Bacterial Inhibition in Waste-Water/Fracking Water Using Copper Ion Solution

by

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ABSTRACT

Natural gas serves as a major energy supply in United States. Hydraulic fracturing is the process of extraction of unconventional natural gas from the tight sandstones, and shale's bed. Hydraulic fracturing uses fracking fluid which consists of large volumes of fresh water, proppants (e.g. Sand), slicking agents, and antimicrobial solutions. These flow back water carries a large amount of bacteria which are responsible for the biofilm formation that can eventually clog the fissures (source) and inhibit gas extraction during down-hole production. Numerous toxic chemicals are used as biocides to eliminate the biofilm. These biocides are toxic and can deteriorate the subsurface environment. There has been exploration of alternative products for viable bacterial control but none are without any human health and/or environmental impacts. To replace these biocides, the potential use of copper solution was examined as a safer alternative to traditional biocides. Copper ions or copper compounds have been used to inhibit bacteria in many forms like solid and, liquids for centuries. Copper solutions can impede the bacterial growth while reducing the harm to the environment. This research was mainly focused on two objectives: i) to test the effectiveness of copper solution against gram positive and gram negative bacteria and ii) to explore the efficacy of copper ion solution as comparison to flow back biocide solutions.

The bacteria *Escherichia coli* and *Staphylococcus aureus* were used to represent gram negative and gram positive cells. The *Staphylococcus epidermis* is an aerobic bacteria present in some fracking wells which can also be experimented on in the culture.

The experiment was performed using 30mL copper ion solution (250ppm Cu^{+2}) and equivalent volume of flow back water, which were added to bacteria cultured vials at 4-6 hours to investigate the ability to inhibit bacterial strains. The growth with copper treatment was impeded for approximately 12 hours for gram negative bacteria and approximately 8-10 hours for gram positive bacteria. However, flow back water was found to inhibit all bacteria growth for up to 48-56 hours as concluded using both turbidity and viable cells count test results. To mimic the flow back water antimicrobial properties, copper ion solution may need to be added at 10-12 hours interval to replace the toxic chemicals used in fracturing fluids. It is not clear what chemicals, concentrations of the chemicals or the cost of the chemical are used in flow back water. Future studies should investigate and optimize the cost and economic viability of the copper solution. Copper and its compound are considered to be benign to the environment and they can also be toxic to deleterious bacteria, this property of copper would not only help fracking industries to remove biofilm problem but also help to preserve the subsurface environment.

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Table of Contents

Table of Figures

List of Symbols

mg	milligram
°C	degree centigrade
hr	Hour
l	liter
mM	millimolar
g/L	gram/liter
N	Normality
F	Fahrenheit
mm	millimeter
nm	nanometer
AU	Absorbance Unit
mL	milli liter
rpm	revolutions per minute
ppm	parts per million
CFU/mL	colony forming unit/milliliter

Chapter 1. Introduction

Natural gas contributes a major supply of energy to meet the United States demands. It has been observed that natural gas production from shale accounted about 50% of all natural gas produced in the US in 2014 (AEO, 2015). Hydraulic fracturing is the process necessary for the extraction of unconventional natural gas from the tight sandstones, shale's bed and some coal beds. It is used in low permeable reservoirs to increase the permeability and make gas production possible. Although it was considered to be uneconomical to exploit such natural resources at a commercial scale, new technologies in the shale gas industry has made it a new thriving industries in the world today (McGlade *et al*., 2013).

Natural gas is an odorless, colorless mixture of short hydrocarbons, primarily methane. It is considered a "bridge fuel" to renewable natural resources because of its low carbon footprint and low concentration of contaminants like nitrogen oxide (NOx), carbon dioxide (CO_2) and sulfur oxide (SOx) than compared to that of coal or petroleum. Horizontal drilling and hydraulic fracturing (hydrofracking or fracking) is the best feasible technology for harvesting this energy source by the use of special recipe fracturing fluids (Entrekin *et al*., 2011).

1.1 Problem Description

Hydraulic fracturing is carried out by using hydrofracking fluid which consists of large volumes of fresh water, proppants (e.g. Sand) and numerous chemical additives such as biociders, friction reducers, and scale reducers. The additives, which make up less than 2 percent of frack fluid by mass, helps improve the gas production by increasing proppant delivery to fractures. This also helps maintain integrity of the well by reducing

the likelihood of microbial fouling, scale building or well scouring. Hydraulic fracturing typically requires one to four million gallons of water for a well where 10-50% of these injected fluids return to the surface (Arthur *et al*., 2009; Paugh *et al*., 2008; Soeder et al., 2009). Flow back fluid is injected into the formation at high pressure, which helps to create fractures whereas added proponents such as sand allow the gas to flow. The operators reuse these volumes of returned flow back fluids so as to reduce the amount of freshwater required for following wells, and the subsequent amount of waste water disposal, which is itself a big management challenge. In addition to flow back wastewater, water is often recovered with natural gas from the subsurface. These large volumes of produced water recovered from the porous reservoir formation from which conventional wells produce gas is typically highly saline commonly referred as brine (Lutz *et al*., 2013). The fracking fluid used for hydraulic fracturing contains wide range of bacteria, attached to a surface which can create biofilms in the well and shale formation (Gaspar *et al*., 2014). Biofilm is a process of undesired growth and proliferation of bacterial species provided favorable habitats in these fracturing operations. The major sources of bacterial contamination in hydro fracking operations can be drilling mud, frac water, proppants and storage tanks. Many of these fracturing fluids contains sugar-based polymers or polyacrylamide and other organic compounds which can serve as a food source for bacteria that are available in highly contaminated source water pond or storage tanks. The high pressure, high temperature downhole conditions in subsurface region are not enough for eliminating bacteria that are presented during the fracturing process. Mass proliferation of microorganisms can result in lined or unlined earthen pits when water is stored prolong prior to use (Dawson *et al*., 2012;

Fitcher *et al*., 2008). Similarly, produced water stored for long time and which is recycled for use in future fracturing operations can help bacteria to thrive (Lysnes *et al*., 2009). Microbial growth is favored by the underground increased temperatures at which fracturing fluids are exposed (Gardner *et al*., 2002); therefore many bacterial species which includes anaerobic species as well that are native to shale formations, may proliferate underground during hydraulic fracturing process (Struchtemeyer *et al*., 2012). The diversity of gram negative and gram positive bacteria include sulfate reducing bacteria (SRB), methanogenic, aerobic and anaerobic acid producing bacteria have all been found in untreated fracking fluid samples. Long storage of flow back water could not only cause bio clogging of formation and inhibit gas extraction but also produce toxic hydrogen sulfide compounds, and introduce corrosion which leads to downhole equipment failure.

Bacterial control in hydraulic fracturing is a necessary operation in order to prevent excessive biofilm formation downhole that may lead to clogging, and consequently obstructing gas extraction. Most of the biocides used for the inhibition of biofilms during the fracturing process are very toxic to aquatic life at any concentration. Glutaraldehyde, which is one of the common biocides currently being used in off-shore hydraulic fracturing operations, is highly toxic to Mollusca and less toxic to others. Despite the fact that they sometime appear less toxic, these biocides are assumed to possess developmental toxicity, mutagenicity, carcinogenicity, and chronic toxicity. Despite little research, the developmental toxicity (i.e, tetratogenicity) has been observed in several lytic biocides that are used for hydraulic fracturing (Kahrilas *et al*., 2014). There has been exploration of alternative products and technologies to enable for

sustainable bacterial control; although none of them are without any human health and /or environmental impacts. The main objective of this research is to find the viability of a copper ion solution for use as a biocide in fracking fluid. Copper solution is commonly used as a water purifier, algaecide, molluscicide, as an anti-bacterial and anti-fouling agent (Borkow *et al*., 2005). The concentration of copper involved in deficiencies, inhibitions and stimulations cover great ranges since their role is varied according to the organisms and conditions concerned. Since copper is an essential minor element, its biological role is varied (Watson *et al*., 1952). However, this research was focused on the inhibiting the aerobic bacterial growth due to the lab constraints.

1.2 Hypothesis

Experiments to determine efficacy of selenium for the treatment of bio fouling in membrane treatment systems was done by Vercellino (2012). The objective of his research at that time was to evaluate the effectiveness of utilizing organo-selenium coated RO membranes to inhibit the development of bacterial biofilms. Laskar (2015) has done similar type of experiment in his/her thesis where two bacterial strains, *Stenotrophomonas maltophilia Oak Ridge Strain 02* and *Pseudomonas sp, PC37,* were studied for selenite resistance. The experimental design used by the Laskar (2015) was to demonstrate the bio remediation of selenite using the two bacteria specimen. Similar experimental design was used for this research however, with different scenario of inhibition of bacteria by copper ion solution in an aqueous wastewater medium was investigated. The copper concentration required to inhibit bacterial growth was measured as compared to the inhibiting level of the fracking fluid. It was hypothesized that the

copper ion solution will be effective in reducing the microbial growth in medium (simulated wastewater) under aerobic conditions similar to the flow back water.

Chapter 2. Literature Review

2.1 Bacteria Present in Hydraulic Fracking

For the oil and gas industry, microbiologically influenced corrosion is a matter of concern, particularly in applications where injection of external water sources (e.g hydraulic fracturing) is involved. Often gathered from surface ponds, hydraulic fracturing process involves the injection of enormous volumes of water. Bacterial contamination of fracturing fluids can lead to various serious problems since these fluids are poorly treated and sulfate reducing bacteria (SRB) and acid-producing bacteria (APB) can create problem downhole in the production lines.

SRB require an oxygen-free (anaerobic) environment and are the most serious offender. In a deep well, micro environments are free of oxygen since all the oxygen are utilized for the metabolic requirements of aerobic bacteria. These bacteria are in latent state until they contacted with a developing biofilm, this condition help SRB to become active. SRB are recognized for reducing available sulfate (thiosulfate) to sulfide resulting ferrous sulfide which can corrode the iron metal through the release of hydrogenase (Roberge, 2000). Similarly, APB which is aerobic or anaerobic in nature can produce organic acid as a byproduct of their metabolism. When these bacteria get into the biofilm, they accumulate at the metal surface and acidify the environment and start accelerating the corrosion (Little *et al.*, 2000). Methanotrophs are other version of bacteria which are abundantly present in fracking related waters and can cause problems due to their high

biofilm forming ability. Methanotrophs usually consume methane or other single carbon compounds to gain energy so as to create biomass. Although most methanotrophs are aerobic and use oxygen to transfer methane to carbon dioxide and water, some of them are anaerobic as well (Sullivan *et al.*, 1998). Apart from these exotic bacteria, sometimes the simple bacteria may survive in the fracking wells since the high pressure, high-temperature down hole conditions in this region are not sufficient for removing these bacteria introduced during the hydraulic fracturing operations. Studies shows that the sources of flow back water are highly contaminated with bacteria and this contamination may be subsequently transferred to the fractured well (Johnson *et al*., 2008). On the other hand, these fluids often contain polyacrylamide or sugar-based polymers. These polymers can be an energy source for injected bacteria. This is the reason why the surface equipment and down hole equipment are put at risk of microbiologically influenced corrosion. Moore *et al*., (2012) did a research on Horn River basin shale gas formation located on British Columbia, Canada. The Horn river basin wells on the production stage and the source water to fracture these wells were studied to discover the viable bacteria present in fracking fluid. From the result it seemed that the source ponds were highly contaminated with bacteria and these bacteria can survive high temperature down hole and establish viable and proliferating communities. The bacterium which was found on well D of Evie Zone was gram positive coccoid identified as *Staphylococcus epidermis*. Staphylococcus being aerobic in nature is one of the rare species that could have been present in hydro fracking well. Despite being rare, if they present just like in Evie zone, they can cause problems because they have higher tendency of forming biofilm.

2.2 Biofilm Development

An expression applied to microbial life in aggregates called biofilm can occur at the solid-liquid, solid-air and liquid-air interface. These are the complex aggregations which can form irreversible attachments to the surfaces of living and nonliving surfaces. Most of the microorganism which are found on the earth can form biofilms and they are living in aggregates (Costerton *et al*., 1987). A feature that is common in the organism are that these organism are embedded in matrix of microbial origin, which consists extracellular polymeric substances (EPS). The natural environment is sound enough for the microorganism to produce EPS which mostly happens both in prokaryotic (bacteria, archae) and in eukaryotic (algae, fungi). EPS mainly comprise of organic matter like DNA, proteins and polysaccharides. Bacterial biofilms which are integrated, multi species communities of cell adhere to almost any surface. Since biofilms has a protected mode of growth, they can easily cope with the hostile environment. Biofilm is usually formed by the structure in which nutrients can circulate easily (Kolter *et al.*, 1998) and in the meantime, different pattern of gene are exhibited by the cells in different region of the biofilm (Whiteley *et al.*, 2001). On the other hand, the biofilm organism get unique advantage from the life embedded into EPS matrix. The synergic micro consortia of different species help the degradation of complex substrates (Wimpenny, 2000). The reason why they can survive under any oligotrophic conditions is that this matrix can keep away this nutrient from the environment for their use (Decho, 2000).

