Efficacy of Clean-in-Place (CIP) Sanitizers on *Pseudomonas* Biofilms During In-Place Cleaning of Food Contact Surfaces

**THESIS**

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

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2015

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Abstract

Sanitizers are used during Cleaning-In-Place (CIP) processes to ensure inactivation of microorganisms. Biofilms, created by microorganisms adhere to surfaces and are notorious for harboring microorganisms resistant to cleaning agents and sanitizers. The overall objective of this research was to evaluate the novel Generally Recognized As Safe (GRAS) sanitizer ProSan LC and compare its sanitation efficacy to sodium hypochlorite and Sanidate 5.0 (a peracetic acid solution), two frequently used sanitizers in the food industry. The comparisons included quantification of the efficacy of inactivating populations of *Pseudomonas* in biofilms formed on stainless steel coupons. *Pseudomonas* was grown for 48hrs on 1.27cm² type-304 stainless steel coupons in 24-well micro plates. To quantify the formation of biofilms on the coupons, a crystal violet assay was performed that stains both extracellular polymeric substances and individual cells which are adhered to the coupon. Sanitizer or normal saline control was applied to a biofilmed coupon prior to staining with crystal violet. To test the efficacy of the sanitizers in inactivating the microbial population in a biofilm, studies were performed on biofilmed coupons using three different concentrations of each sanitizer with varying exposure times (0, 0.5, 5, 10, 15 and 20minutes). After each exposure time, coupons were immersed in neutralizing broth to inactivate each sanitizer. Coupons were then swabbed
and plated on tryptic soy agar. The maximum reductions in microbial population were obtained after 20 minutes exposure time with the highest concentration of each sanitizer. The results of reductions obtained from recommended concentrations include 3.21 log CFU/ml for 230ppm Sanidate 5.0, a reduction of 3.73 log CFU/ml for 7.8ml/L ProSan LC and a reduction of 2.95 log CFU/ml for 200ppm sodium hypochlorite. A biphasic model for non-linear regression that describes two fraction populations of inactivation was used to fit each set of inactivation data. Biphasic parameters showed that *Pseudomonas* has a large susceptible population (>99%) that was quickly inactivated by each sanitizer. This was demonstrated by a large initial kinetic rate of inactivation $k_1$. In contrast a small fraction population was highly resistant and resulted in a much smaller $k_2$ rate of inactivation for all sanitizers. Overall, Sanidate had the lowest $k_1$ reflecting a slower rate of inactivation relative to NaOCl and ProSan. On average, the efficacy of ProSan on *Pseudomonas* biofilms was superior when comparing recommended concentrations. SEM imaging conveyed the visual structure of a *Pseudomonas* biofilm. In addition, images of biofilms exposed to ProSan shows possible evidence of surface interaction and loosening of the biofilm layer.
Acknowledgments

I would like to give my utmost appreciation and gratitude to Dr. Dennis Heldman for his unlimited patience, wisdom and support of me. I am extremely grateful and fortunate to have had him as my advisor throughout my graduate program.

I would like to thank Dr. Hua Wang and Dr. Bala Balasubrahmaniam for being part of my committee and providing support and guidance.

I would like to thank David Phinney and Cheryl Wick for their support and advice throughout my research. Their wonderful input at every weekly meeting they sat through was always helpful and encouraging.

I would like to thank my laboratory colleagues in the Dale Seiberling lab for their advice and input throughout my research.

I would like to thank my parents, Edward and Erin Park for the importance they placed on education and their love and support throughout my life.
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CHAPTER 1: INTRODUCTION

Clean-in-Place (CIP) is a process used to clean and sanitize food processing equipment without the need to dismantle and manually clean and sanitize. The technology was developed in the 1950’s and has become a commercially accepted practice in most sectors of the food and related industries. Prior to CIP, most equipment and pipelines in food processing facilities were manually disassembled, then washed and rinsed by employees with limited training (Seiberling, 2008). Clean-in-place requires individual tanks for storage of cleaning, sanitizer agents and rinse water. These agents are pumped through the processing system of pipes and vessels. Most often, the key steps in the process are controlled by an automated system to reduce human error and ensure reproducibility, uniformity and safety of the cleaning process. The entire process requires significant amounts of water for the rinsing, cleaning and sanitation steps.

In general, the CIP process includes the following steps:

1. Pre-rinse \( \text{H}_2\text{O} \) – initial rinse to remove large soil deposits
2. Alkaline Wash – a key step in soil removal from surfaces and is generally performed using sodium hydroxide or potassium hydroxide
3. \( \text{H}_2\text{O} \) Rinse – rinse any remaining alkali residuals and soil
4. Acid Wash – a step to neutralize alkali residues and remove inorganic scale and soil that may be more soluble in an acid solution

5. H₂O Rinse – rinse any remaining acid residuals

6. Sanitizer – inactivate residual microorganisms

The final step of CIP is sanitization. This step ensures that the processing equipment is free of viable microorganisms. There are many compounds available to effectively accomplish this step such as chlorines, SIP (Steam-in-place), iodophors and various acids.

Biofilms are microbial communities that are embedded within a self-composed matrix of exopolymeric substances that are adhered to a surface. When microorganisms are placed in an environment with minimal nutrients, there is a change in metabolism, and bacteria cells such as *Pseudomonas* begin to adhere to surfaces. The general cycle of biofilm formation involves initial reversible attachment, irreversible attachment, maturation and eventual detachment to begin the cycle anew.

Biofilms are notorious for their high resistance to sanitizers and environmental stresses and represent a different growth phase in bacteria compared to planktonic cells (J W Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995)

There are several proposed explanations for the development of biofilms and the resistance of the microbial cells to external stresses such as sanitizers. These explanations
include (a) resistance due to slow penetration of sanitizer through the exopolymer matrix and (b) metabolism/slow growth of cells and genetic transfer and quorum sensing (Pace, Rupp, & Finch, 2006). In the context of food processing, biofilm formation on equipment surfaces may contribute to increased corrosion and reducing heat transfer in heat exchangers (S. J. Yuan & Pehkonen, 2007).

*Pseudomonads* are a ubiquitous genus of aerobic, gram-negative and flagellated microorganisms that are found in many food processing facilities. These microorganisms are generally considered non-pathogenic but can cause food spoilage. The ability of the *Pseudomonads* to create biofilms on food processing surfaces has been demonstrated (Hood & Zottola, 1997). Due to the ease of growth and rapid development of biofilms, the *Pseudomonads* have been the subject of considerable amounts of biofilm research.

Although other steps of the CIP process contribute to the removal of biofilms from an equipment surface, the sanitizing step ensures inactivation of the microbial population within the biofilm. Due to this important role, the efficacy of sanitizers and the entire sanitation step must be quantified and understood.
CHAPTER 2: LITERATURE REVIEW

2.1 Clean-In-Place (CIP) Overview

Clean-in-Place has been a mainstay of many industries since its development by Dale A. Seiberling more than 50 years ago. Originally, Clean-in-Place was used as a method for cleaning Pyrex glass milking system pipelines (Seiberling, 2008). Since then it has been applied to the food, beverage and biopharmaceutical industries for convenient and efficient cleaning of industrial equipment. Much of the cleaning prior to this was done manually and by the least experienced workers in a facility. This method of manually dismantling equipment is known as clean-out-of-place and is inefficient in comparison to CIP. The system utilizes a number of tanks which contain rinse water and various types of cleaning and sanitizing compounds. These tanks are connected to processing equipment needing cleaning through a series of pipes and a cleaning regime can be electronically automated to carry out a CIP cycle. After a CIP program is completed, the system should be sufficiently cleaned of soil and microbiologically inactivated.

Cleaning programs may be designed to be either single-pass or recirculated. Both programs are considered viable options and have their strengths and weaknesses. A single-pass system only utilizes water once and requires a greater amount of water over
time and therefore has increased waste volume. Cleaning compounds are added by batching or in-line mixing after which it is disposed. This is helpful in a system that may be heavily soiled and the detergent heavily saturated. Although single-use is demanding, it avoids the risk of contamination. A recirculated system design is considered more economical as water or cleaning solution may be reused multiple times with cleaning compounds being recirculated from the processing equipment back into their storage tanks after use thus reducing overall costs and water usage. This option does however introduce the risk of recontamination of processing equipment.

Monitoring and validation techniques are essential to providing information that the CIP program is effective in cleaning to an appropriate level. Each facility will have contain their own specific protocols however in general the system should ultimately be cleaned of any foulant and residues while minimal cleaning compound residues. Parameters such as temperature, flow rate and cleaning sequence should generally be monitored. Residuals may be validated with pH measurements, fluorometry, swab testing and conductimetry (Chisti & Moo-Young, 1994).

The majority of the piping and tanks used in the food industry are composed of high grade, type 304 or 316 stainless steel. The chromium oxide which forms on the surface of high grade stainless steel forms a passive, corrosion resistant layer. Although this layer helps protect the stainless steel, it is not completely resistant and many types of
corrosion such as pitting, stress and crevice corrosions may still occur (Tsutsumi, Nishikata, & Tsuru, 2007). When present these corrosion defects offer attachment sites for processing residues. Cleaning of foulants that accumulate on stainless steel during processing is important to prevent biofilm formation. A study done on modifying stainless steel showed fluorinated nanoparticles deposited onto the surface of type 316L stainless steel heat exchangers reduced foulant accumulation by 97% and improved heat transfer efficiency (Barish & Goddard, 2013).

