1-1-2011

Pure-culture and mixed community biofilm responses to carbon-starvation and UV-C exposure /

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PURE-CULTURE AND MIXED COMMUNITY BIOFILM RESPONSES TO CARBON-STARVATION AND UV-C EXPOSURE

by

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BSc. (Hons) in Environmental Sciences

(University of Lethbridge, 2009)

A thesis

presented to Ryerson University

in partial fulfillment of the

requirements for the degree of

Master of Applied Science

in the program of

Environmental Applied Science and Management

Toronto, Ontario, Canada, 2011

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Abstract

PURE-CULTURE AND MIXED COMMUNITY BIOFILM RESPONSES TO CARBON-STARVATION AND UV-C EXPOSURE

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Master of Applied Science,
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2011

Biofilms are known to contribute to disease through inherent protective mechanisms and propagation strategies. These multi-cellular systems also play essential roles in numerous environmental processes. The current study investigated the responses of a mixed community biofilm to carbon-starvation, and measured the effects of UV-C on pure-culture biofilms at different stages of maturity by monitoring metabolic and cell yield responses. Carbon dioxide production and biofilm-derived planktonic cell yield were used as the measurement parameters. The mixed community rapidly responded to induced carbon-starvation under continuous flow conditions by remaining metabolically inactive throughout the 96 and 120 h starvation periods, only to promptly return to a metabolically active state upon the reintroduction of carbon. The effects of UV-C on pure-culture biofilms was negligible, with no log activation being achieved, and metabolic activity remaining static.

Acknowledgements

I would like to express my sincere gratitude to my supervisor Dr. Gideon Wolfaardt for his support in my academic endeavors and professional development.

I would like to acknowledge Drs. Elanna Bester, Otini Kroukamp and William Yeung for their support and feedback throughout this research. Also to Alexandru Dumitrache and to Romeo Dumitrache for their technical assistance with microscopy and carbon dioxide analysis.

Further, to my family and friends who have supported me from China to Texas, and all places in between – my sincerest gratitude and appreciation for you all. Of particular note are the contributions provided by Isabelle Netto, Julio Rodriguez, Andrew Sousa and Tobin Verbeke.

This research was supported by NSERC, CRC, Bioshield Canada/Advanced Food and Material Network, and Ryerson University.

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Units, Abbreviations and Acronyms

µ	micro
µmax	maximum specific growth rate
CLSM	confocal scanning laser microscopy
CFU	colony forming unit
cm	centimetre
CMR	carbon dioxide monitoring reactor
$C0_2$	carbon dioxide
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DWDS	drinking water distribution system
EPS	extracellular polymeric substance
g	gram
GFP	green fluorescent protein
h	hour
J	joule
L	litre
ln	natural logarithm
m	milli
M	molar
mm	millimetre
min	minute
MSM	minimal salts medium
n	nano
OD	optical density
PCR	polymerase chain reaction
PPM	parts per million
rRNA	ribosomal ribonucleic acid
RNA	ribonucleic acid
RPM	revolutions per minute
s	second
TSB	Tryptic soy broth
UV	ultraviolet
UV-A	ultraviolet range from 315 to 400 nm
UV-B	ultraviolet range from 280 to 315 nm
UV-C	ultraviolet range from 200 to 280 nm
USEPA	United States Environmental Protection Agency
W	watt
WHO	World Health Organization

Chapter 1: Introduction

1.1 Background

Biofilms are generically defined as naturally-occurring heterogeneous microbial communities that adhere to biotic and abiotic surfaces in a wide variety of environments by the secretion of a protective chemical matrix that allows the community to better withstand natural or induced environmental pressures (Costerton *et al.*, 1999; Donlan, 2002). It has been estimated that 80% of the global microbial biomass exists in biofilms (Richards & Melander, 2009a) demonstrating that it is advantageous for bacterial survival to form biofilms. Examples of surfaces where biofilms are found include, but are not limited to human teeth, rocks submerged in rivers (Costerton *et al.*, 1978), in-dwelling medical devices (Costerton *et al.*, 2003), drinking water distribution systems (DWDS) (LeChevallier *et al.*, 1987) as well as both natural and synthetic materials above or below ground (Sauer *et al.*, 2007). The self-synthesized chemical matrix is composed of extracellular polymeric substances (EPS) which are primarily comprised of proteins and polysaccharides, but also includes nucleic acids, lipids and phospholipids (Simões *et al.*, 2010). The EPS also serves as a protective barrier or shield against antimicrobials while allowing the biofilm to mature within a semi-enclosed environment (Sauer *et al.*, 2007).

It is recognized that biofilms allow bacteria to survive the action of various antimicrobials and / or stressors including antibiotics, desiccation, and ultraviolet (UV) irradiation (Gaddy & Actis, 2009), affording resident microorganisms up to 1500 times more resistance to antimicrobials than their planktonic counterparts. Further, the structural organization within a multi-species biofilm permits the mineralization of a wider range of substrates, which the individual members may not be capable of utilizing as discrete entities (Wolfaardt *et al.*, 1994). Biofilms are comprised of both sessile and planktonic cells that have been proven to possess profoundly different phenotypes (Costerton *et al.*, 2003) and metabolic activity (Richards & Melander, 2009a). Sessile cells are those attached to a surface whereas planktonic cells are free-floating in the surrounding environment. The term biofilm encompasses single-species, multi-species or multi-kingdom communities, all of which are heterogeneous because of the presence of more than one species or as a result of different gene expression amongst the same species (Jefferson, 2004; Sauer *et al.*, 2007). Further, according to Donlan (2002), each microbial community or biofilm is considered unique.

Pathogens may exist within the biofilm matrix where they are protected from the effects of antimicrobials. Antimicrobials often fail to reach these protected pathogens whereby propagation and subsequent infection become real possibilities. With antimicrobial resistance being a characteristic of biofilms, a judicious selection of an antimicrobial regime is necessary to reduce biofilm growth. As such, the aim of this study was to investigate the fundamental responses of an environmentally-relevant mixed community biofilm to dual stressors with the ultimate goal of deterring biofilm growth.

Due to the pervasive nature of biofilms and the negative impact imposed on public health and industry, biofilms have been the impetus driving many research initiatives throughout the past three decades. The intent has been to elucidate biofilm characteristics and mechanisms in order to ultimately control their growth and proliferation in undesirable locations. Challenges associated with biofilm persistence in certain environments such as hospitals, clinics, food preparation sites, drinking water distribution systems, ecological and agricultural environments give rise to economic, environmental and public health ramifications (Donlan, 2002).

For the current study a mixed community biofilm isolated from a high traffic washroom sink-drain was examined for its metabolic and cell yield responses to carbon- starvation, in addition to the responses of pure-culture biofilms to UV exposure. Although the scope of this study aimed to contribute to the understanding of the effect of nutrient deprivation and antimicrobial treatment on biofilms as strategies to control their formation in undesirable locations, it is important to acknowledge that biofilms are vital to life on earth. Biofilms form the base of the aquatic food-web and are active participants in biogeochemical cycling (Allan & Castillo, 2007). Additionally, biofilms have an integral role in biosand filtration which allows point of use water treatment in locations not served by piped, treated drinking water (Kubare & Haarhoff, 2010). Furthermore, several biofilm-forming microorganisms have been used to degrade petroleum in soil caused by spills, and have been successfully employed as bio-fungicides on crops (Morikawa, 2006). As asserted by Richards & Melander (2009a) biofilms surely symbolize evolutionary advancement against extreme environmental stresses. To date no study has examined the response of an environmentally-relevant mixed community biofilm to nutrient starvation followed by UV irradiation.

1.2 Study Objectives and Hypothesis

The metabolic, architectural, and planktonic cell yield responses of pure-culture biofilms to carbon deplete and replete conditions were presented by Bester *et al.* (2011). A principle finding from their study was that biofilm-derived planktonic cell yield is a means of microbial propagation; this knowledge in part served as platform of the current study which applied this response measurement to pure-culture biofilm and environmentally-relevant mixed community biofilms that experienced carbon-starvation through the removal and reintroduction of carbon into the media, and to pure-culture biofilms that underwent carbon-starvation and UV exposure. It was hypothesized that under laminar flow, the mixed community biofilm constructed from a sink-drain sample, when subjected to carbon-starvation would exhibit reductions in metabolism and biofilm-derived planktonic cell yield as measured by carbon dioxide production and effluent counts, respectively. The *Pseudomonas aeruginosa* PA01 gfp, pure-culture biofilms were expected to be completely inactivated by UV, as measured by the same parameters.

In order to achieve the overall aim in this study, several objectives were devised:

- Characterization of an environmentally-relevant mixed community using molecular techniques.
- Determination of mixed community biofilm responses to carbon-starvation by measuring metabolic and cell yield responses.
- Evaluation of the effect of UV-C to colonies, planktonic cells, pure–culture biofilms using bench-top and continuous flow system experiments.

Chapter 2: Literature Review

2.1 Biofilm Formation and Attachment

Attachment of cells to a surface is deemed critical in biofilm formation as the interaction between the surface and adherent bacterial cells establishes the foundation for subsequent biofilm-associated cells (Stoodley *et al.*, 2002; Palmer *et al.*, 2007). A commonly accepted 5-stage theory of biofilm development was presented by Stoodley *et al.* (2002) that has been well studied and confirmed by confocal scanning laser microscopy (CSLM).

The first stage is referred to as reversible attachment. In this stage, cells come into contact with a surface and begin to exude EPS. Some cells are still capable of pilus-mediated twitching or gliding, hence being referred to as reversible attachment. Of note is that during the initial stage Brownian motion is in effect whereby the microorganisms can still be easily removed by fluid shear forces, such as rinsing (Palmer *et al.*, 2007). The second stage is referred to as irreversible attachment due to the increase in the number of cells resulting in elevated amounts of EPS being secreted causing the cells to adhere to the surface; upon completion of the second, irreversible step, microorganisms are substantially more difficult to remove from the surface requiring vigorous removal actions such as scrubbing or chemical cleaners. The third stage depicts a change in the overall morphology and topography of the biofilm whereby it becomes a three dimensional (3D) structure. The distinct shape of the biofilm results in increased surface area to maximize nutrient absorption and waste expulsion. Upon maturation, hollow cavities can be seen throughout the biofilm which act as the necessary water, nutrient and waste transport system within the microbial community. The fourth stage is characterized by a continued increase in biofilm complexity coupled with preparation for the release of biofilm cells. Finally the fifth stage results in the detachment of cells from the biofilm. These detached cells are referred to as planktonic revertants, which then move to different locations, ultimately attaching to a suitable surface and repeating the cycle (Richards & Melander, 2009a; Simões *et al.*, 2010). As biofilms house the majority of the world's microbiological communities (Richards & Melander, 2009a) it is easy to understand how bacteria are so successful and difficult to control. The model Stoodley *et al.* (2002) presented is essentially a linear process. Conversely by monitoring cell yield in effluent from biofilms it has been shown that detachment and release

of planktonic revertants is a continuous process throughout biofilm development (Bester *et al.*, 2009).

2.1.1 Factors Affecting Attachment and Biofilm Formation

The initial attachment of planktonic cells to surfaces is dependent on a number of factors pertinent to the adhesion surface, bulk fluid and the cells themselves (Donlan, 2002; Simões *et al.*, 2010). Some of the factors identified by Donlan (2002) and Simões *et al.* (2010) were surface texture, hydrophobicity, conditioning films, charge of the surface and cell, cellular appendages, the EPS, cell-signalling, and gene expression.

As a rule, attachment of microorganisms to surfaces more readily occurs when the surface is rougher, more hydrophobic and coated by surface conditioning films (LeChevallier *et al.*, 1987; Donlan, 2002; Teixeira *et al.*, 2005; Verran & Whitehead, 2005). Conditioning films occur naturally on surfaces as a result of the deposition of organic molecules or polymers from the surrounding environment, whereupon microbial attachment is encouraged, ensued by biofilm formation (Donlan, 2002). Conditioning films derived from humans originate from blood, tears, saliva, or respiratory secretions.

Most bacterial cell surfaces have a net negative charge which keeps them at a short distance from surfaces as a result of electrostatic repulsive forces (Palmer *et al.*, 2007). This can be overcome by hydrophobicity between the cell and attachment surfaces and by extracellular appendages, namely fimbriae or pili, and flagella (Shi & Zhu, 2009; Simões *et al.*, 2010). Of note is that not all cells have fimbriae or flagella. Recall that in the first stages of attachment some cells are still capable of pilus-mediated movement, providing further evidence of the involvement of cellular appendages in the initial stages of attachment.

