

INVESTIGATION OF A CONTROL STRATEGY FOR MANIPULATION AND
PREVENTION OF *PSEUDOMONAS AERUGINOSA* PAO1 BIOFILMS IN
METALWORKING FLUIDS

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Chemical Engineering

MONTANA STATE UNIVERSITY
Bozeman, Montana

May 2018

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DEDICATION

This thesis is dedicated to Gülnur, my beautiful mother, my first role model, and my best friend.

Mom, thank you for your unconditional support and love through the years we have spent apart. You always encouraged me to study hard and thought me the importance of education. You educated me to become an independent adult, pushed me to be my best, and most importantly, you raised me to be a good person. Thank you for being my travel buddy, encouraging me to travel and teaching me the importance of learning about new cultures and languages. Thank you for sending me to college in the United States, supporting me all the way through my undergraduate, and sponsoring for my student visa.

Thank you, mom, for always being strong and for making me stronger. I wouldn't be the person I am today without you.

ACKNOWLEDGEMENTS

I am thankful for the support and guidance from my committee chair, my advisor, Dr. Christine Foreman who had provided me the opportunity to complete a master of science degree in chemical engineering, had a door always open, and had set an example of excellence as a researcher, mentor, and an instructor. I would also thank the other members of my committee, Dr. Phil Stewart and Dr. Jeff Heys, for their valuable time, providing me advice, and helpful suggestions.

I want to thank Dr. Markus Dieser, for his support and encouragement over the past two years, for taking the time to read my thesis, guiding me through the questions I had regarding lab work, and setting a great example for my personal and professional development and success through the graduate school. Markus, we are lucky to have you in the Foreman Research Group.

I also want to thank my professors at the Chemical & Biological Engineering Department and all the wonderful people at the Center for Biofilm Engineering for making this place special. Thank you for helping me understand how to collaborate and grow as a researcher, an engineer, and a person during my undergraduate and graduate over the past six years.

I could not have gotten through this without the continues support my family has given me throughout my time in the U.S. Words are powerless to express my gratitude.

The completion of this work would have not been possible without the funding received from the National Science Foundation (NSF) under NSF 1635347.

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ABSTRACT

Microbial contamination in metalworking fluid (MWF) circulation systems is a serious problem. Particularly water based MWFs promote microbial colonization despite the use of biocides. Inhibiting the quorum sensing mechanism (i.e. cell-cell communication) in bacteria is a promising approach to control and prevent biofilm formation. The objective of this study was (i) to determine the microbial community in MWFs from operational machining shops, (ii) to investigate the effect of well-known quorum sensing inhibitors on controlling biofilm formation, and (iii) to implement experimental data from selected enzymes to a computer simulation biofilm accumulation model (BAM). Planktonic and biofilm samples from two local machining shops in Bozeman, MT, were collected to determine the extent of microbial colonization. In both operations, microbial communities were dominated by *Pseudomonadales* (60.2-99.7%). Rapid recolonization was observed even after dumping spent MWFs and cleaning. Considering the dominance of *Pseudomonadales* in MWFs, the model organism *Pseudomonas aeruginosa* PAO1 was selected for testing the effects of quorum sensing inhibitor compounds on biofilm formation. From a variety of enzymes, natural, and chemical compounds screened for quorum sensing inhibition, Patulin (40 μ M) and Furanone C-30 (75 μ M), were found to be effective in reducing biofilm formation in MWFs when applied as single compound amendments and in combination with the polysaccharide degrading enzyme α -amylase from *Bacillus amyloliquefaciens*. Particularly Furanone C-30, as a single amendment and in combination with α -amylase decreased biofilm formation by 76% and 82% after 48 hours. Putatively identified homoserine lactones in MWFs treated with Furanone C-30 provided evidence for quorum sensing inhibition on biofilm formation. BAM was employed to study the effect of α -amylase (3 Units mL⁻¹) on *P. aeruginosa* PAO1 biofilms in batch reactors for 24 and 48 hours. In the absence of α -amylase, biofilm thickness was predicted to be 23.11 and 31.37 μ m, while its presence reduced thickness to 10.47 and 13.07 μ m after 24 and 48 hours, respectively. The results presented herein highlight the potential effectiveness of quorum sensing inhibition as a strategy to reduce biofilms in MWFs.

CHAPTER ONE

INTRODUCTION

Introduction to Metalworking Fluids

Metalworking or cutting fluids are used in machining, grinding, and milling operations for cooling, reducing friction, removing metal particles, and protecting the workpiece and tools (Benedicto et al. 2017). They are most commonly made of petroleum oil, petroleum sulfonates, and fatty acids (Saha and Donofrio 2012). There are four different types of metalworking fluids (MWFs): insoluble, soluble, synthetic, and semi-synthetic (Whittaker 1997). Insoluble MWFs are oil-based materials that are not soluble in water, and they typically have an amber to brown color. One of the commonly found additives in insoluble MWFs are extreme-pressure agents. Soluble MWFs are oil-based fluids with emulsifiers that allow for stable emulsification when dissolved in water. These fluids have a milky white color and commonly contain additives such as pH stabilizers, biocides, coupling agents, and corrosion inhibitors. Synthetic MWFs are known as water-based fluids that contain no hydrocarbons. They are transparent and often green or other colors. Synthetic fluids commonly have additives similar to soluble MWFs such as lubrication aids, corrosion inhibitors, biocides, water softeners, and anti-foaming agents. Finally, semi-synthetic MWFs contain a small amount of oil. They are usually colorless, translucent, and have the common additives of emulsifiers, pH stabilizers, biocides, anti-foaming agents, and corrosion inhibitors.

According to a recent report, the global market for MWFs was 36.36 million tons in 2014, with demand expected to reach 43.87 million tons by 2022 (Grand View Research 2016). With respect to the United States, more than 1 million machine workers are exposed to MWFs (NIOSH 1998) and more than two billion gallons of MWFs are estimated to be consumed annually (Schwarz et al. 2015). Both usage and treatment of spent MWFs may cause economical, ecological, and occupational safety challenges in work environments; thus, the machining industry is in urgent need of a solution to improve the environmental and health based hazardous issues.

Microbiology of Metalworking Fluids

The microbial contamination of MWFs is a serious problem in the machining industry and particularly water-based MWFs are nutrient-rich environments that promote the growth of microorganisms (Bakalova et al. 2007). Uncontrolled, microbial colonization and biofouling reduce the performance of MWFs, induce corrosion, increase equipment wear, compromise product quality, pose occupational safety risks, and reduce the life-time of the fluids. The use of biocides to combat this contamination can act to impede the biodegradation of spent MWFs (Trafny 2013; Brinksmeier et al. 2015; Schwarz et al. 2015; Najiha et al. 2016; Benedicto et al. 2017). Spoilage is caused by a diverse community of gram negative and positive bacteria (Baecker et al. 1989; Mattsby-Baltzer et al. 1989; Kreis and Cox-Ganser 1997; Van der Gast et al. 2001; Passman and Rossmore 2002; Cheng et al. 2005; Rabenstein et al. 2009; Gilbert et al. 2010) and both aerobic and anaerobic organisms, *Pseudomonas* sp. accounting for 67% of all aerobic bacterial isolates (Baecker et al. 1989). Fungi (Passman and Rossmore 2002; Gilbert et al.

2010; Liu et al. 2010), archaea (Di Maiuta et al. 2017), and potentially pathogenic species including *Legionella*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Alcaligenes faecalis*, and *Escherichia coli* as well as *Mycobacterium* sp. (reviewed in Saha and Donofrio 2012) have also been found in MWFs.

Despite the use of biocides, microbial growth is still observed in computer numerical control (CNC) machines (Trafny et al. 2015), decreasing the life-time of MWFs and the quality of final products. Studies have found that periodic additions of biocide to the MWF system did not have a lasting effect on controlling bacterial cell numbers and implied that change in fluid brand or dilution may impact the microbial levels in MWF systems (Kapoor et al. 2014). Other methods used to prevent microbial contamination in MWFs include: UV-radiation, filtration, ultrasound, pressure, and heat. Often employed as point sources, their efficiency on system wide controls on microbial contaminants is limited (Koch 2014). Most recently, a strategy involving nutrient imbalance with phosphorus depletion was studied within MWF systems as a green alternative to biocides. This work suggests that phosphorus starvation has a potential efficiency in controlling MWF degradation and microbial growth (Azimi and Thompson 2017). Studies also revealed that microbial communities (i.e. *Mycobacterium* and *Pseudomonas*) show high resistance to commonly used industrial biocides (Selvaraju 2005) and rapid recolonization of freshly recharged MWF systems demonstrates the inability to successfully remove microbial contaminants from cutting fluids (Kapoor 2014). In fact, it has been speculated that microbes residing in the form of biofilms in areas of the MWF circulation system that are inaccessible to cleaning are responsible for

the rapid re-contamination (Veillette et al. 2004). Research has also shown that most gram-positive and gram-negative bacteria can survive for months on dry surfaces (Kramer et al. 2006) and biofilm formation increases the survival rate of these microorganisms due to the formation of highly hydrated extracellular polymeric substance (EPS) matrix around the biofilm structure (Espinal et al. 2012).

Potential Health Hazards

Exposure to MWFs most frequently occurs by inhalation or direct skin contact during industrial machining processes. Uncontrolled microbial contamination and exposure to MWFs may impact human health such as respiratory conditions including hypersensitivity pneumonitis, chronic bronchitis, impaired lung function, and asthma (Greaves et al. 1997; Thorne et al. 2006; Rosenman 2009), dermatitis and skin allergies (Geier 2014), or cancer (Calvert et al. 1998). Touching contaminated surfaces, using contaminated equipment, or splashing fluids can also cause skin irritations and allergies (Schwarz et al. 2015). Clearly, while direct exposure to emulsions, cutting fluids, and mist can affect human health, endotoxins produced by microbial species proliferating in MWFs are of major concern (Mirer 2010).

Introduction to Biofilms

In 1676 protists and bacteria were discovered by the father of microbiology, and the inventor of the microscope, Mr. Leewenhoek (Leewenhoek 1677). In his famous letter, a description of protists and bacteria mentioned little animals living in rain water (translated from Dutch to English by Henry Oldenburg, Lane 2015). When Leewenhoek

saw microbial aggregates on scrapings from his teeth (Chandki et al. 2011), the term, biofilm, to describe the lifestyle of microbial aggregates had not been defined yet. In 1978, the term “biofilm” was coined by Dr. Bill Costerton (Costerton et al. 1978). In 2002, a new definition of biofilm structures was offered by Donlan and Costerton stating “a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.” (Donlan and Costerton 2002). Simply, the term biofilm is used to describe microbial communities in which single cells attach to each other as well as to any aquatic surfaces. Biofilm can be classified on the basis of its location and pathogenicity (Ximénez-Fyvie et al. 2000).

Biofilms are complex structures. When the biofilm-associated microbial cells attach to a surface, they start forming colonies, intertwined by EPS matrices, also known as the “house” of biofilm cells (Flemming et al. 2007). Such population wide, social interactions collectively controlling group behavior are regulated by a phenomenon known as quorum sensing (QS) (Frias et al. 2001; Davey and O’toole 2000; Nadell et al. 2009; Watnick and Kolter 2000). QS systems in bacteria could be reviewed in two classes: (1) quorum sensing in gram-negative bacteria, which use N-acyl-homoserine lactones (AHLs) as their signal molecules; and (2) quorum sensing in gram-positive bacteria, which use small peptides as signal molecules (Li and Tian 2016). Chemicals that disrupt QS pathways and reduce the expression of QS controlled genes are called quorum sensing inhibitors (QSI) (Fong et al. 2018). The communication system between

biofilm-associated cells and communities could be jammed by using QSI agents. Studies show that quorum sensing signal production could be inhibited by blocking the communication mechanism of cells with synthetic molecules (O'Loughlin et al. 2013), natural compounds (Tay and Yew 2013), medicinal herbs, and plant extracts (Ding et al. 2011).

Many microorganisms that are found in MWFs, including pathogenic species (i.e. *Pseudomonas aeruginosa*, *Mycobacterium immunogenum*), are capable of biofilm formation (Cook and Gaylarde 1988; Saha and Donofrio 2012; Trafny 2013). Materials used in the MWF systems and machining industry are highly susceptible to biofilm formation due to the nutrient rich environment (Cook and Gaylarde 1988; Lucchesi et al. 2012). However, while biofilms in MWF systems have been causing issues in the machining industry for almost a century, much remains to be learned about microbes in metalworking fluids.