2.2 CIP General Process

Although the CIP process may be customizable in the context of specific industries, most programs utilize water rinses, alkali rinse, acid rinse and sanitizer rinse (Seiberling, 2008). Many factors such as temperature, time, mechanical effects and chemical activity of cleaners contribute to the efficacy of a CIP process. Optimization of these CIP variables for each system based upon the specific foods and processing parameters used is essential for each facility.
2.2.1 Water Rinses

Water rinsing is performed both as a pre-rinse and as intermediate rinses between changing of cleaning compounds. The pre-rinse water serves to remove bulk material from the system prior to alkali detergent wash. This pre-rinse water may be water that has been previously used in intermediate rinse steps and can come from a recovery tank to save costs (Seiberling, 2008). Intermediate rinses of water are performed between changes of cleaning compounds such as alkali and acid rinses. These rinses are carried out using new water that has not been previously used within a CIP system. Potable water may be softened by removing calcium and magnesium to reduce the build-up of scale deposits on piping and tanks.

2.2.2 Alkali Wash

Alkali detergent is used in CIP to remove foulant soil. Many systems undergo some type of heating step such as pasteurization in dairy processing. This thermal challenge often causes fouling onto surfaces due various reactions such as caramelization of sugars, denaturation of proteins and polymerization of fats. Sodium hydroxide (aka caustic soda, NaOH) is the most widely used and effective alkali detergent in CIP systems. Potassium hydroxide (KOH) is another effective detergent but
is more expensive. Rinse water has the ability to solubilize food components such as monovalent salts and sugar but cannot effectively solubilize fats and proteins (Marriott, 1994). Alkalis detergents are able to effectively remove fats, protein and carbohydrate foulants from surfaces. NaOH is considered a surfactant that reacts with fats by separating fats by charge into glycerol fatty acid portions. Alkali rinses alone may be insufficient to fully clean a system however. Alkali does not effectively penetrate soil, has limited suspending ability, cannot clean mineral deposits and has variable efficacy dependent on soil composition (Seiberling, 2008).

Recent developments in CIP have attempted to save costs by reusing alkali solutions. Alkali solution may be recycled using methods such as centrifugation, ultra membrane filtration and decantation (Dresch, Daufin, & Chaufer, 2001). This recycle system may be directly integrated into a CIP system for both single-use and recirculate systems.

2.2.3 Acid Wash

Acid washes are implemented following the alkali wash and an intermediate water rinse. Descaling acid rinses serve to eliminate mainly inorganic scale deposits that may accumulate on surfaces such as calcium, magnesium and other salts. Calcium in particular may react with carbonate to form CaCO₃ deposits on heat transfer
surfaces that reduce the efficiency of heat transfer processes and flow rate. In dairy processing, calcium phosphate salt accumulation is also an issue and forms due to insolubility at higher processing temperature (Visser & Jeurnink, 1997). Thin layers of deposit have been shown to increase the energy consumption by 10% (Energy, 1998). Surfactant ingredients can be added to acid wash solutions to enhance removal of both inorganic and organic scale (Seiberling, 2008). Various acid solutions have been proposed for reducing or removing scale deposits from food processing surfaces. Nitric and phosphoric acid are widely used in CIP systems however organic acids such as citric or tartaric acid may also be used.

2.2.4 Sanitizer Rinse

The application of a sanitizer at the end of a CIP cycle helps to ensure proper food safety by inactivating spoilage and pathogen microorganisms. Alkali and acids are not considered sanitizers and do not sufficiently inactivate microbes, although studies have shown they can remove microbial biofilms from CIP surfaces in combination therefore a compound must be used with the specific objective of eliminating them (Bremer, Fillery, & McQuillan, 2006). Although this step is sometimes considered optional, due to the large risk of microbial contaminations leading to
consumer illness, it is strongly advised that a sanitization step be implemented to ensure proper food safety.

2.2.4.1 Chlorine

Chlorine-based solutions are cheap and widely used sanitizers for households, industry and water systems. As such, it is the standard by which all other sanitizers are measured in efficacy. Sodium hypochlorite was originally created by reacting chlorine gas with soda lye but may also be produced by electrochemical reactions with sodium chloride and chlorine and even by utilizing seawater (Saleem et al., 2012). An aqueous solution may be in three different forms: chlorine (Cl₂), hypochlorous acid (HOCl) and monochlorine monoxide (–OCl). HOCl has the ability to penetrate cellular membranes and damage internal cell structures while –OCl may only oxidize the outer membrane (Fukuzaki, 2006). Cl₂ has low water solubility and therefore is easily released from solution. Chlorines are strong oxidizing agents and its mechanism of microbial destruction has been debated and several explanations have been proposed. The CDC (Rutala, Weber, & Control, 2008) recognizes many mechanisms of inactivation such as oxidation of sulfhydryl enzymes and amino acids, inhibition of protein synthesis, damage to DNA and DNA synthesis or a combination of multiple factors acting at once.
With proteins, it has been shown that chlorine reacts with amino acid side-chains, breaks down protein backbones and creates nitrogen-centered radicals (Hawkins & Davies, 1998). Chlorine concentration has variable efficacy on microorganisms. Concentrations below 260ppm do not induce extensive membrane disruption or protein degradation and the mechanism of inactivation is thought to be through inhibiting DNA synthesis (Mcdonnell & Russell, 1999). Some vegetative bacteria being effected by free available chlorine at levels <5ppm while 1000ppm are required to inactivate M. tuberculosis and 100ppm inactivates >99.9% of *B. atrophaeus* spores (Rutala et al., 2008). A study on persistent pathogens in the food industry found that various *L. monocytogenes* and *E. coli* strains required variable concentrations of sodium hypochlorite (NaOCl) ranging from 5-50 mg/liter to achieve a 5-log reduction (Holah, Taylor, Dawson, & Hall, 2002). As effective as chlorine may be in inactivating microorganisms, its use does have several disadvantages. Due to its strong oxidizing nature, chlorine can lead to passive corrosion of stainless steel (Laycock, Stewart, & Newman, 1997). Sodium hypochlorite is shown to decompose and react by a number of different means:

\[2\text{NaOCl} \rightarrow \text{NaClO}_2 + \text{NaCl}\]

\[\text{NaOCl} + \text{NaClO}_2 \rightarrow \text{NaClO}_3 + \text{NaCl}\]

\[\text{OCl}^- + 2\text{HOC}_1 \rightarrow \text{ClO}_3^- + 2\text{HCl}\]

\[2\text{OCl}^- \rightarrow 2\text{Cl}^- + \text{O}_2\]
The decomposition rates increase with temperature and the active bactericidal compounds (HOCl and \( \cdot \text{OCl} \)) degrade over time (Lister, 1956). Additionally, chlorine is particularly sensitive to UV light which promotes the aforementioned reactions (Solvay Chemicals International, 2005). These issues are likely why the code of federal regulations only allows sodium hypochlorite to be used on food contact surfaces at concentrations of no more than 200ppm (FDA, 2014).

### 2.2.4.2 Peracetic acid

Peracetic acid (\( \text{C}_2\text{H}_4\text{O}_3 \), aka peroxyacetic acid) is a potent sanitizer with a wide range of uses. Its popularity is due to having several key advantages: ability to affect many different microorganisms, enhances removal of organic material, leaves no residues and decomposes into innocuous byproducts such as water and acetic acid (Rutala et al., 2008). Peracetic acid is commercially produced by an ozone catalyzed autoxidation of acetaldehyde (NIH, 2012). The decomposition of peracetic acid follows several different mechanisms: (i) spontaneous decomposition into acetic acid and oxygen (ii) hydrolysis to form acetic acid and hydrogen peroxide and (iii) Various reactions with transition metals (Z. Yuan, 1997). The self-decomposition mechanism in particular is a function of temperature and pH with higher temperatures and higher (more basic) pH.
causes increases in the kinetic rate constant of decomposition (Z. Yuan, 1997). Due to the strong oxidizing nature of peracetic acid, free-radical formation is the main source of bacteria inactivation in bacteria (Clapp, Davies, French, & Gilbert, 1994). The CFR Title 21 (FDA, 2014) restricts peracetic acid to an upper limit of 200ppm for food contact surfaces.