The role of the EPS in biofilm attachment is integral to the success of bacterial surface colonization in natural environments (Costerton *et al.*, 1978). Attachment of microorganisms to a surface stimulates the synthesis of the EPS (Brooks & Flint, 2008). The EPS facilitates the adhesion and cohesion of the biofilm by binding cells and other particulate materials together as well as binding the cells to the surface (Simões *et al.*, 2010). Of note, is that the EPS is negatively charged which certainly contributes to the antimicrobial resistance of biofilms,

whereby positively charged antimicrobials are bound by the EPS rarely reaching the biofilm core (Lewis, 2007).

Quorum sensing (otherwise known as cell-to-cell signalling or bacterial communication) has been identified as an important factor in biofilm formation as well as throughout the biofilm lifecycle (Williams *et al.*, 2007). It is generally described as the phenomenon whereby bacteria communicate amongst themselves by the release of signal molecules into the EPS resulting in a change in bacterial behaviour. As the biofilm grows, increased numbers of signalling molecules are released into the surrounding EPS resulting in an accumulation of these molecules whereby the bacteria are alerted to their increased population density which in turn, elicits a response (Stanley & Lazazzera, 2004; Williams *et al.*, 2007). As suggested by Telgmann *et al.* (2004) the accumulation of signalling molecules is likely a trigger for cell detachment from the biofilm.

2.2 Biofilm Detachment

Similar to biofilm attachment processes, detachment of biofilms (i.e. stage 5) is affected by various physical, chemical and biological factors. Some factors are the presence of matrix-degradation enzymes, gas bubbles produced inherently by microbes, nutrient levels, shear stress, quorum sensing molecules, degradation of the EPS and carbon-starvation (Hunt *et al.*, 2004; McLandsborough *et al.*, 2006; Bester *et al.*, 2009). Of note is that examining biofilm detachment mechanisms and processes is still in the early stages when compared to studies of biofilm attachment and formation (Garny *et al.*, 2008). To this point, the current study strives to augment the knowledge gained by Bester *et al.* (2011) who examined a pure-culture biofilm's detachment response to carbon-starvation, by investigating detachment responses of a mixed community biofilm to carbon-starvation. Garny *et al.* (2008) described four methods of biofilm detachment that may occur simultaneously: erosion, abrasion, sloughing and predator grazing. Erosion results from fluid shear force and abrasion is the collision of particles; both processes refer to the continuous detachment of cells from the overall surface of the biofilm. Sloughing, on the other hand, is the immediate loss of large aggregates which affects the entire biofilm, potentially resulting in entire biofilm loss. Lastly, predator grazing is eukaryotes grazing on the biofilm rendering it weakened or disrupted. Telgmann *et al.* (2004) differentiated erosion from sloughing according to particle size whereby smaller particles are defined to have undergone erosion, and larger pieces of biomass - sloughing. Further, both erosion and sloughing contribute to biofilm

detachment with erosion having a greater influence on overall removal, and sloughing having a greater influence on biofilm morphology (Telgmann *et al.*, 2004).

Contrary to the previously accepted notion of biofilm cell detachment occurring once the biofilm reached maturation, a recent study by Bester *et al.* (2009) found that cells detached throughout the biofilm maturation process, with cells being shown to detach within 6 h of surface attachment by a pure-culture *Pseudomonas sp*. biofilm. This release of cells from the maturing biofilm is termed planktonic cell yield (Bester *et al.*, 2011). Undoubtedly, this will shift the focus of studies examining detachment of biofilm cells and subsequent re-colonization elsewhere. The fact that biofilm cell detachment occurs throughout the biofilm maturation process serves as a basis for continued work by Bester and is a measurement parameter in the current study. By monitoring planktonic cell yield using effluent counts it can be determined when the biofilm reaches a semi steady-state, indicating that the biofilm is starting to mature. Steady-state information was to be used as the baseline in the current study to signal when starve the biofilm by removing carbon from the media.

2.3 Biofilms in Aquatic Environments

As noted by Costerton *et al.* (1978) in early biofilm literature, foundational biofilm experiments were carried out in lotic and lentic environments with further exploratory studies originating within the dental industry. In lotic systems, it was determined that in order to survive in dynamic environmental conditions, microbes converged into aggregates whereupon a network of polysaccharides is secreted to afford the community protection, ensure its adherence and provide structural support. Because of the origin of these experiments, laboratory studies have often strived to mimic these solid-liquid interfaces in order to gain further knowledge on fundamental biofilm properties. As such, much of the biofilm information available in the literature has been put forward from studies at solid-liquid interfaces. The current study adheres to this tendency with the intention of future application of the knowledge gained to solid-air interface models.

When considering biofilms in aquatic environments, especially in lentic systems, it is important to recognize that lentic systems are often classified in terms of nutrient levels i.e. oligotrophic (low nutrients), or eutrophic (excess nutrients). The indigenous microbial populations are certainly affected by the nutrient content, which has been known to alter the

bacterial community profile over time (Christian & Lind, 2007). Of interest to the current study was biofilm response to nutrient starvation. Costerton (1995) asserted that bacteria in oligotrophic environments coalesce into biofilms in order to localize any available nutrients, in addition to their seeming capability of entering quiescence until such time that nutrients once again become available.

2.3.1 Drinking Water Distribution Systems (DWDS)

DWDS encompass a large network of infrastructure, with treatment facilities delivering safe, clean drinking water to the public via an extensive piping network (Bauman *et al.*, 2009). DWDS are colonized by microorganisms which are ubiquitous in water environments and do not pose a public health threat or compromise DWDS integrity when in a planktonic state. However, when these microorganisms are associated with biofilms, problems arise (Bauman *et al.*, 2009). Biofilms in DWDS have long been recognized as problematic in two main ways: biofouling (Flemming, 2002) and microbial contamination of drinking water (LeChevallier *et al.*, 1987; Flemming, 1993; Bauman *et al.*, 2009). In order to counteract the adverse effects of biofilms in DWDS control measures are implemented at several stages of water treatment and distribution. However, one must simultaneously consider the effects of such controls from both a public health and DWDS engineering integrity stance. Both sides aim to remove the attached masses, but dispersal of the biofilm as desired from an engineering standpoint can result in a public health risk, as pathogens trapped within the biofilm are released into the water supply, making biofilm control within these environments complicated (Bauman *et al.*, 2009).

The term 'biofouling' was first introduced by Epstein in 1981, as a specification of the term 'fouling.' Biofouling in general describes the unwanted growth of biofilms on surfaces (Flemming, 2002). Flemming (2002) asserted that biofouling is the direct manifestation of biofilms in water systems. Two distinct environmental problems resulting from biofouling are the need for increased energy consumption to overcome the reduced flow rates in narrowed water transportation pipelines, and the need to alter wastewater treatment regimes to counteract the adverse effects of increased toxic biocide application to control biofilms (Flemming, 1993). Further substantiation of the problems caused by biofouling is presented by Cloete *et al.* (1992) in their extensive review of topics related to biofouling in water systems; biofouling is moreover

further reduced to core issues, namely the physical obstructions in DWDS and microbial induced corrosion (MIC).

Given that biofilms are multi-layered growth forms characterized by three-dimensional structure (Stoodley *et al.*, 2002), it is logical that biofilms could result in physical obstructions by the narrowing of pipelines. This phenomenon occurs regardless of the microbial consortia within the biofilm, and has been detected throughout DWDS (LeChevallier *et al.*, 1987). In effect the decreased diameter of the pipelines increases the friction resistance while consuming the kinetic energy of water, quite simply leading to increased energy demand which is naturally coupled with increased cost (Flemming, 1993). Briefly, MIC occurs because the biofilm creates a specialized microenvironment with the necessary parameters for the electrochemical reactions to occur. MIC results in compromised DWDS structural integrity and causes acidification of the surrounding environment (Cloete *et al.*, 1992). Organisms associated with MIC include sulphate-reducing bacteria, iron - and manganese-oxidizing bacteria and acid forming microorganisms (Cloete *et al.*, 1992; Flemming, 1993). Of note is that none of aforementioned MIC-associated microbes have been selected for the present study because of their specific substrate requirements.

Microbial contamination of water may aesthetically degrade water quality rendering it unpleasant to drink or smell, or it may cause injurious health effects (Block, 1992). Several disruptions, including changes in nutrient concentration and shear force inherent within DWDS were proposed to cause water contamination by releasing cells from the biofilms (Flemming, 2002). Block (1992) also named shear force as a mechanism by which water quality is degraded within DWDS. Microbial contamination becomes a health concern when the released microorganisms are pathogens or opportunistic pathogens. In order to prevent disease or in more extreme instances death, DWDS have a continuous level of disinfectant or biocide present throughout the system (Block, 1992).

Achieving disinfection in DWDS is complicated due to the numerous microenvironments that arise from the heterogeneous system, in addition to the microbial diversity existing within biofilms in these environments (Block, 1992; Simões *et al.*, 2010). Some common bacterial genera in DWDS are *Bacillus, Enterobacter, Klebsiella, Pseudomonas, Staphylococcus, Sphingomonas* and *Acinetobacter (*Simões *et al.*, 2010).Regardless of the inherent complications,

ensuring proper disinfection is imperative to safeguarding public health and safety. Disinfection using chlorine in DWDS was implemented at the turn of the twentieth century and unequivocally remains the most widely used disinfectant in DWDS (Bachmann & Edyvean, 2005). The purpose of disinfection is to reduce the total number of bacteria in bulk water (Bachmann & Edyvean, 2005); however, this does not adequately address the need to control biofilms in DWDS which house an estimated 95% of microbial biomass in biofilms, predominantly attached to pipe surfaces (Simões *et al.*, 2010). Again, what must be taken into account when considering biocide application is the shield against antimicrobials that the biofilm affords to its resident opportunistic pathogens. As such, a variety of biocides with varying mechanisms, target specificity, and dosage requirements are needed to ensure adequate disinfection and compliance with water quality guidelines or standards (Cloete *et al.*, 1992). Of note is that certain biocides are known to cause toxic by-products that result in deleterious effects in aquatic ecosystems and may result in the formation of carcinogenic compounds (Harris *et al.*, 1987; Cloete *et al.*, 1992).

Within this vast infrastructure network there are biofilms present at both solid-liquid and solid-air interfaces. Further, a dynamic environment of wet and dry periods, nutrient deprivation, and disinfection exist, promoting diverse bacterial colonization. To this point, it is important to consider that the terminus of DWDS is sink-drains - which support biofilms rich in diversity and density. These outlets are subject to all the aforementioned conditions; therefore, a community modelled after a sink-drain was used in the current study knowing that its members are able to survive at solid-liquid and solid-air interfaces, under highly variable conditions.

2.4 Biofilms at Solid-Air interfaces

Biofilms at solid-air interfaces constitutes a broad range of potential surfaces in hospital, clinical and food preparation settings. Some examples of these surfaces include floors, sinks, counter/table tops, curtains, beds, medical devices and food processing equipment. Several studies have been carried out in these various environments to characterize and enumerate these biofilms, often with the aim of determining pathogen load in order to relate the biofilms to nosocomial and other infections and / or food spoilage (Roberts *et al.*, 2008; Zorman & Jeršek, 2008). Understandably, there are several parameters affecting the growth and presence of biofilms in these settings including the ventilation system, cleaning methods, location of the ward i.e. intensive care units (ICU) versus surgery ward and age of the buildings, equipment and

construction materials. Biofilms at solid-air interfaces also dominate in the natural environment, such as those found on rocks or soil particles.

2.4.1 Hospital and Clinical Settings

In the attempt to identify pathogen reservoirs in settings housing known immuno-compromised individuals, exhaustive inventories of possible pathogen attachment sites are carried out. The comparison of surface building materials for their bacterial attachment affinity was investigated, showing that vinyl flooring is superior to ceramic tile with regards to reducing bacterial colonization, and stainless steel was proven to be the best materials for counter/bench tops (Yazgi *et al.*, 2009).

When examining a newly added hospital ward to an existing structure it was proven that the changes and establishment of bacteria are closely associated with human presence (Klánová & Hollerová, 2003; Narui *et al.*, 2009). By carrying out an overall bacterial surveillance of both structures (new and old), it was determined that isolates taken from floors, sinks and air changed with the transfer of patients and staff, implying that the movement of people within these environments directly affected the resident bacterial populations in health care facilities.

