 mm wide on the top part and will gradually tighten at the bottom to reach 1.50 mm.

Polyethylene cathode holding rails retain the stainless steel stocking or steel wool to avoid any possibility for shorting between the cathode and the anode mesh. It will also help the cathode to be positioned vertically. The wire that will be used for the electrodes and the DC power source will be 8 gauge wire with the thickness of approximately 3 mm (> 60 A). Wires will be color coated to demote positive and negative charges. There will be a busbar connection on one side of the electrodes to maintain electrical contact. For the anodes, the busbar will be on the same side for both electrodes.

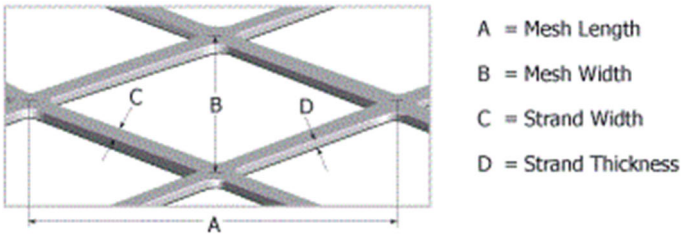

Figure 5.7. Mesh shape and dimensions A=10 mm, B=5.0, C=1.0, D=1.0 (Metakem GmbH, 2018)

A digital DC power supply (Air cooled rectifier 5 - 50 A, Dynapower, S. Burlington, Vermont) will be used to generate the current.

A Honeywell R300-F35-M14-C temperature sensor (Morris plains, New Jersey, United States) will be located at the bottom of the tank which is connected to the rectifier's controller to reduce the voltage and current when the temperature reaches 65°C. After the current reaches the target amps (20 A), it will read no resistance to the solution and the system will automatically stop the electrowinning process.

Immediately after the electrowinning process, the sulfuric acid will be pumped out to another 400 L tank. Since we know that we need 6 gr of $CaCO_3$ for 100 ml of H_2SO_4 to raise the pH to approximately 6, we need 18 Kg of $CaCO_3$ for 300 liters of H_2SO_4. Since there is gas and

foam production due to CO_2 and H_2O generation, 18 Kg $CaCO_3$ will need to be added intermittently. After raising the pH to 6, we can either release the water to the environment or inject it into the irrigation system for irrigation.

5.5. Regeneration of $CuSO_4$

After the electrowinning, a winch will lift the cathode to through a scraper to remove the Cu. The operator will take out the electrodes and will turn the H_2O_2 tank's pump on to add the H_2O_2 to the solution. The 35% H_2O_2 will be housed in a 7000 L polyethylene capsule shape tank with 0.75 m radius and 3 m height.

Since this reaction is exothermic and will generate heat, H_2O_2 will be added intermittently. At the same time that H_2O_2 is added, a Polyethylene agitator will start to agitate the mixture to allow for $CuSO_4$ to dissolve into solution. Approximately 10 minutes of agitation, a new batch of $CuSO_4$ solution will be ready to be pumped back into the footbath. The emptying of the regenerated $CuSO_4$ footbath solution will occur immediately after the used footbath solution is emptied from the footbath. This will allow a continued supply of regenerated $CuSO_4$ footbath solution. Since the ratio of H_2O_2 to the H_2SO_4 and Cu is 3:1, the H_2O_2 tank can supply enough H_2O_2 for seven regenerations.

Figure 5.8 shows the complete system with 1 kW (5 m^2) solar panel, a 7,000 L H_2O_2 tank, DC power source, 400 L electrolysis tank with Cu coiling, a 400 L filtration tank, a 400 L $CuSO_4$ tank, pumps and winch with the stand to pull the cathode out of the tank to remove the Cu. All these parts are sitting on a trailer with a gross weight of approximately 18,000 kg.