This mixed population of biofilm forming organisms is universally distributed not only in the natural soil and aquatic environment like tissue of plants, animals and man but also in the technical system such as filters, reservoirs, plumbing systems, pipelines, ship

hulls, heat exchangers, separation membranes and other porous media. During last couple of years, an increased amount of research has been done on biofilms (Wingender *et al*., 1999). The interesting fact is that the first record of life on earth is fossilized biofilms dating 3.5 billion years ago where they spent successful life in sediments, mineral and plant surfaces which includes some extreme environment as well like as glaciers, hot vent, electrodes and highly radiated parts of nuclear power plants (Satpathy *et al*., 1999). This indicates that there is a potential of microbiologically induced bio clogging due to the formation of biofilms in that high temperature and pressure.

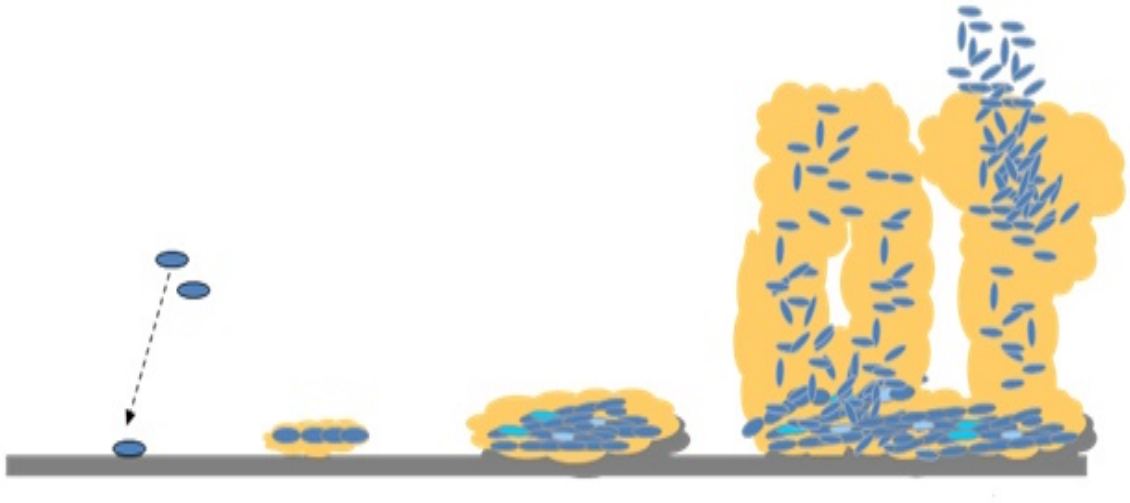

Figure 1: The progression of biofilm formation on a surface. Planktonic cells form monolayer after attaching to a surface and then form a monolayer in matrix of EPS followed by the micro colony which develops into a mature biofilm at the end (Vasudevan 2014).

The *Staphylococcus aureus* is considered to be high biofilm forming bacteria. Biofilm formation requires two consecutive steps which are adhesion of cells to a solid substrate which is followed by cell-cell adhesion and creation of multiple layers of cells at the end. These intercellular adhesions are only possible when there is plenty of polysaccharide intercellular adhesion (PIA). Composed of linear β-1, 6-linked

glucosaminylglycans, this PIA can be synthesized in vitro from UDP-N-acetylglucosamine by products of the intercellular adhesion (ica) locus. In an investigation of a variety of *Staphylococcus aureus* strains, these strains were found to contain the ica locus by which they can form the biofilm (Cramton *et.al.,* 1999). However, the accumulation in multilayered cell clusters after the primary attachment of polymer surfaces leads to biofilm production of *Staphylococcus epidermis* (Mack *et al.,* 1996). Specific antigen of biofilm producing *S. epidermis* characterized by biochemical and nuclear magnetic resonance spectroscopy (NMR) methods helped to illuminate the structure of polysaccharide which is produced by *S. epidermis*. This structural heterogeneity that polysaccharide exhibit are functionally involved in biofilm accumulation.

2.3 Inhibition

2.3.1 Copper Ion Solution

Copper is considered an essential element because it is required by plants, animals and microorganism for normal metabolic process. Although essential at trace levels, at elevated concentrations, this heavy metal is toxic and inhibits microbial activity. The ability of copper ion solution to chelate sulfyhydryl groups and thus interfering with cell proteins or enzymes is what results its anti-bacterial action (Yeager *et al*., 1991).

Copper ions (Cu^{+2}), either alone or in copper complexes, have been used to disinfect liquids and solids for centuries. Thousands years before, the ancient Greek of the pre-Christian era of Hypocrates (400BC) were recognized as the first period to discover the sanitizing power that a copper possess (Borkow *et al*., 2005). At that time, copper was used to be prescribed for purifying drinking water. In Scotland at around 800AD, whisky was produced in copper vessels and this practice is still in use today. In

Hindu culture, Gangajal (sacred river water) is stored in copper utensils due to their anti-fouling and bacteriostatic properties. Japanese soldier used to put pieces of copper in their water bottles so that they can prevent themselves from dysentery during the Second World War. Not only this, the copper complexes with sulfur such as copper sulfate was used by some inhabitants of Africa and Asia for healing sore and skin diseases (Borkow *et al*., 2005)

Copper and copper containing compounds as biocides are most extensively used in agriculture. The biological growth in water from impounding reservoirs, lakes, storage reservoirs, and settling basins can be prevented or eliminated with an algicide such as copper sulfate. Such growth can produce unwanted taste and odors, clog fine-mesh filters, and contribute to the buildup of slime. Several researches have been done to determine the microbial inhibition potential of copper during process of waste water treatment. This research includes the study of inhibitory effect of copper to aerobic heterotrophic bacteria and also killing effect of other microorganism which is involved in sulfate reduction along with other anaerobic degradation processes that includes hydrogen and acetate utilizing methanogens as well (Ahring *et al.*, 1987).

The discovery that many algae are highly receptive to copper sulfate directed to its use by water engineers to prevent bacterial growth (Borkov *et al*., 2005). Even for microorganisms in the same tropic groups, the concentrations of copper (II) to inhibit microbial activity was found to vary widely. For example, 50% inhibitory levels of copper have been observed in sulfate reducing bacteria where concentration was in a range between 0.84mg/l to 200 mg/l. Similarly, the magnitude of concentration varied from 2.2mg/l to 400mg/l m while obtaining the different inhibitory levels of copper in

methanogenic microorganisms present in anaerobic sludge (Ahring *et al*., 1987). These significant variations may be observed due to different experimental conditions employed in various assays (like as pH, composition of the medium, temperature, concentration of copper ligands (sulfide), etc).

2.3.2 Killing Mechanism of Copper

Metallic copper ion solution quickly and proficiently kills bacteria. Although the moist copper ion solution uptake by the microorganism is slower than the dry copper, bacteria cells exposed to copper surfaces accumulates large amount of copper ions. Because of this, cells get extensively damaged in few minutes of the exposure. These finding are more important for illuminating the molecular delicate targets in cells that are fatally challenged by exposure to copper surfaces (Santo *et al*., 2011). In the meantime, this provides a scientific explanation for the purpose of using copper solution as antimicrobial agent.

A small amount of copper is an essential nutrient for aerobic metabolism and as a cofactor in respiration for many microorganisms. But excess copper is highly toxic (Rensing *et al.,* 2003*).* This is due to the intracellular release of free copper ions or accumulation of copper ions which cause cell damage. Hydrogen peroxide is generated in microorganism by the dis mutation of superoxide radical ($O_2^{\bullet-}$) a toxic by-product of aerobic metabolism. It can however, be produced directly by range of oxidase enzymes including glycollate and monoamine oxidases and also by the proximal pathway for β-oxidation of fatty acids (Chance *et al*., 1979, Halliwell *et al*., 2015, Reddy *et al*., 1989, deGroot *et al*., 1989).

Copper and other transition metal readily catalyzes reaction where copper ion reacts with hydrogen peroxide in aqueous solution to lead to the formation of $OH^{\cdot}$ and $HO_2^{\cdot}$ radicals as active intermediates through the Fenton and Haber-Weiss reactions (Halliwell *et al*., 1984, 2015). These oxygen intermediates cause oxidation of proteins and lipid peroxidation. Including oxidases and oxygenase, copper is a common cofactor for many enzymes (Stohs *et al*., 1995*)*. Basic free radical mechanism for the Fenton and Haber-Weiss Reaction (Mwebi, 2005) is shown below.

Fenton Reaction

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + HO^- + HO^{\cdot}$$

Haber-Weiss Reaction (Superoxide Driven Fenton Reaction)

$$H_2O_2 + Cu(I) \rightarrow Cu(II) + HO^- + HO^{\cdot}$$

O_2 $O_2^{\cdot -}$

Haber-Weiss Net Reaction

$$O_2^{\cdot -} + H_2O_2 \xrightarrow{Cu(I)/(Cu(II)} O_2 + HO^- + HO^{\cdot}$$

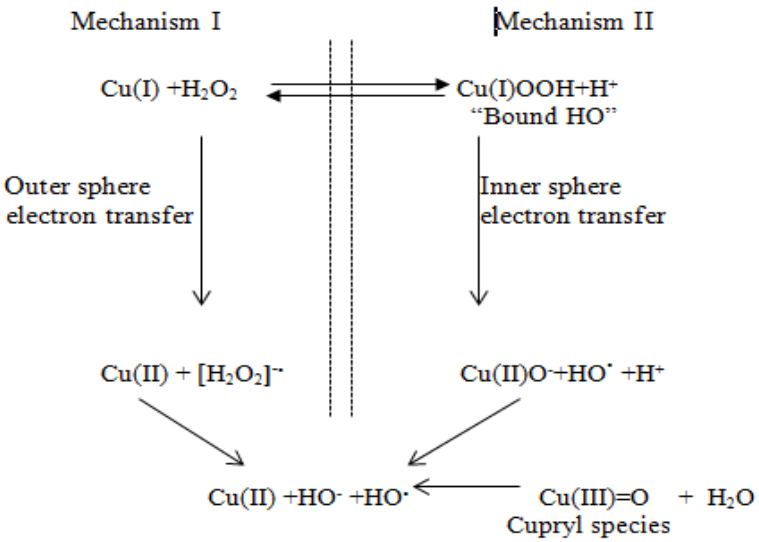

Figure 2: Basic reactions and intermediate involved in the classic Fenton and the metal centered Fenton reactions (Mwebi, 2005)

Free copper ion is capable of oxidizing cysteine-like sulfhydryl group in the cellular redox buffer glutathione or proteins (Helbig *et al*., 2008, Stohs *et al*., 1995). The redox properties of copper and iron complexes of bleomycin, adriamycin, and thio-semicarbazones have been investigated. The common property of these metal complexes is that they were able to be reduced by thiol compounds and can easily be oxidized by iron/ copper or reduced species of iron/copper to generate radicals.

Further studies have shown that in the presence of hydroquinone, copper enhances the establishment of DNA strand breaks (Li *et al*., 1993). Singlet oxygen plays a role in the initiation of DNA strand breaks. Other metal ions including MN (II), Fe (III), Zn (II) and Cd (II) did not improve oxidation of hydroquinone or initiation of DNA strand breaks. Copper may be an important element while damaging DNA in target cells by hydroquinone (Li *et al*., 1993). More specifically, copper ions are found to disable proteins by impairing Fe-S clusters in cytoplasmic hydratases. These are fumarase A in the tricarboxylic acid cycle, dihydorxy-acid dehydratase in branched chain amino acid synthesis pathway, 6-phospogluconate dehydratase in pentose phosphate pathways, and isopropylmalate dehydratase in the leucine-specific branch in *Escherichia coli* which are damaged by copper ions (Macomber *et al*., 2009).

The main objective of the research was to evaluate the efficacy of using a free-copper ion solution as an inhibition agent against Gram-negative and Gram-positive microorganism in secondary wastewaters. The secondary objective was to check its potential use as biocides in fracking fluid so as to replace the toxic chemicals that is currently used. For this purpose the common strains, *Escherichia coli* strain (ATCC #25922), *Staphylococcus aureus* (ATCC #25923) strains and *Staphlyococcus epidermis*

(ATCC#12228) were used for experiments. The main purpose of using these bacteria were to determine how organo-copper reaction affected model gram negative and gram positive organisms. *S. aureus* and *S. epidermis* are strong biofilm former whereas *E.coli* is environmentally relevant microorganisms. *Staphylococcus aureus* and *Staphylococcus epidermis* are a gram-positive bacteria and mainly grown by aerobic respiration. The cell wall and peptidoglycan layer of gram positive bacteria is very thick layer whereas; the gram negative bacteria like *E.coli* has thin peptidoglycan layer and double layer thin cell wall.