Of note is that biofilms at solid-air interfaces can also include the growth of biofilms on medical equipment. As asserted by Richards & Melander (2009b) infections caused by biofilms growing on in-dwelling medical devices (IMD) pose serious health and financial consequences to the patients and health care industry. Essentially once a biofilm has colonized an IMD the only treatment is its removal; this is both costly and only a short-term solution as another IMD must be inserted. As a result, there has been much research into coating the IMDs with antimicrobial agents to prevent biofilm attachment, again possibly only a short-term solution as the microorganisms may adapt and develop resistance to the antimicrobial coating agents (Bak *et al.*, 2009; Richards & Melander, 2009b).

As research in this field advances studies are becoming increasingly specific. For instance, Alfa & Howie (2009) examined build-up biofilms on flexible repeat use endoscopes and determined that the biofilms contain a wide variety of microorganisms despite rigorous cleaning regimes.

2.4.2 Food Processing Settings

Biofilms in the food industry including food preparation, and processing sites are a public health concern as they are known reservoirs for both food-spoilage and pathogenic bacteria (Brooks & Flint, 2008; Shi & Zhu, 2009). Of noted concern to the food industry is that biofilms are becoming increasingly difficult to control and eradicate with current disinfection procedures and technology. This is resulting in much research into new control mechanisms, and control products to ensure compliance with food safety guidelines (Brooks & Flint, 2008).

Several biofilm detection and monitoring methods have been tested in laboratory settings; however, as Brooks & Flint (2008) point out there is a gap between the laboratory testing and the practical application for monitoring biofilms on-site in actual industry settings.

Biofilm formation in the food industry is succinctly explained by Shi & Zhu (2009) as having four basic steps. Organic molecules from a food source are deposited onto the surface resulting in a conditioned surface whereupon microorganisms are attracted to this conditioned surface. Following cleaning and sanitizing, some cells remain that will then initiate growth of the biofilm that is able to expand by gene expression and quorum sensing. Of note is that substratum properties and surrounding environmental factors also affect biofilm formation (Shi & Zhu, 2009). A unique feature of biofilm initiation and formation in the food industry is that often proteins and fats derived from improperly cleaned or sanitized equipment are present on surfaces that provide nutrients and water encouraging biofilm growth (McLandsborough *et al.*, 2006).

Several bacterial pathogens and food spoilage organisms have been identified in the food industry. Interestingly some of these pathogens are the same as pathogens identified in hospital studies; however, some are different. Known bacterial pathogens include members of the genera: *Salmonella, Pseudomonas, Enterobacter, Campylobacter, Escherichia, Staphylococcus, Listeria, Bacillus,* and *Vibrio* (Teixeira *et al.*, 2005; McLandsborough *et al.*, 2006; Brooks & Flint, 2008; Jun *et al.*, 2009; Shi & Zhu, 2009).

There is some conflicting information about preferred biofilm attachment in food industry. Shi & Zhu (2009) reported that more bacterial cells attach to hydrophilic surfaces such as glass and stainless steel, whereas Simões *et al.* (2010) claimed that attachment occurs on

surfaces that are more hydrophobic. Although there is discrepancy in the information, these assertions could be interpreted as being relative statements instead of hard facts.

2.5 Bioaerosols

Bioaerosols are particles of biological origin suspended in the air. Bioaerosols are complex mixtures of live and dead microorganisms, pathogenic and non-pathogenic bacteria, fungi, allergens, pollen, plant fibres and viruses, as well as their respective fragments (Douwes *et al.*, 2003; Zorman & Jeršek, 2008). Of interest is their relationship to the community composition of dry biofilms on solid-air interfaces. Considering that nosocomial infections and food related illness are increasing public health issues, the identification of potential sources of pathogens and mechanisms of pathogen transfer from source to host are imperative to understand. Bioaerosols is one area to be examined and related to the transfer of pathogens from biofilms to host, in addition to determining their sources and reservoirs (Gilbert *et al.*, 2010). Again relating bioaerosols to dry biofilms and disease is an area to be explored in order to gain further understanding into minimizing and controlling various infections which are transmitted in this way. Of note, is that studying this relationship must take into consideration the built environment where it has been proven that ventilation systems transmit pathogen-containing bioaerosols released from individuals, often through coughing or sneezing, to other areas of the building where infection occurs in vulnerable hosts (Beggs, 2003).

Although linking the relationship between bioaerosols and pathogen dissemination are in their infancy, Augustowska & Dutkiewicz's (2006) explained the changes in a hospital ward airborne microbial community over a year by highlighting organisms implicated in nosocomial infections. Roberts *et al.* (2008) demonstrated that an important nosocomial causative agent - *Clostridium difficile* – is indeed spread throughout hospitals by aerial dissemination.

2.6 Biofilm Contribution to Disease

When considering biofilm contribution to disease it is important to recognize the commonly accepted fact that biofilms as a result of their inherent structure can act as reservoirs for pathogens and opportunistic pathogens that can ultimately result in disease and / or infection in a host. Furthermore, biofilms have been implicated as the source of many chronic, persistent infections (Costerton *et al.*, 1999). The increasing frustration of medical personal over the emergence of persistent infections that are immune to antibiotics lead to the important discovery

that the origin of many of these infections are indeed biofilms. The intrinsic structure of biofilms affords its residents immunity to many antibiotics as they are incapable of penetrating the matrix. As a result the antibiotics are effective on planktonic counterparts, but not biofilms themselves – hence the persistent infections (Costerton *et al.*, 2003). Of interest is that many of the culprits in these persistent infections were determined to be common environmental organisms that individuals normally had adequate immunity against. As postulated by Hall-Stoodley & Stoodley (2005) and demonstrated by Bester *et al.* (2011) one mechanism for biofilm contribution to disease is the release or detachment of large numbers of cells which are able to subsequently cause infection elsewhere. Additionally, Hall-Stoodley & Stoodley (2005) mentioned that cells, specifically pathogens, within biofilms are phenotypically heterogeneous whereby the potential exists for the virulent phenotype to survive increasing the opportunity for disease occurrence.

Further, because of the architecture of a biofilm, cells at the core or lower layers are typically more resistant to antimicrobials whether they are antibiotics or microbiocides. Richards & Melander (2009a) state that the failure of antimicrobials to penetrate the biofilm often occurs because they become trapped in the EPS. Understandably, these protective mechanisms against antimicrobials can result in repeat infections as the core pathogen source remains undisturbed (Costerton *et al.*, 1999; Richards & Melander, 2009b).

According to statistics released by the United States of America's National Institute of Health, 65 to 80% of all microbial infections in humans are biofilm-mediated. Known medical issues associated with biofilms include, but are not exclusive to: burn wound infections, ear infections, tooth decay and catheter infections (Richards & Melander, 2009b). Given this information, plus the known astronomical cost of these ailments it is easily understood why there is so much research being carried out to understand all aspects of biofilms.

2.6.1 Nosocomial Infections

Nosocomial infections or hospital-acquired infections are of increasing public health concern because of their magnifying occurrences, and known morbidity, mortality, and economic consequences around the world (Beggs 2003; Gilbert *et al*., 2010). Many bacteria, including *Staphylococcus, Pseudomonas* and *Clostridium,* have been implicated in nosocomial infections. Knowing that the majority of microbial infections are biofilm-related, one can infer that biofilms are also a leading instigator of nosocomial infections. To that point, more than a decade ago it

was estimated that 65% of nosocomial infections were biofilm-associated with a treatment cost of over $1 billion per year (Mah & O'Toole, 2001).

Beggs (2003) notes that nosocomial infections can be spread directly via person-to-person contact, such as from improperly washed hands of hospital staff to patient, or indirectly via a contaminated intermediate object such as endoscope or implant. Recall, that aerial dissemination of *Clostridium* and subsequent infection has been proven (Roberts *et al.*, 2008).

2.6.2 Infectious Diseases

Infectious diseases are caused by pathogenic microorganisms, and can spread directly or indirectly. Examples include: cholera, malaria measles, *C. difficile* and Lyme disease (WHO, 2011). As infectious diseases are caused by pathogens, there is undoubtedly a link between the biofilm mode of growth and infectious diseases. With the global emergence of infectious diseases over the past 20 years, the link between infectious diseases and biofilms is under increasing investigation (Weinstein, 1998) .

2.7 Pathogens and Opportunistic Pathogens

2.7.1 Introduction

Pathogens are disease-causing microorganisms, while opportunistic pathogens are microorganisms that are disease-causing only when the host's resistance is low (Willey, 2011). Costerton *et al.* (1999) claimed that many common environmental organisms which normally cause no adverse effects in individuals become pathogenic when individuals are immuno-compromised i.e. opportunistic pathogens. To this point, many pathogens are deemed opportunistic pathogens in hospitals and clinics where there are a plethora of immuno-comprised patients, with increased vulnerability to the effects of otherwise harmless bacteria. As Hall-Stoodley & Stoodley (2005) asserted, the intrinsic biofilm properties of protection and detachment promote the dissemination of pathogens. As such, it must be considered that biofilms found throughout these environments play an integral role in the transmission of pathogens and / or opportunistic pathogens. The identification and infectious doses of pathogens is of the utmost importance to maintain and promote health integrity. The following list is not exhaustive, but provides a brief overview of common pathogens including their diagnostic characteristics.

2.7.2 Enteric Pathogens- Family: Enterobacteriaceae

Members of the family Enterobacteriaceae are defined as being Gram-negative, rods which are facultative anaerobes, and motile by peritrichous flagella. They do not form endospores or microcysts (*Bergey's manual of systematic bacteriology.* 1984-). Members of this family associated with nosocomial infections include, but are not limited to the genera *Salmonella, Escherichia, Klebsiella* and *Enterobacter.*

Salmonella associated nosocomial infections can originate from infected patients and contaminated foods or devices. Raw eggs are often implicated in *Salmonella* infections, and devices such as gastroscopes and rectal thermometers have been associated with nosocomial outbreaks (Hospital epidemiology and infection control). *Klebsiella* biofilms have been associated with diseases originating from both central venous and urinary catheters (Donlan, 2002). *Enterobacter* as the name implies, are enteric bacteria known to cause nosocomial infections including, but not limited to, urinary tract infections, surgical site infections, bacteremia and nosocomial pneumonia (Chow *et al.,* 1991). *Enterobacter* display a strong affinity for aqueous environments with reservoirs being water, soil, and the human gastrointestinal tract, in addition to proven colonization of drinking water distribution systems, distilled water systems, humidifiers and soft tissues throughout the body (Hospital epidemiology and infection control; Simões *et al.*, 2010)

2.7.3 Non-Enteric Pathogens

2.7.3.1 Genus *Staphylococcus*

Are characterized as being spherical shaped cells that can be arranged singly, in pairs or clustered; Gram-positive, non-motile; facultative anaerobes, although known to grow best under aerobic conditions (*Bergey's manual of systematic bacteriology.* 1984-).

Problems associated with *Staphylococcus sp.* namely, *Staphylococcus aureus* include its resistance to the beta-lactam antibiotic, methicillin. Commonly referred to as methicillin resistant *Staphylococcus aureus* (MRSA), it has been proven to cause nosocomial infections by infecting sites such as damaged skin, and is prevalent in healthcare facilities around the world (Beggs, 2003).

2.7.3.2 Genus *Pseudomonas*

Straight or slightly curved rod shaped cells; Gram-negative aerobes, non-fermenting; motile by 1+ polar flagella (*Bergey's manual of systematic bacteriology.* 1984-). Several pseudonomads have been used as model organisms due to their prevalence in biofilms in natural conditions and the ease of flowcell culturability in laboratory conditions (Heydorn *et al.*, 2000). Pseudomonads are some of the best modelled organisms for studying biofilm formation due to the non-fastidious growth requirements (Richards & Melander, 2009b).

Pseudomonas aeruginosa is ubiquitous in hospitals and water systems (Ayliffe *et al.*, 1974; Lakretz *et al.*, 2010). Costerton (1987) opined that *P. aeruginosa* is the most widely used organism employed in studying biofilm formation, and Sauer *et al.* (2004) asserted that it is the most medically important biofilm-forming species. *P. aeruginosa* is known to cause incurable infections in the lungs of cystic fibrosis patients (Lewis, 2007; Richards & Melander, 2009a) and is one of the four most common Gram-negative nosocomial pathogens (Weinstein, 1998)

In addition to *P. aeruginosa, Pseudomonas fluorescens* and *Pseudomonas putida* are important opportunistic pathogens. *P. fluorescens* commonly encountered in soil and water habitats that has been implicated in nosocomial infections (Baum *et al.*, 2009). *P. putida* is commonly associated with plants (Richards & Melander, 2009b; Kaplan, 2010).