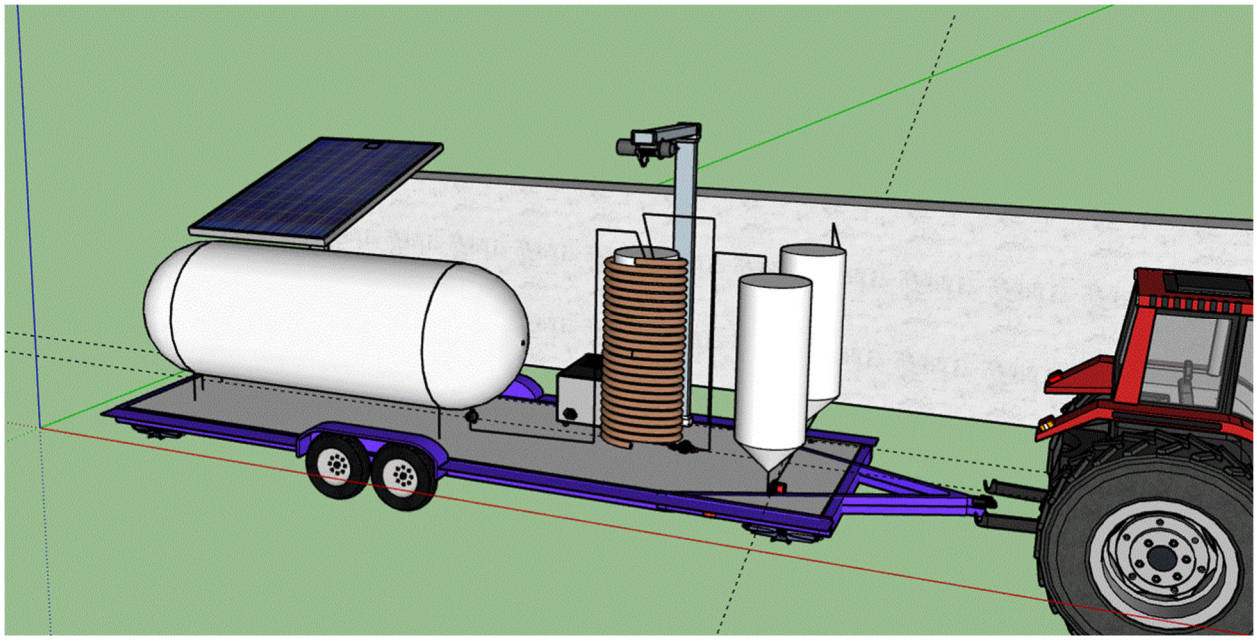

Figure 5.8. View of the complete regenerating system

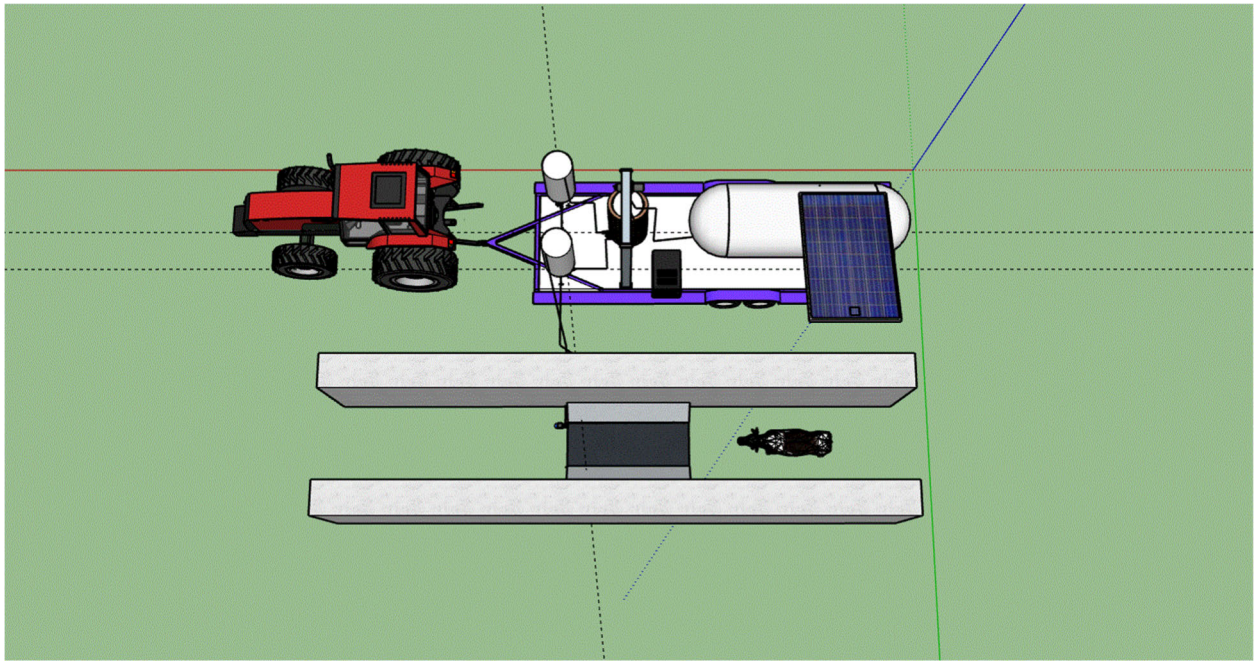

Figure 5.9. Top view of the complete $CuSO_4$ regenerting system

5.6. Economic Analysis

There are two different aspects that were considered during the design of this systems: the economic aspect, and the environmental impact. In the Cu refining industry, the cost of

electro refined Cu from a 0.5% Cu ore plus 10% fixed capital investment is $33,000 per annual metric ton of Cu (Schlesinger et al., 2011). Ore grade has a direct influence on the amount of investment. An open-pit mine with high-grade ore operations with the capacity of 250,000 tons per day needs an investment of more than 7.5 billion dollars. Figure 5.10 shows a table of fixed investment costs of Cu extraction and mining facilities per annual per ton of Cu production.

Facility	Fixed investment cost ($U.S. per tonne of Cu per year)
Mine (open pit)	10,000
Concentrator (including water acquisition and recycle)	10,000
Smelter (Outotec flash furnace smelting/converting), including sulfuric acid plant	9,000
Electrolytic refinery (excluding precious metals refinery)	1,000
Total	30,000

Figure 5.10. Fixed investment costs of copper extraction and mining facilities per annual per ton of copper production (Schlesinger et al., 2011)

Copper extraction is profitable when the price per kg of Cu is above $6/kg, and expansion of the industry is encouraged. When the price goes below $3/kg, some operations will be closed. Underground ores have about 1.5 % Cu that is attainable, as are open-pit ore bodies containing about 0.5% Cu (Schlesinger et al., 2011). It must be noted that costs of operation are five times higher in underground mining in comparison to open-pit mining. Figure 5.11 shows price quotes for high grade Cu (USD/lbs.) as of end of day April 5th, 2018, which is $6.75/kg (NASDAQ, 2018).