Chapter 3. Material and Methods

3.1 Experimental Set Up and Conditions

The effect of copper ion solution on the inhibition of bacteria was investigated mainly using three basic methods; first one is to measure the turbidity using the spectrophotometer to examine the growth curve of the bacteria, second is heterotrophic plate count of serial dilutions using spread plate method (viable count). The third is the quantification of copper using Inductively Coupled Plasma Atomic Emission Spectrometer.

Figure 3: Experimental set up for turbidity test of the samples by spectrophotometry.

Initially, a single colony was inoculated into secondary waste water medium a day before the experiment was started. As soon as the culture was ready which was noticed from the increased turbidity of the cultures, different specimen of sample were transferred into the flask. Appropriate amount of bacterial culture so as to get proper growth curve was added to respective flask to monitor the growth. Some of these flasks

to see the growth inhibition were added with maximum possible copper concentration of the range that had been used before; i.e 10- 300mg/l (Ochoa-Herrera *et al*., 2011) to examine the potential amount of copper required enough to inhibit the bacteria. Using a spectrophotometer at 600nm, the absorbance of bacteria growth in solution was monitored at 2 hours intervals for first 10 to 12 hours; after 12 hours the sample reading were taken at an interval of 4 to 6 hours. The samples were left incubated at 37˚C for 48-56 hours in hot water bath during the entire growth monitoring. Samples were separately used every 4 hour, centrifuged to separate pellet and supernatant, digested and analyzed for copper content by ICP. Centrifuge was run at 300 rpm for 15 minutes using IEC CU-5000 Centrifuge. The supernatant and pellets were separated and poured into new tube. They were stored at -10˚C till all the samples were collected.

The feed media used in the experiments was mimicking of secondary waste water and LB broth. The first test solution was flow back water, which was collected from an operation in the local region; the second solution was a proprietary copper ion solution obtained from Environmental Water Solution, Cleveland. The bacteria was first grown using an enriched synthetic wastewater medium.

3.2 Bacterial Strains

Experiment was processed using Gram-negative and Gram-positive bacteria. Initially two bacterial strains *Escherichia coli* strain (ATCC #25922) and *Staphylococcus aureus* (ATCC #25923) was used. After the successful lab testing on them, a hydro fracking relevant bacterium *Staphylococcus epidermis* (ATCC#12228) was also tested.

3.3 Feed Media Preparation

3.3.1 Synthetic Wastewater

The chemicals used to prepare synthetic wastewater were based on secondary effluent quality outlined by Herzberg *et al*., 2007. Diluted LB Broth along with high carbon and high energy source were added to wastewater media to obtain an enriched biofilm behavior. The preparation of synthetic wastewater mainly consisted of deionized water (DI) supplemented by ten times of each 1.16mM Sodium citrate, 0.45mM KH_2PO_4, 0.94mM NH_4Cl, 0.5mM $CaCl_2.2H_2O$, 0.5mM $NaHCO_3$, 2.0mM NaCl and 0.6mM $MgSO_4.7H_2O$. For every 1L of DI water, 1mL of LB broth was added to get final pH of 7.4 and ionic strength of 14.6mM. The solution was diluted 10 times to make the working solution for bacteria growth (Herzberg *et al*., 2007).

3.3.2 Luria Bertani (LB) Agar

LB broth was prepared by using ready mixed 10g/l casein peptone, 5g/L yeast extract, 10g/L sodium chloride, 12g/L agar purchased from Fisher Scientific and de ionized water. The solution was then adjusted to pH 7.2 by adding 10N NaOH. The solution was thoroughly stirred and autoclaved to temperature about 250F for 15 minutes and then poured in individual petri dishes having diameter 100mm. LB agar was selected for the biofilm experiments to use it as control media which represent a nutrient rich environment that would help biofilm formation.

3.4 Optical Density-Absorbance Test

The growth of bacteria on each flask was observed using a Spectrophotometer (Bausch and Lomb Spectronic 1001 UV-VIS). The machine was turned on and allowed to warm up for at least 30 minutes to stabilize the source and detector. The wavelength was set for 600nm.The meter was initially set to 100% transmittance and 0% absorbance

with a clean cuvette with de-ionized water (no bacteria) as a blank to standardize the spectrophotometer. Since this tube containing de-ionized water is the blank, it should have 100% of the light waves pass through and thus will standardize the upper limit of spectrophotometer. The spectrophotometer was re-standardized with the blank if a significant period of time passed between readings or if the wavelength changed. The sample tube contained the bacterial solution is thus called the sample tubes. Sample tube was inserted into the sample holder and read individually at the appropriate time.

3.5 Viable Cell Count

For this viable cell count, 1 ml of sample from each flask was transferred into test tube containing in 9ml broth to make 10^{-1} dilution. Then 1ml of the 10^{-1} dilution was transferred to a second test tube containing 9 ml broth to make 10^{-2} dilution. This process was continued to get dilution up to 10^{-6}. A volume of 0.1 ml of each dilution was plated onto Luria-Bertani (LB) agar petri plates. For each bacteria growth hour, there were 5 to 6 plates with different dilutions made. 10^{-4} and 10^{-5} dilutions sample plating were done for the hour 0, 4, 10 and 16 and next 24, 34, 48, 56 hours sample were plated with 10^{-5} and 10^{-6} dilutions. The samples were then incubated for 24 hours at 37 ˚C. The colonies that appeared on the spread plate were then counted using a colony counter.

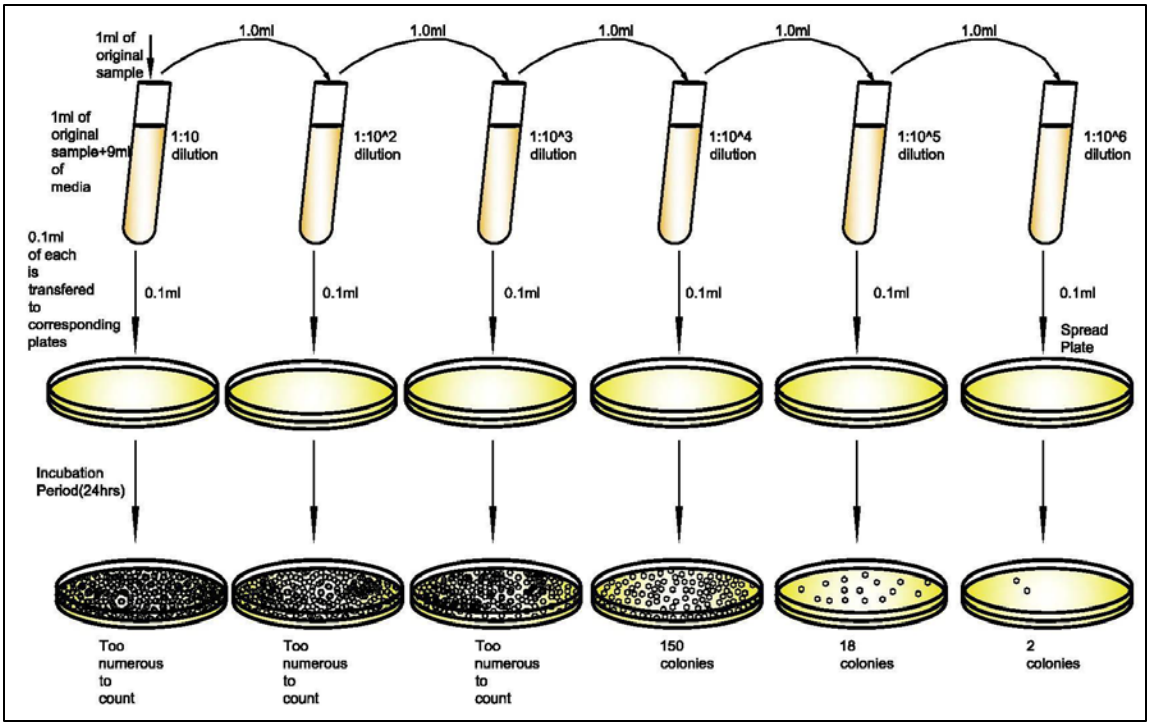

Figure 4: A schematic drawing of serial dilution method for viable cell count (Hester *et al*., 2014).

3.6 Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES)

ICP-AES was performed to determine the concentration of copper content present in the solution used for the bacterial inhibition (provided by Environmental Water Solution). The copper content in the feed media was measured and then feed media with added copper was digested and measured for residual copper.

3.6.1 Sample Preparation

Sample were collected at points where there was sharp changes in the absorbance values in the growth and inhibition curves obtained from turbidity test to measure the amount of copper present in growth medium(supernatant). Sample of sterile growth media was collected at the zero hour and at 48th hour and was tested for copper concentration as negative controls.

3.6.2 Nitric Acid Digestion

The metal associated with particulates can get converted to a form that can be determined by inductively coupled plasma spectroscopy. However, to reduce further intervention of metal ion by organic matter; one of the least rigorous digestion method which may be compatible with analytical method so that the metal can be analyzed, was used. Since nitric acid can digest most samples adequately and nitrate is an acceptable matrix for ICP, nitric acid digestion was used. A well-mixed 5mL sample was transferred into the sample bottle and 5mL of nitric acid was added for storage and preservation. This 10mL acid preserved sample was transferred into flask or beaker and 5mL of concentrated HNO_3 was added. The sample was boiled and evaporated slowly to bring it to the lowest volume possible (about 10mL) before precipitation occurred. The sample was heated with additional volumes of concentrated HNO_3 as necessary until digestion was complete as shown by light colored, clear solution. The flask or beaker walls were washed with DI water and then filtered. The filtrate was transferred to a 100mL volumetric flask with two 5mL portions of water. The sample is then cooled, diluted to mark and mixed thoroughly. This sample was analyzed by ICP AES for copper determinations (Lenore *et al*., 1995)

3.6.3 Preparation of Calibration Standard

Single calibration standards were prepared containing the concentration range between 1 and 300 ppm copper by combining appropriate volume of stock solution in 100mL volumetric flasks. Two milliliters of 1:1 HNO_3: DI and 10mL 1:1 HCL: DI was added and diluted to 100mL with water.

Chapter 4. Results

4.1 Growth Curve/Inhibition Results by Absorbance

4.1.1 Growth and Inhibition Curve of *Escherichia coli*

The growth curve of bacterial strains of *E.coli* inoculated in secondary waste water with the dilution ratio of 1:15 was determined for triplicates of the sample. The absorbance was measured in absorbance units (AU) and was plotted in Y axis whereas the incubation time in hours was plotted in X axis (Figure 5). The copper ion solution was added 4 hours after inoculation since some growth momentum was achieved at that point. The data was taken at an interval of 2 hours for first 12 hours and next 44 hours were taken at an interval of 4 hours and 6 hours. The growth of *Escherichia coli* without copper (positive control) demonstrated typical growth curve with log or exponential growth phase and a prominent stationary phase and a small death phase at the end (Figure 5 solid blue line with solid diamond points). There was no outstanding lag phase in the positive control. The experiment was put to an end as soon as the curve started declining slowly with an assumption that the death phase might have started. Absorbance started from 0.16 units and increased up to 0.37 units after 6 hours and it started to level out up to 16 hours. This was caused because all the glucose present in media was consumed in 6 hours and after 16 hours it started using the lactose present in the media. After 30 hours it starts to level off. There was no color change except that it turned from clear to off white which indicated cell growth. For the negative control, the same experiment was conducted but without bacteria inoculation so as to ensure that no potential contamination occurred during the experiment. The data showed that there were no changes in the absorbance of the blank media since there was no color change in the medium (Figure 5 green line with green triangle points). The line with square points showed in Figure 5 was

the absorbance of *E.coli* cells supplemented with approximately 250ppm copper ion solution which was actually 30ml of 525 ppm stock copper solution provided by Environmental Water Solution.