2.7.3.3 Genus *Acinetobacter*

A genetically diverse group of rod shaped cells that become spherical in stationary phase of growth. Members of *Acinetobacter* are Gram-negative, aerobic, non-fermenting bacteria, which have been isolated from soil, sewage and skin (*Bergey's manual of systematic bacteriology.* 1984-; Beggs, 2003; Gaddy & Actis, 2009). *Acinetobacter baumannii* and *Acinetobacter calcoaceticus* are opportunistic pathogens demonstrating multidrug resistance. Both organisms have been implicated as infectious agents of diseases such as pneumonia, urinary tract infections, septicaemia and wound infections (Beggs, 2003; Gaddy & Actis, 2009; Richards & Melander, 2009a).

2.7.3.4 Genus *Clostridium*

Rod shaped cells; usually Gram-negative; obligate anaerobes that form endospores (*Bergey's manual of systematic bacteriology.* 1984-). *Clostridium difficile* has been implicated in nosocomial infections, specifically as the causative agent of nosocomial infectious diarrhoea

(Hospital epidemiology and infection control). It is known that *C. difficile* produces two toxins: an entertoxin and a cytotoxin. The minimum lethal dose (MLD) of the entertoxin in mice was determined to be 1 x 10^{6} MLD/ mL (Fu *et al.*, 2004).

C. difficile is the cause of *Clostridium difficile*-associated diarrhoea (CDAD), "a frequently occurring healthcare-associated infection, which is responsible for significant morbidity and mortality amongst elderly patients in healthcare facilities" (Roberts *et al.*, 2008).

2.7.3.5 Genus *Chryseobacterium*

A six species genus, possessing Gram-negative, bacillus-shaped and non-fermentative diagnostic characteristics, that was recently classified from the parent genus *Flavobacterium* because of proven phylogenetic differences (Lambiase *et al.*, 2007). Reservoirs of *Chryseobacterium* are primarily in soil and water (Kirby *et al.*, 2004; Lambiase *et al.*, 2007; Lin *et al.*, 2010).

Select *Chryseobacterium* species are known opportunistic pathogens shown to cause bacteremia in newborns and immuno-comprised patients (Kirby *et al.*, 2004), in addition to being isolated in conjunction with *P. aeruginosa* from the lungs of cystic fibrosis patients (Lambiase *et al.*, 2007). As Kirby *et al.* (2004) noted, *Chryseobacterium* have a relatively low degree of pathogenicity; however, their prevalence is sufficient enough to warrant their inclusion in a worldwide antimicrobial bacteria surveillance program to record susceptibility to common antibiotics administered in hospitals.

Of importance is that environmental studies have revealed *Chryeobacterium's* capability of surviving in chlorine-treated water distribution systems going on to colonize sink drains, raising particular concern in hospitals (Kirby *et al.*, 2004).

2.7.3.6 Genus *Sphingomonas*

Sphingomonas species are Gram-negative, obligate aerobes with single polar flagellum when motile (Balkwill *et al.*, 1997). Genus members are noted as strong candidates for bioremediation and waste treatment as a result of possessing broad catabolic capabilities including an aptitude at degrading chlorinated biphenyls, toluene, naphthalene, xylene and polyaromatic hydrocarbons (Fredrickson *et al.*, 1995). Further lending *Sphingomonas* to the

aforementioned tasks is the origins in soil, water, sediments and sub-surface geological formations (Fredrickson *et al.*, 1995; Balkwill *et al.*, 1997).

2.7.4 Conclusion

Of interest to the current study was the environmental origins –soil, water, sediments – of many bacterial genera which are now known residents of clinical environments and causative nosocomial infection agents. The link between the environmental origins of many bacteria and medically-relevant and industrial settings is important to consider when entertaining biofilm reduction strategies.

2.7.5 Infectious Doses of Common Pathogens

Understanding the infectious dose of pathogens or their toxins requires consideration that it is a subjective term which is dependent upon the "immunological status of the host and the natural infectivity of the organism" (Bhunia, 2008). Table 1 describes the infectious doses of some common nosocomial infectious agents.

Table 1: Infective doses of common organisms.

Organism	Infective Dose	Source
Salmonella sp.	15-20 cells Dependent on age, health of host and strain May vary from 1 to 10^9 cfu/g. Tests on humans suggest a range of 10^5 to 10^{10} to cause disease	http://www.fda.gov/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/ucm069966.htm;(Bhunia, 2008)
Staphylococcus aureus	Toxin dose of less than 1.0µg in contaminated food produces symptoms on intoxication-toxin level reached when populations exceed 100000/g	http://www.fda.gov/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/ucm070015.htm
Campylobecter jejuni	400-500 bacteria can cause illness in some people, however far greater numbers are needed in other individuals 500-10000 organisms with intensity of attack correlating to dosage	http://www.fda.gov/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/ucm070024.htm; (Bhunia, 2008)
Listeria monocytogenes	<1000 total organisms in susceptible persons 100cfu/g to 1011 cfu/g have been associated with the gastrointestinal form of disease	http://www.fda.gov/Food/FoodSafety/FoodborneIllness/FoodborneIllnessFoodbornePathogensNaturalToxins/BadBugBook/ucm070064.htm;(Bhunia, 2008)
Bacillus cereus	10^5 to 10^8 viable cells or spores per gram to cause intoxication or toxicoinfection	(Bhunia, 2008)
Vibrio cholera	10^4 to 10^{10} cfu/g	(Bhunia, 2008)
Vibrio parahaemolyticus	$2x10^5$ to $3x10^7$ cfu	(Bhunia, 2008)
Clostridium perfringens	10^7 to 10^9 cells Causes infection	(Bhunia, 2008)

2.8 Biofilm Response to Adverse Conditions

2.8.1 Persister Cells

Persister cells are a sub-population of cells, both planktonic and biofilm-associated, that are adept at defying the deleterious effects of antibiotics or microbiocides (Lewis, 2007; Richards & Melander, 2009a). These cells represent an altruistic behavior whereby in the unlikely event that all cells within the population are killed the survivors are able to pass along their genetic information to re-establish the community. In essence, this evolutionary mechanism explains the difficulty in overcoming chronic infections. Lewis (2007) highlighted the obstacles faced by clinical microbiologists in overcoming these persister populations in medical settings (i.e. antibiotic resistance). Although persister cells represent a small percentage of the entire population it is of the utmost importance to consider this adaptation when implementing a biofilm eradication scheme. Lewis (2007) estimated that no more than 1% of the entire biofilm population is comprised of persister cells indicating that the persister state is not the preferred mode of growth.

Although persister cells exist in all the places where biofilms can be found, the eradication of persister cells is possible through the use of antiseptics (Lewis, 2007). Understandably, antiseptics cannot be applied systemically to eradicate biofilms, as they are toxic to all cells whether they are commensal, pathogenic or beneficial. Therefore, multiple-tiered efforts to eradicate biofilms causing chronic infections must be teased out experimentally and determined to not harm the patient. With this in mind, the current study aimed to shed insight into a multiple stage biofilm control method that could most certainly be applied to in-dwelling medical devices before their insertion or upon removal to inhibit biofilm growth or cause death.

2.8.2 Nutrient Starvation

From the onset of presenting the theory of the biofilm mode of growth, the role of nutrient availability was predicted to have a marked effect on the orientation of microorganisms within the biofilm and upon biofilm development itself (Costerton *et al.*, 1978). Several studies suggest that the biofilm mode of growth is the result of a lack of nutrients in an environment, causing planktonic cells to coalesce into biofilms whereby an ecological advantage is achieved through the optimized use of limited resources (Brown *et al.*, 1977; Flemming, 1993). Since

biofilms are naturally-occurring, they are often found in oligotrophic environments, such as aquatic ecosystems (McBain *et al.*, 2003; Simões *et al.*, 2007). As such, it can be inferred that the biofilm allows microorganisms to persevere in oligotrophic conditions. What needs to be considered is that not only do these oligotrophic conditions exist in natural environments, but in fact these low-nutrient conditions are present in medical and food processing facilities, homes, and water distribution networks, to name but a few (Shi & Zhu, 2009; Brooks & Flint, 2008; LeChevallier *et al.*, 1987). These settings as a result of sound hygienic practices and the promotion of public health and welfare are repeatedly being disinfected, sanitized or sterilized. However, the problem of pathogen transmission arises as a result of the biofilm's inherent protective mechanisms that shield microorganisms embedded in the matrix from the intended effects of antimicrobials. As a result, the application of bactericidal agents does not eradicate the biofilm in its entirety, but allows cells (potentially pathogens) to remain unaffected by these treatments. In practice, health and safety measures have proven futile in many instances of deterring pathogen transmission and subsequent infection, hence the magnitude of research devoted to biofilm eradication.

Imposing nutrient starvation, starting with carbon upon biofilms may be integral to developing effective infection control measures. By carrying out such investigations under controlled laboratory conditions it becomes possible to gain insight into a biofilm's fundamental behavioural strategies under imposed environmental stresses.

Of importance when examining biofilms under nutrient starved conditions is the ratio of sessile and planktonic cells (Costerton *et al.*, 2003; Hall-Stoodley & Stoodley, 2005), where planktonic cells are constantly being dispersed by biofilms at low rates as part of the biofilm's natural cycle (Sauer *et al.*, 2004). To this end, Delaquis *et al.* (1989) reported a detachment of cells by way of active emigration in addition to normal cell detachment activities in a pure-culture *Pseudomonas fluorescens* biofilm under glucose starvation. Similar results were obtained by Gjermansen *et al.* (2005) who observed a global dissolution of a pure-culture *Pseudomonas putida* biofilm within minutes of imposed carbon starvation, as measured by confocal laser scanning microscopy. Additional support for the detachment of cells from a biofilm under nutrient starvation conditions is presented by Hunt *et al.* (2004) who reported a 90% detachment of cells from a *Pseudomonas aeruginosa* biofilm upon induced carbon starvation. Further, Hunt

et al. (2004) quantified that 2-7% of biofilm present tenaciously remained attached well into induced starvation conditions, demonstrating that starvation in and of itself is not sufficient for biofilm eradication; recall the theory of persister cells. With the insight that a certain percentage of cells remain attached to a given substrate, Bester *et al.* (2011) tested the robustness of a mature pure-culture *Pseudomonas aeruginosa* CT07::*gfp* biofilm to induced carbon-starvation conditions followed by the reintroduction of carbon into the system. An immediate recovery of the starved biofilm towards pre-starvation levels was observed using planktonic cell yield, and measures of biofilm metabolism and architecture.

2.8.3 Ultraviolet (UV) Radiation

Ultraviolet (UV) light ranges from 100 to 400 nm on the electromagnetic spectrum, and is sub-divided into UV-A (315 – 400 nm), UV-B (280 – 315 nm), UV-C (200 – 280 nm) and vacuum UV (100 – 200 nm) (Fig.1). In the context of the current study UV-A, UV-B and UV-C will be considered. UV irradiation is a physical disinfectant that has long been used to disinfect drinking water and decontaminate treated wastewater (Harris *et al.*, 1987; Hijnen *et al.*, 2006). UV, specifically UV-C or germicidal UV, has known efficiency at inactivating viruses, bacteria, pathogens and protozoa in addition to being able to treat large volumes at a relatively low cost (Hijnen & Medema, 2005). Furthermore, UV can also be used to control biofouling and in water treatment processes without producing detrimental by-products (Dykstra *et al.*, 2007; Lakretz *et al.*, 2010). Interestingly, a huge environmental benefit of using UV is its lack of residual by-product(s) formation (many of which are known to cause injurious effects to aquatic ecosystems); however, as noted by Dykstra *et al.* (2007), the lack of residual disinfectant results in the need to use UV in conjunction with a secondary disinfectant, such as chlorine dioxide, to achieve biologically stable water i.e. safe drinking water, which negates the benefits described by using UV. Importantly, Lakretz *et al.* (2010) demonstrated UV as a preventative strategy to biofilm control in the disinfection of water.

UV is produced by the sun. UV-A and UV-B are able to penetrate the earth's ozone layer to reach the earth's surface where they trigger necessary Vitamin D production and result in skin effects such as tanning, sun burns, or in more severe instances skin cancers (Oppenländer, 2007). Conversely, UV-C is unable to penetrate the ozone layer and must be synthetically produced on earth (Elasri & Miller, 1999). UV-C was applied in the current study because of its known

inclusion of the germicidal or pathogenic bacteria inactivation at a value of 254 nm (Elasri & Miller, 1999; Hijnen & Medema, 2005). At this wavelength direct DNA damage is caused as pyrimidine dimers are formed that inhibit replication and transcription of the DNA, ultimately preventing the cell from multiplying (Harris *et al.*, 1987; Hijnen *et al.*, 2006; Li *et al.*, 2010).