Figure 5.11. High grade copper (USD/lbs.) as of end of day April 5th, 2018

The 1,200 head dairy operation in northern Colorado that was used to obtain our samples has two footbaths. 22.6 kg of $CuSO_4$ is being used for each footbath twice a day. This brings the total use of $CuSO_4$ to 90.7 kg/day of $CuSO_4$, which is an annual consumption of more than 33,106 kg (33 metric tons). Since 25% of the $CuSO_4$ is Cu, 8,276 kilograms (8.2 metric tons) of pure Cu are used every year by this operation. With the price of $6.75/kg of Cu, the annual amount of Cu used in this operation is worth approximately $56,000.

Based on several economic variables, there are three different options to select when extracting Cu form $CuSO_4$ footbaths. One option would be to extract the Cu and sell the extracted pure Cu and H_2SO_4. Another option would be to add calcium carbonate to H_2SO_4 and make gypsum, which could be sold or used as fertilizer. The third option would be to regenerate the $CuSO_4$ by adding a strong oxidizer like 35% H_2O_2 to the Cu and H_2SO_4 solution.

In the proposed design, the footbath (300 liters) is smaller and would require 15 kg of $CuSO_4$ instead of 22 kg to make a 5% footbath solution. With the assumption of regeneration

after 150 head cows, we need to change the footbath three times per day per footbath. Since we are using 15 kg of $CuSO_4$, the expected extracted Cu per footbath will be 3.75 kg. With the extraction efficiency of 80%, approximately 3 kg of Cu would be extracted per extraction. With these assumptions, approximately 9 kg of Cu could be extracted on a daily basis, which amounts to an annual value of $22,000. The direct operating costs per kg of Cu is shown in Table 5.1. Fixed investment costs can be found in Table 5.2.