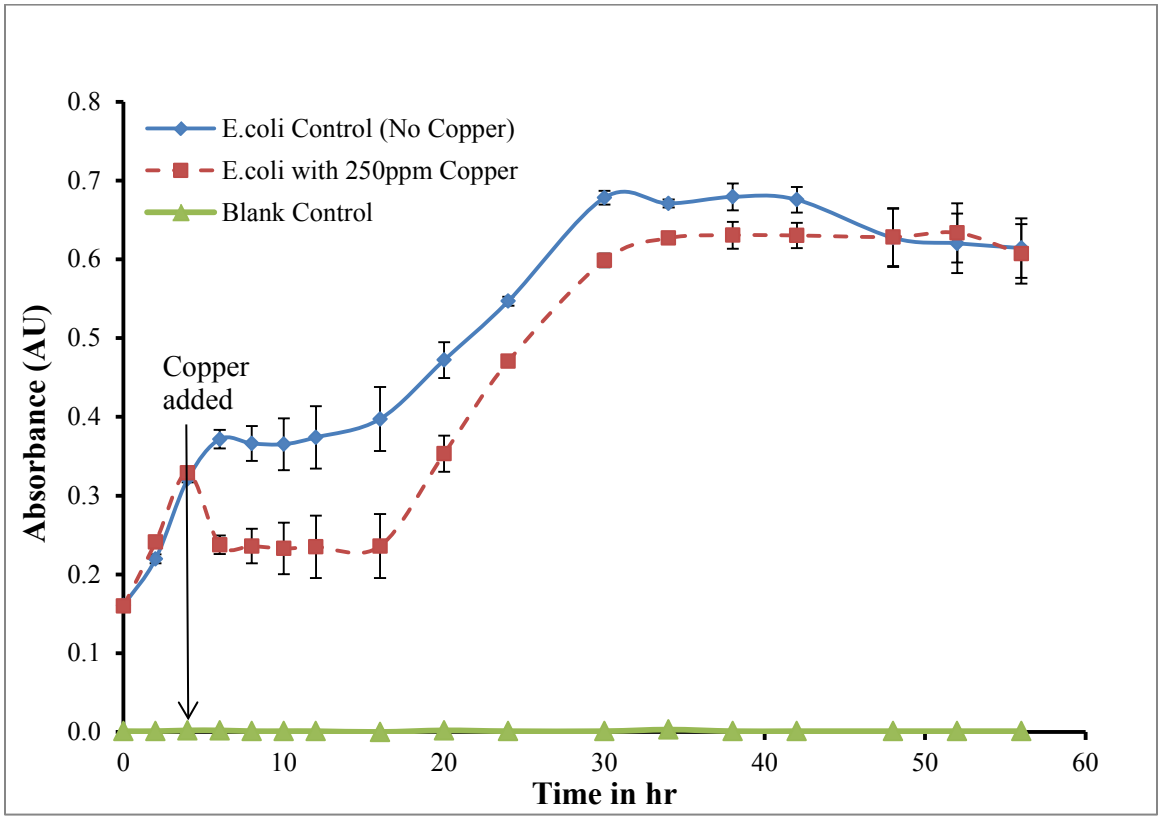

Figure 5: The growth curve, inhibition curves due to copper, of *E. coli* expressed as absorbance in AU units. The error bar represents the 95% confidence interval and N-1 degrees of freedom from 3 different experiments.

Absorbance of all replicate vials was same for first four hour. Then, just after the addition of copper ions solution, the off white color changed into blue but didn't show any remarkable difference in absorbance just color change. Then, after two hour, there was significant drop in absorbance which indicates inhibition of bacterial growth. The absorbance then leveled off for at least 10 hours signifying continual inhibition of bacterial growth. Then, the absorbance started rising up past the standard growth curve. The highest absorbance reached was 0.634 AU for inhibition curve which started to

decline only after 52 hours. Despite the fact, the copper ion solution was effective for at least 12 hours for gram negative bacteria like *E. coli.*

To compare the inhibition properties of flowback, same volume of flowback was supplemented to another vial of sample after 4 hours of inoculation. The flow back solution was able to give more prominent inhibition curve than copper ion solution. Absorbance of all replicate vials was same for the first four hours. Then just after the addition of flowback solution, the off white color changed into yellow. Like copper, there was no any change in absorbance just the color change. There was significant drop in first two hours just after the flow back was added (Figure 6 dash line with square points). The inhibition remained constant up to the 35th hour. Hence, the fracking fluid was able to inhibit bacteria for an extended amount of time.

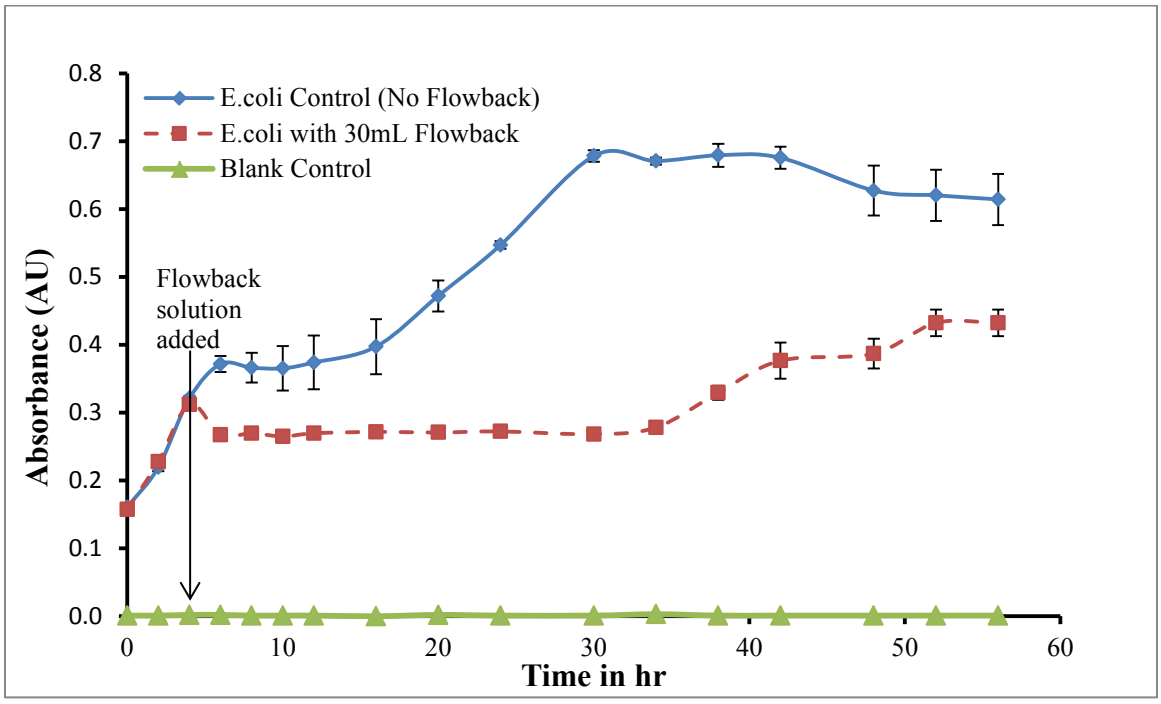

Figure 6: The growth curve, inhibition curves due to flowback, of *E. coli* expressed as absorbance in AU units. The error bar represents the 95% confidence limits of 3 different experiments.

4.1.2 Growth and Inhibition Curve of *Staphylococcus aureus*

The colorimetric test of *S. aureus* was done for the standard growth curve and to examine its inhibition using concentration of copper ions solution first. The first 12 hours of data was taken at an interval of 2 hours and next 44 hours data were taken in various time intervals. The ratio of bacterial cultures to secondary waste water was 1:15. The bacterial control curve of *S. aureus* demonstrated typical growth curve with more prominent exponential growth phase, stationary phase and some death phase (Figure 7, diamond points). The absorbance started from 0.108AU and then increased to reach 0.549AU after 34 hours. There was a substantial growth of the bacteria in this period which seemed to be level off for about the next 20 hours. This tendency was stopped after 52^{nd} hour where the curve started to decline. The highest absorbance achieved was 0.558AU.

The inhibition curve for *S. aureus* due to 250ppm copper ion solution was also plotted (Figure 7, red squares points). It seems that there was some inhibition for a while, from 8^{th} hour to 16^{th} hour. This curve declined slightly for 8 hours and started increasing dramatically and then remained constant.

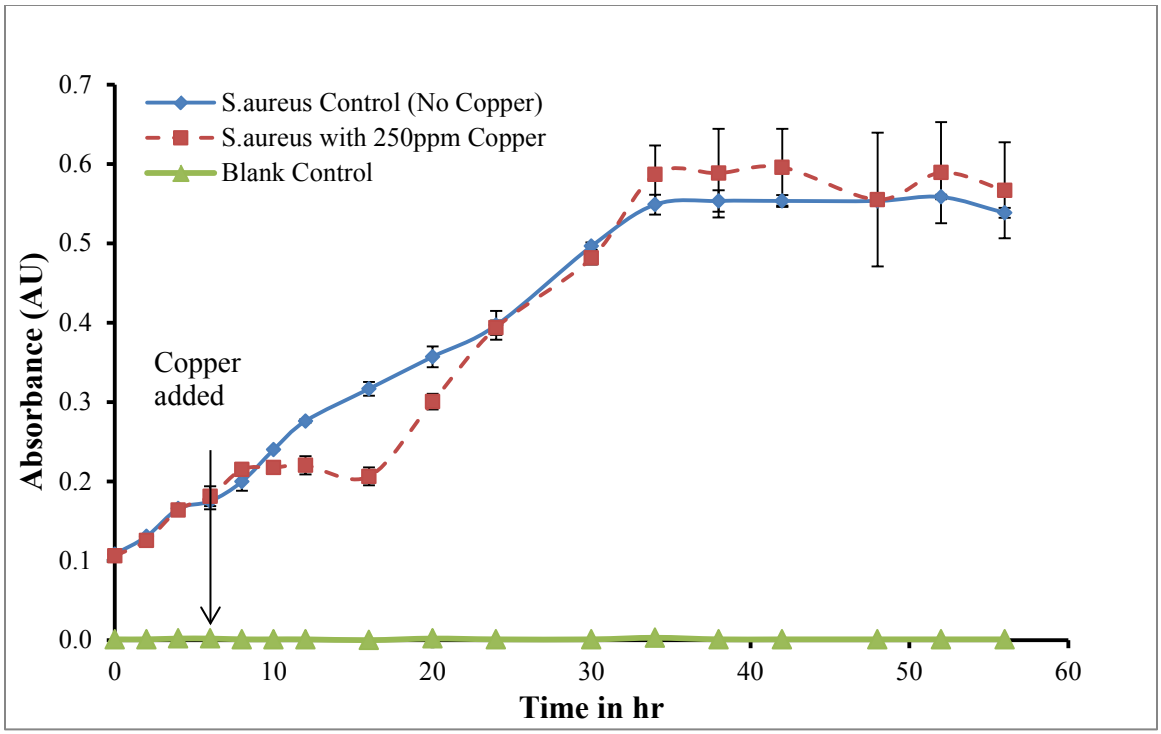

Figure 7: The growth curve, inhibition curve due to copper, of *S. aureus* expressed as absorbance in AU units. The error bar represents the 95% confidence interval of 3 replicate experiments and N-1 degrees of freedom.

The same process was done to see the inhibition due to flowback solution. The 30 mL flowback solution equivalent to the volume of copper used was added after 6 hours since gram positive bacteria took more time than gram negative to get momentum to grow (lag phase). There was no sharp decline of curve seen with the addition of copper. However, the inhibition was gradual and lasted to end of end of the testing period (56 hours). The absorbance started from 0.105AU was ended to 0.348 AU after 56 hours in case of flowback solution. In contrast, the growth control curve with the copper solution went up to nearly 0.60AU. The blank as always remain unaffected by the surrounding environment.

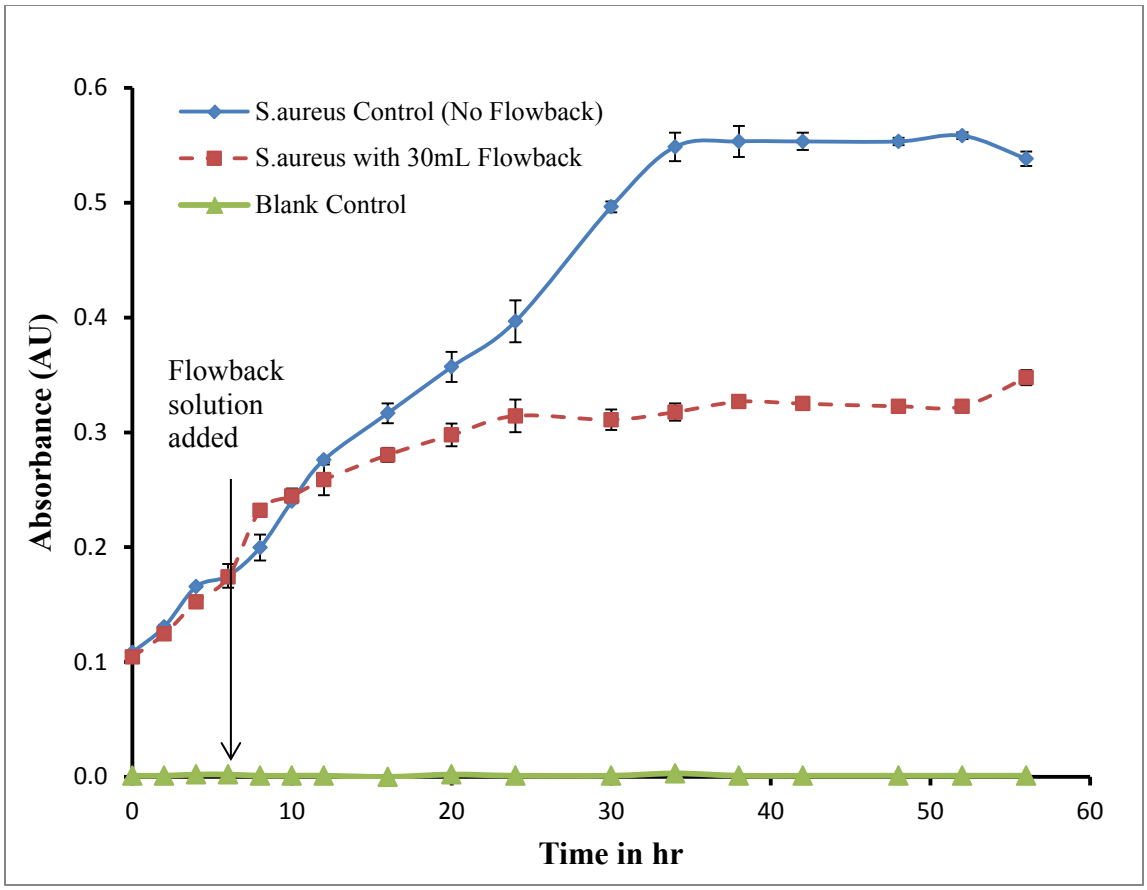

Figure 8: The growth curve, inhibition curve due to flowback, of *S. aureus* expressed as absorbance in AU units. The error bar represents the 95% confidence interval of 3 replicate experiments and N-1 degrees of freedom.