With regards to the necessary dose or fluence to achieve bacterial inactivation, several values can be found in the literature, depending on the application. Bak *et al.* (2009) stated that the dose required for effectively killing 99.9% of viable planktonic cells known to form biofilms on in-dwelling medical devices was between 1 – 30 mJ/cm^2. Whereas Pozos *et al.* (2004) cited the effective dose range required in the United States of America (USA) for potable water as being 7.7 – 259 mJ/cm^2. What is important to keep in mind is that dose requirements for eliminating planktonic cell populations versus biofilm eradication are remarkably different. Table 2 provides bacterial inactivation values of common water-borne organisms.

Table 2: Required UV-C destruction doses for common bacteria.

Organism	**UV-C Dose Required for Destruction (mJ/cm^2)**	**Source**
Salmonella typhi	2 -10	(Hijnen *et al.*, 2006)
Staphylococcus aureus	6.6	www.uvp.com
Campylobacter jejuni	0.5 – 6	(Hijnen *et al.*, 2006)
Escherichia coli	6.6	(Hijnen *et al.*, 2006); www.uvp.com
Clostridium perfringens	48 – 64	(Hijnen *et al.*, 2006)
Pseudomonas aeruginosa	10.5	www.uvp.com
Pseudomonas fluorescens	6.6	www.uvp.com
Vibrio cholera	0.6 – 4	(Hijnen *et al.*, 2006)
Legionella pneumophilia	1 - 12	(Hijnen *et al.*, 2006)

Several investigations have used UV in conjunction with other disinfection processes to increase the efficacy of treatment when aiming for clean drinking water (Lakretz *et al.*, 2011). In the current study the objective was to determine the effect of employing UV while biofilm cells are stressed with the hope of destroying the biofilm, compared to the effect when they have

recovered from a critical stress state. The application of such a tiered process has far-reaching implications when considering effective antimicrobial treatments to remove biofilms in unwanted places such as on water distribution filters and in hospitals and homes.

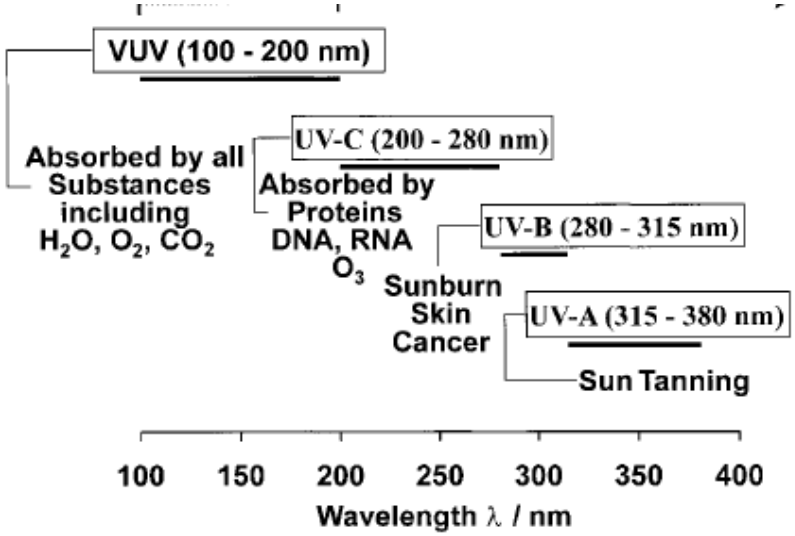

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Figure 1: The sub-divisions of UV light. Source: Oppenländer, 2007.

2.9 Control

Understandably with the known public health and economic implications associated with biofilms their control and eradication is a burgeoning area of research. There are numerous incentives to find practical, low cost solutions to biofilms in all impacted industries. Studies range from examining control methods on specific bacterial species (Gaddy & Actis, 2009) to control methods on entire microbial communities (Richards & Melander, 2009a; Richards & Melander, 2009b; Simões *et al.*, 2010). As a result of all the research being carried out, in addition to the economics of biofilm control there is an ever increasing selection of commercially available products targeting biofilm control or inhibition.

It has been determined that the initial attachment of planktonic cells to surfaces usually exhibits a lag until growth starts; however, when cells from the biofilm detach and colonize further downstream the cells behave like biofilm cells whereby reattachment occurs quickly and no lag is observed (Brooks & Flint, 2008). This can complicate the use of control products that are meant to attack biofilms once attached; timing is an issue in these cases. Further, binding properties of products to a wide variety of surfaces in affected industries has been difficult to

obtain. For example, a product that binds to stainless steel, may not readily bind to ceramic surfaces which can be adjacent to the stainless steel. With regards to the control product itself, it must be ensured that by-products are non-toxic and that the product is easy to use, safe and efficient. As noted by Boyce (2009) two compounds commonly found in antimicrobial disinfectants are formaldehyde and chlorine dioxide. Both of which have proven effective in eradicating microorganisms; however, potentially toxic end products are produced that require specific disposal techniques. As a result of the associated toxicity, replacement products are being developed and tested. Lastly, depending on the industry and country there are different standards and regulations for the acceptable levels of pathogens. This can create problems when trying to test products for control to be exported, additionally it has been especially problematic in the food industry where meats and products are constantly being exported and must not only meet the guidelines in the country of origin but also those of the final destination (Roberts *et al.*, 2008).

Chapter 3: Experimental

3.1 Introduction

Globally, the past two decades have seen an increase in the number of nosocomial infections as well as antibiotic resistance in bacteria (Weinstein, 1998; Clark & de Calcina-Goff, 2009). Annexed to unprecedented international travel and changing environmental conditions, microbes are being transported and are surviving in new locations contributing to the worldwide increase in infectious diseases (Wilson, 1995). Biofilms are known causative agents contributing to a wide variety of persistent infections and chronic diseases in both healthy and immuno-compromised individuals (Costerton *et al.,* 1999; Richards & Melander, 2009a).

With an estimated 80% of the world's microbial biomass housed in biofilms, it is apparent that forming these attached heterogeneous communities shrouded in a self-secreted matrix is advantageous to their propagation and survival (Richards & Melander, 2009a). Further, the surrounding matrix composed of exopolymeric substance (EPS) has been proven to aid in shielding biofilm community members from a variety of antimicrobial treatments, in addition to functioning as the biofilm's scaffolding (Gaddy & Actis, 2009; Kaplan, 2010). As such, biofilms have been the focus of many investigations aiming to understand the mechanisms that allow them to withstand various antimicrobial treatments, in addition to their role in harbouring and transmitting pathogens. Determining reservoirs for pathogens in domestic, medical and industrial settings is of increasing importance in order to reduce the transmission of disease in these environments. Ultimately, eradication of these disease-harbouring biofilms in undesirable locations is the goal.

Nutrients are integral to bacterial survival and may determine the community structure within the biofilm (Costerton *et al.*, 1978). The inherent biofilm architecture results in some cells being subjected to nutrient limitation, restricting these cells to a slow-growing or dormant state (Costerton *et al.*, 1999; Mah & O'Toole, 2001). It is well known that biofilms survive in oligotrophic environments or settings characterized by nutrient fluxes such as drinking water distribution systems (LeChevallier *et al.,* 1987; Costerton *et al.,* 1995). Bester *et al.* (2011) demonstrated that the removal of carbon from the growth medium sustaining a pure-culture biofilm resulted in an immediate decrease in biofilm metabolism. The biofilm remained metabolically inactive throughout the carbon-starvation period, only to promptly return to pre-

starvation activity upon the reintroduction of carbon. This finding instigated the investigation into whether the application of a second stress to a metabolically inactive biofilm would be successful in deterring biofilm propagation. Knowing that ultraviolet (UV) light has long been used as a disinfectant in water and wastewater industries (Hijnen *et al.*, 2006), it was selected as the second stress to be applied to the carbon-starved pure-culture biofilms. UV, specifically UV-C, is used in large-scale industrial operations because of its capabilities of disinfecting large volumes of water without producing detrimental chemical by-products (Dykstra *et al.* 2007). UV-C directly damages the deoxyribonucleic acid (DNA) of bacteria and other microorganisms, rendering them incapable of replication (Li *et al.*, 2010). Additionally, UV is emerging as a preventative strategy against biofilm-related problems in water disinfection (Lakretz *et al.*, 2010). The current study measured the number of cells released by the biofilm (biofilm-derived planktonic cell yield) and metabolic responses of an environmentally-relevant mixed community biofilm to carbon-starvation and the effects of UV exposure on pure-culture biofilms.

3.2 Materials and Methods

3.2.1 Mixed Community Isolation and Characterization

3.2.1.1 Sink-drain mixed community isolation

A sink-drain sample was retrieved from a high traffic university public washroom by swabbing 5 cm from the top of the sink outlet using a Quick Swab (3M, London, ON, Cat. # 6433). The swab was placed into the letheen broth and transported to the laboratory to be further processed. In the laboratory, the Quick Swab was vortexed at top speed for 1 min to ensure the release of bacterial cells from the swab. The suspension was then added to 50 mL of minimal salt medium (MSM) which contained (grams per liter): NaCl (2.0), NH_4Cl (1.0), $MgSO_4 \cdot 7H_2O$ (0.12), Na_2HPO_4 (4.24), KH_2PO_4 (2.7); and trace element solution (1mL/L): $CaCl_2$ (0.99), $FeSO_4 \cdot 7H_20$ (0.5), $MnSO_4 \cdot 2H_20$ (0.2), $NaMoO_4 \cdot 2H_20$ (0.25). The two carbon sources were sodium pyruvate and starch (Caldwell & Lawrence, 1986; Delaquis *et al.*, 1989; Wolfaardt *et al.*, 1994). The sample was then incubated with 250 rpm shaking for 24 h at room temperature (24˚C ±2˚C). Serial dilutions in sterile 0.9% sodium chloride were prepared from 1mL of sample, with 100 µL of dilution spread-plated onto MSM, R2A and 10% tryptic soy broth (TSB) media. The plates were inverted and left at room temperature for 7 days to encourage growth.

Ten visually distinguishable colonies (labelled as SD 1-10) were isolated from plates from all three media types for further characterization. Single colonies were picked and re-streaked three more times to ensure the purity of the isolates.

3.2.1.2 DNA extraction and 16S rRNA gene PCR amplification

Genomic DNA from the isolates was extracted from an overnight culture using the Genelute™ Bacterial Genomic DNA Kit (Sigma-Aldrich Cat. # NA2110-1KT, Oakville, ON). The extracted DNA was frozen at -20˚C until PCR amplification.

In each PCR reaction a volume of 50 µl was prepared consisting of 1.0 µl of U341GC#2 (5'-CCTACGGGAGGCAGCAG-3') as the forward primer, 1.0 µl of U758 (5'-CTACCAGGGTATCTAATCC-3') as the reverse primer, 8.0 µl of 1.25 mM dNTPs, 0.625 µl of BSA, 32.375 µl of dH_2O, 5.0 µl of buffer, 0.5 µl of rTaq polymerase (New England Biolabs, Canada) and 1.0 µl of DNA from each sample.

An Eppendorf PCR machine was heated to 96˚C for 5 min followed by thermocycling at 94˚C for 1 min with the annealing temperature set at 65˚C, which decreased by 1˚C every cycle, for 10 cycles. Each cycle was followed by an elongation for 3 min at 72˚C (Muyzer & Smalla, 1998).

PCR products were visualized after electrophoresis on a 1% agarose gel. PCR products were prepared by mixing 1 µl of SYBR® Safe (Invitrogen, Burlington, ON) dye with 5 µl of PCR product. A total of 5 µl was loaded into each of the wells along with a well of 100 bp ladder (Fermentas, Cat. No. SM0241, Burlington, Ontario). The gel was run for 40 min at 85 volts (V). Upon completion, the gel images were captured with UVP BioDoc-It™ Imaging System, and DNA concentrations were determined using a Nanodrop 2000c.

3.2.1.3 Sequencing and identification

The PCR products were prepared for sequencing using a Gel/PCR DNA Fragments Extraction Kit (IBI Scientific, Peosta, IA, Cat. No. IB47020). DNA sequencing and subsequent species identification were performed using BLAST from the sequence information obtained from The Centre for Applied Genomics at the Hospital for Sick Children, Toronto, Canada.

3.2.2 Culture medium and growth conditions

MSM was used for subsequent cultivation of the isolates and the lab strain *P. aeruginosa* PA01 gfp (PAO1).