2.2.4.3 Quaternary Ammonia

Quaternary ammonia (QACs) sanitizers are cationic compounds that contain a nitrogen atom with four covalently bonded alkyl R groups which disrupts proteins, enzymes and cell membranes (Rutala et al., 2008). They are popular for their flexibility, low toxicity and efficacy against gram positive bacteria (Schmidt, 1997). QACs are used in the food industry on a variety of surfaces such as floors, piping and tables and are able to coat surfaces thus creating a protective film to prevent bacterial growth (Marriott, 1994). They are however not effective against bacteriophages or gram-negative bacteria and lose their efficacy in the presence of hard water and anionic detergents (Schmidt, 1997). QACs are additionally classified as cationic surfactants which are highly dependent upon the composition of the various R-groups attached to the nitrogen (Block, 2001). Synergistic blends of various R-groups are able to provide a
higher hard water resistance while maintaining proper microbial sanitation. This is observed for instance by utilizing 2,4,6-trimethylbenzyldimethyl ammonium chloride (which has a water hardness tolerance of 1300ppm) and mesitylenyl or pseudocuminyl quaternary compounds that have excellent antimicrobial properties (Block, 2001). The efficacy of QACs on bacteria is shown to be variable. A study (Feliciano, Li, Lee, & Pascall, 2012) showed ≥5 log reductions in populations of *E. coli* and *Listeria innocua* on tableware but only approximately a 3 log reduction in norovirus populations from 200ppm QAC. Another study on the populations of *E. coli* and *Salmonella* on harvesting knives demonstrated 3.51 and 3.42 log reductions with use of 200ppm QAC, respectively (Tapp, Gragg, Brooks, Miller, & Brashears, 2013). Issues in utilizing QACs for a CIP system would likely arise with hard water used in rinse steps as well as the potential for residual alkali detergent, both of which may reduce the potency of QACs. The CFR Title 21 (2015) restricts use of quaternary ammonia compounds to 200ppm on food contact surfaces.

### 2.2.4.5 Organic Acids

Organic acids such as citric, acetic and lactic acid have been shown to have antimicrobial activities and are generally considered GRAS status compounds
(which demonstrate little to no public hazard) by the FDA which makes them more attractive as disinfectants than other chemicals (FDA, 2014). As primary acid compounds in fermented foods and fruits, organic acids have for a long time been identified as inhibiting or inactivating agents for microorganisms. Organic acids are a general term for a variety of compounds but the main mechanism of inactivation is thought to be similar with pH being a major factor. The efficacy of organic acids on pathogens has been widely studied. An in vitro study on Campylobacter jejuni found that organic acids alone or in combination had strong bactericidal effects achieving as much as >4 log reductions in overall population in under 2 hours at a pH of 4.0 and in 8 hours at a pH of 5.5 (Chaveerach, Keuzenkamp, Urlings, Lipman, & van Knapen, 2002). Interactions between citric acid and pH in another study demonstrated that the kinetics of Listeria monocytogenes inactivation depended on both pH and citric acid concentration however low levels of citric acid demonstrated a protective effect on Listeria (Buchanan, Golden, & Whiting, 1993). Organic acids however do not always inactivate microorganisms and may only slow down growth. The novel sanitizer Prosan, contains organic acids as well as surfactant ingredients that effectively inactivate bacteria. Research on the use of ProSan is sparse due to the novelty of the compound however a study used Prosan and ProSan LC to examine the efficacy on inactivating planktonic and biofilm cells of L. monocytogenes (Wassinger, 2011). Results from these experiments showed that both
were very effective on planktonic cells but failed to achieve more than a 2 log reduction on biofilm populations.

2.3 Biofilm Formation

Biofilms are aggregates of microorganisms contained within a self-composed extracellular polymeric substance (EPS) that is attached to a surface. The EPS may be composed of various materials such as polysaccharides, proteins and DNA. Biofilms are found in numerous ecosystems and are generally much more resistant to environmental stresses than their planktonic cell counterparts (J W Costerton, Stewart, & Greenberg, 1999). Biofilms are considered to not only be a special circumstance in the life cycle of bacteria, but a normal phase of growth that bacteria develop through to better survive in harsh, fluctuating and low-nutrient environments (Hall-Stoodley, Costerton, & Stoodley, 2004). The physiology and metabolism of bacteria found in a biofilm are noticeably different compared to planktonic cells and this is partially reflected by the formation of a mature biofilm. Many different factors affect the overall development of a biofilm including organism, medium, flow, nutrient content etc. which ultimately influence the genetic expression of the microorganisms found within a biofilm (Hall-Stoodley et al., 2004).
2.3.1 Adherence and Attachment

Biofilms must first be initiated by the attachment of a microbe onto a surface. The process of adherence involves two phases: first a reversible rapid adhesion then a non-reversible molecular and cellular adhesion of planktonic cells in the first phases of biofilm development (An, 2000). Both motile and non-motile bacteria exhibit adhesion and initial biofilm formation by producing EPS that help adhere irreversibly to surfaces and adjacent cells (Götz, 2002). *P. aeruginosa* in particular is a motile species that produces a mainly alginate-based EPS in a continuous culture biofilm and the algC gene that regulates alginate production was shown to be expressed in as little as 15 minutes upon exposure to a glass surface (Davies & Geesey, 1995). Several key factors affect the adhesion of bacteria onto a surface such as the environment, substrate of adherence and the bacteria itself. A study on *S. epidermidis* found that levels of CO₂ and type of medium both significantly affected the production of slime and adherence onto a polystyrene surface (Hussain, Wilcox, White, Faulkner, & Spencer, 1992). An investigation of the effects of temperature found greater growth of *L. monocytogenes* at 18°C on stainless steel coupons compared to 4°C and 30°C after 48hrs (D. E. Norwood & Gilmour, 2001). Starvation and environmental stresses both are known to cause biofilm
formation and increased resistance in biofilms (Fux, Costerton, Stewart, & Stoodley, 2005). Different surface types have been shown to affect adherence of different bacteria. An investigation on the attachment of *L. monocytogenes* to stainless steel, Teflon, polyester and nylon found that biofilm formation was greatest on polyester (40% of surface area) and least on nylon (3%) (Blackman, Frank, & Frank, 1996). A study on pre-conditioning surfaces for reduced adhesion found that aqueous cod muscle extract could be applied to stainless steel to decrease the adhesion of *P. fluorescens* (Bernbom et al., 2009). The ability of adherence is variable not just between different genus and species, but differs considerably between strains within a species as well (Chae, Chae, Schraft, & Schraft, 2000).

### 2.3.2 Maturation

After adherence to a surface, microorganisms begin the process of maturing into a complex multi-layered structure biofilm. The biofilms created may be mushroom-like, porous, flat or stream-like depending on a number of factors. These structures were shown in a continuous flow chamber to contain as much as 98% EPS and space indicated the presence of nutrient/water flow channels essential to the growth and maturation of a biofilm (Lawrence, Korber, Hoyle, Costerton, & Caldwell, 1991). As the
biofilms mature, microcolonies of daughter cells develop with high densities of EPS within each colony but are sparse in inter-colonial spaces. Each colony’s water channels act almost as a multi-cellular organism that delivers nutrients to individual cells while excreting metabolic byproducts (J. W. Costerton et al., 1994). The exact infrastructure developed is however highly dependent upon the organism and environment (Hall-Stoodley et al., 2004). A study on biofilm formation under high shear stress such and those found in streams or processing equipment matured as “streamer” shaped with strong cohesiveness this study also suggests that biofilms in these situations are younger and mature slowly (Rochex, Godon, Bernet, & Escudié, 2008)(Hall-Stoodley et al., 2004). This is in constrast to biofilms matured under lower shear stresses which tend to mature as mound-shapes and are generally not as strong or cohesive (Stoodley, P. Cargo, R. Rupp, CK Wilson, S Klapper, 2002). These differences in maturation patterns are reflected on a metabolic and phenotypic level. A study on the metabolic response of biofilms to shear stress found a correlation between high shear stress grown biofilms and higher dehydroxenase activity with lower growth rates. This may indicate that biofilms respond to shear stress by regulating both catabolic and anabolic pathways (Liu & Tay, 2001).
2.3.3 Detachment

Once a biofilm matures, bacteria within a biofilm begin detaching and dispersing in order to survive and colonize new environments. The phenomena of detachment may occur due to environmental shear forces, or enzymatic degradation of EPS the microorganisms for release. A study on shear-induced detachment found that a $P. \text{aeruginosa}$ biofilm grown for 6-days at 0.03 ms$^{-1}$ rapidly detached from a surface when flow was adjusted to 1ms$^{-1}$ while a biofilm grown at 1ms$^{-1}$ did not have detachment until 2.5ms$^{-1}$ (Stoodley, P. Cargo, R. Rupp, CK Wilson, S Klapper, 2002). An alginate lyase enzyme expressed by a mucoid strain of $P. \text{aeruginosa}$ induced detachment/sloughing of cells from agar with an increase of activity when the biofilm is in an unfavorable growth environment (Boyd & Chakrabarty, 1994).

2.4 Methods for Imaging and Sampling Biofilms

Numerous methods have been utilized for biofilm visualization and sampling including - crystal violet staining, electron microscopy and scanning confocal laser microscopy (SCLM).
2.4.1 Biofilm Imaging Methods

Crystal violet staining of biofilms is a high throughput method that can easily be done in micro plates. The crystal violet stains both cells and EPS revealing the extent of growth of biofilms on a surface (Lawrence et al., 1991). Although easy to perform, this method is unable to directly quantify the microbial population since no actual plating estimates are provided.

Electron microscopy is a popular method of visualizing biofilms on surfaces and is capable of very high magnifications. Several variations of electron microscopy exist: Scanning, Transmission and Environmental Scanning. Scanning electron microscopy (SEM) utilizes dried and metallized samples that are observed in a vacuum sealed chamber and scanned with an electron beam. Transmission electron microscopy (TEM) is similar to SEM but transmits electrons through a thin sample. Environmental scanning electron microscopy (ESEM) is a variation of SEM but allows samples to contain up to 10 torr water vapor (Surman et al., 1996). Although electron microscopy may be useful, it has several drawbacks due to the extensive sample preparation that may alter the native state of a biofilm sample such as shrinkage by dehydration (Flemming, Szewzyk, & Griebe, 2000).