Quorum Sensing

Cell-cell communication or quorum sensing is a well-known phenomenon in bacterial cells that is facilitated through chemical signaling molecules. In natural environments, biofilms are commonly seen microbial lifestyles. Their functions depend on social interactions and these interactions have specific mechanisms such as regulations of specific gene expressions (Processor 1999; Chandki et al. 2011). In biofilms, bacterial communication has been shown to be an essential tool for motility (Schuster and Greenberg 2006), surface attachment (Dunne 2002), EPS matrix production (Flemming and Wingender 2003), social interactions, and building a well-structured and organized

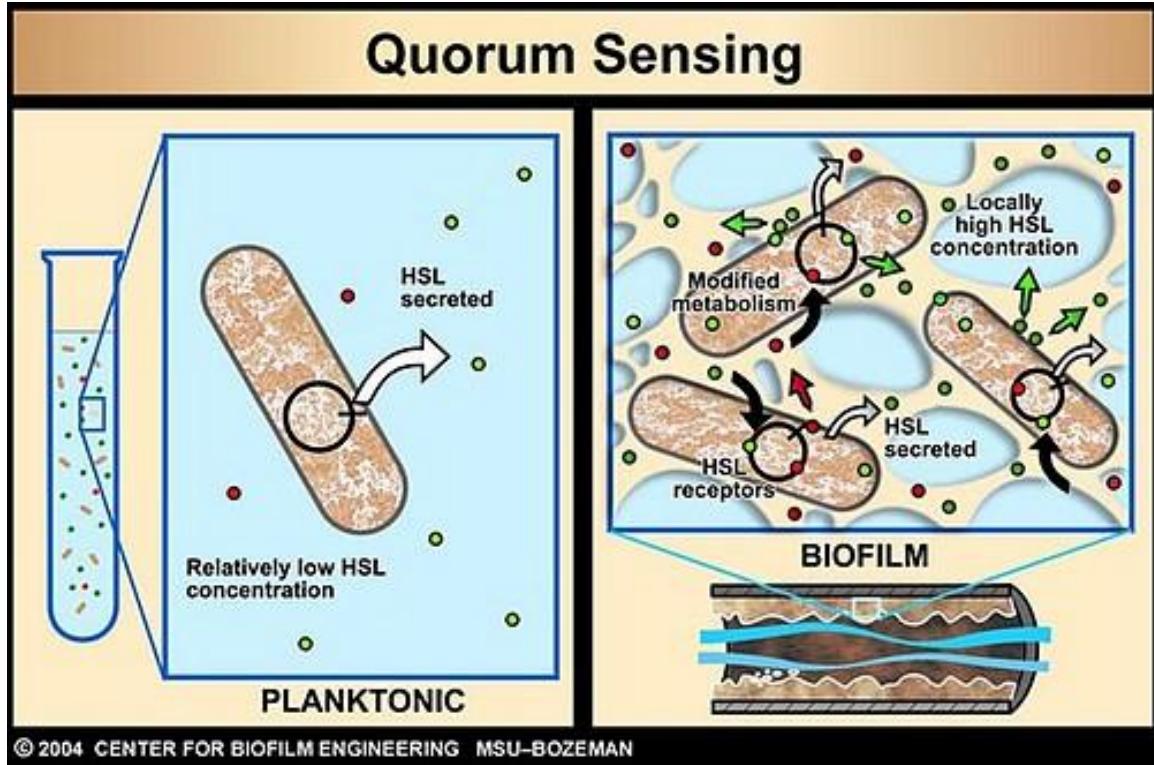


Figure 1.1 Planktonic cells secrete homoserine lactones (HSLs), however, low concentrations of signal molecules do not change gene expression. In dense populations (e.g. biofilm communities) HSL secretion attains higher concentrations. HSL molecules trigger changes in genetic activity on a population/community level. (Image courtesy Center for Biofilm Engineering-Montana State University).

community (Hammer and Bassler 2003). Quorum sensing is also known as a cell density dependent signaling communication method within or between species, where the increase in cell population (Figure 1.1) density would also cause an increase in releasing chemical signal molecules termed autoinducers (Miller and Bassler 2001). In 1970, a study on a gram-negative bacterium, *Vibrio fischeri*, revealed that bacteria could use chemical signaling to communicate and co-ordinate group activities through the secretion of autoinducers (Nealson et al. 1970). Quorum sensing in gram-negative bacteria is through acyl-homoserine lactones (AHLs) and these lactone molecules are secreted as

their signal molecules (Li and Tian 2016). Acylated homoserine lactone synthesis is dependent on regulatory proteins called LuxI and LuxR (Miller and Bassler 2001). When these two proteins are bound to autoinducers, LuxR behaves as a transcriptional activator to specific DNA elements and activates target gene transcription (Fuqua and Greenberg 2002). Quorum sensing in gram-positive bacteria is accomplished through small autoinducing peptides, which consist of 5-25 amino acids (Li and Tian 2016). This two-component-type QS mechanism produces and secretes oligopeptide autoinducers by an autoinducing peptide and a signal transduction system (Dunny and Leonard 1997).

Quorum Sensing Inhibition

Anti-quorum sensing molecules have emerged as a manipulation strategy for disabling the quorum sensing circuits, and thus, disrupting and decreasing biofilm formation within bacterial communities (O'Loughlin et al. 2013; Ding et al. 2011). Substantial mitigation of membrane biofouling by QS inhibition has been demonstrated in wastewater treatment systems (Oh et al. 2012; Weerasekara et al. 2014; Lee et al. 2016), seawater desalination tanks (Katebian et al. 2016), and reverse osmosis operations (Oh et al. 2017). Studies revealed that enzymatic quenching of bacterial quorum sensing is a proven approach for control biofouling in membrane bioreactors for advanced wastewater treatment systems (Yeon et al. 2009). Enzymes that inactivate QS signals are called quorum quenching (QQ) enzymes (Fong et al. 2018). In another study, (QQ) enzyme was successfully immobilized onto a nanofiltration membrane to manipulate biofouling in a nanofiltration process (Kim et al. 2011). Given the growing resistance of MWF microbial communities to commonly applied biocides (Mattsbj-Baltzer et al.

1989; Veillette et al. 2004; Lodders and Kämpfer 2012; Kapoor et al. 2014), my proposed strategy to remove biofilm in MWFs using QSI molecules provides an attractive approach to reduce the microbial load, improve product performance, and lower occupational safety risks in MWF systems and machining industries.

Research Goal

Water and oil based working fluids are used in machining applications to conduct heat, lubricate contact and work spaces, and remove metal particles (Trafny 2013). These fluids are also highly suitable to both planktonic and biofilm contamination, adversely affecting the properties of these liquids and potentially increasing health problems in work environments. However, meticulous cleaning, the use of biocides, and other hygiene practices have proven ineffective in preventing re-colonization and combating the microbial load. The goal of this research was to investigate quorum sensing inhibition as a strategy to prevent biofilm formation in machining and grinding operations. My results highlight a promising method using quorum sensing inhibitor molecules in *Pseudomonas aeruginosa* PAO1 biofilms for developing an effective biofilm manipulation strategy in MWFs. These results may increase the life-time of MWF, quality of the final product, and may provide easy post-cleaning for circulation systems. Moreover, these results may reduce the safety hazards associated with microbial contaminations in closed work environments, protect the environment from excessive amount of waste (i.e. used MWF), and decrease the usage of chemical additives in MWFs.

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CHAPTER TWO

QUORUM SENSING INHIBITION AS A PROMISING METHOD TO
CONTROL BIOFILM GROWTH IN METALWORKING FLUIDS

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

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Environmental Science & Technology

Status of Manuscript:

- ☒ Prepared for submission to a peer-reviewed journal
- ☐ Officially submitted to a peer-reviewed journal
- ☐ Accepted by a peer-reviewed journal
- ☐ Published in a peer-reviewed journal

QUORUM SENSING INHIBITION AS A PROMISING METHOD TO CONTROL BIOFILM GROWTH IN METALWORKING FLUIDS

The following work is currently in progress to be submitted for publication to

Environmental Science and Technology

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Abstract

Microbial contamination in metalworking fluid (MWF) circulation systems is a serious problem. Inhibiting quorum sensing mechanism (i.e. cell-cell communication) in bacteria is a promising approach to control and prevent biofilm formation. This study aimed to determine the microbial community in MWFs from two machining shops and investigate the effect of quorum sensing inhibitors on biofilm growth. In both operations, biofilm associated and planktonic microbial communities were dominated by *Pseudomonadales* (60.2-99.7%). Rapid recolonization was observed even after dumping spent MWFs and meticulous cleaning. Using *Pseudomonas aeruginosa* PAO1 as a model biofilm organism, Patulin (40 μ M) and Furanone C-30 (75 μ M) were found to be effective in reducing biofilm formation in MWFs. Particularly Furanone C-30 reduced biofilm by 76% and 82% over 48 hours. Putatively identified homoserine lactones in MWFs treated with Furanone C-30 indicated the effect of quorum sensing inhibition on biofilm formation. The results presented herein highlight the effect of quorum sensing inhibition as a potential strategy to manipulate biofilms in MWFs.

Introduction

Metalworking fluids (MWF) are used in machining, grinding, and milling for cooling, reducing friction, removing metal particles, and protecting the workpiece and tools (Benedicto et al. 2017). They are most commonly made of petroleum oil, petroleum sulfonates, and fatty acids (Saha and Donofrio 2012) and categorized as insoluble, soluble, synthetic, or semi-synthetic fluids. Application dependent, a single MWF may consist of more than 300 different components including lubricants, corrosion inhibitors, emulsifiers, chelating agents, detergents, odorants, anti-foaming and anti-mist agents, pH buffers, abrasives, and biocides (Brinksmeier et al. 2015). According to a recent report, 36.36 million tons of MFWs were used globally in 2014, with usage expected to reach 43.87 million tons by 2022 (Grand View Research 2016). Forecasts for the United States predict a demand of 550 million gallons of synthetic lubricants and functional fluids by 2018 (Fredonia 2014). With increasing demand and treatment of spent MWFs placing an economical burden on the machining industry, as well as creating environmental and occupational safety risks, there is a need for improved MWF formulations that not only improve work piece quality, but also extend the lifetime of in-use MWFs.

Microbial contamination of MWFs is a fundamental problem, affecting the quality of the product and the lifetime of MWFs (Matsby-Baltzer et al. 1989; Veillette et al. 2004; Kapoor et al. 2014; Trafny et al. 2015; Gilbert et al. 2010; Lodders and Kämpfer 2012), and potentially increasing occupational safety risks (Mirer 2010; Burton et al. 2012; Duquenne et al. 2013; Grigoriev et al. 2017). Water-based MWFs, in particular, are nutrient-rich environments that promote the growth of microorganisms (Bakalova et al.

2007; Lidders and Kämpfer 2012). Measures to combat microbial growth in MWFs typically involve the use of biocides, and other measures such as UV-radiation, filtration, ultrasound, pressure, and heat (Alkawareek et al. 2012; Koch 2014). Alternative, more environmentally friendly approaches compared to commonly applied biocides have also been investigated (Ashraf et al. 2014; Azimi and Thompson 2017). Nonetheless, these measures are insufficient at eliminating the microbial load in MWF circulation systems (Matsby-Baltzer et al. 1989; Veillette et al. 2004; Lidders and Kämpfer 2012; Kapoor et al. 2014). In fact, certain bacterial strains present in MWFs (e.g. *Mycobacterium* and *Pseudomonas*) have been shown to be particularly resistant to industrial biocides (Selvaraju et al. 2005; Falkinham 2009; Sun et al. 2012; Trafny 2013; Trafny et al. 2015). Rapid recolonization of freshly recharged MWF systems directly after emptying and extensive cleaning is a strong indication for the ineffective removal of microbial colonizers (Veillette et al. 2004; Kapoor 2014); contaminants which are believed to persist in the inaccessible parts of these machines in the form of biofilms (Saha and Donofrio 2012; Trafny 2013). While increasing biocide dosage could improve biofilm disruption in MWFs (Lucchesi et al. 2012), such attempts would be counterproductive to current trends in the MWF industry, by raising costs, occupational safety and environmental risks, and impeding the biodegradation of spent MWFs.

Intercellular communication, a phenomenon termed quorum sensing, has been identified across diverse bacterial taxa (Li and Tian 2016) and enables complex, population wide interactions among bacteria (Miller and Bassler 2001). Amongst many physiological processes, the formation of biofilm, a well-organized and structured

microbial community enclosed in a self-produced polymeric matrix and adherent to a surface, is regulated by quorum sensing (Davey and O'toole 2000; Watnick and Kolter 2000; Frias et al. 2001; Nadell et al. 2009). In its basics, quorum sensing is mediated by the production, release, sensing of, and response to small, diffusible signal molecules by bacterial cells (i.e. autoinducers) related to cell density (Kaplan and Greenberg 1985; Seed et al. 1995; Latifi et al. 1996; Pesci et al. 1997; Wiley-Blackwell 2009; Rutherford and Bassler 2012). However, this dependency on signal molecules to coordinate a translational behavior on a population level provides ideal targets to disrupt bacterial communication (e.g. QS-signal cleavage, competitive inhibition, blockage of signaling receptors) and, hence, biofilm formation (Costerton et al. 2003; Ding et al. 2011; O'Loughlin et al. 2013; Tay and Yew 2013). Some large- scale applications on wastewater treatment systems (Oh et al. 2012; Weerasekara et al. 2014; Lee et al. 2016), seawater desalination tanks (Katebian et al. 2016), and reverse osmosis operations (Oh et al. 2012) have implemented this strategy (i.e. quorum sensing inhibition), successfully mitigating biofilm contamination.

Given the emerging resistance of MWF microbial communities to biocides, we hypothesize that quorum sensing inhibition can reduce biofilm formation in MWF circulation systems and hence, minimize their associated adverse effects. The aim of this study was to determine the effect of commercially available quorum sensing inhibitor compounds on biofilm reduction in a water soluble MWF, using the model organism *Pseudomonas aeruginosa* PAO1.

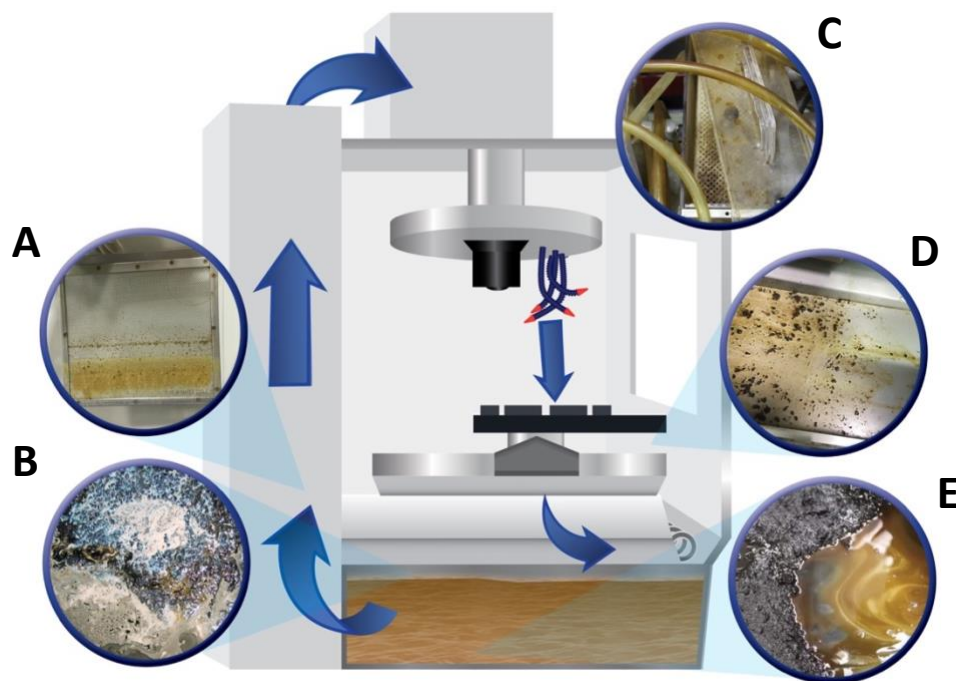


Figure 2.1 Schematic with images of biofilm in a MWF circulation system. (A) particle filtration screen (B) bottom (C) tubing (D) metal shaving and (E) surface of fluid reservoir. (Image courtesy Foreman Research Group-Montana State University).