3.2.3 Continuous-flow culture of biofilms

3.2.3.1 Flowcell

A four-channel conventional Plexiglass flowcell (flowcell) was milled as previously described by Wolfaardt *et al.* (1994) and Bester *et al.* (2011), with channel dimensions of 40 mm x 5 mm x 4 mm; length, width and height respectively. A continuous-flow of sterile medium was delivered to the flowcell at a flow rate of 17 mL h-1, and critical dilution rate of 21.25 h^{-1}, (Reynolds number 1.03), using a Watson-Marlow 205U peristaltic pump at a tension of 2.8, via silicone tubing (VWR International Cat #: 60985-714; inside diameter (ID), 0.062 mm) and Tygon tubing (R-3603). Tygon tubing was used over the pump and immediately upstream of the flowcell to impede microbial growth. The flowcell was covered with two side-by-side quartz slides (Chemglass Life Sciences, Vineland, NJ) that each measured 24 x 50 x 1mm (Fig.2). The

flowcell was maintained at a 90˚ angle, instead of the previously described 0˚ to encourage the release of gas from the channels.

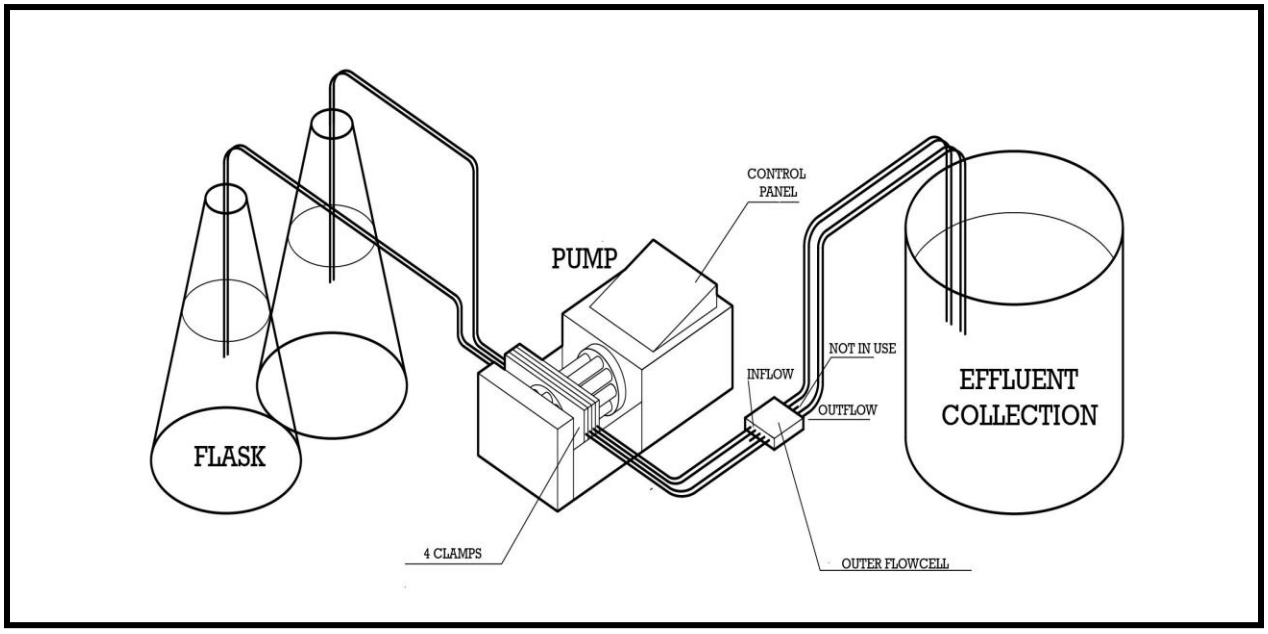

Figure 2: Schematic diagram of the experimental continuous flow set-up. MSM medium was delivered through the network of silicon and Tygon tubing via a peristaltic pump to the four-channel flowcell. Effluent was delivered to a waste receptacle via silicon tubing.

3.2.3.2 Disinfection and inoculation

The system was disinfected with a continuous flow of 10% sodium hypochlorite (v/v) solution for 3 h, followed by an overnight flow of sterile distilled water (dH_2O). The water was replaced with sterile growth medium for at least 0.5 h, at which time the medium flow was stopped in preparation for inoculation. An overnight 5 mL pre-culture in sterile medium was prepared prior to inoculation; 100 µL per channel of the pre-culture was then inoculated into the flowcell using a sterile 1 mL HSW Henke Sass Wolf GmbH syringe (National Scientific Company, Rockwood, TN) and 25G 1½ ” needle (VWR, Cat # CA15003-024). The pump was restarted 1 h post-inoculation, having allowed the bacteria to start biofilm formation.

3.2.4 Enumeration of biofilm-derived planktonic cell yield

Effluent samples (1mL) were collected from the flowcell at 24 h intervals. Samples were then serially diluted in 0.9% sterile saline solution, with duplicate spread-plates being prepared

from 100 µl dilutions. After 48 h incubation at room temperature colonies were counted and reported as CFU mL^{-1}.

3.2.5 Carbon dioxide production

The carbon dioxide monitoring reactor (CMR) was set up using the open loop configuration as previously described by Bester *et al.* (2010). The setup (Fig. 3) included a 20 mL serum vial with a butyl stopper perforated by four individual 16G ½ inch needles: in-flowing and out-flowing liquid (effluent), and in-flowing and out-flowing gas. All measurements were taken using the Mass Flow Controller GFC Carbon Dioxide Analyzer (Aaloborg, Orangeburg, NY) and captured by LI-820 v.2.0.0. software. CO_2 measurements were taken for up to 2 h at 24 h intervals.

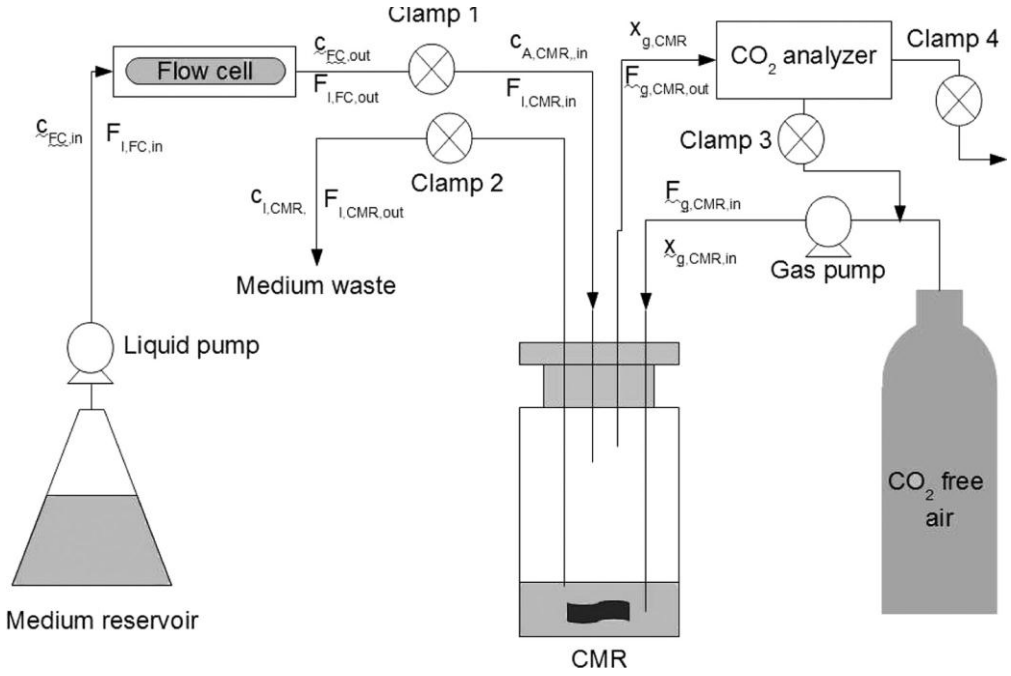

Figure 3: A schematic diagram of the experimental setup showing the flow cell and the CMR. Source: Bester *et al.*, 2010.

3.2.6 Determining effluent cell origin

In order to verify that planktonic cell replication in the flowcell chambers did not contribute to biofilm-derived planktonic cell yield numbers, the maximum specific planktonic growth rate ($\mu max_{\ planktonic}$) for each organism was determined from at least three replicate batch cultures. The doubling time of each organism was calculated as follows:

$$Doubling\ time = \frac{\ln(2)}{\mu max} \qquad (E1)$$

As the critical dilution rate for the continuous flow system (D = 21.25 h^{-1}) greatly exceeded the $\mu max_{planktonic}$ for all organisms, it can be concluded that cells in the effluent originated from the biofilm and were not independently replicating planktonic cells (Bester *et al.*, 2010).

3.2.7 Ultraviolet (UV) radiation

UV radiation was applied using a 4 Watt UVP handheld lamp (UVGL-15 Compact UV Lamp) (UV lamp) possessing both UV-A (365 nm) and UV-C (254 nm) wavelength settings. The lamp was positioned 5 cm above agar plates, and 0.8 cm above the flowcell. The dose (or fluence) delivered by the UV lamp was 292 mJ/cm^2, as calculated from radiometer readings and residence time in the flowcell. The dose delivered exceeded the United States Environmental Protection Agency (USEPA) recommended range used for disinfecting potable water (Pozos *et al.*, 2004).

3.2.7.1 Determining the most effective UV lamp setting

The inhibition of bacterial growth on streak plates was used to determine whether to use the 254 nm or 365 nm setting. Each of the plates was divided with one half being exposed to UV light and the other half blocked from receiving UV light. The agar was directly exposed to UV immediately after being streaked.

3.2.7.2 The extent of UV-C penetration through borosilicate glass and quartz cover slips

Spread and streak plates were subjected to UV-C with suspended borosilicate or quartz glass cover slips above the agar. UV-C exposure times were 0.5 min, 1 min, 2 min, and 5 min. Unexposed UV-C plates served as the reference. Plates were UV-C exposed immediately after being spread or streaked, and bare agar was exposed to the UV-C directly.

3.2.7.3 Planktonic Culture

1/10 (v/v) and 1/1000 (v/v) *Pseudomonas aeruginosa* PA01 gfp cultures were passed through the flowcell simultaneously, while being subjected to UV-C. Both cultures were inoculated into sterile medium. Sterile flasks were constantly agitated and kept cool in order to deter further growth in the flask. Effluent samples were obtained from downstream of the flowcell at 15 min and 1 h, serially diluted and used to determine cell number using traditional plating techniques. Residence time in the flowcell is 169 sec.

3.3 Results and Discussion

3.3.1 Mixed Community Isolation and Identification

The ten morphologically distinct colonies that were isolated from the sink-drain were grown on R2A, TSB and MSM agar, which ultimately yielded BLAST results from the amplified 16S rRNA gene sequence to five species closely related to isolates from other drain and wastewater environments (Table 3). The five isolates selected to create an artificial mixed drain community for flowcell experiments were SD-1, SD-4, SD-5, SD-6 and SD-8.

An artificial sink-drain mixed community was constructed for the proposed dual-stress regime using these 5 isolates for their individual relevancy to public health and / or industrial applications. For decades, pathogens and opportunistic pathogens have been known to colonize sink-drains in hospitals (Whitby & Rampling, 1972) and homes (Finch *et al.*, 1978). To this point, sink-drains are under increasing scrutiny as pathogen transmission sights in hospitals (Doring *et al.*, 1991), and homes (McBain *et al.*, 2003). Hota *et al.* (2009) demonstrated that biofilms in hospital room sinks transfer pathogens to healthcare workers through hand-washing, or to areas surrounding the sink. In this instance *Pseudomonas aeruginosa* was transferred as a result of the poor sink-drain design and placement.

Further, biofilms of varying thickness and community composition have been detected throughout water distribution networks (LeChevallier *et al.*, 1987). Additionally, bacteria isolated from biofilms found in several sink-drains and throughout water distribution networks are known to cause biofouling of engineered surfaces leading to the increased use of disinfectants in these systems which may directly or indirectly cause adverse environmental and health impacts (Flemming, 2002).

Sink-drains and piping networks, being aqueous environments intermittently supplied with nutrients, are favourable sites for microbial colonization and biofilm formation (Brooke, 2008). McBain *et al.* (2003) asserted that sink-drains possess sufficient available substrate providing ample nutrients for the formation of thick biofilms. Moreover, the standard sink-drain design lends itself to the accumulation of microorganisms within its convoluted piping network with areas of low turbulence preceded by high turbulence (Charaf, 1997).