4.1.3 Growth and Inhibition Curve of *Staphylococcus epidermis*

Figure 9 represents the growth of hydraulic fracturing relevant gram positive bacteria *S. epidermis* alone and with copper ion solution in two separate experiments. The positive control culture contained fresh sterile medium which was inoculated with an overnight culture of *S. epidermis* in a ratio of 1:15. There was a prominent growth curve with well distinguished exponential growth and stationary phase. The death phase wasn't that much significantly achieved since we stopped the experiment after 56th hour. There wasn't significant color change except the off-white color changed into white signifying the bacterial growth. The absorbance at zero hour was 0.168AU which went high up to

0.821AU at 24 hours showing the exponential growth of the bacteria which was leveled off until termination of the experiment.

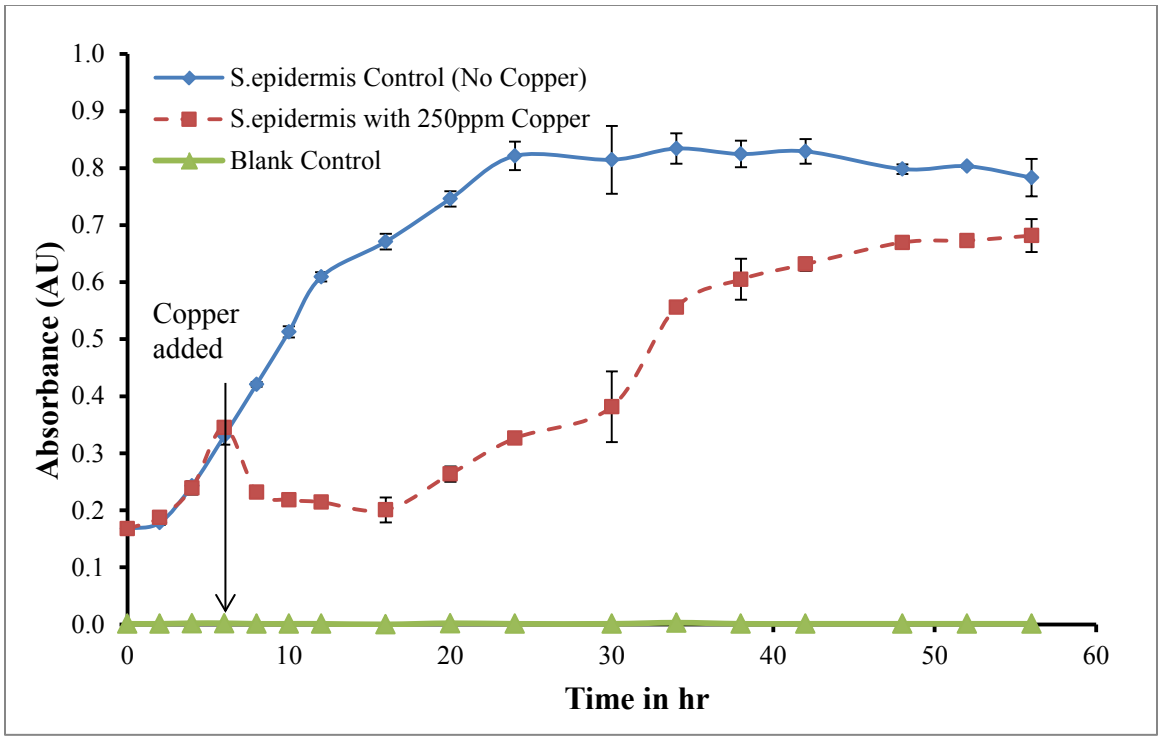

Figure 9: The growth curve, inhibition curve due to copper, of *S. epidermis* expressed as absorbance in AU units. The error bar represents the 95% confidence interval of 3 replicate experiments and N-1 degrees of freedom.

There was dramatic decline in growth soon after the copper solution was added in the sample (Figure 9 dash line with square points). There was some gradual decrease in absorbance up to 16th hour which was then increased gradually for next 12 hours. As always, the copper solution wasn't able to inhibit the bacteria for so long. The difference between first two turbidity tests with this test was that there was a wide gap between growth and inhibition. The growth in inhibition curve due to copper was steady which resembles to the fact that copper ion solution was more effective in *S. epidermis* than other two bacteria.

However, there was remarkable inhibition due to flowback solution. The flow back water was kept into some sample which had same media inoculated with bacteria. No sooner the flowback water was added into the sample solution, the inhibition was steady and gradual. There was sharp decrease in absorbance soon after the fracking fluid was added. But after that, curve was almost leveled off.

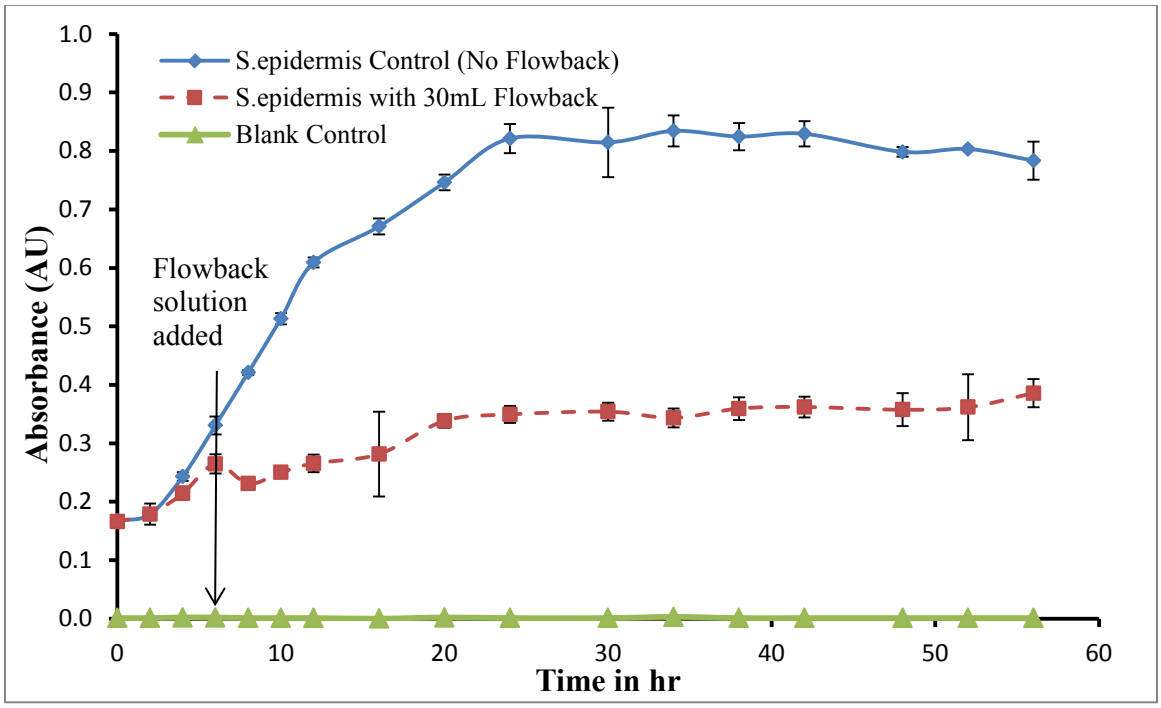

Figure 10: The growth curve, inhibition curve due to flowback, of *S. epidermis* expressed as absorbance in AU units. The error bar represents the 95% confidence interval of 3 replicate experiments and N-1 degrees of freedom.

4.2 Viable Cell Count

To confirm that the turbidity measured with the spectrophotometer was a measure of live cells, viable cell count was done. Samples were taken at the points where there were major changes in the values of absorbance in optical density-absorbance test, appropriate dilutions were plated on LB agar medium to measure the viable cells.

4.2.1 Growth and Inhibition curve of *Escherichia coli*

The viable cell count growth curve and inhibition curve of *E. coli* due to application of copper ion solution and flowback solution are similar to the optical density-absorbance curves (Figures 11 and Figure 5 and 6). The growth curve of *E. coli* shows prominent exponential, stationary and death phase. There was sharp increase in number of cells between 0 and 24 hours and then curve get leveled off. After 48 hour, the number of cells decline. After the copper ion solution was added at 4 hours of incubation, the growth trend was impeded and the curve declined sharply and continued for some time. During this 12 hour decline or inhibition period, the number of cell count was less than the cell count at the 4th hour. The inhibition didn't last long similar to that of turbidity test. The curve started to rise abruptly after the inhibition period, showing the decreasing effect of copper ion solution. The trend was somewhat different in case of flow back solution. The number of colonies counted decreased soon after the flowback solution was added and the curve stayed at a depressed level with slight increment in colonies at the end similar to what was seen in the turbidity analysis.

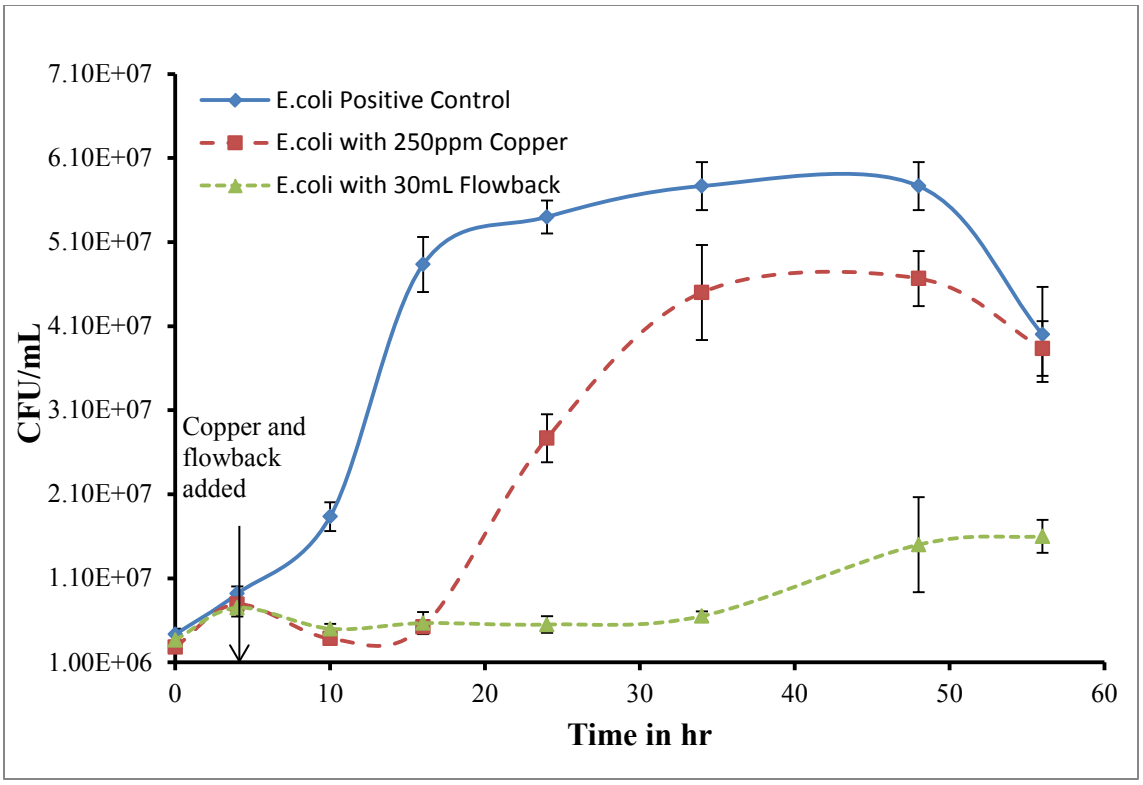

Figure 11: Growth, inhibition curves of *E. coli* expressed as CFU/mL vs time (hr). The error bar represents the 95% confidence interval of 3 replicate experiments and N-1 degrees of freedom.