Scanning confocal laser microscopy (SCLM) analysis of biofilms utilizes fluorescent probing techniques to visualize the shape and architecture of a biofilm. This
type of microscopy is helpful when investigating spatial differences, revealing water channels and depth of a biofilm and may be constructed into a 3D map (J W Costerton et al., 1995). SCLM also has the option of using non-toxic negative staining techniques that allow unhindered development and thereby observe biofilm maturation (Caldwell, Korber, & Lawrence, 1992).

2.4.2 Biofilm Sampling Methods

Sampling biofilms for viable count may be performed using numerous methods, each with their own advantages and disadvantages. Some popular methods include swabbing/scraping, sonication, and direct pour agar enumeration. Direct mechanical force removes a biofilm from a surface for direct enumeration of a population. This can be done by utilizing metal scrapers, swabs, glass beads or flat knives to manually remove a biofilm. This type of method is ideal for smooth surfaces however porous or rough surfaces that have crevices would likely yield inaccurate microbial populations (Flemming et al., 2000). Swabbing/scraping methods may be easy to perform but are potentially inaccurate as portions of the biofilm may still remain giving inaccurate counts (Wirtanen, Salo, Helander, & Mattila-Sandholm, 2001).
Sonication disrupts biofilms and removes them from a surface without lysing cells. It is a good alternative to scraping and has been shown to more comprehensively remove biofilms and enumerate more bacteria versus conventional swabbing/scraping (Bjerkan, Witsø, & Bergh, 2009). It does however require a higher capital investment due to additional equipment costs. Another method for enumerating biofilm populations is by direct pouring of agar onto sample surfaces. Unlike the previous two methods, direct agar enumeration does not manually attempt to remove biofilms from a surface instead directly the agar is directly poured over a sample for enumeration. This allows for an accurate representation of a biofilm population but only allows for sampling small residual numbers of bacteria (Fatemi & Frank, 1999).

2.5 Methods for Biofilm Growth and Development

Methods used for biofilm growth depend on the ultimate goal and objectives of the research.- A method may be orientated towards a high throughput design that generally only requires short development periods or a long-term growth development with larger time investments. Method designs may also be differentiated by the presence
of flowing or static medium for growth. All these factors contribute to the overall characteristics of a developing biofilm such as structure and metabolism.

O’Toole (2011) developed a high throughput bench top method of analyzing biofilms by employing micro titer plates to incubate and grow microbes. This assay has many advantages including relative ease of performance, little equipment requirements and allows an investigator to quantify viable cell counts, visualize biofilms and perform microscopy of living cells. The disadvantage of such a method is that in a real world environment, many biofilms are developed in flowing systems such as water/sewage and food processing systems and thus the method may not be representative of a practical situation. Another study utilized static biofilm development with 3-week long growth periods with daily sanitizer exposure to investigate the increased resistance of *L. monocytogenes* biofilms over time (Pan, Breidt, & Kathariou, 2006). The study justified the use of a static growth system by arguing that many biofilms in food processing persist in areas that do not see high intensity shear forces but instead areas of minimal exposure such as dead-ends, joints and corners.

Many different devices exist that provide a controlled continuous flow environment for biofilms. A few examples include RotoTorque, constant-depth film fermenter, rotating disc reactor etc. The RotoTorque consists of an inner rotating cylinder with slides attached to a stationary outer cylinder for biofilm accumulation (Flemming et
al., 2000). The constant-depth fermenter utilizes “plugs” which are set at a constant-depth flowing system and allowed to develop over long periods of time after which the samples are taken out and scraped (D E Norwood & Gilmour, 2000). Rotating disk reactors contain a rotating disc “CD” centered in a device that spins to develop biofilms with exposure to shear stress while providing nutrient flow (Horn et al., 2007).

2.6 *Pseudomonas* spp. Biofilms

*Pseudomonas* is a genus of gram-negative, aerobic and rod-shaped microbes prevalent in many environments including the food industry as the major spoilage microorganism and is widely studied in biofilm related research. The main avenue of spoilage in food generally occurs due to protease and lipase activity which results in rancidity and off-odors (Huis In’t Veld, 1996). *Pseudomonas* has been studied extensively in the medical and food industry due to its tenacity and resistance to antibiotics and environmental stresses. This is at least partially due to the production of alginate that acts as a protective barrier without restricting nutrient influx (Montie, 1998). Alginate is a polysaccharide composed of 1→4 linear linked polymers of β-D-mannuronic acid and α-L-guluronic acid that has been isolated in *Pseudomonas* species *P. putida*, *P. fluorescens* and *P. mendocina* among others (Govan, Fyfe, & Jarman,
Additional polysaccharides have been identified in *P. aeruginosa* such as levan (polymer of β–polyfructan), cellulose and PsI (polymer of β-D-mannose) which contribute to the overall biofilm matrix (Mann & Wozniak, 2012). The overall structure of a biofilm as discussed earlier, is dependent upon many environmental factors. As a model for *Pseudomonas* biofilm structures, *P. aeruginosa* has been shown to have a distinct mushroom-like structure in its mature biofilm. The “stalk” of the structures was found to be non-motile while the “heads” contained motile subpopulations that were preparing for potential migration and detachment (Klausen, Aaes-Jørgensen, Molin, & Tolker-Nielsen, 2003).

### 2.7 Efficacy of Sanitizers on Biofilm Inactivation

Biofilms, in contrast to planktonic cells, are difficult to inactivate due to their aforementioned resistance mechanisms and much research has been done investigating the efficacy of various sanitizers on a variety of microorganisms.

*L. monocytogenes* has been a well-studied organism in biofilm research due to its pathogenicity and ability to form biofilms on food contact surfaces. A study on the resistance of *L. monocytogenes* to hydrogen peroxide, QAC and chlorine found that a 3-week old biofilm had increased resistance to each sanitizer as maturation time increased.
Initial decreases ranged from 1-2.5 log CFU/cm² however by day 5, only reductions of 0.3 log CFU/cm² were observed (Pan et al., 2006). The cause of resistance was attributed to the ability of EPS to block the sanitizing action of each compound used. Similar results were obtained by a study on a 28-day old multi-species biofilm consisting of *L. monocytogenes*, *S. xylosus* and *P. fragi* for resistance to sodium hypochlorite. The results demonstrated high resistance to inactivation with a 2-log reduction from free chlorine concentrations of 1000 ppm (D E Norwood & Gilmour, 2000). A 4-day old *L. monocytogenes* cocktail exposed to 50mg/l sodium hypochlorite was found to have a 3.09 log CFU/cm² reduction observed over a 10 minute period (Vaid, Linton, & Morgan, 2010). Biofilms of *E. coli* and *L. monocytogenes* grown on lettuce leaves were used to evaluate ozone, chlorine and organic acid efficacy of removal and inactivation. It was found that none of the treatments effectively removed cells. A 6-hour biofilm was reduced by 3-3.5-logs with all three sanitizers however a 24-hour biofilm saw only 2-2.5-log reduction across all three sanitizers (Ölmez & Temur, 2010).

*Pseudomonas spp.* are a common bacteria that can form biofilms and many studies on sanitizer efficacy exist. One study utilized confocal laser scanning microscopy to visualize real-time effects of peracetic acid (PAA) and benzalkonium chloride (BAC) on *P. aeruginosa* biofilms. The investigation found that PAA uniformly penetrated a biofilm while BAC efficacy was first localized at the biofilm periphery and slowly spread
internally and exemplified the differences in resistance of different sanitizers (Bridier, Dubois-Brissonnet, Greub, Thomas, & Briandet, 2011). Another study tested a variety of sanitizers on *P. fluorescens* with 5-minute exposure times. It was found that a peracetic acid/ hydrogen peroxide solution reduced populations by approximately 3-log after 5 minutes but only 1-2 logs with iodophor and chlorohexidine gluconate. This was in contrast to planktonic cells which had 5-log reductions (Lindsay & von Holy, 1999).

2.8 Biofilms in the Food Industry

Biofilms on food contact surfaces are potentially dangerous and persistent sources of contamination in the food industry. It is therefore important to design efficient cleaning systems in food facilities to prevent accumulation and growth of biofilms. CIP systems generally do adequately in removing soil and deposits from food processing surfaces. Cleaning processes are particularly important to break up particles and biofilm matrices that can build-up. These problems may be exacerbated by corrosion and pits on surfaces which are harder to clean and therefore retain soil and debris after cleaning (Leclercq-Perlat & Lalande, 1994). Cleanability and surface adherence to varying surface types should also be considered. A study of *Salmonella spp.* and *L. monocytogenes* on attachment onto stainless steel, rubber and polytetrafluorethylene found that attachment
and sanitizer efficacy varied considerably between surface types. It was found that stainless steel was less adherent and a recommended surface type compared with the other two however polytetrafluorethylene had the easiest cleanability and sanitation (Sinde & Carballo, 2000).