Sink-drains are a terminus of water distribution networks; therefore, biofilms whether in the sink-drain proper or dispersed along the piping network should be taken into account when considering the role of pathogen propagation and dispersal from sink-drains (LeChevallier *et al.*, 1987; Anaissie *et al.*, 2002).

Since it has been established that biofilms in hospital drains can act as pathogen reservoirs, a natural extension to these studies would be to investigate domestic drains as potential pathogen reservoirs. *P. aeruginosa*, a well-known pathogen in immuno-compromised patients, has been isolated from both domestic and hospital sinks; however, Whitby & Rampling (1972), as well as Ayliffe *et al.* (1974) found that *P. aeruginosa* contamination was far less frequent in domestic environments. This can be attributed to the fact that *P. aeruginosa* is ubiquitous in hospitals. To this end, it is worthwhile to note that *Pseudomonas* is the most abundant bacteria in drinking water distribution systems (Lakretz *et al.*, 2010; Simões *et al.*, 2010).

It is well known that sink-drains harbour a wide variety of microorganisms. This knowledge has been the incentive driving several investigations whose aim has been to identify and characterize specific microorganisms present in hospitals and domestic sink drains and water distribution networks in order to promote infection control (Perryman & Flournoy, 1980; LeChevallier *et al.*, 1987; McBain *et al.*, 2003; Brooke, 2008).

As expected several bacterial genera have been isolated from hospital and domestic sinks as well as water distribution networks. The majority of studies focus on one environment; however, trends of organisms common to the three environments have emerged, whereupon more in-depth research to understand these microorganisms has been carried out. For example species of *Moraxella, Enterobacter,* and *Pseudomonas* have been isolated from all three environments, whereas *Staphyloccocus* and *Chryseobacterium* were isolated from domestic sinks, *Serratia* and *Acinetobacter* were isolated from hospital sinks and water distribution networks and *Sphingomonas* was isolated from hospital and domestic sinks (Finch *et al.*, 1978; Perryman & Flournoy, 1980; LeChevallier *et al.*, 1987; Charaf, 1997; McBain *et al.*, 2003; Brooke, 2008). Based on this information and what could be isolated from the drain sample, a sink-drain model mixed community biofilm comprised of five members was cultivated. Microorganisms were selected based on their lasting presence in the sink-drain by comparing

two samples taken six months apart, and performing denaturing gradient gel electrophoresis (DGGE) to match DNA bands. Additionally, selection was based upon the inclusion of representative genera as confirmed by relevant literature.

Table 3: Identification of organisms from a sink-drain sample as determined by alignment with the 16S rRNA amplified gene sequence, with BLAST. DNA extraction, PCR and sequencing were carried out to obtain organism identification.

Identification	**Closest Match**	**% Match to BLAST**	**Common Drain Organisms**
SD-1	*Chryseobacterium sp.*	99	(McBain *et al.*, 2003; Kirby *et al.*, 2004)
SD-2	*Enterobacter asburiae*	99	(McBain *et al.*, 2003)
SD-3	*Delftia (tsuruhatensis)*	99	
SD-4	*Pseudomonas putida*	99	(Perryman & Flournoy, 1980; Charaf, 1997)
SD-5	*Enterobacter sp.*	100	(Perryman & Flournoy, 1980)
SD-6	*Sphingomonas sp.*	98	(Charaf, 1997; Brooke, 2008)
SD-7	*Sphingobacterium sp. (multivorum)*	99	(McBain *et al.*, 2003)
SD-8	*Pseudomonas aeruginosa*	99	(Whitby & Rampling, 1972; Ayliffe *et al.*, 1974; Perryman & Flournoy, 1980; Döring *et al.*, 1991; Charaf, 1997; Brooke, 2008; Hota *et al.*, 2009)
SD-9	*Moraxella sp.*	99	(McBain *et al.*, 2003)
SD-10	*Acidovorax sp.*	97	(Furuhata *et al.*, 2009)

3.3.2 Maximum Specific Growth Rate and Doubling Time of Mixed Community Members

The maximum specific growth rates (μmax) of individual community members as obtained from the natural logarithm (ln) of their respective growth curve optical density 600 nm values are displayed in Table 4, as well as the doubling time of each organism in MSM.

Table 4: The maximum specific growth rate (μmax) obtained from the natural logarithm of optical density (600nm) growth curves. Doubling time was calculated from the μmax.

Organism	Maximum Specific Growth Rate μmax (h^{-1})	Doubling Time (h)
SD-1	0.32 ± 0.02	2.17
SD-4	0.60 ± 0.01	1.16
SD-5	0.22 ± 0.02	3.15
SD-6	0.60 ± 0.05	1.16
SD-8	0.28 ± 0.04	2.48

3.3.3 Mixed Community Response to Carbon-Starvation

The average biofilm-derived planktonic cell yields from two mixed community flowcell inoculations were used to determine when the biofilms had transitioned from exponential growth into stationary growth (Fig. 4). From these two trials it was observed that biofilm steady-state according to planktonic cell yield was achieved between 72 – 96 h. It was decided that future mixed community biofilms grown in the flowcell were to be starved, or receive UV application at 72-74 h, based on these observations.

The response of a five member mixed community biofilm to 96 h carbon-starvation (Fig. 5a) was determined by measuring carbon dioxide production (ppm) and biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}). Carbon starvation was induced at 73 h (Fig. 5b) until 169 h when carbon was reintroduced into the system (Fig. 5c). All induced carbon-starvation and reintroduction responses were monitored by carbon dioxide production in real-time. The industrious response of the mixed community biofilm to carbon reintroduction after 96 h of carbon-starvation lead to the investigation of a mixed community's response to 120 h of carbon-starvation (Fig. 6a) using the same measurement parameters. The pre-determined carbon-starvation time of 73 h was used as the reference point to induce starvation upon the biofilm (Fig. 6b). Carbon was reintroduced into the system at 193 h (Fig. 6c).

Although both mixed community biofilms were starved at 73 h, their $C0_2$ production levels differed, which may be attributed to the different origins of inocula (Kroukamp *et al.*, 2010) despite each community being inoculated at identical OD_{600} and comprised of the same members: SD-1, SD-4, SD-5, SD-6 and SD-8. Whether the biofilm was carbon-starved for 96 h

or 120 h the responses of the mixed community biofilms to the reintroduction of carbon is prompt with increased $C0_2$ production occurring within 1 h. The rapid metabolic response of the biofilms demonstrates that at least some of the community members within the flowcell are able to withstand prolonged periods of starvation, suggesting that the biofilm acts a reservoir for nutrients affording its members protection under adverse environmental conditions. Considering that the sink-drain origin of the mixed community members is characterized by nutrient fluxes, variability in water availability and episodes of disinfection or sanitization, investigating the effects of these parameters individually is important to understanding the overall biofilm mechanisms of survival in constantly changing environments. With similar rapid metabolic responses of two mixed community biofilms to carbon-starvation and carbon reintroduction, in addition to the known metabolic responses of a pure-culture biofilm presented by Bester *et al.* (2011), the resiliency of biofilms under nutrient deprivation conditions is further exemplified.

As expected in both mixed community biofilms an increase of biofilm-derived planktonic cells accompanied the first 72 h of growth, until a steady-state was entered. With induced carbon-starvation, biofilm-derived planktonic cell yields decreased during carbon-starvation by less than a log reduction, demonstrating that despite imposed stress, and minimal metabolic activity, the biofilm is capable of releasing cells into the surrounding environment at relatively constant rates. The constant release of biofilm-derived cells has far-reaching implications when considering the dissemination of pathogens. For example, planktonic revertants from a sink-drain may become aerosolized and be transported several meters from the original source, whereupon contact with a surface or vector may initiate pathogenesis. Conversely, the released cells may come into direct contact with an immuno-compromised patient in a hospital resulting in nosocomial infection, or a hospital outbreak.

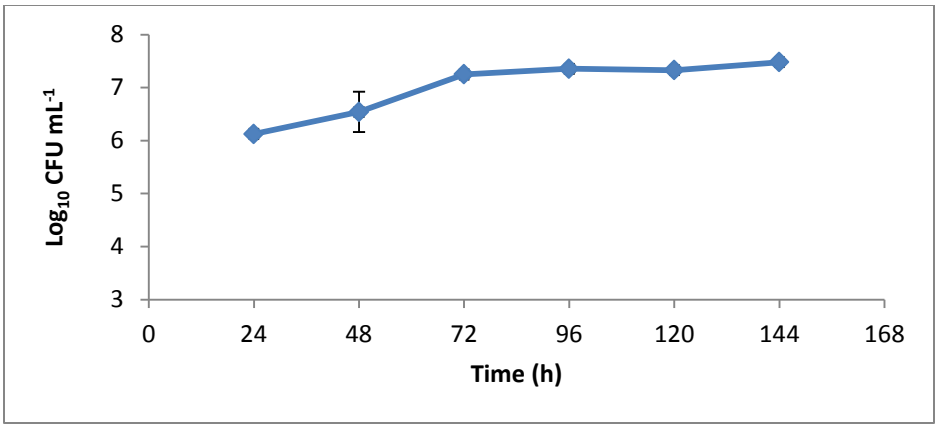

Figure 4: Determination of average five-member mixed community biofilm-derived planktonic cell yield as enumerated from multiple channel flowcell effluent.

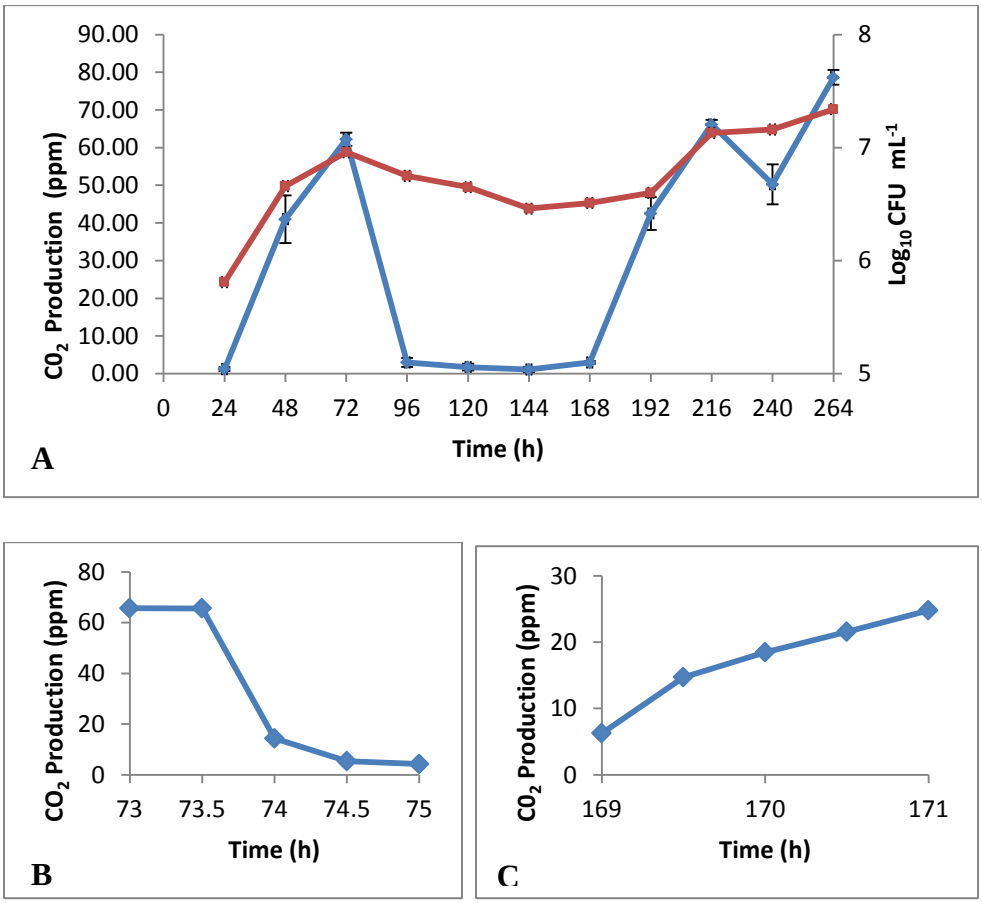

Figure 5: A) Mixed community biofilm response to 96 h carbon starvation as measured by (◆) carbon dioxide production (ppm) and (■) biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}). B) Induced carbon-starvation at 73 h in a mixed community biofilm as measured by carbon

dioxide production (ppm). C) Reintroduction of carbon at 169 h into media, sustaining a mixed community biofilm after 96 h of carbon-starvation as measured by carbon dioxide production (ppm).