Materials and Methods

Determination of Microbial Communities in MWF Systems

Planktonic and biofilm samples were collected from two local machine shops in Bozeman, Montana, USA (herein referred to as Shop 1 and Shop 2). MWF ‘Brand A’ (S-500-CF, Hangsterfer’s) a water-soluble fluid, was used at both shops. At the time of sample collection, this MWF had been in continuous use under routine fluid management practices for one year. Additionally, samples were collected from Shop 2 after a commonly followed industrial dumping, cleaning, and recharging process. Samples from this cleaned and recharged system were collected after one day of operation with the new

fluid. Samples for microbial community analysis were collected from the MWF reservoir of two computer numerical control (CNC) machines, biofilm from screens and shavings, and mist (Figure 2.1). Samples from the MWF reservoir of CNC machines (~100 mL) were filtered onto 47 mm Supor® 200 PES membrane filters with a pore size of 0.2 µm using a peristaltic pump. Biofilm from screens and shavings were scraped with a sterile spatula directly from CNC machine compartments into sterile 50 mL Falcon tubes. Mist from inside the CNC machine working chamber during operation was collected onto 47 mm Supor® 200 PES membrane filters with a pore size of 0.2 µm. Membrane filters were transferred to sterile tubes and all samples were stored at -80°C until further processing. DNA was extracted using the PowerWater® DNA Isolation Kit (14900-S, MO BIO Laboratories) following the manufacturer's instructions. Extracted DNA was quantified by using the high sensitivity Qubit DNA Assay Kit with a Qubit® Fluorometer (Thermo Fisher Scientific).

DNA Amplification and Sequencing Analyses

A polymerase chain reaction (PCR) was performed using primer pairs specific to the V3-V4 regions of the 16S rRNA gene (D'Andrilli et al. 2017). Primer complexes included the Illumina adaptor sequences followed by either the universal primers 341F (5'acactctttccctacacgacgtcttccgatctCCTACGGGNGGCWGCAG-3') or 805R (5'gtgactggagttcagacgtgtgcttccgatctGACTACHVGGGTATCTAATCC-3') (UW Biotechnology Center, Madison, WI, USA). Each 25 µL PCR reactions contained extracted DNA template, 0.1 µM of each primer, a 1X final concentration of Bull's Eye PREMIUM Taq 2X Mix (Midwest Scientific, St Louis, MO, USA), and an adjusted

volume of nuclease free water. The amplification protocol consisted of an initial denaturation at 95°C for 3 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension step at 72°C for 5 min. PCR was performed in an Eppendorf Mastercycler pro S. Confirmation of PCR products size was performed by band visualization in 1% agarose gel stained with GelRed™. PCR amplicons were submitted to the University of Wisconsin-Madison Biotechnology Center. A blank sample for the extraction procedure was included in the sequence libraries. Paired end, 250 bp sequencing was performed using an Illumina MiSeq Sequencer and a MiSeq 500 bp (v2) sequencing cartridge.

For joining the forward and reverse sequence libraries the Quantitative Insights Into Microbial Ecology (QIIME) toolkit (Caporaso et al. 2010) was used. Quality refinement of contigs was subsequently performed using the Mothur platform v.1.34.4 by excluding sequences containing ambiguous bases, homopolymers >8 bases, and an average quality score below 35 over a 50 bp window (Schloss et al. 2011). Processed sequences were aligned against the SILVA Gold database in Mothur. Chimeric sequences were removed using UCHIME (Edgar et al. 2011) with the SILVA Gold database (Pruesse et al. 2007) and a second chimera check using sequences from the present study as a database. Sequences were classified with a Bayesian method (Wang et al. 2007) using the Mothur formatted version of the RDP classifier. Operational taxonomic unit (OTU) was defined at $\geq 97\%$. Raw sequences libraries were deposited to GenBank NCBI database under the accession number SRA PRJNA398788.

Agents

Four natural agents, two enzymes, and two chemicals were tested for biofilm reduction and their effect on quorum sensing inhibition. Natural agents Emodin (E7881), Gingerol (G1046), and Patulin (P1639), enzymes α -amylase *Bacillus amyloliquefaciens* (A7595) and Acylase I from porcine kidney (A3010), and the chemical agents Furanone C-30 (53796) and Phenyl disulfide (169021) were purchased from Sigma-Aldrich. Garlic was extracted and purified adopting the protocol from Rasmussen et al. (2005).

Culture Conditions

Pseudomonas aeruginosa PAO1 (ATCC: 15692), a model biofilm forming organism, was grown in tryptic soy broth (TSB) at 37°C while shaking at 125 rpm for 16 hours. Cultures were harvested and centrifuged at 10,000xg for 2 min. Pellets were resuspended in 2X TSB.

Screening for Compound Applicability

P. aeruginosa PAO1 enrichments were transferred into 96 wells microtiter plates at an optical density (OD) of 0.03 at 600 nm. Wells were supplemented with TSB (1X final conc.) and the agents shown in Table 2.1. Eight replicates per agent and control were tested. Quantitative analysis in microtiter plates was performed following the method described in Pitts et al. (2003). Plates were incubated at 37°C for 48 hours.

Table 2.1 Compounds tested for reducing *P. aeruginosa* PAO1 biofilm formation grown in 96 wells microtiter plates in TSB. Efficiency is presented as percent biofilm reduction.

Types	Agent	% Biofilm Reduction		
		≥ 80	≥ 50	No Effect
Natural	Emodin (10 – 1000 µM)			x
	Garlic extract (0.5% - 15% v/v)			x
	Gingerol (350 uM)			x
	Patulin (40 uM)	x		
Enzymes	α -amylase <i>Bacillus amyloliquefaciens</i> (1 Unit)	x		
	α -amylase <i>Bacillus amyloliquefaciens</i> (≥ 10 Units)			x
	Acylase 1 from Porcine kidney (0.5 - 20 Units)			x
Synthetic/	Furanone C-30 (75 uM)		x	
Chemicals	Phenyl disulfide (0.02 – 1 mM)			x

At selected time points (i.e. 0, 24, 48 hours), plates were washed five times in DIW to remove planktonic cells. Wells were stained with 300 µL of 1:3 diluted crystal violet solution for 20 min at 22°C. Excess stain was washed off five times in DIW.

Subsequently, 300 µL of 95% ethanol was added to each well, and de-colorization of the wells was allowed for 15 min. Light absorbance data were read at 540 nm in a BioTek FL600 plate reader.

CDC Biofilm Reactors

Compounds that demonstrated the highest percentage in biofilm reduction (i.e. α -amylase from *Bacillus amyloliquefaciens*, Patulin, and Furanone C-30) were further investigated. CDC Biofilm Reactors were purchased from Biosurface Technologies

Table 2.2 Single amendment treatments and in combination with α -amylase applied in CDC biofilm reactors on *P. aeruginosa* PAO1 biofilm.

Type	Agent
Single Agent	Patulin (40 μ M) α -amylase <i>Bacillus amyloliquefaciens</i> (3 Units mL ⁻¹) Furanone C-30 (75 μ M)
Combinations	α -amylase <i>Bacillus amyloliquefaciens</i> (3 Units mL ⁻¹) and Patulin (40 μ M) α -amylase <i>Bacillus amyloliquefaciens</i> (3 Units mL ⁻¹) and Furanone C-30 (75 μ M)

(Bozeman, MT). These reactors consist of a glass vessel, eight polypropylene coupon holders (rods) each holding three coupons (biofilm growth surface) 12.7 mm in diameter, and a lid with a gas exchange port. CDC biofilm reactors were prepared following standard operating procedures (Goeres et al. 2005). Reactors were assembled with stainless-steel coupons and autoclaved for 30 min. Subsequently, 300 mL of 1X TSB media were poured into the reactor and enriched with 2 mL of an overnight *P. aeruginosa* PAO1 culture. Treatments were applied as both single compound amendments and in combination (Table 2.2). Experiments were performed as batch reactors on a stir plate at 125 rpm at 37°C for 48 hours.

At two time points (i.e. 24 and 48 hours), one rod holding three stainless-steel coupons was removed from untreated and treated reactors. Coupons were dipped in 1X phosphate-buffered saline (PBS) to remove unattached, planktonic cells. Each coupon was placed in sterile falcon tubes filled with 5 mL of 1X PBS and vortexed for 10 sec. Ten-fold dilution series were prepared using sterile 1X PBS. An electronic pipetter (ThermoLabsystems) was used for high accuracy and precision. Five drops (10 μ L each)

per dilution were pipetted on TSA plates (Herigstad et al. 2001). Colony forming units (CFUs) of biofilm were enumerated after 16 hours of incubation at 37°C.

Multi-well Plate Assays with MWF

Overnight enrichments of *P. aeruginosa* PAO1 were harvested by centrifugation at 10,000xg for 2 min, washed twice in autoclaved tap water, and transferred into 12 wells microtiter plates (08-772-3A, Falcon™ Polystyrene Microplates) at an OD of 0.03 at 600 nm. Wells were supplemented with 7% (final conc.) MWF ‘Brand A’ in autoclaved tap water (i.e. recommended operational concentration, Hangsterfer’s Laboratories Inc.) and agents shown in Table 2.2. A stainless-steel coupon in each well was used as the biofilm growing surface. Coupons were autoclaved for 30 min prior to the experiment. Agents and controls were tested in triplicate. Plates were incubated at 37°C for 48 hours. Biofilm formation was investigated after 24 and 48 hours. Quantitative analyses were performed by applying the drop-plating method described above.

Microscopy

Epi-fluorescence microscopy (Nikon Eclipse E-800) was performed to visualize biofilm on untreated and treated sample coupons. Coupons were pulled out from CDC reactors after 48 hours and dipped in filter sterilized 1X PBS to remove planktonic cells from the coupon surface. Biofilm was stained with LIVE/DEAD™ BacLight™ Bacterial Viability Kit (L7012, Invitrogen by Thermo Fisher Scientific) in the dark at 22°C for 30 min. Sample coupons were carefully rinsed with filter sterilized DIW prior to imaging to

remove excess dye from coupon surfaces. Samples were imaged under 60x magnification with the MetaVue version 7.8.13.0 (Molecular Devices, LLC.) software package.

Random images (n=10) were taken per coupon. Interferences between MWF and epi-fluorescent signals prevented imaging of coupons treated in MWF.

Metabolite Extraction

Triplicate biofilm samples were grown in 3 mL TSB on stainless-steel coupons for an untreated control, Patulin (40 μ M) and Furanone C-30 (75 μ M) amendments, and in 3 mL MWF supplemented with Furanone C-30 (75 μ M) in 12-well plates at 37°C for 24 hours. Triplicate stainless-steel coupons were pooled in sterile falcon tubes with 6 mL of 1X PBS and vortexed for 10 sec. Subsequently, cell pellets were collected by centrifugation at 10,000xg for 2 min. Supernatant was discarded and cell pellets were suspended in 1X PBS.

Metabolites were extracted according to the modified procedure adapted from Carlson et al. (2018). Samples were kept on ice at all times. Tubes were centrifuged at 10,000xg at -9°C for 15 min. Supernatant was discarded and the cell pellet was suspended in x3 volumes of sterilized water. Tubes were vortexed until a homogenous mixture of cell pellet and water was achieved. A sonication step was performed by using an Ultrasonic Homogenizer (Biologics, Inc.) at 60% duty cycle, power 5 for 5 min per sample. Equal volumes of 100% methanol were added and tubes were vortexed for 30 sec. Samples were centrifuged at 20,000xg at -9°C for 15 min. Supernatants (metabolite extracts) were collected into sterile microcentrifuge tubes. Acetone precipitation was performed with 5:1 v/v acetone: metabolite extract at -80°C for 16 hours. Tubes were

centrifuged at 20,000xg for 5 min and supernatants were transferred to fresh, sterile microcentrifuge tubes. Samples were dried in a speed vacuum (Vacufuge plus, Eppendorf) for 2.5 hours, resuspended in 50% methanol and stored at -80°C until liquid chromatography-mass spectrometry (LC-MS) was performed.

Hydrophilic Interaction Chromatography (HILIC)

Extracted metabolites were screened for homoserine lactones (HSLs) by aqueous normal phase analysis on a Cogent Diamond Hydride HILIC 150 mm x 2.1 mm column (MicroSolv, Eatontown, NJ) for liquid chromatography (LC) separation with a flow rate of 600 $\mu\text{L min}^{-1}$. Solvent A consisted of 0.1% formic acid in water, while solvent B consisted of 0.1% formic acid in acetonitrile. The elution gradient consisted of 95% solvent B for 1 min (with first min going to waste to avoid contaminating the source with excess salt), to 70% solvent B over 13 min, held, and returned to 95% in 0.5 min, with a total run time of 15 min using an Agilent 1290 UPLC (Agilent, Santa Clara, CA) system connected to an Agilent 6538 Q-TOF Mass Spectrometer (Agilent, Santa Clara, CA).