Some novel solutions for biofilm prevention have been studied in recent years with particular emphasis on surface conditioning. Aqueous protein extracts were used as a conditioning agent on stainless steel. It was found that coating the surface of the stainless steel in a layer of protein helped prevent microbial adhesion for up to 21 days for *P. fluorescens* (Bernbom et al., 2009). Another study found that the compound nisin had the ability to adsorb onto stainless steel while maintaining its anti-microbial activity to prevent biofilm formation (Guerra et al., 2005).

Food industry biofilms containing multiple species exhibit different dynamic properties compared to monospecies biofilms. A multi-species biofilm such as *L. monocytogenes/Pseudomonas sp.* mixed-cultures has been found to lend protective properties to the *L. monocytogenes* populations. This mixed-culture biofilm was evaluated for resistance to peracetic acid and chlorine. Although *Pseudomonas* populations dominated the mixed-culture, *L. monocytogenes* was still present in a 48-hour incubation and survived a 5 minute exposure of 160ppm peracetic acid (Fatemi & Frank, 1999).
CHAPTER 3: OBJECTIVE

The overall objective of this research project was to evaluate the efficacy of sanitizers used during the Clean-in-Place process for food contact surfaces in a food processing facility. The specific objectives were as follows:

a. To measure the efficacy of sanitizers in the inactivation of *Pseudomonas spp.* biofilm populations on stainless steel surfaces.

b. To compare the efficacy of three different sanitizers, including Sodium hypochlorite, Sanidate 5.0 (a peracetic acid solution) and Pro-San LC, a novel GRAS sanitizer.

c. To explore the mechanisms of sanitizer action, with specific attention to biofilm removal as compared to inactivation of the microbial population.
CHAPTER 4: MATERIALS AND METHODS

4.1 *Pseudomonas* Isolation and Identification

Beef and milk samples purchased from a local grocery store were used for isolating *Pseudomonas* colonies. Beef samples were placed in a whirl pack with 10ml of 0.1% peptone and masticated with a Stomacher 80 (Seward, Worthing, UK) for 60 seconds on high then serially diluted in 0.1% peptone. Milk samples were directly serially diluted. Pseudomonas isolation agar and tryptic soy agar (BD, Franklin Lakes, New Jersey) were plated and incubated at 30°C for 24hrs. Isolates were gram-stained to identify morphology. A -80°C culture was prepared for each isolate by growing each isolate overnight in 1ml tryptic soy broth (TSB) then adding 1ml of a 60% glycol solution. Samples were labeled and stored in -80°C freezer.

Polymerase chain reaction (PCR) of the 16s rRNA gene was performed on isolates for genus identification using MJMini thermocycler (BioRad, Hercules, CA). Single colonies were isolated and placed in a vial with 100ul of dH2O and glass beads using sterilized toothpicks. To extract DNA, vials were shaken for 3 minutes with a Mini Beadbeater (Biospec, Bartlesville, OK), boiled for 10 minutes in water and placed in an ice bath overnight at -20°C. Samples were then centrifuged at 10000 rpm for 1 minute
and 10-20ul of supernatant (DNA template) was placed into separate tubes. A PCR mastermix (New England Biolabs Inc., Ipswich, MA) was employed to provide the materials necessary for replication. Sterile water was used as a negative control for each PCR run. 1% agarose gel electrophoresis was performed on PCR products for verification of successful production and stained with ethydium bromide for observation under UV light. Samples were then purified using Qiagen Qiaprep Kit (Venlo, Germany) and sent for sequencing to the Plant-Microbe Genomics Facility (Ohio State University, Columbus, OH).

4.2 Growth and Attachment Assay by Crystal Violet Staining

Rate of growth of Pseudomonas spp. strains were assessed using a Spectronic 20 Genesys spectrophotometer (Thermo Fish Scientific, Waltham, MA) to measure optical density. Cultures were revived from -80°C into 1ml TSB and transferred twice before growth assessment to ensure log phase. 100ul of culture was then transferred into 10ml TSB and incubated with agitation at 30°C. The O.D. at 600nm was measured every 2 hours to monitor population growth.
Figure 1. Growth curves of *Pseudomonas* spp. at 30°C

|  |  
|---|---|
| **Table 1.** Time required for growth (Approx. $10^8$ log CFU/ml) by O.D. at 600nm in *Pseudomonas* spp. grown at 30°C |
| Pseudomonas spp. | Time to Achieve O.D. 0.5 (hrs) |
| Strain B1 | 6.35 |
| Strain M2 | 6.78 |
| Strain M6 | 6.45 |
| Strain L3 | 6.57 |
A rapid attachment assay utilizing crystal violet staining was performed to determine initial biofilm forming capabilities of each isolate in polystyrene tubes. Crystal violet has the ability to stain both EPS and bacterial cells. Bacteria rapidly attach to the tube walls and may be quantified through staining. Cultures were revived from -80°C into 1ml TSB and incubated overnight at 30°C. Cultures were transferred twice more before a 1% inoculation into 25ml of TSB then incubated overnight at 30°C. Each Pseudomonas spp. was centrifuged, washed with saline, and re-suspended to O.D. 0.5 at 600nm in a 10⁻¹ dilution TSB medium. 2ml aliquots of each culture was then dispensed into each tube and incubated for 4hrs at 30°C. 500ul of 0.1% crystal violet (Thermo Fisher Scientific) was added to each tube and allowed to stain for 15 minutes at room temperature after which each was rinsed with 3x4ml H₂O to remove planktonic cells. The tubes were allowed to air dry to remove residual water then crystal violet was de-stained with 4ml 95% ethanol (Thermo Fisher Scientific) and absorbance read at 600nm with spectrophotometer. All samples were performed in triplicate with two separate trials.
Figure 2. Rapid attachment crystal violet assay of *Pseudomonas spp.* on polystyrene tubes

Letters signify significant difference (*P*<0.05)

4.3 Efficacy of Sanitizers on Planktonic Cells

Cultures were revived from -80°C and plated on trypticase soy agar (TSA). Single colonies were transferred to 10ml of TSB and then transferred once more prior to transferring to 500ml of TSB. All transfers were incubated at 30°C overnight. Cultures were then centrifuged at 5000rpm for 10 minutes at 10°C, re-suspended in saline, and centrifuged once again. Pellets were then re-suspended in 10⁻¹ diluted TSB and O.D. adjusted to 0.5 (approx. 10⁸ CFU/ml). 1ml of culture was then added to a 2ml centrifuge tube and 1ml of the lowest concentration of each sanitizer (100ppm HOCl, 3.95ml/L Pro-
San and 80ppm Sanidate) added to each and allowed to incubate at room temperature for 1 minute. Tubes were then centrifuged at 13,000 rpm for 1 minute and supernatant removed and replaced with 1 ml of Dey-Engeley (D/E) neutralizing broth. Appropriate serial dilutions were performed and samples were plated on TSA. All analyses were performed in triplicate.

4.4 Crystal Violet Staining Assay on Stainless Steel

Biofilms were grown on 1.27 cm$^2$ round type 304-stainless steel coupons (Ohio State Machine Shop, Columbus, OH) to quantify and visualize biofilm development after sanitizer and saline rinses. Cultures were revived from -80°C and plated on TSA. Single colonies were transferred to 10 ml of TSB and then transferred once more prior to transferring to 500 ml of TSB. All transfers were incubated at 30°C overnight. Cultures were then centrifuged at 5000 rpm for 10 minutes at 10°C, re-suspended in saline, and centrifuged once again. Pellets were then re-suspended in 10$^{-1}$ diluted TSB and O.D. adjusted to 0.5 (approx. 10$^8$ CFU/ml). 1 ml of culture was then transferred into each well of a 24-well micro plate, each well containing a stainless steel coupon. Biofilms were allowed to develop over a 48 hour period at room temperature. After 48 hours, each well was aspirated and then exposed to 1 ml of either saline (control) or a sanitizer for 5 or
20-minute period after which 1 ml of D/E neutralizing broth was added to each well and allowed to incubate for 1 minute to neutralize the sanitizer effects. The D/E neutralizing broth was aspirated and followed by a saline rinse, the coupons were then exposed to 500 ul of 0.1% crystal violet and left to stain for 10 minutes at room temperature after which the coupons were rinsed several more times and left to air dry for 10 minutes. Crystal violet was de-stained with 4 ml of 95% ethanol and the absorbance read @ 600 nm in a spectrophotometer. Each sample was assayed in triplicate.

4.5 Efficacy of Sanitizers on Biofilms Grown on Stainless Steel

The method for sampling biofilms developed on coupons was similar to that of the crystal violet assay on coupons. Biofilms were allowed to develop over a 48 hours period at room temperature. Sanitizers were prepared as needed on sampling days with three concentrations chosen for each: 100, 200 and 300 ppm sodium hypochlorite (Ricca, Arlington, TX), 80, 230 and 460 ppm Sanidate 5.0 (Biosafe Systems, Hartford, CT), 3.9 ml/L, 7.8 ml/L and 15.6 ml/L Pro-San LC (liquid-concentrate) (Microcide, Detroit, MI). The first concentration was chosen based off of manufacturing recommendations and FDA regulatory limitations on food contact surfaces. Then two concentrations, one below and one above the recommended concentration were chosen. After 48 hours, each
well was aspirated and then exposed to 1ml of either saline (control) or a sanitizer solution with varying exposure times (0, 0.5, 5, 10, 15 and 20 minutes) after which 1ml of DE Broth was added and incubated at room temperature for 1 minute to neutralize the sanitizer effects. Each coupon was then swabbed with a calcium alginate swab (Puritan, Guilford, ME) dipped in 0.1% peptone tween-80 (Fisher, Fairlawn, NJ) and placed in 1ml of 0.85% saline for serial dilutions. Dilutions were then plated on TSA and incubated for 24 hours at 30°C. Each variable set was performed in triplicate.