Table 5.1. The direct operating costs per kg of Cu extracted from the used footbath

Activity	**Direct Operating Cost (USD/Kg of Cu)**
Electricity consumption	$0.3
Labor and overhead	$1.0
Total	**$1.3**

Table 5.2. Fixed investment costs for the complete Cu regeneration system

Parts	**Cost (USD)**
340 liters, 316 L stainless steel footbath	$1,000
Trailer (GVWR 7000 kg)	$5,000
Polyethylene tank (2000 liters)	$1,000
3 Polyethylene tanks (400 liters)	$2,000
Dynapower rectifier 110VAC 0-50A	$4,500
Cathode copper sheet 1.0 m x 0.4 m (thickness of 1.58 mm)	$100
2 platinized titanium mesh 2.5 μm, 0.8 m x 0.3 m	$2,000
AMT Self-Priming Solids Handling Pump – 440 liters/min	$1,000
2 Grundfos CM15-2 A-S-I-E-AQQE Centrifugal Pump	$3,000
TrailMaster's TM300	$200
Wiring and plumbing	$2,000
Pulling electric winch 500 kg	$500
Solar panel 1 kW (2.5m x 2m)	$1,500
10% installation	$2,380
Total	**$26,180**

Tables 5.3 and 5.4 show fixed investment costs for complete $CuSO_4$ regeneration system and direct operating costs for $CuSO_4$ regenerating machine per liter of 4% $CuSO_4$, respectively.

Table 5.3. Fixed investment costs for complete $CuSO_4$ regeneration system

Parts	**Cost (USD)**
340 liters, 316 L stainless steel footbath	$1,000
Trailer (GVWR 18000 kg)	$10,000
Polyethylene tank (7000 liters)	$1,000
3 Polyethylene tanks (400 liters)	$2,000
Dynapower rectifier 110VAC 0-50A	$4,500
Cathode copper sheet 1.0 m x 0.4 m (thickness of 1.58 mm)	$100
2 platinized titanium mesh 2.5 µm, 0.8 m x 0.3 m	$2,000
AMT Self-Priming Solids Handling Pump – 440 liters/min	$1,000
3 Grundfos CM15-2 A-S-I-E-AQQE Centrifugal Pump	$4,500
Grundfos 98500195, dosing tank station DTS 52GT 0004FEF0A0H	$1,000
TrailMaster's TM300 livestock counting sensor	$200
Wiring and plumbing	$2,000
Pulling electric winch 500 kg	$500
Solar panel 2 kW (2.5m x 2m)	$1,500
10% installation	$3,130
Total	**$34,430**

Table 5.4. Direct operating costs for the $CuSO_4$ regenerating machine per liter of 4% $CuSO_4$

Activity	**Direct Operating Cost (USD/Liter of 4% $CuSO_4$)**
Electricity*	< 0.01
Labor** and Overhead	0.02
Cost of H_2O_2***	0.6
Total	**$0.62**

* Assuming that the electricity cost is $0.12/ kWh.
** Calculated with the salary of $20/ hour
*** %35 H_2O_2 with the price of $200/metric ton.

5.7. Conclusion

By reviewing our proposed design, extracting Cu and selling the extracted Cu has a solid return on investment. With approximately $27,000 fixed investment costs and $5.3 per kg of Cu including the labor and overhead, the producers will still be able to make a profit from direct Cu sales to pay for $CuSO_4$ purchases. They can also either add $CaCO_3$ to produce gypsum and use the water for irrigation or collect the H_2SO_4 in the 7000 liters tank and sell it to the consumers and use some of it for their own use in the dairy.

For the regeneration of $CuSO_4$ solution, there is a higher fixed investment cost in comparison to the Cu extraction only. The $CuSO_4$ regenerating system seems to be more integrated; however, there is a downside which is the large amount of H_2O_2 that should be used as the powerful oxidizer to convert the Cu back into $CuSO_4$. If alternative oxidizing agents could be used that were environmentally more powerful than H_2O_2 this method will be more practical than the currently proposed.