Mass spectrometry analysis was conducted in positive ion mode, with a cone voltage of 3500V and a fragmentor voltage of 120V. Drying gas temperature was 350°C with a flow of 8 L min^{-1} and the nebulizer was set to 60 psig. Spectra were collected at a rate of 2.52 per sec with a mass range of 50 to 100 m/z. The mass analyzer resolution was 18,000 and post calibration tests had a mass accuracy of approximately 1 ppm.

Data files from the LC-MS were converted to MZxml format using the Masshunter Qualitative software provided with Agilent instruments (Agilent, Santa Clara, CA). Analysis of LC-MS data was done using the features and identification

function in MZmine version 2.31. Procedures, together with parameters used for the alignment of features and identification in MZmine, were as follows: LC-MS files were imported into MZmine, followed by data set filtering to remove the first min of elution data for HILIC analysis, and the first 2 min of elution data for reverse phase analysis. A minimum intensity cutoff of 5000 and a minimum elution time window of 0.1 min were used to create molecular feature lists. Lists included retention time (R/T) adjusted with a tolerance of 0.2 min or less. These R/T-adjusted lists were aligned into one mass list and gap-filled to add missing peaks not detected in all runs with an m/z tolerance of 15.0 ppm. The m/z peak intensities were normalized by normalizing the total ion intensity of each run to the most intense total ion intensity. Significance was determined using a two-tailed t -test

Statistical Analysis

Statistical analysis was performed on CFU log reductions (LR) in R (R Core Team 2013). t -tests with unequal variances were performed to determine the efficiency of individual treatments compared to untreated controls. Linear mixed effects models and follow-up t -tests using Kenward Roger degree of freedom approximation were applied to determine the repeatability of experiments (Tilt and Hamilton 1999). Standard deviations (SD) for repeatability of ≤ 0.5 are accepted (Parker and Hamilton 2011). The pairwise comparisons of LR were performed by Welch t -tests with a Bonferroni multiple comparison correction for each agent. The MetaboAnalyst version 4.0 (Xia and Wishart 2016) was adopted for creating heatmaps for HILIC analysis.

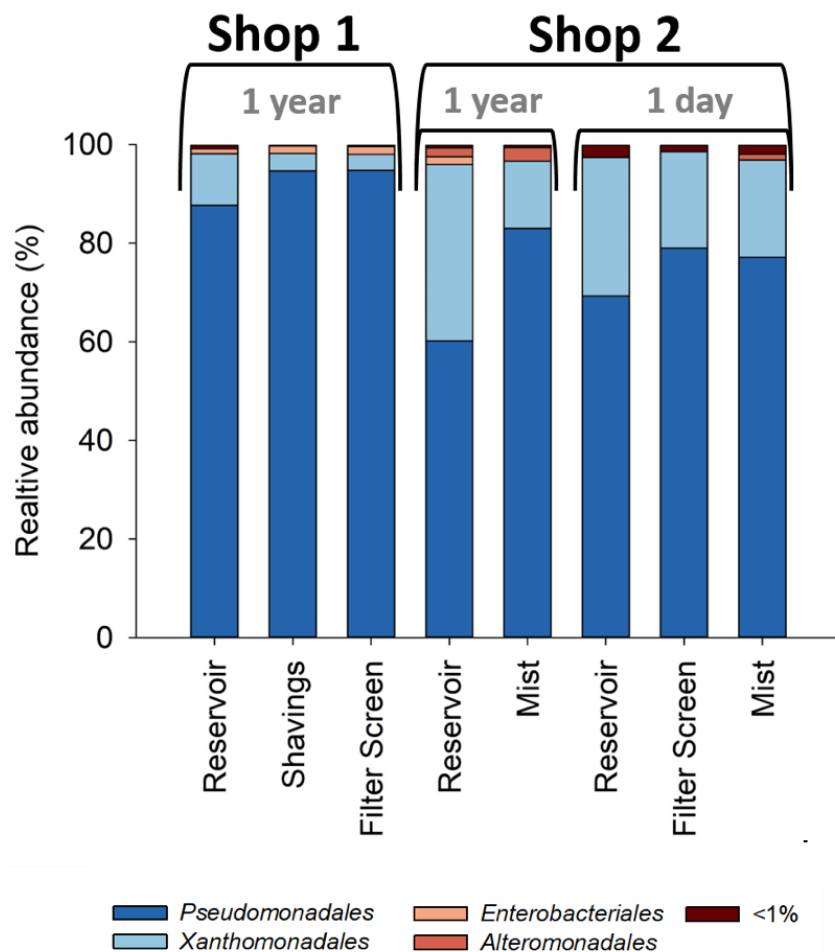


Figure 2.2 Relative abundance of the microbial contamination (Order level) present in two local machine shops (Bozeman, MT).

Results

Determination of Microbial Communities

Quality refinement (including removal of blank OTUs and singletons) resulted in 98162 ± 40475 sequences per library, which clustered into 46 OTUs. All samples collected tested positive for bacterial contamination, dominated by *Pseudomonadales* (60.2-99.7%; Figure 2.2). MWF 'Brand A' was used at two different shops over a similar

period; however, compared to Shop 1 dominated by *Pseudomonadales* (92±4%), the MWF circulation system at Shop 2 was colonized by both *Pseudomonadales* (74±9%) and *Xanthomonadales* (23±9%). After dumping and cleaning the MWF circulation system at Shop 2, infection of the newly recharged MWF had occurred within the first day, with *Pseudomonadales* driving recolonization.

Effectiveness of Agents on Biofilm Reduction

Various enzymes, natural, and chemical compounds were screened for their effect on biofilm formation both as single compound amendments and in combination (Table 2.2). Screening for compound applicability showed that emodin (10-1000 µM), garlic extract (0.5%-15% v/v), gingerol (350 µM), α-amylase from *Bacillus amyloliquefaciens* (≥10 Units), Acylase 1 from Porcine kidney (0.5-20 Units), and phenyl disulfide (0.02-1 mM) had no effect on eradication of *P. aeruginosa* PAO1 biofilms. Conversely, Patulin (40 µM), Furanone C-30 (75 µM), and α-amylase from *Bacillus amyloliquefaciens* (1 Unit; herein refer to as α-amylase) were effective in reducing biofilm formation (≥50-80% reduction). These three agents were selected for further investigation of *P. aeruginosa* PAO1 biofilm formation.

When cultivating *P. aeruginosa* PAO1 biofilm in TSB applied to CDC batch reactors (Figure 2.3A), α-amylase significantly decreased biofilm formation over 48 hours ($p=0.009$ using a *t*-test with unequal variances). Furanone C-30 and Patulin demonstrated an initial treatment effect after 24 hours ($p<0.05$ using a *t*-test with unequal variances).

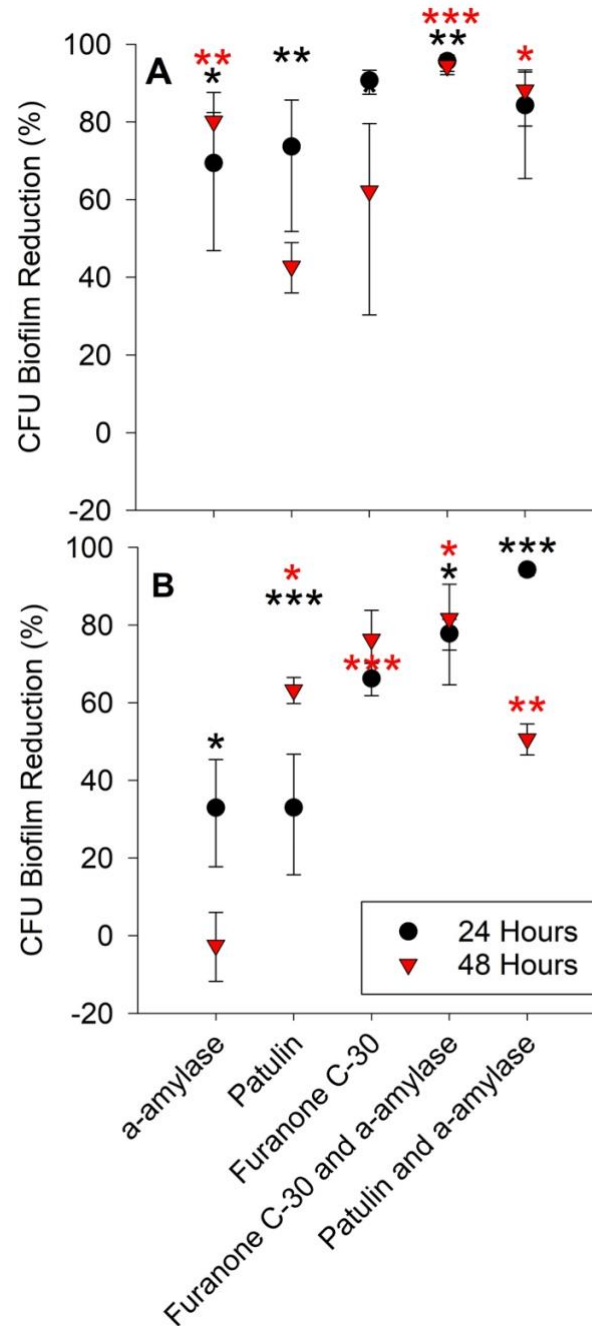


Figure 2.3 (A) Effect of QS inhibitors as single amendments and in combination with an enzyme on *Pseudomonas aeruginosa* PAO1 biofilm formation in 1X TSB media in CDC batch reactors. (B) Effects of QS inhibitors on *Pseudomonas aeruginosa* PAO1 biofilm formation in 7% (final conc.) water soluble MWF in twelve well plates. Circular and triangle shape data points represent CFU biofilm reduction (%) over a period of 24 and 48 hours respectively. Significant levels are shown as * ≤ 0.05 , ** ≤ 0.01 , *** ≤ 0.001 .

However, these agents were less effective after 48 hours and resulted in an increase in biofilm of 28.5 and 30.8%. Both Furanone C-30 and Patulin combined with α -amylase showed a significant difference in biofilm formation when compared to an untreated reactor after 48 hours ($p<0.001$ and $p=0.012$ respectively, using a t -test with unequal variances; Figure 2.3A). Furanone C-30 in combination with α -amylase caused ~95% in biofilm reduction for both time points investigated. Calculated standard deviations (SD=0.335) provided statistical evidence for the repeatability of these experiments.

The same agents were applied to multi-well plates assays with 7% MWF 'Brand A' over a period of 48 hours (Figure 2.3B). Similar to biofilm reduction in TSB, the combination of Furanone C-30 and α -amylase significantly decreased biofilm formation after both 24 and 48 hours ($p=0.014$ and $p=0.022$ respectively, using a t -test with unequal variances) by $79.6\pm3\%$ on average. The combination of Patulin and α -amylase reduced biofilm by 94.3% after 24 hours ($p<0.001$ using a t -test with unequal variances).

However, after an additional 24 hours of incubation, biofilm appears to have regrown, with a reduction of only 50.7% as compared to the control. In 7% MWF 'Brand A', both single treatments of Furanone C-30 and Patulin led to biofilm reduction over 48 hours ($p=0.012$ and $p=0.001$ respectively, using a t -test with unequal variances). No effect on biofilm reduction was detected with α -amylase after 48 hours (Figure 2.3B). Statistical analysis revealed the repeatability of these trials (SD=0.161).

Treatment effects on *P. aeruginosa* PAO1 biofilm formation were also visualized by epi-fluorescent microscopy (Figure 2.4). A dense network of biofilm was clearly

observed on control coupons with no treatment after 48 hours (Figure 2.4A). The combined treatment of Furanone C-30 and α -amylase was visually (Figure 2.4B) and

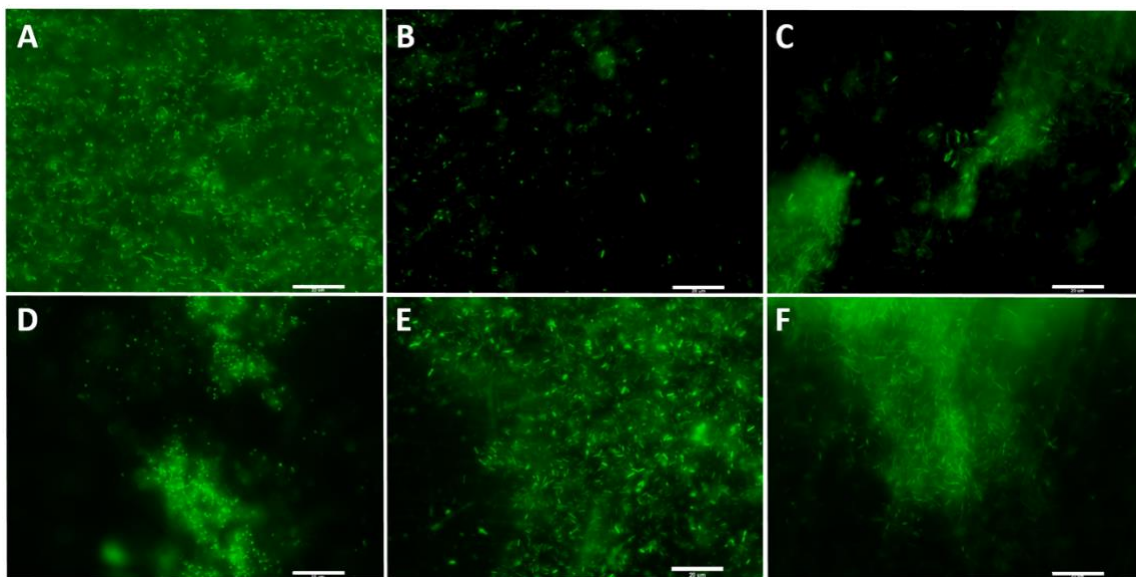


Figure 2.4 Epi-fluorescent images of *Pseudomonas aeruginosa* PAO1 biofilm on stainless-steel coupons. (A) Untreated biofilm from CDC reactors at 48 hours. Treated coupon with (B) combination of Furanone C-30 and enzyme α -amylase, (C) combination of Patulin and enzyme α -amylase, (D) α -amylase, (E) Furanone C-30, and (F) Patulin. Samples were imaged under 60x magnification. Scale bar = 20 μ m.

quantitatively (Figure 2.4B) the most effective treatment followed by the combination of Patulin and α -amylase (Figure 2.4C). For single compound amendments, α -amylase exhibited the least biofilm coverage on stainless steel coupons (Figure 2.4D). Although dead cells (i.e. those with compromised membranes) are not shown, images showed the biofilm associated cells on the coupon surfaces were viable (i.e. green cells).