Similar procedures were performed when conducting experiments with agitation. After incubation for 48 hours, each well’s medium was aspirated and replaced with the recommended concentration of each sanitizer or saline. Samples were then placed inside of a shaker and allowed to shake for 1 minute at 100rpm then 1ml D/E broth neutralizing aliquoted to each well. Plating and dilutions were performed as mentioned above.

Figure 3. Stainless steel coupons incubated with *Pseudomonas* culture medium
4.6 Kinetics and Modeling of Inactivation

The traditional interpretation of a microbial survival curve assumes a first-order kinetic reaction and follows a log-linear regression with a general model: \( N = N_0 \exp(-kt) \). The reality may however be quite different from an ideal log-linear model of inactivation. It is particularly important in the food industry to use accurate predictive models as they are ultimately used to establish sanitation parameters just has temperature, concentration and time required to reach appropriate log reductions. In some cases, inactivation may observe a considerable tailing as time increases in an inactivation process, a phenomena that has been researched for some time (Cerf, 1977). The biphasic model has the general formula:

\[
\log_{10}(N) = \log_{10}(N_0) + \log_{10}(f e^{-k_{max1}t} + (1-f) e^{-k_{max2}t}) \quad (1)
\]

This model was used to fit the data points obtained from inactivating *Pseudomonas* biofilms on stainless steel coupons and essentially splits the inactivation process into two separate portions, each with a different k-constant and population fraction. Variables \( N \) and \( N_0 \) are defined as the population at time \( t \) and initial population, respectively. The \( f \)-value is defined as the fraction population and the two “k” constants represent rate constants in each respective phase. The model was fit into 3 data points at each time point.
through GinaFit Excel add-in program. Data from the population and time obtained from each concentration automatically outputted a regression curve and biphasic parameters.

4.7 Biofilm Observation by Scanning Electron Microscopy (SEM)

SEM imaging is utilized in microbiology to better visualize a target population. In this particular project, SEM is used for identifying the successful formation of a biofilm on stainless steel as well as exploring the morphological and structural effects that sanitizers may have on a biofilm. Biofilms were prepared as previously mentioned in section 4.3.4. Sample coupons were then exposed to saline or sanitizer at recommended concentrations (100ppm sodium hypochlorite, 230ppm Sanidate 5.0 and 3.9ml/L Pro-San LC) and neutralized by D/E broth for 1 minute. After a H2O rinse, each coupon was immersed in a fixative solution (10ml 25% gluteraldehyde, 3.42g sucrose, 50ml 0.2M phosphate buffer pH 7.4 and ddH2O) and allowed to fix overnight. Sample coupons were rinsed in 0.1M phosphate buffer 3x5 minutes each and then dehydrated using an ethanol series from 50% to 100% with 10 minute incubation times at each concentration. Coupons were stored overnight in the final 100% ethanol before critical point drying and platinum coating at the Molecular and Cellular Imaging Facility (Wooster, OH) followed by SEM imaging performed on a Hitachi S-3500N scanning electron microscope.
4.8 Statistical and Data Analysis

Statistical analysis was performed via SPSS Statistics 22 (IBM, Armonk, NY) using ANOVA and t-tests. GInaFit 1.6 (Katholieke Universiteit Leuven, Leuven, Belgium), an excel add-in program used for modeling non-linear regression curves, was used to fit regression curves into each set of data on sanitizer inactivation. SAS Enterprise Guide 6.1 (SAS Institute, Cary, NC) Graphs were created using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA).
CHAPTER 5: RESULTS AND DISCUSSION

5.1 Inactivation of Planktonic Cells

Each sanitizer was evaluated for their efficacy of inactivating planktonic (free-living) cells that are not attached to a surface. Results in Figure 4 show that in comparison to a saline control, each sanitizer at a given concentration effectively reduced the population by at least 4 logs in only 1 minute of exposure. 100ppm NaOCl had a mean reduction of 4.87 log cfu/ml, 80ppm Sanidate saw reductions of 4.89 log cfu/ml and 3.95ml/L ProSan had observed reductions of 5.99 log cfu/ml. Paired t-tests indicate a significant difference ($P<0.05$) between saline and all sanitizers. ProSan was significantly more effective against planktonic cells when compared to Sanidate and NaOCl. The contrast in resistance is important to note as it demonstrates the need to prevent biofilm formation and establish proper strategies to prevent contamination in a processing facility. Previous research shows inactivation of planktonic $B.\ cereus$ cells with 200ppm sodium hypochlorite observed a 5.78 log CFU/ml reduction after a 60 second exposure time (Peng, Tsai, & Chou, 2002). $B.\ subtilis$ and $P.\ fluorescens$ bacteria saw a 2-log differential in efficacy when comparing peracetic acid inactivation against planktonic and biofilm cells (Lindsay & von Holy, 1999). An additional study on the resistance of $L.$
plantarum to various organic acids found that in all tests, cells in a biofilm had higher survival compared to planktonic cells in concentrations above 5% of lactic, citric and acetic acid (Kubota, Senda, Tokuda, Uchiyama, & Nomura, 2009). In contrast however, another study found that P. aeruginosa did not show increased resistance to antimicrobials whether it was in its planktonic or biofilm states of growth, attributing “persister” cells, that constituted a small minority of the total population, as the sole survivors (Spoering & Lewis, 2001).

Figure 4. Inactivation of Isolate B1 planktonic cells with 1 minute exposure time to sanitizer or saline (control)

Different letters signify significant difference ($P<0.05$)
5.2 Crystal Violet Assays

Crystal violet reversibly stains both EPS and bacterial cells in a biofilm. The amount of stain taken up by the biofilm may be quantified as a rapid method of detecting bacterial attachment to surfaces. Optical density measurements at 600nm quantify the amount of crystal violet that remained after multiple rinses of water. Each sanitizer’s ability to remove biofilms from a stainless steel surface is shown in Table 5. Results represented in Figure 6 indicate no significant difference in removal between each of the sanitizers at both 5 and 20 minutes of exposure ($P<0.05$). However, each sanitizer was significantly different in removal when compared to saline ($P<0.05$). These results may be due to the conditions of exposure. Turbulence was kept to a minimum during wash changes which may have minimized removal regardless of sanitizer used. The corresponding plate counts shown in Figure 7 indicate that both saline and sodium dodecyl sulfate (SDS) had little to no efficacy on the inactivation of Isolate B1 while all sanitizers showed reductions of at least 2 log cfu/ml. Based on both crystal violet and plate count results, we see that the observed log reductions are likely not due to manual removal of the biofilm from the surface but from direct inactivation from each sanitizer.
ProSan’s ingredients include SDS, an anionic surfactant which is used to remove dirt from fruit/vegetable surfaces and is generally used in conjunction with light scrubbing and brushing. We can infer from comparing the log reductions by SDS to ProSan that the observed inactivation in ProSan is unlikely to have been caused by a significant amount of removal. While surfactants are theoretically able to remove particles from a surface,
this is generally done with agitation or heat which could explain why SDS was ineffective in properly removing biofilms from the stainless steel coupons.

Figure 6. Crystal violet assay of sanitizer rinsed Isolate B1 biofilm on stainless steel coupons

Different letters signify significant difference ($P<0.05$)
Figure 7. Inactivation of Strain B1 biofilm with 5min exposure times

Different letters signify significant difference (P<0.05)

5.3 SEM Imaging

SEM images were taken at OARDC (Wooster, OH) to observe the presence, morphology and structure of a *Pseudomonas* biofilm.

Due to the many rinsing steps involved in the sample preparation, portions of the biofilm were shown to be sloughed off. This seems to be a common occurrence in many SEM
images published in literature (Pan et al., 2006) Each coupon however preserved large portions of a good biofilm layer for imaging. These images are shown in Figures 8-12. Figure 8 shows an early SEM image taken of a biofilm grown in an agitated beaker with a stir-bar revolving at 200rpm. The main purpose of the two images was to show how the stainless steel coupon appears after SEM preparation as well as to help support conclusions found in the crystal violet assay (Figure 6). The contrast between surfaces and bacteria is apparent and is also present in the bare spots on Figures 8A and 8B.

A. 

B. 

![SEM image of stainless steel coupon surface with sparse populations of *Pseudomonas*](image)

**Figure 8.** SEM image of stainless steel coupon surface with sparse populations of *Pseudomonas*
Images of coupons exposed to saline indicate extensive coverage of the surface with *Pseudomonas* biofilm (Figure 9). The saline does not appear to substantially affect the biofilm layer which can be seen in both 5 and 20-minute exposure times.

Figure 9. SEM image of *Pseudomonas* biofilm exposed to saline at 5 minutes (A and B) and 20 minutes (C and D)
SEM images of coupons exposed to Sanidate and NaOCl (Figures 10 and 11) show extensive preservation of a dense biofilm layer in the areas captured. Based on these images, we can support the conclusions found in the crystal violet assays that found no substantial mechanical removal of the biofilm layer due to sanitizer exposure.