It should be remembered that the primary goal of this research was to prevent the heavy metal contamination in the soil due to $CuSO_4$ footbath use in dairies which we believe is feasible

based on the data presented in this dissertation. Future research is encouraged to make this system more efficient and sustainable.

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APPENDICES

APPENDIX A

CHAPTER 2 - MISCELLANEOUS EXPERIMENTS AND NOTES

After our proof of concept experiments, we went to another dairy in northern Colorado. This dairy was milking 1200 head and had two footbaths on both sides of the milking parlor. Each of the footbaths held approximately 550 liters of the footbath. They scheduled their footbath program to add 22.7 kg of $CuSO_4$ in each footbath twice a day.

In the beginning, we obtained the samples just prior to the time they were changing the footbath for the next shift. The viscosity of the sample was high due to the significant amount of organic matter contamination. We transferred 250 ml of the footbath sample to a 400 ml beaker and we measured the initial pH which was 3.6. As soon as we began electrolysis, foam buildup occurred.

The next day I transferred the used footbath solution to six 50 ml conical tubes (Falcon 50mL conical centrifuge tubes) and centrifuged the samples at 1,200 x *g* for 15 min at room temperature prior to electrolysis. After centrifugation, I add 250 ml of the sample to a 400 ml beaker and began electrolysis at 20V and 15A. As soon as we start the electrolysis, it began to generate foam.

I have also tried different compounds to stop the solution to generating foam. One of the compounds that we used was vegetable oil. We added 10 ml of vegetable oil to our spent footbath solution (250 ml) started the electrolysis with 20V and 15A for 15 min, but it did not inhibit foam build up. Another compound that I tried was an anti-foaming agent from Birko (Birko, 2015). Birko Antifoam 10, Birko Corporation, Henderson Colorado, which was a

silicone antifoam agent. We added 5 ml of the solution to our spent footbath solution (250 ml) and ran the electrolysis at 20V and 15A for 15 min. Birko Antifoam 10 inhibited foam buildup but our extraction efficiency went down substantially.

We obtained footbath samples from another dairy which was using zinc sulfate ($ZnSO_4$) in their footbaths. For this experiment, we changed our cathodes from Cu to zinc (Zn). The electrodes were Frey Scientific Zn electrode strips, 12.7 cm long, 1.9 wide, with a thickness of 0.11 cm. We used 7.14 ml of the initial premade zinc sulfate and added 243 ml of deionized water to reach the total volume of 250 ml. The initial pH of the solution was 6. We began electrolysis with 15 A and 20 V for 10 minutes. After analyzing all solutions for Zn concentrations (ICP analysis) we calculated a greater than 95% extraction efficiency (Figure A-1).

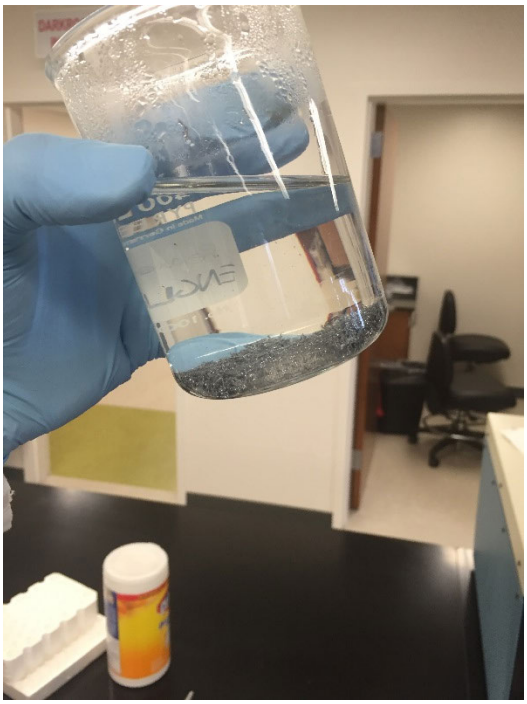

Figure A.1. Extracted zinc sitting at the bottom of the beaker after electrolysis of the footbath (Jalali and Engle, 2016)