LC-MS: Screening for HSLs

LC-MS data were filtered to identify reported m/z values of produced HSLs by *P. aeruginosa* PAO1 (Cataldi et al. 2009). Putative identification indicated the presence of

C₄-HSL (N-Butyryl-DL-homoserine lactone), 3-oxo-C₁₀-HSL (N-(3-Oxodecanoyl)-L-homoserine lactone), and 3-oxo-C₁₂-HSL (N-(3-Oxo-dodecanoyl) homoserine lactone).

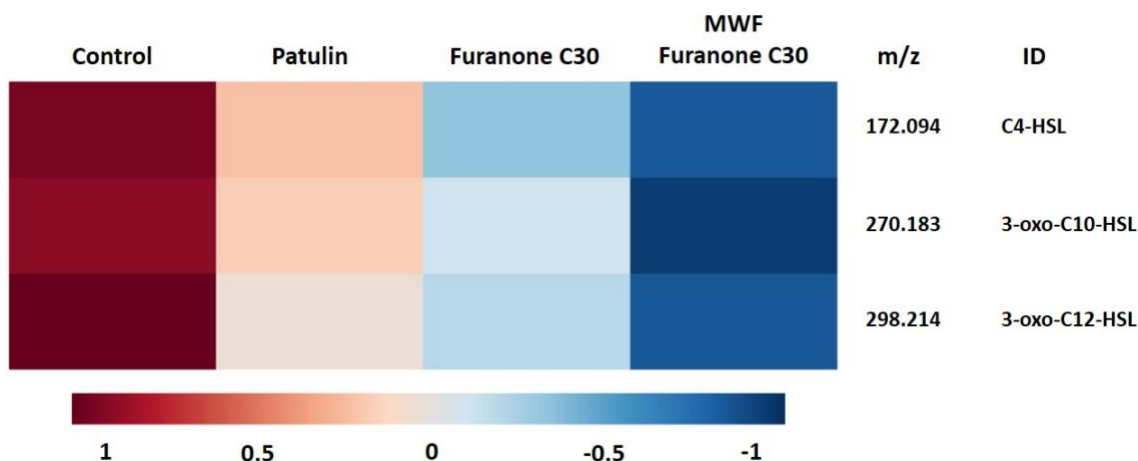


Figure 2.5 Heatmap of putative identified homoserine lactones (HSLs) intensities found in *P. aeruginosa* PAO1 untreated (control) and treated biofilm samples at 24 hours in TSB and metalworking fluid (MWF).

In parallel with CFU biofilm reduction (Figure 2.3), samples amended with quorum sensing inhibitor agents (i.e. Patulin and Furanone C-30) showed a lower expression of HSLs when compared to the untreated control in TSB (Figure 2.5). Furanone C-30 in MWF expressed almost none of the major HSLs found in *P. aeruginosa* PAO1 indicating a successful treatment for quenching the quorum sensing mechanism of PAO1.

Discussion

Planktonic and biofilm samples collected from two local machine shops tested positive for microbial contamination, and were dominated by *Pseudomonadales* (Figure 2.2). Previous studies on microbial contaminants in MWFs confirm the dominance of this Order (Baecker et al. 1989; Van der Gast et al. 2001; Rabenstein et al. 2009; Gilbert et al.

2010). *Pseudomonas* spp. may account for up to ~90% of bacterial sequence libraries (Di Maiuta et al. 2017) with biomass as high as $>10^8$ CFUs mL⁻¹ in some MWFs (Mattsby-Baltzer et al. 1989). Significant in this study was the rapid recolonization after a commonly practiced dumping, cleaning, and recharge process at Shop 2. With residual microbes residing in inaccessible parts of MWF circulation systems escaping these hygiene practices, recolonization is an inherent issue associated with the MWF industry (Veillette et al. 2004; Marchand et al. 2010; Kapoor et al. 2014). Most sequences classified within the Order *Pseudomonadales* were phylogenetically related to a single OTU. This OTU had 99% sequence identity to the 16S rRNA gene of *Pseudomonas stutzeri*, a species frequently associated with biodegradation activity in MWFs (Muszynski et al. 2007; Gilbert et al. 2010; Saha et al. 2010; Moscoso et al. 2012; Di Maiuta et al. 2017). In preliminary experiments, *P. stutzeri* was unable to form a stable biofilm in the CDC reactors. Thus, *P. aeruginosa* PAO1, another known MWF contaminant (Jakšić et al. 1998; Van der Gast et al. 2001; Karadzic et al. 2006) and model strain for biofilm formation (Kato et al. 2008) was selected for quorum sensing inhibition experiments.

In the present study the commercially available quorum sensing inhibitory compounds Furanone C-30 and Patulin were tested for biofilm prevention of *P. aeruginosa* POA1 in MWFs. Synthetic Furanone C-30 decreases acyl-homoserine lactone-based signaling by interacting with transcriptional regulators that propagate the quorum sensing response (Hentzer et al. 2003). Patulin, a mycotoxin, produced by fungi of the genera *Aspergillus* and *Penicillium* (Bergel et al. 1943) has been shown to inhibit

up to 80% of quorum sensing regulated genes, including the repression of the *lasB* promoter required for encoding elastase and the *rhlAB* operon for rhamnolipid production (Hentzer et al. 2003; Rasmussen et al. 2005). Other genes of the central part of the quorum sensing circuit (e.g. *lasR*, *rhlR*) have been suggested to be regulated at the post-transcriptional level (Hentzer et al. 2003; Rasmussen et al. 2005). Importantly, both elastase LasB (i.e. major virulence factor) and rhamnolipids are crucial in biofilm development of *P. aeruginosa* PAO1 (Yu et al. 2014). α -amylase was tested in combination with quorum sensing inhibitor agents as it hydrolyses polysaccharides and glycosidic linkages; thus, degrading major constituents of the extracellular polymeric substance (EPS) matrix of biofilms (Craigien et al. 2011; Kaplan et al. 2003; Izano et al. 2008; Fleming et al. 2017). It is noteworthy that as a mycotoxin, Patulin could also exhibit antibiotic properties and impair growth when administered at high concentrations. However, inhibitory effects on cell growth of *P. aeruginosa* PAO1 have not been reported (Rasmussen et al. 2005).

No significant treatment effect was found between single compound amendments (i.e. Patulin and Furanone C-30) and their co-administration with α -amylase after 48 hours ($P \geq 0.146$), indicating a diminishable contribution of α -amylase to biofilm reduction in MWFs overtime. As EPS secretion of *P. aeruginosa* PAO1 is controlled by quorum sensing (Nadell et al. 2008), interference with the quorum sensing circuitry would be expected to reduce the amount of EPS produced, and thus, the available substrates for α -amylase hydrolysis. Diggle et al. (2006) showed that biofilm produced by *P. aeruginosa* PAO1 on stainless-steel is orchestrated by quorum sensing. Ren and Wood (2004)

demonstrated that *Desulfotomaculum* populations could be regulated by Furanones, thereby reducing corrosion of mild steel. In support of a quorum sensing strategy Patulin (40 μM) and Furanone C-30 (75 μM) showed a strong positive effect in preventing biofilm formation on stainless-steel coupons in MWF, reducing biofilm by ~63% and 76% on average, respectively (Figure 2.3). In comparison, Zhou et al. (2017) reported that *P. aeruginosa* PAO1 grown in TSB treated with eight-fold higher (325 μM) concentrations of Patulin only reduced biofilm by 52%. Higher concentrations of Furanone C-30 (100 μM) employed in a different study reduced biofilm formation by only 63% (Kim et al. 2015). However, quorum sensing is highly sensitive to the chemical and biological makeup of the environment, and as such, comparing biofilm removal efficiencies should be viewed conservatively. Patulin and Furanone C-30 have also been shown to render *P. aeruginosa* biofilm more susceptible to biocide treatment (Hentzer et al. 2003; Rasmussen et al. 2005; Christensen et al. 2012). With biocides commonly used to combat microbial growth in MWFs, their combination with quorum sensing inhibitor agents awaits investigation for preventing detrimental biofilm formation. Further, while Patulin and Furanone C-30 were only administered at the beginning of the 48 hours of incubation in the present study, improved biofilm reduction may be achieved by periodic amendments of quorum sensing inhibitors (Hentzer et al. 2003; Wu et al. 2004).

It is well known that *P. aeruginosa* uses N-Acyl homoserine lactone signal molecules to coordinate attributes such as motility, virulence factors secretion, and biofilm formation (Chan et al. 2015). The quorum sensing system of *P. aeruginosa* typically shows a multilayered, hierarchical network (Lee and Zhang 2014), with N-(3-

oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) playing a critical role in biofilm growth and regulating genes involved in *las* and the activation of the subordinate *rhl* and *pqs* system, while N-butanoyl-L-homoserine lactone (C4-HSL) is the most significant autoinducing molecule (Davies et al. 1998; de Kievit and Iglewski 2000; Cataldi et al. 2009; Kušar et al. 2016). Shorter chain length HSLs such as 3-oxo-C10-HSL are rare, but may be produced when one of the enzymatic steps in the 3-oxo-C12-HSL biosynthetic pathways becomes limited by 3-oxo-acyl- carrier proteins (ACPs) precursors (de Kievit and Iglewski 2000; Hoang et al. 2002). These proteins have been shown as carriers of acyl intermediates and their involvement in the quorum sensing circuit has been proposed (Hentzer et al. 2003; Rasmussen et al. 2005; White et al. 2005; Zhu et al. 2008; Jimenez et al. 2012); although, recent *in vitro* evidence opposes these reports (Ma et al. 2017). Furanone C-30 and Patulin downregulate the transcription of genes encoding 3-oxo-ACPs (Hentzer et al. 2003; Rasmussen et al. 2005) which may explain the putative detection of 3-oxo-C10-HSL. Importantly, expression of genes in the *P. aeruginosa* quorum sensing system are auto-regulated and depends on the concentration of signaling molecules, which is related to cell density (Seed et al. 1995; Latifi et al. 1996; Pesci et al. 1997). In fact, Hentzer et al. (2003) demonstrated that despite high doses of exogenous signaling molecules added to the starting inoculum (i.e. simulating the build-up of extracellular molecules), expression of genes involved in quorum sensing lagged, but could be induced in response to high cell densities. In agreement with these reports, the expression profile of putatively identified HSLs corresponded with biofilm cell density in this study (Figure 2.4, Figure 2.5). Importantly in this study, the expression of HSLs was

maximal in the untreated controls, but lower in samples treated with quorum sensing inhibitors, supporting the idea of HSL accumulation and cell density. The lowest expression of C4-HSL and 3-oxo-C12-HSL was detected in MWF. With Patulin and Furanone C-30 manipulating the quorum sensing circuit, the reduced amplification of quorum sensing molecules would have affected a cascade of genes, including those involved in biofilm formation. Although much remains to be learned about quorum sensing/inhibition in biofilm formation, maintenance, and dispersal in MWF circulation systems, data presented herein provides a promising strategy for battling microbial contaminants.

Acknowledgments

Funding for this research was provided by National Science Foundation (NSF) under NSF 1635347. Any opinions, findings, or conclusions expressed in this material are those of the authors and do not necessarily reflect the views of NSF.

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CHAPTER THREE

APPLICATION OF BIOFILM ACCUMULATION MODEL (BAM)
ON CDC BATCH REACTORSMathematical Modelling of Biofilms

Modeling of biofilms was first began in the 1970s to demonstrate substrate utilization and mass transport within the biofilms (Harris and Hansford 1976; Rittmann and McCarty 1980; Rittmann and Dovantzis 1983; Harremoës 1976; LaMotta 1976; Williamson and McCarty 1976). The first models followed the chemical engineering principles of mass transfer, diffusion, and biochemical reactions (Harris and Hansford 1976) to formulate the mathematical description of biofilm formation. While predicting the behavior of biofilms through models, a couple of assumptions were made. The first models assumed a simple geometry with uniform biomass and biofilm thickness distribution within the film (Williamson and McCarty 1976; Rittmann and McCarty 1980). Studies of the first biofilm models determined the importance of change in substrate concentration inside the biofilm, showing that the substrate concentration decreases dramatically while biofilm associated cells consume the nutrient-rich environment resources (Rittmann and McCarty 1980). In the 1970s, investigations modelling biofilm kinetics also assumed that the mass or thickness of biofilm would maintain steady-state (no accumulation) kinetics for a given microorganism. Substrates were allowed to vary, and mixed models consisting of two substrates were also

introduced (Harris and Hansford 1976; Williamson and McCarty 1976; Rittmann and McCarty 1980).

In the 1980s, models became more complicated and investigators began predicting non-uniform biofilm structures with mixed bacterial culture biofilms (Kissel and Street 1984; Wanner and Gujer 1986; Rittmann and Manem 1992) while still assuming the simple biofilm geometry seen in the 1970s. In addition to maintaining the simple geometry and steady-state kinetics, new research began investigating modeling parameters for mixed cultures inside biofilms. In the 1990s, improved models were developed to predict the behavior of biofilms in multi-dimensions (e.g. 2 and 3D modelling of biofilm structures), including different terms and mechanisms to existing equations in order to study resistance to antibiotics, biocide actions, rate of detachment, and substrate rate limiting (Rittmann and Dovantzis 1983; Wanner and Gujer 1986; Stewart 1994; Stewart et al. 1996). In 1991, a computer simulation biofilm accumulation model (BAM) was developed with a component allowing for the introduction of biocides, by researchers at the Center for Biofilm Engineering (Stewart 1994; Stewart et al. 1996, version 1.0). BAM is based on descriptions and mathematical formulations of Wanner and Gujer (1985). Chapter 3 presents my research using BAM to model the effects of a single treatment of α -amylase on a *Pseudomonas aeruginosa* PAO1 biofilm grown in a CDC biofilm reactor.