Figure 10. SEM image of *Pseudomonas* biofilm exposed to Sanidate at 5 minutes (A and B) and 20 minutes (C and D)
Figure 11. SEM image of *Pseudomonas* biofilm exposed to NaOCl at 5 minutes (A and B) and 20 minutes (C and D)
The finals images are those captured from coupons exposed to ProSan. From the four images seen in Figure 12, we can see the formation of the biofilm is less compact and more fractured in comparison to the previous two sanitizers. This may indicate the surfactants role in loosening the adherence of the biofilm layer to the stainless steel surface. We could speculate from this qualitative evidence that this loosening could have influenced the rapid inactivation seen in the application of ProSan to the coupons.

![Figure 12. SEM image of Pseudomonas biofilm exposed to ProSan at 5 minutes (A and B) and 20 minutes (C and D)](image-url)
5.4 Efficacy of Sanitizers on Biofilms Grown on Stainless Steel

In evaluating the efficacy of each sanitizer, three separate concentrations were used; one at the recommended/legal limits concentration for food contact surfaces, one below and one above this concentration. Figure 13 shows the experimental data for three different concentrations of Sanidate.

![Experimental Data for Sanidate](image)

Figure 13. Inactivation of Isolate B1 by Sanidate 5.0 with error bars represent log CFU/ml±SD

The survivor curve shows a large contrast in efficacy: 80ppm Sanidate fails to achieve more than a 0.9 log reduction. At the food contact use concentration limit of 230ppm, inactivation is rapid relative to 80ppm but the initial rate of inactivation is even greater at 460ppm. The ineffectiveness of 80ppm Sanidate may be due to the intrinsic traits of *Pseudomonas spp.* Peroxygens generally perform better on bacteria that are gram-
positive or do not synthesize peroxidases (Mcdonnell & Russell, 1999). Since

*Pseudomonads* are gram-negative and synthesize catalase it may resist inactivation to

lower concentrations of Sanidate 5.0. Additionally, species such as *P. aeruginosa*

contains highly resistant outer membranes with LPS (lipopolysaccharides) that prevents

access into the cytoplasm. Assuming our *Pseudomonas* isolates have similar intrinsic

factors, this may explain the high level of resistance in a biofilm where alginates and

various polysaccharides prevent penetration of a sanitizer.

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**Figure 14. Inactivation of Isolate B1 sodium hypochlorite error bars represent log CFU/ml±SD**

Figure 14 shows inactivation by three concentrations of NaOCl. The initial rate of

inactivation for all concentrations used is fairly rapid while inactivation after 5 minutes is
slowed considerably. The results indicate that NaOCl is relatively effective in this general range of concentration.

Figure 15. Inactivation of Isolate B1 by ProSan LC error bars represent log CFU/ml

Figure 15 represents the inactivation of *Pseudomonas* in a biofilm by three different concentrations of ProSan. Notice that inactivation at 3.95ml/L is not nearly as efficient as that of 7.8ml/L and 15.6ml/L which are very similar in their inactivation.
Similar research is wide and varied in the results on the efficacy of sanitizers on biofilms. A six-day old biofilm of *P. aeruginosa* grown on type 304-stainless steel was used to evaluate the efficacy of sodium hypochlorite and sodium hypochlorite-hydrogen peroxide (Ox-B) combination sanitizer. Ox-B was found to reduce populations by 7-8 logs in 5 minutes while sodium hypochlorite produced a 4-6 logs. No cells were recovered after 20 minutes of treatment (DeQueiroz & Day, 2007). Biofilms that have had regular exposure to sanitizers were shown to become more resistance as they aged in research performed on *L. monocytogenes* biofilms exposed to peroxide, quaternary ammonia and chlorine every 24 hours for 21 days (Pan et al., 2006). Younger biofilms showed between 1-3 log reductions but decreased to only 0.3 log reductions or less within one week of growth with daily exposure to a particular sanitizer. Another study used a constant-depth film fermenter to form a 28-day old mixed-species biofilm containing *L. monocytogenes*, *Ps. fragi* and *S. xylosus* (D E Norwood & Gilmour, 2000). The mature biofilm was shown to only achieve a 2 log reduction with exposure to 1000 ppm sodium hypochlorite. In the context of the food industry, this concentration would exceed the 200 ppm limit on food contact surfaces and reflects the importance of preventing biofilms early in order as older biofilms will resist sanitizers to a greater extent as they mature. In contrast, a study on *P. putida* biofilms grown for 1,2 and 3 days showed no difference in treatment resistance.
when sanitizing with a liquid hypochlorite solution or a non-foaming acid sanitizer (Chumkhunthod, Schraft, & Griffiths, 1998). A study on the efficacy of ProSan on L. monocytogenes biofilms showed that less than a 2 log reduction was achieved in a 30 minute period (Wassinger, 2011).

The large variety of results may be indicative of the complexity involved with biofilms and its research. Variability in methods, organisms, and sanitizers may all be contributing factors.

The biphasic model of microbial inactivation utilizes two k-values to distinguish two different inactivation rates for different fractions of a population and helps to further compare the efficacy of each sanitizer. The general formula as previously stated is:

\[
\log_{10}(N) = \log_{10}(N_0) + \log_{10}(f*e^{-k_{max1}t} + (1-f)*e^{-k_{max2}t})
\] (1)

In equation (1), the f-value is defined as a particular fraction of a population. Based upon the general biphasic equation, a fraction of the population follows one kinetic constant and another fraction a second constant. In the case of our sanitizers, the majority (>90%) of the microbial biofilm population follows the first kinetic rate constant (Table 2).
### Table 2. Biphasic model parameters

<table>
<thead>
<tr>
<th>Sanitizer</th>
<th>Concentration</th>
<th>f±SE</th>
<th>$k_1$±SE (log reduction/min)</th>
<th>$k_2$±SE (log reduction/min)</th>
<th>R²</th>
<th>Estimate log reduction at 20min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sanidate</strong> 3.0</td>
<td>80 ppm</td>
<td>0.93±0.487</td>
<td>0.16±0.12</td>
<td>0±0.28</td>
<td>0.93</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>230 ppm</td>
<td>0.999±8.9E-4</td>
<td>0.74±0.06</td>
<td>0±0.05</td>
<td>0.99</td>
<td>3.02</td>
</tr>
<tr>
<td></td>
<td>460 ppm</td>
<td>0.9971±1.7E-3</td>
<td>7.3±1.08</td>
<td>0.16±0.03</td>
<td>0.97</td>
<td>3.94</td>
</tr>
<tr>
<td><strong>Sodium Hypochlorite</strong></td>
<td>100 ppm</td>
<td>0.9919±2.7E-3</td>
<td>6.39±0.67</td>
<td>0.009±0.02</td>
<td>0.98</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>200 ppm</td>
<td>0.9938±4.1E-3</td>
<td>8.37±1.48</td>
<td>0.09±0.04</td>
<td>0.93</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>300 ppm</td>
<td>0.9988±3.4E-4</td>
<td>13.27±0.87</td>
<td>0.04±0.02</td>
<td>0.99</td>
<td>3.30</td>
</tr>
<tr>
<td><strong>ProSan LC</strong></td>
<td>3.95 ml/L</td>
<td>0.9946±4.8E-3</td>
<td>1.11±0.21</td>
<td>0.08±0.06</td>
<td>0.95</td>
<td>2.92</td>
</tr>
<tr>
<td></td>
<td>7.8 ml/L</td>
<td>0.9973±1.5E-3</td>
<td>7.35±1.02</td>
<td>0.13±0.03</td>
<td>0.97</td>
<td>3.68</td>
</tr>
<tr>
<td></td>
<td>15.6 ml/L</td>
<td>0.9972±1.1E-3</td>
<td>8.48±0.76</td>
<td>0.12±0.02</td>
<td>0.98</td>
<td>3.61</td>
</tr>
</tbody>
</table>

The $f$-value as discussed previously is defined as the fraction population. This fraction is large (>99% of total population in most cases) and the inactivation rate is defined by $k_1$. This implies that the majority of the population is inactivated within the time-frame of the first kinetic rate constant. In all cases $k_1$ increases with increasing concentration (Figure 16) indicating an increase in inactivation rate with respect to concentration. Notice however the trends within each sanitizer. Sanidate has a very small $k_1$ for both 1x and 0.5x recommended concentration while 2x is more comparable to NaOCl and ProSan large. For Prosan, 0.5x is minimal but is comparable for 1x and 2x. For NaOCl the
rate of inactivation increases steadily over the concentration range. These values further support the fact Sanidate may be less effective in inactivating *Pseudomonas* biofilm populations in comparison to the other two sanitizers. These observations may relate to the mechanism of inactivation and the ability of *Pseudomonas* to produce oxidase enzymes that neutralize the sanitizing compounds in Sanidate. The $k_2$ value for all sanitizers is considerably smaller than the $k_1$ and essentially plateaus with little to no change occurring. The large $k_1$ of coupons exposed to ProSan may be influenced by mechanical removal as seen in the SEM images of loosened biofilm layers. This is in contrast to Sanidate and NaOCl which showed tight and compact biofilm layers.