APPENDIX B

CHAPTER 3 – R CODES

```
setwd("/Users/home/Desktop /Sam/anova_aug_2017")



dat <- read.csv("data.csv",header=T)

head(dat)

dat <- dat[,-c(11:20)]

head(dat)



dat2 <- filter(dat,Final_pH < 4)

dim(dat)



dev.new()

qplot(Num_of_Cows,Final_pH, data=dat,

        geom=c("boxplot","jitter"),fill=Num_of_Cows)

ggsave("images/finalpH_by_cows.pdf")
```

```
qplot(Num_of_Cows,Final_pH, data=dat,
        geom=c("boxplot","jitter"),fill=Num_of_Cows)
ggsave("images2/finalpH_by_cows2.pdf")

qplot(Num_of_Cows,Final_pH, data=dat2,
        geom=c("boxplot","jitter"),fill=Num_of_Cows)
ggsave("images2/ifinalpH_by_cows2.pdf")

        #test this
mylm <- lm(Final_pH ~Num_of_Cows, data=dat2)
anova(mylm)
xtable(anova(mylm))
xtable(TukeyHSD(aov(mylm))$Num_of_Cows)

        #without the blank included in "0"
qplot(Cows_and_Blank,Final_pH, data=dat,
        geom=c("boxplot","jitter"),fill=Cows_and_Blank)
ggsave("images2/finalpH_by_cows_and_blank2.pdf")
```

```
        ##
        # break it down by treatment


qplot(Num_of_Cows,Final_pH, data=dat,
        geom=c("boxplot","jitter"),fill=treat)
ggsave("images2/finalpH_by_cows_by_treat2.pdf")


qplot(Num_of_Cows,Final_pH, data=dat2,
        geom=c("boxplot","jitter"),fill=treat)
ggsave("images2/finalpH_by_cows_by_treat3.pdf")


qplot(treat,Final_pH, data=dat,
        geom=c("boxplot","jitter"),fill=treat)
ggsave("images2/finalpH_by_treat2.pdf")


#test this
mylm <- lm(Final_pH~treat, data=dat)
anova(mylm)
```

```
xtable(anova(mylm))

xtable(TukeyHSD(aov(mylm))$treat)



ggplot(data=dat,aes(x=treat, y=Final_pH,fill=treat))+geom_boxplot() +

        theme(axis.ticks.x=
element_blank(),axis.text.x=element_blank())+facet_grid(.~Num_of_Cows)

ggsave("images2/finalpH_by_cows_by_treat4.pdf")



ggplot(data=dat2,aes(x=treat, y=Final_pH,fill=treat))+geom_boxplot() +

        theme(axis.ticks.x=
element_blank(),axis.text.x=element_blank())+facet_grid(.~Num_of_Cows)

ggsave("images2/finalpH_by_cows_by_treat5.pdf")



#test this

        #test this

mylm <- lm(Final_pH ~Num_of_Cows * treat, data=dat)

anova(mylm)

xtable(anova(mylm))

xtable(TukeyHSD(aov(mylm))$Num_of_Cows)
```

```
        # filter by cows


zero <- dat %>% filter(Num_of_Cows ==0)

qplot(treat,Final_pH, data=zero,

        geom=c("boxplot","jitter"),fill=treat, main="0 Cows")

ggsave("images2/zero2.pdf")


zero <- dat2 %>% filter(Num_of_Cows ==0)

qplot(treat,Final_pH, data=zero,

        geom=c("boxplot","jitter"),fill=treat, main="0 Cows")

ggsave("images2/zero3.pdf")


mylm <- lm(Final_pH~treat, data=zero)

anova(mylm)

xtable(anova(mylm))

xtable(TukeyHSD(aov(mylm))$treat)


one_fifty <- dat %>% filter(Num_of_Cows ==150)
```

```
qplot(treat,Final_pH, data=one_fifty,
      geom=c("boxplot","jitter"),fill=treat, main="150 Cows")
ggsave("images2/one_fifty2.pdf")

mylm <- lm(Final_pH~treat, data=one_fifty)
anova(mylm)
xtable(anova(mylm))
xtable(TukeyHSD(aov(mylm))$treat)

three_hun <- dat %>% filter(Num_of_Cows ==300)
qplot(treat,Final_pH, data=three_hun,
      geom=c("boxplot","jitter"),fill=treat, main="300 Cows")
ggsave("images2/three_hun2.pdf")

mylm <- lm(Final_pH~treat, data=three_hun)
anova(mylm)
xtable(anova(mylm))
xtable(TukeyHSD(aov(mylm))$treat)
```

```
four_fifty <- dat %>% filter(Num_of_Cows ==450)

qplot(treat,Final_pH, data=four_fifty,

        geom=c("boxplot","jitter"),fill=treat, main="450 Cows")

ggsave("images2/four_fifty2.pdf")


mylm <- lm(Final_pH~treat, data=four_fifty)

anova(mylm)

xtable(anova(mylm))

xtable(TukeyHSD(aov(mylm))$treat)


six_hun <- dat %>% filter(Num_of_Cows ==600)

qplot(treat,Final_pH, data=six_hun,

        geom=c("boxplot","jitter"),fill=treat, main="600 Cows")

ggsave("images2/six_hun2.pdf")


mylm <- lm(Final_pH~treat, data=six_hun)

anova(mylm)
```

```
xtable(anova(mylm))

xtable(TukeyHSD(aov(mylm))$treat)
```