Materials and Methods

Experimental Methodology

Pseudomonas aeruginosa PAO1 (ATCC: 15692), a model biofilm forming organism (Kato et al. 2008), was grown in tryptic soy broth (TSB) at 37°C while shaking at 125 rpm for 16 hours. Cultures were harvested and centrifuged at 10,000xg for 2 min. CDC Biofilm Reactors were purchased from Biosurface Technologies (Bozeman, MT). These reactors consist of a glass vessel, eight polypropylene coupon holders (rods) each holding three coupons (biofilm growth surface) 12.7 mm in diameter, and a lid with a gas exchange port. Reactors were prepared following standard operating procedures (Goeres et al. 2005) and experiments were performed as batch reactors on a stir plate at 125 rpm at 37°C for 48 hours. Media (300 mL) of 1X TSB was poured into an untreated (control) and treated reactor with 2 mL of an overnight *P. aeruginosa* PAO1 culture. α -amylase from *Bacillus amyloliquefaciens* (A7595, Sigma-Aldrich) was applied to the treated reactor at a concentration of approximately 3 Units mL⁻¹ as a single amendment. At two time points (i.e. 24 and 48 hours), one rod holding three stainless steel coupons was removed from untreated and treated reactors. Coupons were dipped in 1X phosphate-buffered saline (PBS) to remove unattached, planktonic cells. Each coupon was placed in sterile falcon tubes filled with 5 mL of 1X PBS and vortexed for 10 sec. Ten-fold dilution series were prepared using sterile 1X PBS. An electronic pipetter (ThermoLabsystems) was used for high accuracy and precision. Five drops (10 μ L each) per dilution were pipetted on TSA plates (Herigstad et al. 2001). Colony forming units (CFUs) of biofilm were enumerated after 16 hours of incubation at 37°C.

Microbial Growth Kinetics

Microorganisms have four different phases to complete growth during batch culture. These phases are lag phase where cells increase in size without cell division; log phase where cell division occurs at a constant rate; stationary phase when the number of cells undergoing division is equal to the number of cells that are dying; and the death phase when a decrease in population size occurs mostly due to running out of nutrients (Navarro Llorens et al. 2010; Rolfe et al. 2012). These phases are analyzed to understand growth in microorganism. The fastest multiplication of the microorganisms occurs during the log phase of growth. Since bacteria grow exponentially, it is useful to plot the logarithm of the population size (or absorbance) versus time to analyze the growth rate of the related microorganism (Zwietering et al. 1990).

One of the most common ways to measure the specific growth rate of a microorganism is to generate a growth curve. In this experiment, *P. aeruginosa* PAO1 growth kinetics were measured in batch culture. Full strength TSB media (50 mL) was inoculated with 200 μ L of overnight *P. aeruginosa* PAO1 cell culture in an Erlenmeyer flask. Subsequently, inoculated media was incubated at 37°C while shaking at 125 rpm. To measure the increase in cell density over time the inoculated flask was removed from the incubator and 1 mL of inoculum was pipetted into a cuvette inside a sterile hood. A Thermo Scientific Genesys 10uv spectrophotometer was set to a wavelength of 600 nm to collect absorbance values, often referred to as optical density (O.D.) measurements. OD₆₀₀ measurements were recorded every 30 min for 6 h. At each time point, a cuvette with full strength TSB was measured to blank the spectrophotometer. Generation time and growth rate values of *P. aeruginosa* PAO1 were calculated from semilog plot of

absorbance at 600 nm versus time plot (Powell 1956). Cell division occurs at a different rate and time for each microorganism. This time is defined as the generation time or doubling time and can be calculated by

$$\frac{dX}{dt} = \mu X \quad \text{Equation 3.1}$$

where X is the number of cells, t is the time and μ is the specific growth rate (Painter 1975; Maier 2009). This expression shows the linear portion of the semilog plot, which can be solved by integration

$$\frac{dX}{X} = \mu dt \quad \text{Equation 3.2}$$

$$\int_{X_0}^X \frac{dX}{X} = \mu \int_0^t dt \quad \text{Equation 3.3}$$

$$\ln X = \ln X_0 + \mu t \quad \text{Equation 3.4}$$

Equation 3.4 can also be written in exponential form as

$$X = X_0 e^{\mu t} \quad \text{Equation 3.5}$$

where t is the doubling time. Generation time can be expressed for the cell mass, X, to be doubled by

$$\frac{X}{X_0} = 2 \quad \text{Equation 3.6}$$

where X_0 is the initial mass of cells and X is the final cell mass when cell division is completed. When Equation 3.6 is substituted in Equation 3.5, generation time can be expressed by

$$2 = e^{\mu t} \quad \text{or} \quad t = \frac{\ln 2}{\mu} \quad \text{Equation 3.7}$$

where t is the generation time. Equation 3.7 can be solved for specific growth rate, μ , or μ can be calculated by simply taking the slope of the semilog plot of absorbance/ growth rate vs time (Painter 1975).

Biofilm Accumulation Model (BAM)

BAM, was used to model the effect of a single agent treatment of α -amylase at a concentration of 3 Units mL⁻¹ to reduce *P. aeruginosa* PAO1 biofilms. α -amylase was tested in this study with quorum sensing inhibitor agents as it hydrolyses polysaccharides and glycosidic linkages; thus, degrading major constituents of extracellular polymeric substance (EPS) matrixes of biofilms (Craigien et al. 2011; Kaplan et al. 2003; Izano et al. 2008; Fleming et al. 2017). This computer model applies the principles of conservation of mass to biofilm and bulk fluid simultaneously as well as applying material balances on biomass and substrate by following a series of differential equations (Wanner and Gujer 1986; Stewart 1994; Stewart et al. 1996).

Wanner and Gujer (1986) determined their solution to biofilm models through a series of differential equations using numerical techniques, a method called the *method of lines*, in which partial differential equations were transformed to a set of ordinary differential equations by spatial discretization. The developers of BAM used this technique through the FORSIM VI simulation package. To investigate the general behavior and sensitivity of this model more than 200 simulations were conducted, varying input parameters such as biofilm thickness, biocide concentration, biocide rate coefficient, biocide dose duration, and biocide reaction rate coefficient (Wanner and Gujer 1986; Stewart et al. 1995). Authors also used statistical analyses to interpret data from the

simulation experiment. The quality of the mathematical model was measured by the correlation coefficient between the predicted value and the computer simulation value across the simulations (>200) conducted (Stewart et al. 1995).

General material balances on the bulk fluid compartment in BAM can be expressed by

$$\frac{dX}{dt} = \mu \max \frac{S}{K_S + S} X - bX - k_1 XSC + k_2 \varepsilon \rho L_f^2 \frac{A}{V} - \frac{Q}{V} X \quad \text{Equation 3.8}$$

where X is the cell density, $\mu_{\max}$ is the maximum specific growth rate of microorganism, S is the substrate concentration, K_S is the rate coefficient for substrate, b is the specific death rate, k_1 is the rate coefficient for eradication, C is the concentration of treatment, k_2 is the rate coefficient of detachment, ε is the cell volume fraction, ρ is the cell density, L_f is the film thickness, A is the biofilm growth area, and $\frac{Q}{V}$ is the dilution rate. The terms in this equation represent (from left to right) accumulation, growth in bulk fluid, death in bulk fluid, eradication, detachment, and flow out of the reactor (Wanner and Gujer 1986; Stewart et al. 1996).

A material balance in the biofilm compartment of BAM on live cells can be expressed by

$$\frac{\partial \epsilon}{\partial t} = \mu_{\max} \frac{S}{K_S + S} \epsilon - \frac{\partial(v\epsilon)}{\partial z} - b\epsilon - k_r C \epsilon \quad \text{Equation 3.9}$$

where ϵ is the fraction of biofilm volume, $\mu_{\max}$ is the maximum specific growth rate of microorganism, S is the substrate concentration, K_S is the rate coefficient for substrate, v is the advective velocity, b is the specific death rate, k_r is the treatment rate coefficient, and C is the concentration of treatments. The terms in this equation represent

accumulation, growth, advection, death, and treatment from left to right (Stewart et al. 1996; Wanner and Gujer 1986).

Moreover, the concentration of growth media in the biofilm (Stewart et al. 1996) is described in BAM by

$$\frac{\partial S}{\partial t} = D \frac{\partial^2 S}{\partial z^2} - \frac{\mu}{Y} \times \frac{S}{K_S + S} \times \epsilon \rho \quad \text{Equation 3.10}$$

where S is the substrate concentration, D is the diffusion coefficient of substrate, μ is the growth rate of microorganism, Y is the substrate yield coefficient, K_S is the substrate Monod coefficient, ϵ is the biofilm fraction, and ρ is the biofilm cell density; with the terms of accumulation, diffusion, and consumption from left to right.

In addition to the material balances above, advection also participates in the growth of biofilms. When describing transport processes within the biofilms, advection should be considered along with diffusion and growth. The advective velocity can be described by

$$\frac{\partial v}{\partial z} = \mu_{max} \frac{S}{K_S + S} \times \frac{\epsilon}{\epsilon_c} \quad \text{Equation 3.11}$$

where $v = 0$ at $z = 0$ for $t > 0$ (Stewart et al. 1996). The thickness of the biofilm also depends on growth and detachment. The change in film thickness in BAM can be expressed by

$$\frac{\partial L_f}{\partial t} = \mu_{max} \frac{S}{K_S + S} \epsilon - k L_f^2 \quad \text{Equation 3.12}$$

where L_f is the film thickness, S is the substrate concentration, K_s is the rate coefficient for substrate, and k is the detachment rate; with the terms of accumulation, growth and detachment from left to right.

BAM is built on multiple definitions of geometry, substrate, particulate (i.e. organisms), reaction, boundary conditions, and initial conditions. This computer model can also simulate several reactors in series. In the model, “Geometry Definitions” lets the user pick a definition for a number of units. My model is defined as one unit: Unit 1, which is run as one batch reactor with a constant reactor volume. “Substrate Definitions” applies the diffusivity of the substrate(s) with its molecular diffusivity in water. In this section of substrate definitions, a biocide is defined as the last substrate. Biocide addition is defined as the initial start time for the addition of a biocide, having options for treatment introduction as either pulse or step-wise additions. Diffusivity of the biocide, duration of the treatments, and inlet concentration are also indicated in this section of definitions. “Particulate Definitions” are made up of two compartments, these compartments can be defined as live cells and dead cells, with biofilm phase density. In the “Reaction Definitions” section of the model, two reactions can be defined: growth and eradication of biofilm. For defining growth kinetics in BAM, the rate law of the defined reaction (i.e. growth and eradication) needs to be selected. In this model, Monod kinetics (Monod 1949) was adopted for the reactions mentioned above. Monod kinetics is defined in this model following the Monod equation (Equation 3.13) and growth reaction (Equation 3.14) by

$$\mu = \mu_{\max} \times \frac{S}{K_S + S} \quad \text{Equation 3.13}$$

$$r = X \times \mu_{\max} \times \frac{S}{K_S + S} \quad \text{Equation 3.14}$$

where μ is the specific growth rate of microorganism, $\mu_{\max}$ is the maximum specific growth rate of *P. aeruginosa* PAO1, S is the substrate concentration, K_S is the rate coefficient for substrate, r is the reaction rate, and X is the cell concentration. Equation 3.14 is a first order reaction with respect to the cell concentration while substrate concentration is modeled on Monod kinetics. The stoichiometric coefficient for cell concentration is 1.0 since the reaction rate is based on cell concentration. On the other hand, the stoichiometric coefficient for cell concentration in the eradication reaction is -1.0, since α -amylase is modeled as a biocide and the reaction rate is based on cell concentration. In the “Boundary Conditions” section of BAM, detachment is defined as a

Table 3.1 Biofilm accumulation model (BAM) input parameters.

Parameter	Value	Source or reference
Maximum specific growth rate ($\mu_{\max}$)	0.32 h ⁻¹	Measured value
Initial biofilm thickness (L_f)	0.015 μm	Estimated value
Biofilm phase density (ρ)	2.5x10 ⁷ g m ⁻³	Enumerated value
Media diffusion coefficient (D)	6.7x10 ⁻⁵ m ² day ⁻¹	(Stewart 2003)
Biocide diffusion coefficient (D_b)	73.872x10 ⁻⁶ m ² day ⁻¹	(Stewart 2003)
Biofilm area (A)	4.05x10 ⁻⁴ m ²	Calculated value
Void fraction of water in film (ϵ_w)	0.90	(Stewart 1994)
Biofilm-bulk diffusivity ratio (τ)	0.5	(Stewart 2003)
Rate coefficient for biocide (k_f)	10.6 g L ⁻¹	(Hernández-Heredia and del Moral 2016)
Biocide inlet concentration (C)	6400 g m ⁻³	Tested value
Detachment rate coefficient (k)	5000 μm^{-1} day ⁻¹	Adjusted value

function of biofilm thickness. Inlet flow table values were set to no flow because the behavior of *P. aeruginosa* PAO1 biofilms was modelled in a batch reactor. The final

section of the model parameters to be defined is the “Initial Conditions, which defines the initial biofilm thickness, substrate inlet concentration, bulk concentrations, and film void fraction. All input parameter values for biofilm modeling are shown in Table 3.1.

In this model, laboratory experimental data was compared to model simulations by comparing the percent reduction of CFUs after 24 and 48 hours. The percent error was calculated for each case, control and treatment reactors by

$$\% \text{ Error} = \frac{|E-S|}{E} \times 100 \quad \text{Equation 3.15}$$

where E is the percent CFU biofilm reduction from laboratory experiments and S is the percent biofilm thickness reduction from the BAM simulation. Percent error was calculated at each time point separately.