4.2.2 Growth and Inhibition curve of *Staphylococcus aureus*

The experimental process was repeated with *S. aureus*. The growth and inhibition curve for *S. aureus* was drawn where viable cell count (CFU/mL) was plotted in Y-axis and time (hrs.) in X-axis (Figure 12). There seems some lag phase but more prominently exponential growth and stationary phase were achieved for control. There wasn't significant death phase achieved since the experiment was stopped at the 56th hour. The copper ion solution showed similar results to the turbidity results for cell growth. The copper ion solution was able to hinder the growth for at least 8-10 hours. This was a longer inhibition phase that what was observed with turbidity method. Flowback solution was efficient in inhibition of the bacteria for extended amount of time similar to turbidity

method of analysis. The largest growth spikes occurred early in 16th hour, and then the curve leveled off for rest of the time period.

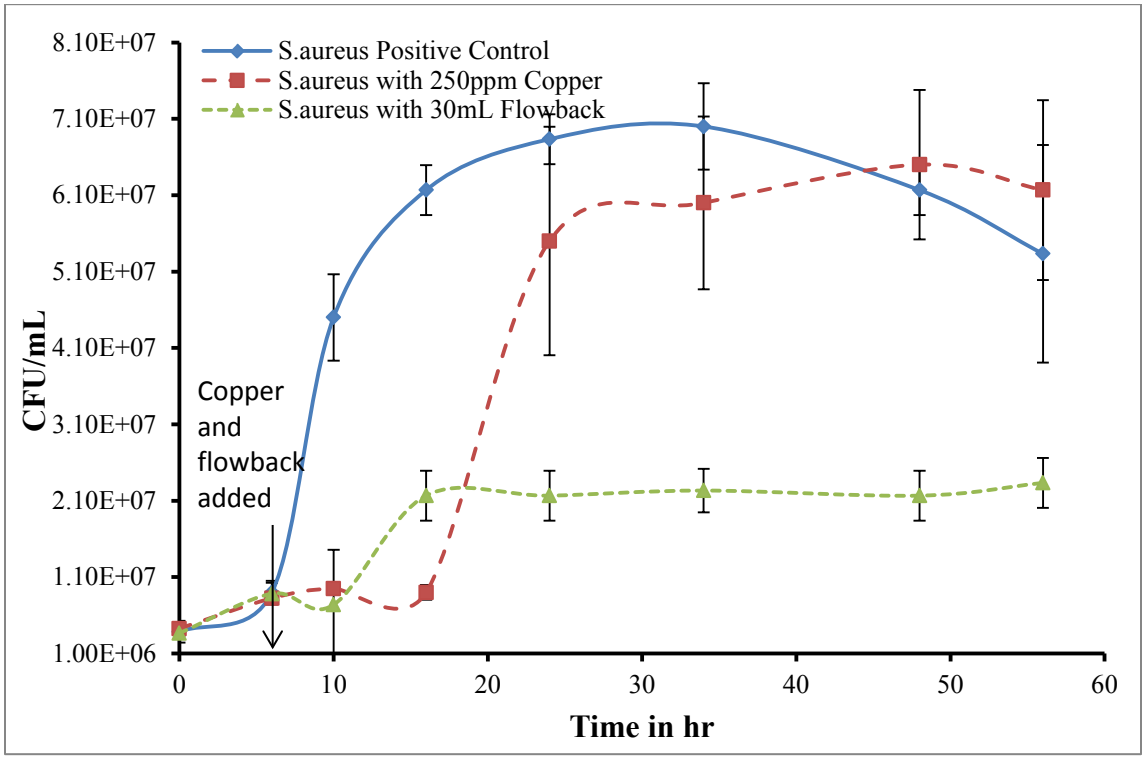

Figure 12: Growth, inhibition curve of S. aureus expressed in CFU/mL vs time (hr). The error bar represents the 95% confidence interval of 3 replicate experiments and N-1 degrees of freedom.

4.2.3 Growth and Inhibition curve of *Staphylococcus epidermis*

Viable cell count of *S. epidermis* was consistent with the other two bacteria growth curves. The graph was plotted with colony count (CFU/mL) in Y-axis and time (hr) in X-axis (Figure 13). The growth control curve has distinctive exponential phase and stationary phase and beginning of a death phase. The growth was achieved primarily in the first 24 hours and was leveled off and declined after 48 hours.

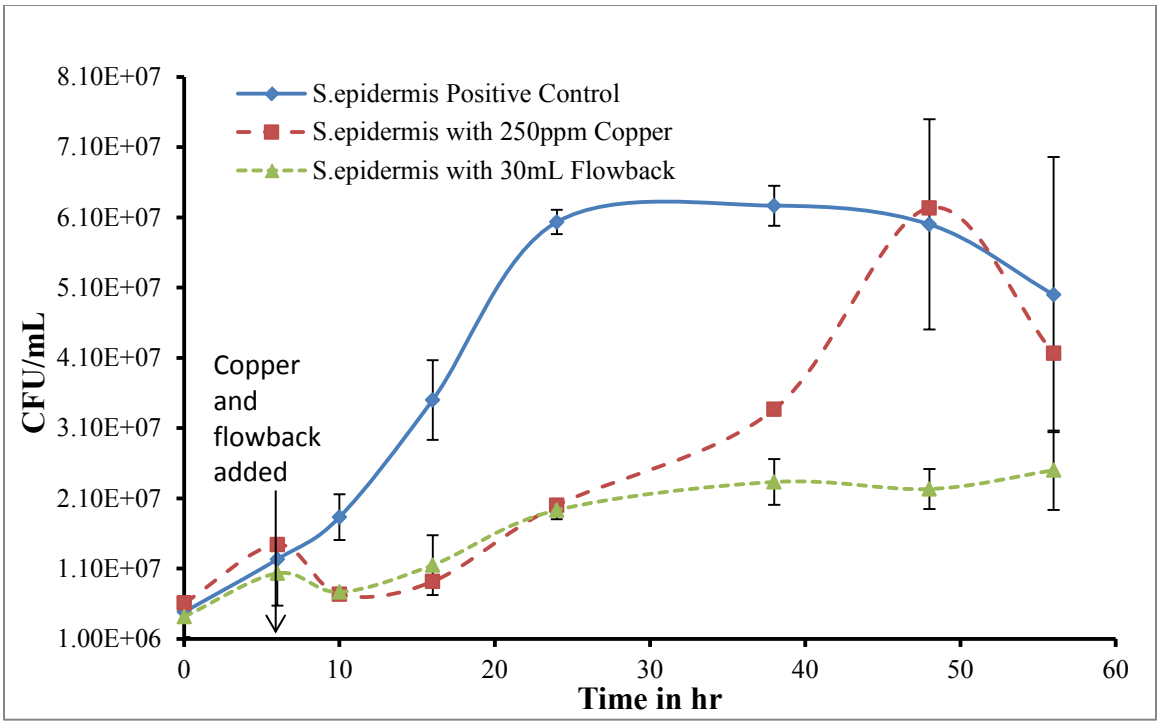

Figure 13: Growth, inhibition curve of *S. epidermis* expressed in CFU/mL vs Time (hr). The error bar represents the 95% confidence interval of 3 replicate experiments and N-1 degrees of freedom.

The inhibition curve due to copper ion solution followed the same pattern as the turbidity analysis with the growth control curve for first 6 hours (Figure 13). After the addition of copper on 6th hour, growth started declining rapidly and then remains almost leveled off for 8 hours. This portion of the curve would be better referred as, inhibition curve due to copper ion solution, which is similar to those, observed in the turbidity test method. After the inhibition phase, the curve started increasing indicating some bacteria recovery. Unlike the other two bacteria, there was a gradual increase of the growth curve after 10 hour of inhibition. This might be due to the slowed metabolism of the cells before all copper has become inactive. The death phase of the curve seems to start after 48 hour which is much earlier than the other two bacteria and not consistent with the turbidity method. There was a large amount of variability in the replications noted by the large error bars which could be the reason for observed decline (Figure 13). The

inhibition curve due to flowback solution as usual slightly inclined without any sharp increase in viable cell count then levels off without a secondary growth phase. Inhibition of *S. epidermis* was similar for both copper ion solution and flowback solution for the first 24 hours then the flow back solution provided longer inhibition of bacteria growth.

4.3 Measurement of Copper Concentration using ICP

The ICP was calibrated using standard solution of copper at concentration 0ppm to 300ppm. From the linear regression analysis, highest R^2 value was obtained at wavelength of 3247nm. The concentration of copper of unknown samples was produced in ppm.

Concentration of Copper in Bacteria

The graph below was plotted based on the data produced from ICP analysis. The time in hours was plotted in X-axis and concentration of copper (ppm) was plotted in Y-axis (Figure 14). This curve represents the copper consumption in sterile medium as well as with each bacteria species. As the bacteria grow they consume nutrients including copper. The sterile control medium had no change in concentration from beginning to end of the test. The concentration of copper was detected soon after it was added at 5^{th} hour in growth medium inoculated with *E.coli* strains. The concentration of copper measured on frequent interval of time showed that copper was consumed as long as it was available in the medium. There was a noticeable drop in copper concentration from 20^{th} to 24^{th} hour. Overall for *E.coli*, there was a gradual drop before 20^{th} hour and obvious drop after 24^{th} hour which is consistent with the growth curves that saw inhibited growth before 20 hours and secondary growth around 24 hours.

The concentration of copper in growth medium after it being consumed by *S. aureus* did not observe a large decline in the copper concentration for the first 24 hours similar to *E. coli*.

The concentration of copper in supernatant for the bacterial strain *S. epidermis* indicated a slightly steeper decline in copper over the first 20 hours, unlike *E. coli* and *S. aureus*, This was followed by a sharp decrease in concentration of copper at 24 hours then at the end of 48 hour lab experiment. Like *E.coli*, this bacterium samples also has sudden drop of concentration of copper at the middle of the experiment (24 hours).

Copper ions inhibited all three bacterial strains for 8-12 hours as seen with lower cell counts, which would result in lower copper consumption. This cell count was increased after these bacteria produce anti-oxidant enzymes helping them to resist or adapt to the copper ion solution.

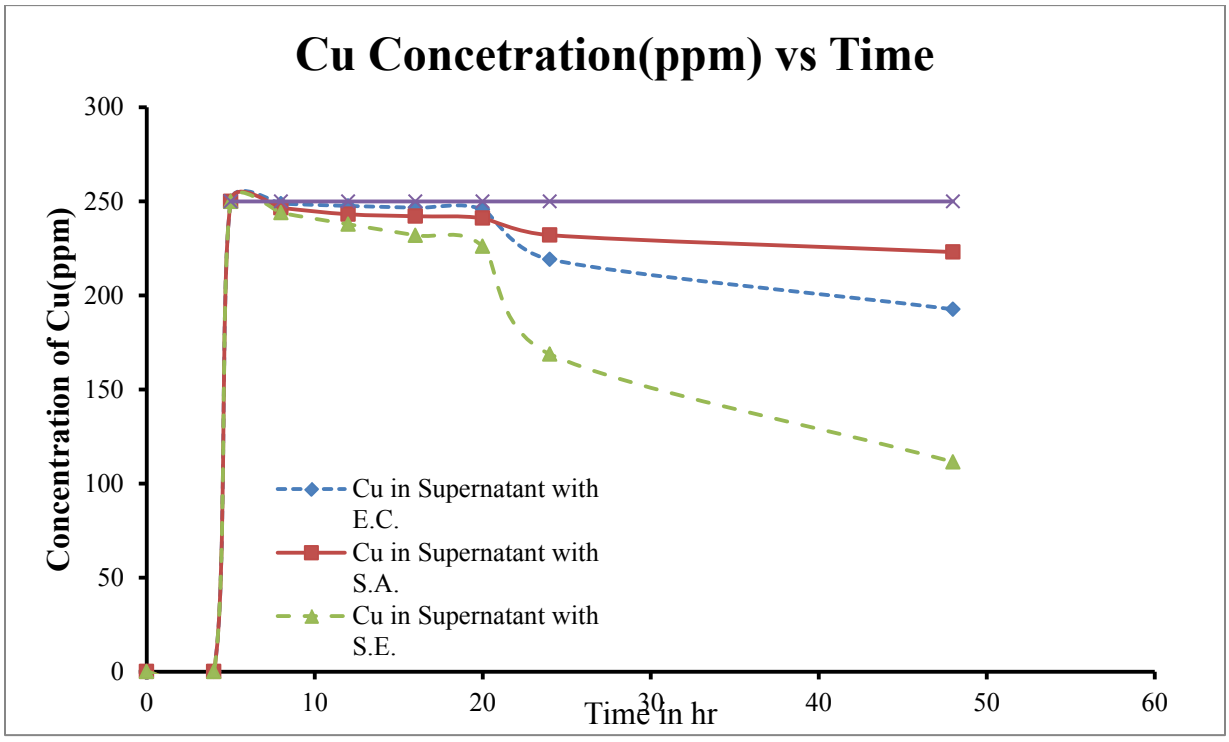

Figure 14: Concentration of copper in supernatant inoculated by three different bacterial strains. The graph is from average value of 2 replicates. Copper was added after 5 hours of incubation. E.C. is *E.coli*, S.A is *S. aureus* and S.E. is *S. epidermis.*

Chapter 5. Discussions

The research objective was to test effectiveness of copper ion solution on gram positive and gram negative bacteria and to find the efficacy of copper solution that would inhibit the formation of bacteria (biofilm) in frack related water. The effect of copper was explored on gram positive and gram negative bacteria. The common bacteria *E.coli* is gram negative and *S. aureus* is gram positive bacteria, were first selected to establish the experimental design. *S. epidermis*, fracking relevant gram positive bacteria, was added after design was established. The result showed that there wasn't significant difference in the ability of copper ion solution to impede growth of gram positive and gram negative bacteria despite some variation on tendency of curve. For the gram negative bacteria like *E.coli*, the inhibition due to copper ion solution continued for 12 hours whereas for gram positive bacteria like *S. aureus* and *S. epidermis*, the effectiveness was 8-10 hours. Meanwhile, flowback was able to inhibit all three bacterial strains for an extended time as compared from viable cell count and copper concentration analysis. Viable cell count showed a clear picture how copper reduced the cell growth. From turbidity and viable cell count for all three bacteria, it appeared that copper ion might have killed or inhibited these bacteria.