APPENDIX C

CHAPTER 4 – R CODES AND DIAGNOSTIC PLOTS FOR THE EXPERIMENTAL MODEL

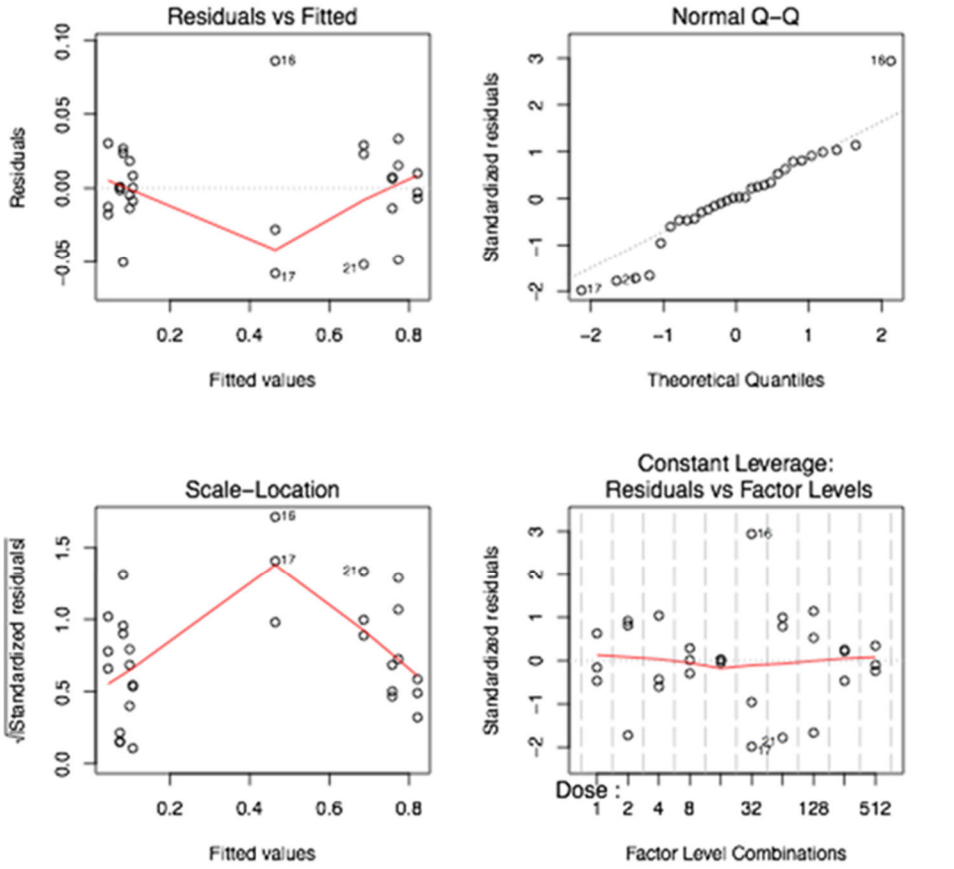

Figure C.1. Diagnostic plots

```
dat <- read.csv("dat.csv", header=T)

names(dat) <- c("Trt","Dose","finalRead","percentInhib","initialCu")


dim(dat)
```

```
        # 180 rows by 5 columns

str(dat)



dat$Trt <- as.factor(dat$Trt)

dat$Dose <- as.factor(dat$Dose)



dat$treat <- as.factor(c(rep(c(rep("Auto+Cent+",3),rep("Auto+Cent-",3),rep("Auto-

Cent+",3),rep("Auto-Cent-",3)),5),"Blank","Blank","Blank"))

dat$treat <- factor(dat$treat, levels=dat$treat)

dat$Num_of_Cows <- as.factor(dat$"Cow..")

dat[32,"Final.pH"] <- 1.76

dat$Final_pH <- as.numeric(as.character(dat$"Final.pH"))

dat$Cows_and_Blank <- as.factor(c(dat$Num_of_Cows[1:60],"Blank","Blank","Blank"))




library(ggplot2)



plot.new()

qplot(dat$finalRead, fill = dat$Trt)
```

```
ggplot(dat, aes(x=Trt, y=finalRead, fill=Trt)) +
                stat_boxplot() +
                stat_summary(fun.y=mean, geom="point", shape=5, size=4) +
                ggtitle("Final Readings")
ggsave("finalReadings.jpg")
ggplot(dat, aes(x=Trt, y=percentInhib, fill=Trt)) +
                stat_boxplot() +
                stat_summary(fun.y=mean, geom="point", shape=5, size=4) +
                ggtitle("Percent Inhibition")
ggsave("percentInhib.jpg")


dev.new()



ggplot(dat, aes(x=Trt, y=finalRead, fill=Dose)) +
                stat_boxplot() +
                ggtitle("Final Readings by Dose")
ggsave("finalReadings_by_Dose.jpg")
```

```
ggplot(dat, aes(x=Trt, y=percentInhib, fill=Dose)) +
        stat_boxplot() +
        ggtitle("Percent Inhibition by Dose")