![Figure 16. Comparison of $k_1$-value of sanitizers relative to concentration](image)

Figure 16. Comparison of $k_1$-value of sanitizers relative to concentration
The same however is untrue in \( k_2 \) (Figure 17) which does not show a clear relation between concentration and rate constant. Unlike \( k_1 \) no clear trend can be established in comparing sanitizers and their concentrations. It is of particular importance to note however that all \( k_2 \) values are extremely small relative to \( k_1 \).

![Figure 17. Comparison of \( k_2 \)-value of sanitizers relative to concentration](image)

This indicates a plateauing of inactivation. If assuming the population of the biofilm is not homogeneous in the resistance to sanitizers, this fraction population is highly resistant and likely could survive indefinitely at theses concentrations. Overall inactivation and biphasic parameters reflect the relative action of the sanitizers and are unlikely due to physical removal of the biofilm. This conclusion is supported by the crystal violet assay
performed on each coupon discussed in the previous section that found minimal biofilm removal due to sanitizer exposure.

The f-value of the biphasic model indicates fraction population as previously mentioned.

Figure 18 shows that for all sanitizers and respective concentrations, the value is relatively similar. This was an expected result as the same strain of *Pseudomonas* was used throughout the experiment. We can deduce however that this f-value would vary with organism, biofilm age and many other factors. If for instance we applied these sanitizers to an older, multi-species biofilm, we could expect more resistance and a smaller fraction population that is susceptible to rapid inactivation.

![Figure 18. Comparison of f-value of sanitizers relative to concentration](image)

Figure 18. Comparison of f-value of sanitizers relative to concentration
Comparing the efficacy of each sanitizer can be interpreted in several different ways from this model. First, the final population at 20min of each sanitizer’s inactivation process may be compared. For all sanitizers, the highest concentration resulted in the highest overall log reduction. Prosan at 15.6ml/L was shown to have the highest overall log reduction and Sanidate at 80ppm the lowest log reductions. Secondly, we can compare efficacy based on rate of inactivation. Constant $k_1$ provides the most dramatic rate for comparing sanitizers. NaOCl at 300ppm has the highest rate at $k_1=13.27$ log reduction/min while once again Sanidate at 80ppm is the least efficacy with $k_1=0.16$ log reduction/min. It should be noted that while the rate constants are large for $k_1$ the period in which the inactivation occurs in relatively small and can be determined as the time required to achieve a log reduction of the corresponding $f$-value. It is difficult to predict inactivation past the designated time points that were sampled in these experiments, however based up the results, we can speculate that the time points further out than 20 minutes would likely result in minimal log reductions. The $R^2$ value is high indicating good fit for all concentrations to the model. This is further reflected by the accurate log reductions estimated by the biphasic model in comparison to the experimental values.

To further compare the efficacy of each sanitizer with the biphasic parameters, each concentration was calculated for the time required to achieve a 2 log reduction with the
biphasic model equation (Table 3). Although each concentration has a different f-value, a normalized value of 0.99 was chosen for the population fraction N/N₀. Calculations were performed assuming k₁ as the only inactivation rate constant. We can see that Sanidate overall takes much longer to achieve this log reduction in comparison to NaOCl and ProSan. We also observe that all concentrations of NaOCl are comparatively rapid in reduction. ProSan at 3.95ml/L is fairly slow in comparison to higher concentrations which are rapid. These results further support the conclusion that Sanidate is inferior to both NaOCl and ProSan in inactivating *Pseudomonas* biofilm populations.

**Table 3. Time required to achieve 2 log reduction on Strain B1 biofilms (N/N₀=.99)**

<table>
<thead>
<tr>
<th>Sanitizer</th>
<th>Concentration</th>
<th>Time (seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sanidate</td>
<td>80ppm</td>
<td>348</td>
</tr>
<tr>
<td></td>
<td>230ppm</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>460ppm</td>
<td>7.6</td>
</tr>
<tr>
<td>Sodium Hypochlorite (NaOCl)</td>
<td>100ppm</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>200ppm</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>300ppm</td>
<td>3.9</td>
</tr>
<tr>
<td>ProSan LC</td>
<td>3.95ml/L</td>
<td>53.2</td>
</tr>
<tr>
<td></td>
<td>7.8ml/L</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>15.6ml/L</td>
<td>6.5</td>
</tr>
</tbody>
</table>
5.5 Inactivation with Agitation

Biofilms grown statically were tested with sanitizers or saline control in conjunction with an agitation step. This was performed to explore the efficacy of each sanitizer when applying agitation; whether or not agitation aids in the removal or inactivation of the biofilm population. Results in Table 4 show that in comparison to an estimate log reduction based off of the biphasic model at 1 minute, the experimental log reduction is greater than the expected estimates. We find that each sanitizer is significantly different from the saline control and that on average ProSan provided the largest log reductions in a 1 minute period. We can infer from these results that in all cases, a certain portion of the biofilm was removed from the surface of each coupon. The plate counts suggest that both inactivation and mechanical removal resulted in the overall population sampled. This is supported by the fact that coupons exposed to saline still contained a significant portion of biofilm left. ProSan was shown to have the greatest mean log reduction but was not statistically different from NaOCl. We may speculate that the surfactant ingredient in ProSan could have aided in the removal of biofilm from the coupon surface or that agitation caused increased penetration of sanitizers into a biofilm, thereby increasing efficacy. This is further supported by SEM images of coupons exposed to ProSan that show a loosening of the biofilm layer (Figure 12, Appendix
Figures 1 and 2). In similar research performed on several single-species biofilms found that mechanical agitation improved the inactivation of NaOCl (Sena et al., 2006).

**Table 4. Comparison of log reductions relative to saline exposure of Isolate B1 biofilm with agitated sanitizer exposure with estimate values at 1 minute of a non-agitated coupon**

<table>
<thead>
<tr>
<th>Sanitizer</th>
<th>Agitated 1min (Log CFU/ml)</th>
<th>Biphasic Estimate 1min (Log CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>ProSan 7.8ml/L</td>
<td>4.04±0.08</td>
<td>2.52</td>
</tr>
<tr>
<td>Sanidate 230ppm</td>
<td>3.09±0.49</td>
<td>0.32</td>
</tr>
<tr>
<td>NaOCl 200ppm</td>
<td>3.35±0.55</td>
<td>2.23</td>
</tr>
</tbody>
</table>

Letters indicate significance (*P*<0.05) (n=2)
CHAPTER 6: CONCLUSIONS

Three CIP sanitizers were compared for efficacy against *Pseudomonas* biofilms grown on stainless steel coupons:

1. Sanidate 5.0, sodium hypochlorite and a novel GRAS sanitizer, ProSan LC, were evaluated and it was found that all three reduce the biofilm population by approximately 3 logs or more when using the recommended concentration. ProSan on average had the highest log reduction after 20 minutes at recommended concentration.

2. A biphasic model was used to describe the inactivation data and generate parameters to compare the efficacy of each sanitizer and concentration. The model parameter $k_1$ increased with increasing concentration. Sanidate had the smallest $k_1$ relative to the other sanitizers which indicates a slower inactivation rate and is therefore less effective. $k_2$ was extremely small in comparison which conveys the resistance of a small fraction of the *Pseudomonas* population to sanitizer inactivation.
3. A comparison of biofilms exposed to agitated sanitizer indicated that ProSan LC was more effective in removing/inactivating biofilm populations when compared to saline and the other two sanitizers.

4. SEM images showed that stainless steel coupons exposed to sanitizers or saline had considerable biofilm remaining on the surface. Some images suggest that ProSan’s surfactant ingredient may aid in mechanically loosening biofilm layers from the surface. This is supported by results from the agitated sanitizer experiment that showed ProSan had a lower average population than an estimate population calculated from the biphasic model and in comparison to the other two sanitizers as well.
CHAPTER 7: FUTURE STUDIES

Future studies could improve upon methods used to evaluate the efficacy of sanitizers. The current model created with the experimental data should be validated through follow-up experiments. This may be done by performing experiments at longer time periods and at varying concentrations than those used in the research. The sanitizers should be evaluated at longer time periods to support the argument of a plateau in inactivation. A drying step of the coupons prior to sanitizer exposure to observe changes in sanitizer efficacy would also be of potential interest. A flowing bench-top fermenter such as an annular reactor may be used as the next step in simulating a flowing system such as a CIP process. From there, a pilot-scale experiment may be performed by growing biofilms within stainless steel pipes walls. Temperature as a variable may also be explored. Older biofilms may be studied using these methods and as such we can investigate how age and regular sanitizer exposure changes the efficacy of each sanitizer. As previously mentioned, NaOCl and peracetic acid decompose in presence of heat and light. It would be interesting to explore ProSan’s capabilities at variable temperatures and whether or not inactivation or removal is increased.
LIST OF REFERENCES


monocytogenes and Escherichia coli. Symposium Series (Society for Applied Microbiology), 11S–120S. doi:10.1046/j.1365-2672.92.5s1.18.x


APPENDIX: Additional SEM Images
Figure 19. SEM image of flaking/sloughing of biofilm layer by ProSan
Figure 20. SEM image of coupon exposed to ProSan for 20min showing possible surface detachment of biofilm layer