A copper concentration of 250 ppm has some inhibitory or toxic effect on both gram positive and gram negative bacteria with a difference in the length of time of the effectiveness. From the observations, for 10 to 12 hours, the copper ion solution overwhelms the inside of the cell. The cell metabolism, a biochemical reactions needed for life is obstructed by the copper ion. Metabolic reactions are carried out and catalyzed by enzymes. But when copper ions bind to these enzymes, their activity gets halted. This

bacterium could no longer eat, breathe, digest and create energy. A change in enzyme's activity and metabolism is one effect of heavy metals on bacterial cells. The growth inhibition and inhibition of replication are due to cell lysis (Silver *et al.,* 1994). But the trend of inhibiting didn't last long, which may signify that these bacteria were able adapt or overcome the copper's toxicity. Some bacteria like *S. aureus* have mechanism of adapting to high levels of environmental copper via increased oxidative stress resistance (Baker *et al*., 2010). Similarly, some microorganisms are capable of resisting the effects of heavy metals by forming the antioxidant enzyme superoxide dismutase or by the reduction of metals ions (Singh *et al*., 2013).

The ICP test was done at the end to see whether these copper was being used as nutrient or digested by the bacterial cells. The result obtained from copper analysis suggest that copper was consumed by all three bacterial strains as long as copper was available as indicated by the lowering of the copper concentrations as population grew. However, during the inhibition period, this copper might have been reluctantly consumed by bacteria since copper was highly toxic for them at that time. After adaptation period, copper was still consumed but its effect on bacterial growth was reduced due to the antioxidant enzyme produced by the bacteria. In general, the amount of bacteria especially in the first 15-20 hours was less therefore consumption of the copper was less rapid. After 20 hours, copper was greatly consumed by the increased population of bacteria. In the turbidity test and viable cell count, there is sharp increase in growth as soon as inhibition ends for *E. coli* and *S. aureus* which however was different for *S. epidermis*. Unlike *E. coli* and *S. aureus*, *S. epidermis* decelerated growth due to the copper ion solution extended after its 8-10 hour inhibition phase. This is consistent with

the results from the copper concentration analysis that showed there was more consumption of copper from supernatant (lower copper concentration in media) than in any other bacterial strains.

Future Work

The research was done only by studying the population growth of bacteria instead of growing the biofilm mass in the sample test. As we know improved biofilm monitoring is essential in order to progress the fundamental research on biofilm behavior. For the quantitative biofilm analysis, mass density measurements should have been done (Bakke *et al.,* 2001).

Copper ion solution is the simplest ion that copper forms in solution. This solution typically appears in blue color in the form of hexaaquacopper(II)-$[Cu(H_2O)_6]^{2+}$. We used a proprietary copper ion solution provided by the company, literally unknown about the chemical composition, in our research and focused on finding potential use of it in inhibiting the bacteria responsible for biofilm formation. The result wasn't as per our expectation. Had we tried to do same research using exact copper salts like ($CuSO_4$, $CuNO_3$, $CuSO_4.5H_2O$) instead, then we might have become able to inhibit bacteria for so long as done by the flow back solution (Chudobova *et al.,* 2015*).* On the other hand, we got this result by directly using 250ppm of copper ion solution. This may be more accurate by experimenting with wide range of copper concentration. We may increase the copper concentration to get long time inhibition.

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Appendix

Absorbance data of growth and inhibition curves of *Escherichia coli*

S.No.	Sample Vials	0	2	4	6	8	10	12	16	20	24	30	34	38	42	48	52	56
1	WW+*E.coli*	0.159	0.215	0.318	0.361	0.347	0.337	0.334	0.356	0.449	0.542	0.670	0.666	0.665	0.660	0.591	0.582	0.577
2	WW+*E.coli*	0.161	0.225	0.325	0.382	0.386	0.395	0.399	0.420	0.486	0.552	0.685	0.675	0.695	0.679	0.654	0.642	0.641
3	WW+*E.coli*	0.162	0.219	0.320	0.372	0.366	0.364	0.389	0.416	0.482	0.547	0.680	0.672	0.678	0.688	0.637	0.637	0.625
	Average	0.161	0.220	0.321	0.372	0.366	0.365	0.374	0.397	0.472	0.547	0.678	0.671	0.679	0.676	0.627	0.620	0.614
	Standard Deviation	0.002	0.005	0.004	0.011	0.020	0.029	0.035	0.036	0.020	0.005	0.008	0.005	0.015	0.014	0.033	0.033	0.033
	Confidence Interval	0.002	0.006	0.004	0.012	0.022	0.033	0.040	0.041	0.023	0.006	0.009	0.005	0.017	0.016	0.037	0.038	0.038
4	WW+*E.coli*+30ml Copper	0.160	0.243	0.336	0.240	0.240	0.235	0.238	0.237	0.360	0.482	0.603	0.637	0.637	0.635	0.631	0.638	0.621
5	WW+*E.coli*+30ml Copper	0.161	0.241	0.330	0.238	0.235	0.233	0.235	0.236	0.349	0.462	0.598	0.627	0.630	0.631	0.625	0.633	0.601
6	WW+*E.coli*+30ml Copper	0.159	0.239	0.321	0.235	0.233	0.231	0.232	0.235	0.352	0.468	0.595	0.617	0.625	0.625	0.629	0.630	0.599
	Average	0.160	0.241	0.329	0.238	0.236	0.233	0.235	0.236	0.353	0.471	0.599	0.627	0.631	0.630	0.628	0.634	0.607
	Standard Deviation	0.001	0.002	0.008	0.003	0.004	0.002	0.003	0.001	0.005	0.010	0.004	0.010	0.006	0.005	0.003	0.004	0.012
	Confidence Interval	0.001	0.002	0.009	0.003	0.004	0.002	0.003	0.001	0.006	0.012	0.005	0.011	0.007	0.006	0.003	0.005	0.014
7	WW+*E.coli*+30ml Flowback	0.157	0.231	0.315	0.271	0.273	0.270	0.274	0.274	0.274	0.273	0.271	0.281	0.336	0.395	0.401	0.452	0.452
8	WW+*E.coli*+30ml Flowback	0.157	0.223	0.312	0.267	0.271	0.265	0.270	0.271	0.268	0.271	0.269	0.279	0.333	0.385	0.395	0.425	0.426
9	WW+*E.coli*+30ml Flowback	0.158	0.229	0.310	0.264	0.265	0.260	0.265	0.270	0.271	0.273	0.265	0.275	0.319	0.350	0.365	0.420	0.419
	Average	0.157	0.228	0.312	0.267	0.270	0.265	0.270	0.272	0.271	0.272	0.268	0.278	0.329	0.377	0.387	0.432	0.432
	Standard Deviation	0.001	0.004	0.003	0.004	0.004	0.005	0.005	0.002	0.003	0.001	0.003	0.003	0.009	0.024	0.019	0.017	0.017
	Confidence Interval	0.001	0.005	0.003	0.004	0.005	0.006	0.005	0.002	0.003	0.001	0.003	0.003	0.010	0.027	0.022	0.019	0.020
10	30mL WW	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.000	0.002	0.001	0.001	0.003	0.001	0.001	0.001	0.001	0.001

Absorbance data of growth and inhibition curve of *Staphylococcus aureus*

S.No.	Sample Vials	0	2	4	6	8	10	12	16	20	24	30	34	38	42	48	52	56
1	WW+ *S. aureus*	0.108	0.131	0.166	0.173	0.190	0.241	0.278	0.325	0.370	0.415	0.501	0.561	0.567	0.561	0.555	0.561	0.542
2	WW+ *S. aureus*	0.109	0.129	0.166	0.167	0.199	0.239	0.275	0.315	0.353	0.390	0.495	0.545	0.548	0.549	0.550	0.558	0.532
3	WW+ *S. aureus*	0.108	0.132	0.165	0.185	0.210	0.240	0.275	0.310	0.348	0.385	0.493	0.540	0.545	0.550	0.555	0.556	0.541
	Average	0.108	0.131	0.166	0.175	0.200	0.240	0.276	0.317	0.357	0.397	0.496	0.549	0.553	0.553	0.553	0.558	0.538
	Standard Deviation	0.001	0.002	0.001	0.009	0.010	0.001	0.002	0.008	0.012	0.016	0.004	0.011	0.012	0.007	0.003	0.003	0.006
	Confidence Interval	0.001	0.002	0.001	0.010	0.011	0.001	0.002	0.009	0.013	0.018	0.005	0.012	0.014	0.008	0.003	0.003	0.006
4	WW+*S.aureus*+30ml copper	0.107	0.126	0.160	0.169	0.214	0.224	0.232	0.218	0.310	0.401	0.489	0.623	0.642	0.641	0.640	0.639	0.625
5	WW+*S.aureus*+30ml copper	0.106	0.125	0.164	0.185	0.215	0.214	0.215	0.201	0.298	0.395	0.480	0.562	0.545	0.556	0.501	0.528	0.520
6	WW+*S.aureus*+30ml copper	0.105	0.126	0.168	0.190	0.216	0.214	0.214	0.200	0.293	0.385	0.475	0.575	0.578	0.590	0.524	0.600	0.555
	Average	0.106	0.126	0.164	0.181	0.215	0.217	0.220	0.206	0.300	0.394	0.481	0.587	0.588	0.596	0.555	0.589	0.567
	Standard Deviation	0.001	0.001	0.004	0.011	0.001	0.006	0.010	0.010	0.009	0.008	0.007	0.032	0.049	0.043	0.075	0.056	0.053
	Confidence Interval	0.001	0.001	0.005	0.012	0.001	0.007	0.011	0.011	0.010	0.009	0.008	0.036	0.056	0.048	0.084	0.064	0.060
7	WW+*S.aureus*+30ml Flowback	0.105	0.124	0.156	0.175	0.232	0.243	0.266	0.283	0.305	0.326	0.317	0.310	0.327	0.324	0.321	0.325	0.351
8	WW+*S.aureus*+30ml Flowback	0.104	0.124	0.150	0.174	0.230	0.240	0.265	0.284	0.300	0.316	0.314	0.322	0.328	0.326	0.323	0.323	0.341
9	WW+*S.aureus*+30ml Flowback	0.104	0.125	0.151	0.173	0.234	0.251	0.245	0.274	0.288	0.301	0.302	0.321	0.325	0.325	0.324	0.320	0.351
	Average	0.104	0.124	0.152	0.174	0.232	0.245	0.259	0.280	0.298	0.314	0.311	0.318	0.327	0.325	0.323	0.323	0.348
	Standard Deviation	0.001	0.001	0.003	0.001	0.002	0.006	0.012	0.006	0.009	0.013	0.008	0.007	0.002	0.001	0.002	0.003	0.006
	Confidence Interval	0.001	0.001	0.004	0.001	0.002	0.006	0.013	0.006	0.010	0.014	0.009	0.008	0.002	0.001	0.002	0.003	0.007
10	30mL WW	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.000	0.002	0.001	0.001	0.003	0.001	0.001	0.001	0.001	0.001