Results and Discussion

This model describes the change in biofilm thickness with, and without, the addition of α -amylase in a batch reactor system. As discussed in Chapter 2, untreated (control) and treated CDC biofilm batch reactors were run at 37°C for 48 hours. When no treatment was applied to the control reactor, a cell count was found to be 4.00×10^7 CFUs per biofilm growth surface (coupon) at 24 hours, as described in Chapter 2. When enumerated CFUs were converted to an estimation of biofilm thickness at 24 hours, this yielded a thickness of approximately 20 μm per coupon. This approximation was made by using the coupon area and biofilm volume fraction (i.e. 10% of its original value) from Table 3.1 with an estimation of 1 μm bacterial cell diameter. In this model, initial film

thickness was estimated to be $0.015\ \mu\text{m}$ at time point zero and the bulk concentration of full strength TSB media was taken as $30\ \text{g L}^{-1}$. Since the growth media was mostly made of water, it was assumed that the void fraction of water within the film was 90%.

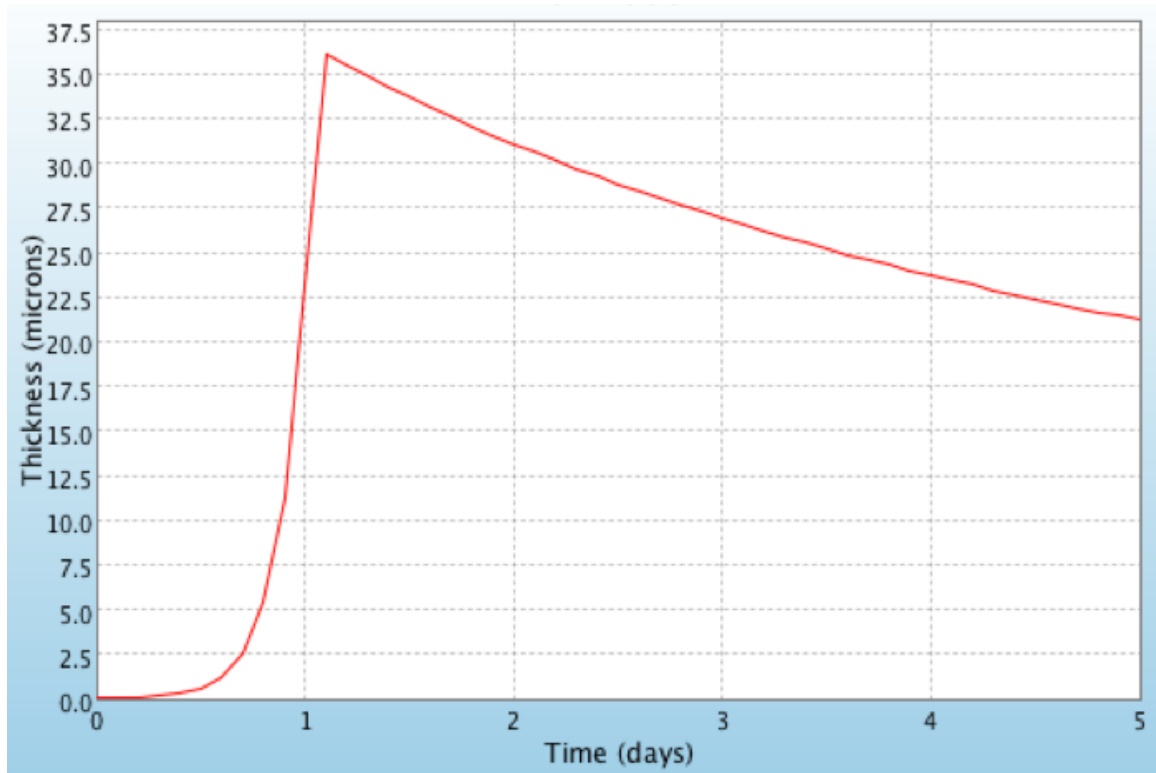


Figure 4.1 Thickness of *P. aeruginosa* PAO1 biofilm was found to be $23.14\ \mu\text{m}$ at 24 hours in biofilm accumulation model (BAM) on control (untreated) coupons in batch reactor mode.

When BAM was run for a control reactor with no treatment, the thickness of the *P. aeruginosa* PAO1 biofilm was found to be $23.14\ \mu\text{m}$ at 24 hours (Figure 4.1). This data shows a correlation with the calculated film thickness of $20\ \mu\text{m}$ at 24 hours. When the simulation was run for 48 h, it was determined that the film thickness on the stainless-steel coupons was $31.37\ \mu\text{m}$. A plot of biofilm thickness versus time (Figure 4.1) shows an exponential increase in the film thickness until the first half of day 2. This simulation

shows a correlation between the bulk density versus time (Figure 4.2) showing that by the end of 1.5 days the microorganism runs out of media, which is a typical outcome when running batch reactors. The simulation run length was set for 5 days in BAM even though

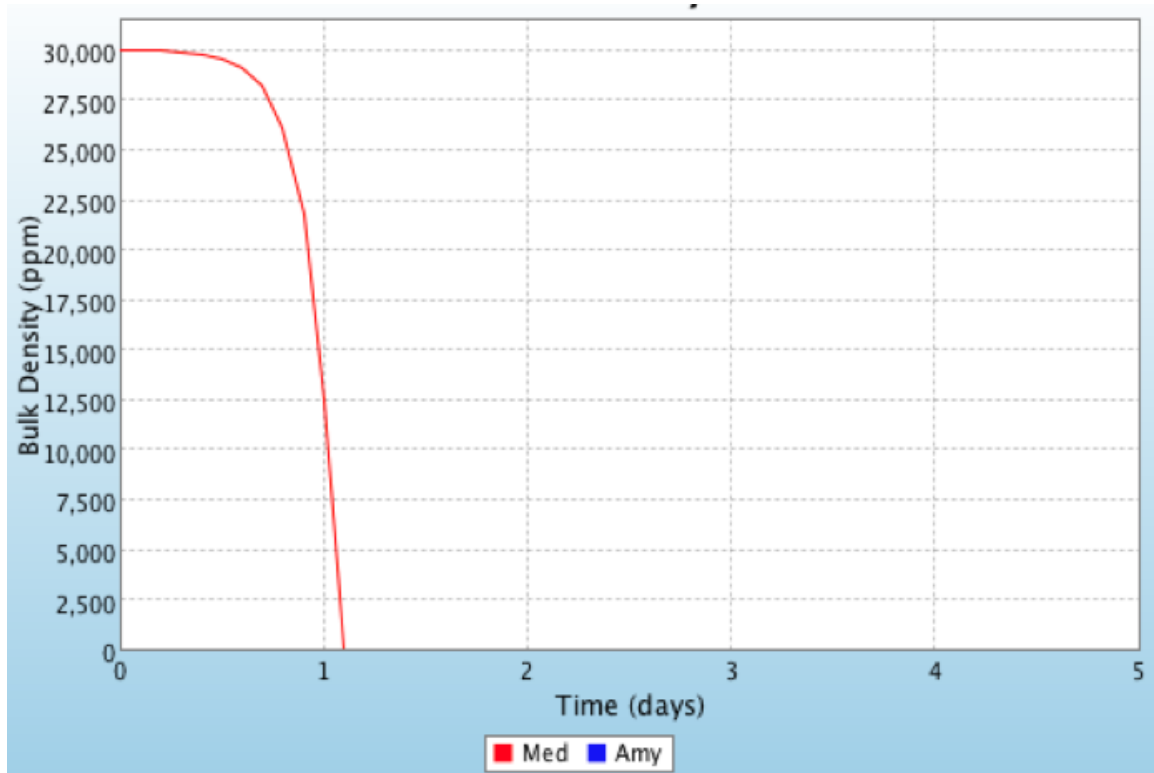


Figure 4.2 The change in bulk density (i.e. media) with time in untreated (control) batch reactors, simulated in biofilm accumulation model (BAM).

laboratory experiments were only run for 2 days. This approach was adopted in order to allow the simulation to reach steady state conditions.

When a single amendment of α -amylase at a concentration of 3 Units mL⁻¹ was introduced to the batch reactor, it was anticipated that there would be a decrease in CFUs per coupon, as well as film thickness. In lab experiments, it was determined that when α -amylase treatment was applied there was a 60% reduction in biofilm CFUs after 24

hours. BAM was run for the treated CDC batch reactor, following the same assumptions explained above, defined with α -amylase at a fixed concentration of 3 Units mL⁻¹.

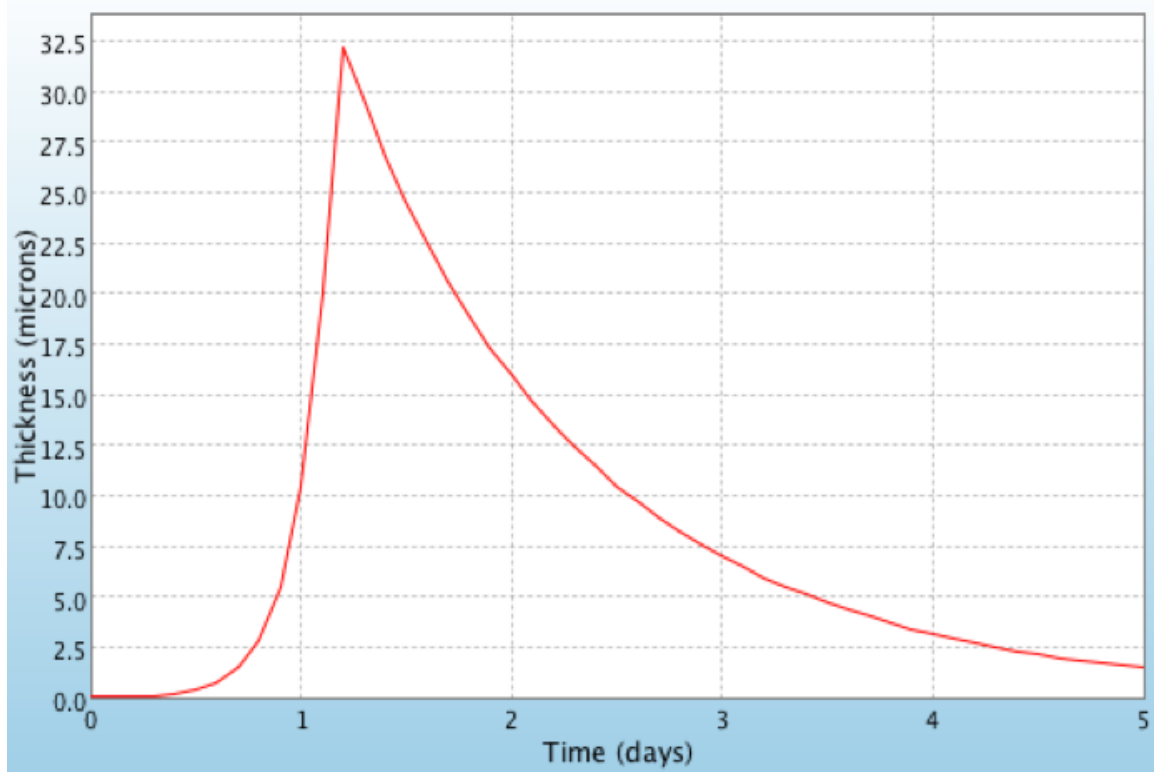


Figure 4.3 Thickness of *P. aeruginosa* PAO1 biofilm was found to be 10.47 μm at 24 hours in biofilm accumulation model (BAM), when treated with α -amylase in batch reactor mode.

A biofilm thickness of 23.14 and 31.07 μm was obtained with the absence of α -amylase, while the presence of α -amylase resulted a biofilm thickness of 10.47 and 16.07 μm over a period of 24 and 48 hours respectively (Figure 4.3), showing the microorganism runs out of media by the end of 1.5 days and the concentration of α -amylase does not change in time (Figure 4.4). If compared, yields would be 55 and 58% reduction in film thickness at 24 and 48 hours respectively. Moreover, lab experiments resulted in 69.5 and 80% of CFU biofilm reductions with α -amylase treatment in batch reactors at 24 and 48 hours

respectively. When model yields compared to lab experiments, the percentage error was calculated and determined to be 21 and 27% (Equation 3.15).