ggsave("percentInhib_by_Dose.jpg")


    ##
    #  individual plots
    ##
one <- filter(dat, Trt=="1")
ggplot(one, aes(x=Dose, y=finalRead, fill=Dose)) +
        stat_boxplot() +
        ggtitle("Final Readings by Dose - Trt 1")
ggsave("finalRead_Trt1.jpg")


for (i in 1:6) {
    temp <- filter(dat, Trt==i)
    ggplot(temp, aes(x=Dose, y=finalRead, fill=Dose)) +
        stat_boxplot() +
```

```
            ggtitle(paste("Final Readings by Dose - Trt",i))

        ggsave(paste("finalRead_Trt", i, ".jpg", sep=""))

        }

        #  Run an ANOVA on Dose


one <- filter(dat, Trt=="1")

model <- aov(finalRead ~ Dose, data=one)

summary(model)

        # treatment IS significant

        #   p-value < 2e-16


TukeyHSD(model)


Fit <- lm(finalRead ~ Dose, data=one)

summary(Fit)

par(mfrow=c(2,2))

pdf("images/fit_resid.pdf")

plot(Fit)
```

```
dev.copy(pdf,"images/fit_resid.pdf")

dev.off()



dev.new()




        #  Run a two-way ANOVA to determine whether an effect exists

        #    for Treatment and the other variables



model <- aov(finalRead ~ Dose*Trt , data=dat)

summary(model)

        #   again...treatment is significant

        #     p-value < .0000000000000226

TukeyHSD(model)



anova(lm(finalRead~Trt*Dose, data=dat))

anova(lm(finalRead~Dose*Trt, data=dat))
```

```
        # Do the appropriate Type 3 SS
library("car")
options(contrasts=c("contr.sum","contr.poly"))
fit <- lm(finalRead ~ Trt*Dose, data=dat)
model <- Anova(fit, singular.ok =T, type="III" )
summary(model)
model
xtable(model)



Anova(lm(finalRead ~Trt * Dose, data=dat, contrasts=list(topic=contr.sum,sys=contr.sum),
type=3))
Anova(lm(finalRead ~Trt * Dose, data=dat, type=2))
```