Absorbance data of growth and inhibition curves of *Staphylococcus epidermis*

S.No	Sample Vials	0	2	4	6	8	10	12	16	20	24	30	34	38	42	48	52	56
1	WW+*S.epidermis*	0.169	0.175	0.236	0.338	0.418	0.505	0.601	0.663	0.754	0.844	0.854	0.855	0.846	0.850	0.804	0.805	0.801
2	WW+*S.epidermis*	0.169	0.182	0.249	0.315	0.420	0.512	0.612	0.665	0.733	0.800	0.755	0.809	0.805	0.812	0.790	0.801	0.799
3	WW+*S.epidermis*	0.165	0.178	0.244	0.339	0.425	0.522	0.615	0.685	0.753	0.820	0.835	0.839	0.823	0.826	0.801	0.804	0.750
	Average	0.168	0.178	0.243	0.331	0.421	0.513	0.609	0.671	0.746	0.821	0.815	0.834	0.825	0.829	0.798	0.803	0.783
	Standard Deviation	0.002	0.004	0.007	0.014	0.004	0.009	0.007	0.012	0.012	0.022	0.053	0.023	0.021	0.019	0.007	0.002	0.029
	Confidence Interval	0.003	0.004	0.007	0.015	0.004	0.010	0.008	0.014	0.013	0.025	0.059	0.026	0.023	0.022	0.008	0.002	0.033
4	WW+*S.epidermis*+copper	0.169	0.178	0.239	0.340	0.235	0.210	0.207	0.185	0.252	0.318	0.400	0.554	0.641	0.640	0.678	0.675	0.711
5	WW+*S.epidermis*+copper	0.169	0.187	0.229	0.345	0.225	0.221	0.219	0.222	0.275	0.328	0.320	0.550	0.581	0.620	0.665	0.666	0.670
6	WW+*S.epidermis*+copper	0.165	0.197	0.249	0.351	0.235	0.224	0.217	0.195	0.264	0.333	0.425	0.565	0.593	0.635	0.666	0.678	0.664
	Average	0.168	0.187	0.239	0.345	0.232	0.218	0.214	0.201	0.264	0.326	0.382	0.556	0.605	0.632	0.670	0.673	0.682
	standard deviation	0.002	0.010	0.010	0.006	0.006	0.007	0.006	0.019	0.012	0.008	0.055	0.008	0.032	0.010	0.007	0.006	0.026
	confidence interval	0.003	0.011	0.011	0.006	0.007	0.008	0.007	0.022	0.013	0.009	0.062	0.009	0.036	0.012	0.008	0.007	0.029
7	WW+*S.epidermis*+Floowback	0.169	0.171	0.213	0.273	0.230	0.246	0.263	0.211	0.338	0.347	0.351	0.348	0.351	0.355	0.345	0.390	0.397
8	WW+*S.epidermis*+Flowback	0.165	0.168	0.205	0.248	0.224	0.252	0.254	0.299	0.329	0.338	0.342	0.328	0.348	0.351	0.341	0.304	0.361
9	WW+*S.epidermis*+Flowback	0.164	0.197	0.225	0.273	0.239	0.252	0.280	0.335	0.349	0.363	0.369	0.355	0.379	0.380	0.386	0.391	0.399
	Average	0.166	0.179	0.214	0.265	0.231	0.250	0.266	0.282	0.338	0.349	0.354	0.344	0.359	0.362	0.357	0.362	0.386
	standard deviation	0.003	0.016	0.010	0.014	0.008	0.003	0.013	0.064	0.010	0.013	0.014	0.014	0.017	0.016	0.025	0.050	0.021
	confidence interval	0.003	0.018	0.011	0.016	0.009	0.004	0.015	0.072	0.012	0.014	0.016	0.016	0.019	0.018	0.028	0.057	0.024
10	30 ml WW	0.001	0.001	0.002	0.002	0.001	0.001	0.001	0.000	0.002	0.001	0.001	0.003	0.001	0.001	0.001	0.001	0.001

Viable cell count data of growth and inhibition curves of *Escherichia coli*

S.No	Sample Vials	0	4	10	16	24	34	48	56
1	*E.coli* +WW	5.00E+06	1.00E+07	2.00E+07	4.50E+07	5.50E+07	6.00E+07	6.00E+07	3.50E+07
2	*E.coli* +WW	4.00E+06	8.50E+06	1.80E+07	5.00E+07	5.20E+07	5.80E+07	5.50E+07	4.00E+07
3	*E.coli* +WW	4.00E+06	9.00E+06	1.70E+07	5.00E+07	5.50E+07	5.50E+07	5.80E+07	4.50E+07
	Average	4.33E+06	9.17E+06	1.83E+07	4.83E+07	5.40E+07	5.77E+07	5.77E+07	4.00E+07
	Standard Deviation	5.77E+05	7.64E+05	1.53E+06	2.89E+06	1.73E+06	2.52E+06	2.52E+06	5.00E+06
	Confidence Interval	6.53E+05	8.64E+05	1.73E+06	3.27E+06	1.96E+06	2.85E+06	2.85E+06	5.66E+06
4	*E.coli* +WW+ Copper	3.00E+06	9.00E+06	3.50E+06	5.50E+06	3.00E+07	5.00E+07	4.50E+07	4.00E+07
5	*E.coli* +WW+ Copper	3.00E+06	8.20E+06	4.00E+06	5.00E+06	2.80E+07	4.00E+07	4.50E+07	4.00E+07
6	*E.coli* +WW+ Copper	2.50E+06	6.50E+06	4.00E+06	5.20E+06	2.50E+07	4.50E+07	5.00E+07	3.50E+07
	Average	2.83E+06	7.90E+06	3.83E+06	5.23E+06	2.77E+07	4.50E+07	4.67E+07	3.83E+07
	Standard Deviation	2.89E+05	1.28E+06	2.89E+05	2.52E+05	2.52E+06	5.00E+06	2.89E+06	2.89E+06
	Confidence Interval	3.27E+05	1.44E+06	3.27E+05	2.85E+05	2.85E+06	5.66E+06	3.27E+06	3.27E+06
7	*E.coli* +WW+Flowback	4.00E+06	8.00E+06	4.50E+06	5.00E+06	6.00E+06	7.00E+06	2.00E+07	1.50E+07
8	*E.coli* +WW+Flowback	2.50E+06	7.50E+06	5.00E+06	5.00E+06	6.00E+06	6.50E+06	1.50E+07	1.80E+07
9	*E.coli* +WW+Flowback	4.50E+06	7.00E+06	5.50E+06	7.00E+06	4.50E+06	6.00E+06	1.00E+07	1.50E+07
	Average	3.67E+06	7.50E+06	5.00E+06	5.67E+06	5.50E+06	6.50E+06	1.50E+07	1.60E+07
	Standard Deviation	1.04E+06	5.00E+05	5.00E+05	1.15E+06	8.66E+05	5.00E+05	5.00E+06	1.73E+06
	Confidence Interval	1.18E+06	5.66E+05	5.66E+05	1.31E+06	9.80E+05	5.66E+05	5.66E+06	1.96E+06

Viable cell count data of growth and inhibition curves of *Staphylococcus aureus*

S.No	Sample Vials	0	6	10	16	24	34	48	56
1	*S.aureus* +WW	5.00E+06	1.00E+07	4.00E+07	6.00E+07	7.00E+07	7.00E+07	6.00E+07	4.00E+07
2	*S.aureus* +WW	4.00E+06	9.50E+06	5.00E+07	6.50E+07	7.00E+07	7.50E+07	6.00E+07	5.50E+07
3	*S.aureus*+WW	2.50E+06	7.50E+06	4.50E+07	6.00E+07	6.50E+07	6.50E+07	6.50E+07	6.50E+07
	Average	3.83E+06	9.00E+06	4.50E+07	6.17E+07	6.83E+07	7.00E+07	6.17E+07	5.33E+07
	Standard Deviation	1.26E+06	1.32E+06	5.00E+06	2.89E+06	2.89E+06	5.00E+06	2.89E+06	1.26E+07
	Confidence Interval	1.42E+06	1.50E+06	5.66E+06	3.27E+06	3.27E+06	5.66E+06	3.27E+06	1.42E+07
4	*S.aureus* +WW+ Copper	5.00E+06	8.00E+06	1.00E+07	1.00E+07	7.00E+07	7.00E+07	7.00E+07	5.00E+07
5	*S.aureus*+WW+ Copper	4.30E+06	8.50E+06	9.00E+06	8.50E+06	5.00E+07	6.00E+07	7.00E+07	6.50E+07
6	*S.aureus*+WW+ Copper	3.50E+06	8.20E+06	9.50E+06	8.50E+06	4.50E+07	5.00E+07	5.50E+07	7.00E+07
	Average	4.27E+06	8.23E+06	9.50E+06	9.00E+06	5.50E+07	6.00E+07	6.50E+07	6.17E+07
	Standard Deviation	7.51E+05	2.52E+05	5.00E+05	8.66E+05	1.32E+07	1.00E+07	8.66E+06	1.04E+07
	Confidence Interval	8.49E+05	2.85E+05	5.66E+05	9.80E+05	1.50E+07	1.13E+07	9.80E+06	1.18E+07
7	*S.aureus* +WW+Flowback	4.00E+06	9.00E+06	1.20E+07	2.00E+07	2.00E+07	2.00E+07	2.00E+07	2.50E+07
8	*S.aureus* +WW+Flowback	3.50E+06	7.50E+06	1.50E+05	2.00E+07	2.50E+07	2.20E+07	2.00E+07	2.00E+07
9	*S.aureus*+WW+Flowback	3.50E+06	1.00E+07	1.00E+07	2.50E+07	2.00E+07	2.50E+07	2.50E+07	2.50E+07
	Average	3.67E+06	8.83E+06	7.38E+06	2.17E+07	2.17E+07	2.23E+07	2.17E+07	2.33E+07
	Standard Deviation	2.89E+05	1.26E+06	6.34E+06	2.89E+06	2.89E+06	2.52E+06	2.89E+06	2.89E+06
	Confidence Interval	3.27E+05	1.42E+06	7.18E+06	3.27E+06	3.27E+06	2.85E+06	3.27E+06	3.27E+06

Viable cell count data of growth and inhibition curves of *Staphylococcus epidermis*

S.No.	Sample Vials	0	6	10	16	24	38	48	56
1	*S.epidermis* +WW	5.00E+06	1.50E+07	2.00E+07	4.00E+07	6.00E+07	6.50E+07	5.00E+07	4.00E+07
2	*S.epidermis*+WW	5.00E+06	1.20E+07	2.00E+07	3.50E+07	6.20E+07	6.00E+07	5.50E+07	4.00E+07
3	*S.epidermis* +WW	4.50E+06	1.00E+07	1.50E+07	3.00E+07	5.90E+07	6.30E+07	7.50E+07	7.00E+07
	Average	4.83E+06	1.23E+07	1.83E+07	3.50E+07	6.03E+07	6.27E+07	6.00E+07	5.00E+07
	Standard Deviation	2.89E+05	2.52E+06	2.89E+06	5.00E+06	1.53E+06	2.52E+06	1.32E+07	1.73E+07
	Confidence Interval	3.27E+05	2.85E+06	3.27E+06	5.66E+06	1.73E+06	2.85E+06	1.50E+07	1.96E+07
4	*S.epidermis* +WW+ Copper	5.00E+06	1.50E+07	8.00E+06	1.00E+07	2.00E+07	3.50E+07	5.50E+07	5.00E+07
5	*S.epidermis*+WW+ Copper	6.80E+06	1.42E+07	8.00E+06	9.00E+06	2.20E+07	3.10E+07	6.70E+07	4.00E+07
6	*S.epidermis* +WW+ Copper	6.50E+06	1.40E+07	6.00E+06	8.50E+06	1.80E+07	3.50E+07	6.50E+07	3.50E+07
	Average	6.10E+06	1.44E+07	7.33E+06	9.17E+06	2.00E+07	3.37E+07	6.23E+07	4.17E+07
	Standard Deviation	9.64E+05	5.29E+05	1.15E+06	7.64E+05	2.00E+06	2.31E+06	6.43E+06	7.64E+06
	Confidence Interval	1.09E+06	5.99E+05	1.31E+06	8.64E+05	2.26E+06	2.61E+06	7.28E+06	8.64E+06
7	*S.epidermis* +WW+Flowback	7.00E+06	1.50E+07	8.00E+06	1.50E+07	1.80E+07	2.00E+07	2.00E+07	2.50E+07
8	*S.epidermis* +WW+Flowback	3.50E+06	8.50E+06	7.50E+06	1.20E+07	2.00E+07	2.50E+07	2.20E+07	2.00E+07
9	*S.epidermis* +WW+Flowback	2.00E+06	7.50E+06	7.50E+06	7.50E+06	2.00E+07	2.50E+07	2.50E+07	3.00E+07
	Average	4.17E+06	1.03E+07	7.67E+06	1.15E+07	1.93E+07	2.33E+07	2.23E+07	2.50E+07
	Standard Deviation	2.57E+06	4.07E+06	2.89E+05	3.77E+06	1.15E+06	2.89E+06	2.52E+06	5.00E+06
	Confidence Interval	2.90E+06	4.61E+06	3.27E+05	4.27E+06	1.31E+06	3.27E+06	2.85E+06	5.66E+06

Inductively coupled plasma test results

S.No.	Sample	0	4	5	8	12	16	20	24	48
1	Sterile medium with Copper	0	0	250.000	250.000	250.000	250.000	250.000	250.000	250.000
2	Cu in supernatant with S.A.	0	0	250.000	248.812	247.624	246.673	245.722	219.190	192.657
3	Cu in supernatant with E.C.	0	0	250.000	246.590	243.180	242.121	241.063	232.079	223.095
4	Cu in supernatant with S.E.	0	0	250.000	243.933	237.866	232.005	226.145	168.830	111.516