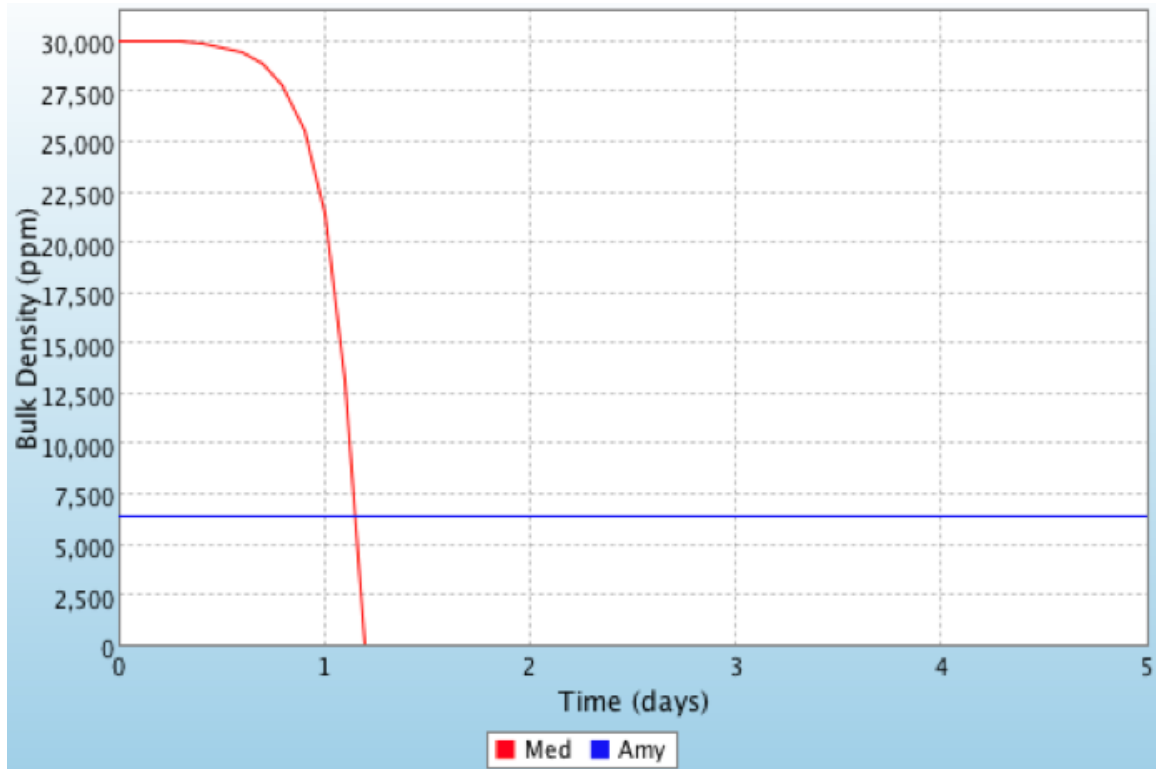


Figure 4.4 The change in bulk density (i.e. media) with time in treated (control) batch reactors with α -amylase, simulated in biofilm accumulation model (BAM).

Microorganisms are very complicated creatures and can present unpredictable behaviors. Therefore, the percent error calculations of 21 and 27% at 24 and 48 hours are within the acceptable range (personal communication, P. Stewart). In the simulation, a biofilm thickness of 23.14 μm confirmed the calculated film thickness (i.e. 20 μm) with a percent error of 15% at 24 hours (Equation 3.15). This indicates that the estimated biofilm thickness of 20 μm was accurate at 24 hours. The calculated and obtained thickness from lab experiments and BAM was used to check the behavior of model at the end of day 1. This provided a correlation between the experimental data and BAM.

Model Limitations

BAM simulations with batch phase reactors for the biofilm formation of *P. aeruginosa* PAO1 was limited to a couple of parameters. Even though the laboratory experiments, described in Chapter 2, tested multiple agents in multiple combinations, simulation modeling using BAM was limited to only α -amylase. This computer model depends upon several differential equations that are already introduced to the system and cannot be replaced with one another. In the “Reactions Definitions” section of the model the user is prompted to select the type of rate law for both growth and treatment compartments. In this case, reaction kinetics (i.e. Monod) is considered with kinetics parameters of agents (e.g. rate coefficient). Since α -amylase is an enzyme and has been used in many different studies (Craigen et al. 2011; Ashraf et al. 2014; Fleming et al. 2017), the correct parameters for the kinetics of α -amylase were already published and available (Hernández-Heredia and del Moral 2016). On the other hand, Furanone C-30 and Patulin, the quorum sensing inhibitor agents that were tested for prevention of PAO1 biofilm formation in laboratory experiments, did not have kinetics data available. Synthetic furanones (Hentzer et al. 2003) and Patulin (Hentzer et al. 2003; Rasmussen et al. 2005) are known to be quorum sensing inhibitors, homoserine lactone (HSL) inhibitors and do not undergo enzymatic reactions. Quorum sensing in gram-negative bacteria (e.g. *P. aeruginosa*) occurs through acyl-homoserine lactones (AHLs) and these lactone molecules are secreted as their signal molecules (Li and Tian 2016). The behavior of action of these agents was explained in detail in Chapter 2.

Another limitation of simulating CDC biofilm batch reactors in BAM was to estimate an initial biofilm thickness for the system. In real experiments, batch reactors of

constant volume with equal amount of cell density ($OD_{600} = 0.03$) were run with no initial biofilm inside the reactors or on the biofilm growth surface, coupons. Treatments were introduced to the growth media with planktonic cells at the same time, aiming to prevent or manipulate the formation of biofilm structures. However, the simulation studied in this chapter had to introduce an initial biofilm thickness of $0.015\ \mu\text{m}$ to the system to avoid convergence issues of the model. If the simulation was set with an initial biofilm thickness of $0\ \mu\text{m}$, the computer model would not be converged and calculated in BAM due to the running differential equations in the background of the simulation.

Conclusion

This simulation studied the effect of a single amendment treatment of α -amylase on *P. aeruginosa* PAO1 biofilms in batch reactors for 24 and 48 hours. This model assumed that the reactors were running at a constant volume, were well mixed, and the initial film thickness was very close to zero, $0.015\ \mu\text{m}$, due to model limitations. In the absence of α -amylase, the biofilm thickness was found to be 23.11 and $31.37\ \mu\text{m}$ at 24 and 48 hours respectively; while the biofilm thickness was found to be 10.47 and $13.07\ \mu\text{m}$ at 24 and 48 hours respectively, in the presence of α -amylase. When simulation results were compared to the laboratory experimental data, percent error was calculated to be 21 and 27% at 24 and 48 hours respectively.

To conclude, a single treatment of α -amylase at a constant concentration of 3 Units mL^{-1} reduced *P. aeruginosa* PAO1 biofilm formation after both 24 and 48 hours in batch phase CDC biofilm reactors. A computer simulation run in BAM confirmed that

the biofilm thickness was reduced by 55 and 58% at 24 and 48 hours respectively; while the lab experimental results showed a CFU biofilm reduction of 70 and 81% at 24 and 48 hours respectively (Figure 2.3, Chapter 2).

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CHAPTER FOUR

PROJECT SUMMARY

The main focus of my thesis was to develop a control strategy for manipulating and reducing the formation of biofilms in MWF circulation systems by quenching the quorum sensing (communication) mechanism of bacterial cells. In other words, as described in detail in Chapter 2, we used commercially available quorum sensing inhibitor compounds to test water soluble MWFs for prevention of *Pseudomonas aeruginosa* PAO1 biofilm formation within these fluids. Knowing that the application of quorum sensing inhibitors have an important impact on biofilm formation in gram-negative bacteria in tryptic soy broth (TSB), we adopted the current literature upon quorum sensing mechanisms and quorum sensing inhibition techniques and applied it to solve an existing, fundamental problem in water soluble nutrient rich MWFs to combat contamination issues of machining industry.

In this study, two commercially available quorum sensing inhibitors (i.e. Patulin and Furanone C-30) were tested as single compound amendments and in combination with a commercially available enzyme α -amylase from *Bacillus amyloliquefaciens* on biofilm manipulation and reduction in water soluble MWF, using the model microorganism *Pseudomonas aeruginosa* PAO1. In Chapter 2, microbial communities in MWF systems were determined based upon collected planktonic and biofilm samples from two local machine shops in Bozeman, MT by DNA extraction, amplification, and sequencing analyses. Various natural and commercially available chemical compounds

were screened for compound applicability in micro-titter plates before testing selected compounds, that demonstrated highest percentage in biofilm reduction, in CDC biofilm reactors. Further experiments were conducted in multi-well plates with selected compounds (i.e. Patulin, Furanone C-30, and α -amylase) in 7% (final conc.) water soluble MWF, following quantitative and statistical analysis.

All samples, collected from two local shops, tested positive for microbial contamination and were dominated by *Psuedomonadales* (60.2-99.7%) at Shop 1 and *Pseudomonadales* (74 $\pm$ 9%) and *Xanthomonadales* (23 $\pm$ 9%) at Shop 2; which lead us to use the model microorganism *Pseudomonas aeruginosa* PAO1 in this study. Sample collection also concluded that the MWF samples from newly recharged circulation system contaminated within the first day and tested positive for *Psuedomonadales*. Screening for compound applicability provided that Patulin (40 μ M), Furanone C-30 (75 μ M), and α -amylase (3 Units mL⁻¹) showed $\geq$ 50-80% effectivity in CFU biofilm reduction compared to an untreated control over a period of 48 hours. Application of Furanone C-30 and α -amylase significantly reduced the biofilm formation in 7% water soluble MWF after both 24 and 48 hours. For both time points investigated, Furanone C-30 in combination with α -amylase caused $\sim$ 95% in CFU biofilm reduction. Additionally, single compound amendments of Furanone C-30 and Patulin resulted an initial treatment effects after 24 hours and demonstrated an increase in biofilm after 48 hours, while no effect on biofilm reduction was detected with α -amylase after 48 hours. These results concluded that commercially available quorum sensing inhibitors could be used in MWF circulation systems in order to prevent and decrease the formation of biofilm

contaminates. Moreover, visualized effects of each treatment by epi-fluorescent microscopy supported the work done on biofilm reduction. The combined treatment of Furanone C-30 and α -amylase was visually and quantitatively the most effective treatment followed by the combination of Patulin and α -amylase, clearly showing biofilm manipulation while control (untreated) coupon showing a dense network of biofilm.

Metabolites from untreated and treated samples were extracted for further analysis and screened using Hydrophilic Interaction Chromatography (HILIC) to detect the absence or presence of HSLs. Identified m/z values of produced HSLs by *P. aeruginosa* PAO1 were filtered from liquid chromatography-mass spectrometry (LC-MS) data. Screening for HSLs in treated samples with Furanone C-30 and Patulin indicated lower expressions of C₄-HSL, 3-oxo-C₁₀-HSL, and 3-oxo-C₁₂-HSL compared to the untreated control in TSB. Furanone C-30 in MWF expressed almost none of the major HSLs found in *P. aeruginosa* PAO1 concluding a successful treatment of quenching the quorum sensing.

In Chapter 3, lab studies were applied to a computer simulation biofilm accumulation model (BAM) that was developed in 1991 by researchers at the Center for Biofilm Engineering (Bozeman, MT). This model studied the behavior of *P. aeruginosa* PAO1 inside untreated and treated (i.e. α -amylase of 3 Units mL⁻¹) CDC biofilm batch reactors for 24 and 48 hours. Growth phases of *P. aeruginosa* PAO1 was analyzed in batch culture and generation time with growth rate values were calculated. Subsequently, these parameters were used to model the biofilm batch reactors in BAM with the effect of a single agent treatment of α -amylase at a concentration of 3 Units mL⁻¹. In the lab

studies, when no treatment was applied to the reactors a cell count was found to be 4.00×10^7 CFU per growth surface (coupon) at 24 hours. Enumerated CFU was converted to an estimation of biofilm thickness at 24 hours and yielded a thickness of approximately 20 μm per coupon while the thickness of biofilm was found to be 23.14 μm in BAM with a calculated percent error of 15% at 24 hours. Lab experiments showed that the application of single amendment of α -amylase yielded a 60% reduction in biofilm CFU after 24 hours. A biofilm thickness of 23.14 and 31.07 μm was obtained with the absence of α -amylase, while the presence of α -amylase resulted a biofilm thickness of 10.47 and 16.07 μm over a period of 24 and 48 hours respectively. When compared, yields concluded a 55 and 58% reduction in film thickness at 24 and 48 hours respectively as discussed in detail in Chapter 3. When model yields compared to lab studies, an error of 21 and 27% was calculated due to complicated and unpredictable behaviors of microorganisms. This model was limited to several parameters and was adjusted in its best way by applying assumptions as described in Chapter 3.

My contribution to existing control strategies for MWF circulation systems was to investigate the effectiveness of commercially available quorum sensing inhibitor compounds in MWFs and to show the possibilities of reducing the contamination issues within the industrial, closed work environments by the usage of these agents. My results may impact the post-cleaning procedures by reducing the number of contaminants within the MWF circulation systems and application surfaces, manipulate and decrease the amount of biofilm produced by bacterial cells, increase the lifetime of cutting fluids while simultaneously increasing the quality of final products (e.g. less or no contaminants on

the final product), and most importantly, may decrease the environmental pollution by reducing the amount of waste (i.e. used MWF) within the production plants. Increase in lifetime of MWFs could result in a lower number of cleaning and recharging procedures per circulation system.

Future Work

This thesis has been primarily focused on quenching the quorum sensing mechanism of bacterial cells by applying commercially available quorum sensing inhibitors in MWF circulation systems. Future work with quorum sensing inhibitor agents, introduced in Chapter 2, should be tested for larger volumes of experimental samples with *P. aeruginosa* PAO1 biofilms in same (300 mL) or increased volumes of CDC biofilm reactors with single or mixed cultures species (e.g. *Pseudomonadales* and *Xanthomonadales*) to investigate the effect of Patulin and Furanone C-30 inside mixed culture biofilms. Also, a combination between quorum sensing inhibitors and biocides is recommended. Further analyses of detailed N-acyl-homoserine lactone detection, additional to the work performed in Chapter 2, such as chemical analyses, characterization, quantification, and concentration measurements in untreated and treated samples with tandem mass spectrometry (LC-MS/MS) are suggested. An alternative approach could be performed by thin layer chromatography following by MS analyses with presence of HSL reporter strains. *Gfp*-based AHL sensor systems could also be used for the detection of communication between the individual cells present in mixed communities. In addition to listed suggestions above, detailed studies of bacterial gene

expressions could be provided by testing production of HSLs in knockout mutants of *P. aeruginosa* PAO1 species in MWFs.

Thereafter, this study should be tested in our full-scale CNC mini mill model in the Foreman Lab. Initially, trials in CNC mini mill system should be run with single species cultures of biofilms in MWF. Temperature, pH, and oxygen concentration throughout the experiments could be monitored through sensors installed in the CNC mini mill system. Samples should be collected from submerged coupon holders (i.e. stainless-steel coupons), so that the collected data could be compared to CNC biofilm batch reactors. Mist and biofilm on shavings should be collected and tested for further analyses, following the methodology in Chapter 2, over a period of designated process times. Once the inhibition of quorum sensing mechanism is confirmed by detailed analyses of HSL detection in CNC mini mill (lab scale) model, this study should be brought to the field for further investigations at different machining shops.

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