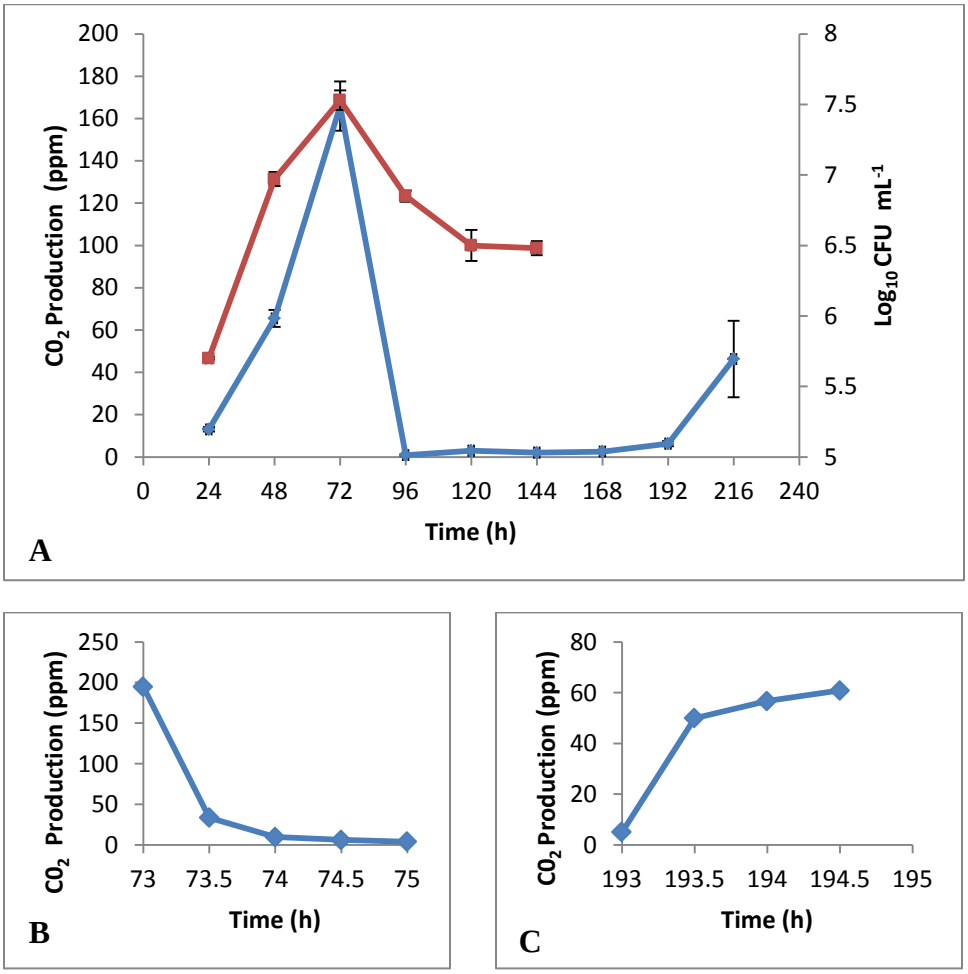

Figure 6: A) Mixed community biofilm response to 120 h carbon starvation as measured by (◆) carbon dioxide production (ppm) and (■) biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}). B) Induced carbon-starvation at 73 h in a mixed community biofilm as measured by carbon dioxide production (ppm).

3.3.4 Pure-Culture Response to Carbon Starvation

Responses of a PA01 biofilm to 72 h carbon-starvation (Fig. 7a) were measured by carbon dioxide production (ppm) and biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}). The carbon dioxide (ppm) production of PA01 at induced carbon-starvation (Fig.7b) and

subsequent reintroduction (Fig.7c), were measured by the same metabolic and cell yield parameters. Responses of a SD-1 biofilm under identical conditions, and using identical measurement parameters were obtained (Figs.8 a-c).

PA01 and SD-1 metabolic and cell yield responses were as expected when relating the data to that presented by Bester *et al.* (2011), especially since the carbon-starvation time in the current study was 48 h less than that presented by Bester *et al.* (2011). Notable differences between the current study and that of Bester *et al.* (2011) were the current study's use of sodium pyruvate and starch as opposed to citrate as a carbon source. Further Bester *et al.* (2011) carried out their study on *Pseudomonas* sp. strain CT07::*gfp*, while *Pseudomonas aeruginosa* PA01 gfp and SD-1 were used in the current study.

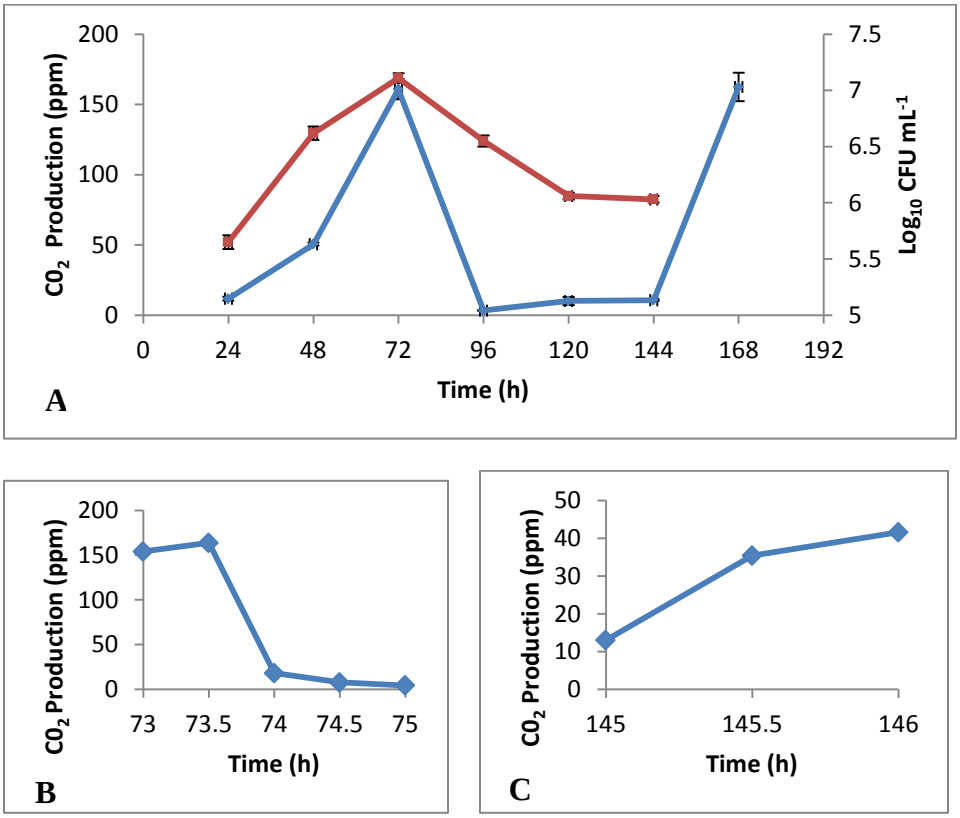

Figure 7: A) *Pseudomonas aeruginosa* PA01 gfp response to carbon-starvation between 96 – 144 h as measured by (■) carbon dioxide production (ppm) and (◆) biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}). B) *Pseudomonas aeruginosa* PA01 gfp carbon dioxide production (ppm) response to induced carbon-starvation at 73 h. C) *Pseudomonas aeruginosa* PA01 gfp carbon dioxide production (ppm) response the reintroduction of carbon into media at 145 h.

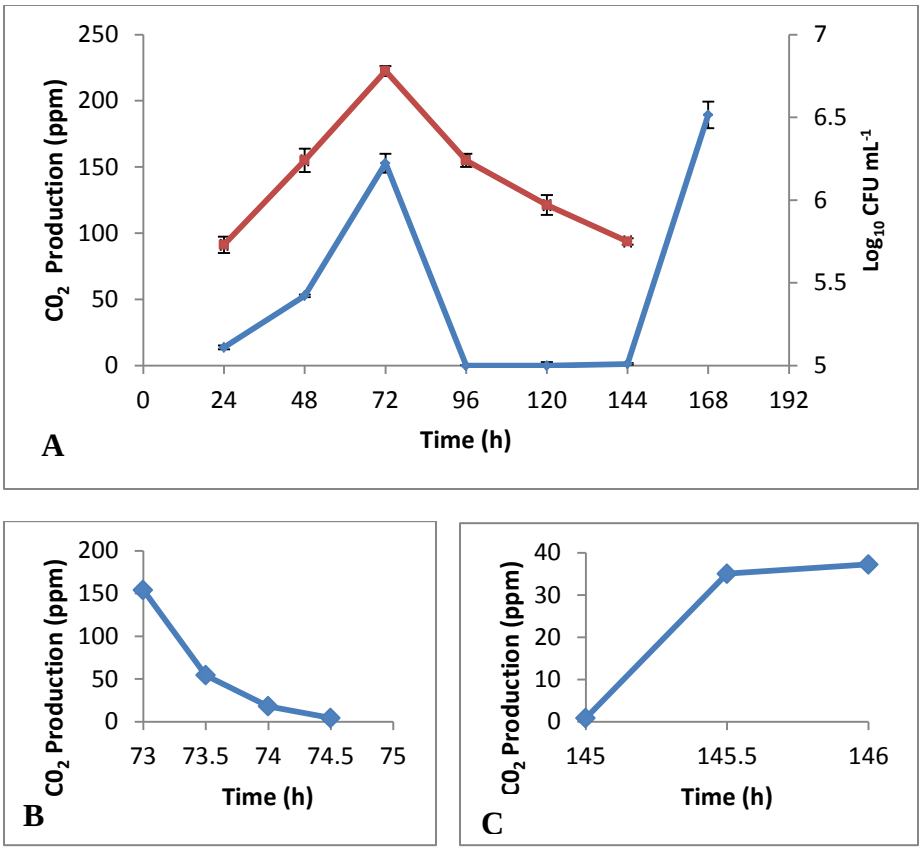

Figure 8: A) SD-1 response to 72 h carbon-starvation as measured by (◆) carbon dioxide production (ppm) and (■) biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}). B) SD-1 carbon dioxide production response (ppm) to induced carbon starvation at 73 h. C) SD-1 carbon dioxide production response (ppm) to carbon reintroduction into the media at 145 h.

3.3.5 Ultraviolet (UV) Light

3.3.5.1 Determining the Optimal UVP Handheld Lamp Setting

Streak plates of all constructed community members were exposed to UV-A (365 nm) light for 45 min, based on exposure times provided by Mori *et al.*, (2007). SD-6 is displayed in Figure 9, the left half of the plate (a) was exposed to UV-A, while the right half (b) was covered to block UV-A penetration. In a separate experiment, constructed community streak plates were exposed to 5 min UV-C (254 nm), with SD-1 shown in Figure 10. The left half (a) of the plate was exposed to UV-C, while the right half (b) was covered.

UV-C is commonly accepted as the optimal range for the inactivation of bacteria and other microorganisms (Pozos *et al.*, 2004; Hijnen & Medema, 2005). However, UV-A has also

been successfully applied to inactivate bacteria, and is subject to an increasing number of investigations into its sterilization efficiency (Mori *et al.*, 2007; Yagi *et al.*, 2007). As such, determining which setting on the lamp had the most efficient inactivation capacity for bacterial growth provided the foundation for continued UV experiments with the selected light source. From the images provided in Figures 9 and 10, it is clear that with the given light source, UV-C inhibits bacterial growth on agar plates.

It is important to note that according to the USEPA, UV-C applications in continuous flow systems, such as the flowcell, are more complex than applications in mixed batch reactors (USEPA, 2006). It is possible that some cells would not receive the UV-C due to their orientation in the flowcell channel in relation to the UV-C source. As such, the UV-C application for continued experiments exceeded the USEPA recommendations for the treatment of potable water with regards to dose (7.7 – 259 mJ/cm^2), with a dose delivered of 292 mJ/cm^2.

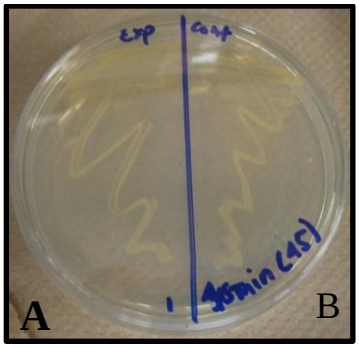

Figure 9: 45min UV-A exposure to an SD-6 streak plate. A was UV-A exposed, B was blocked from UV-A light.

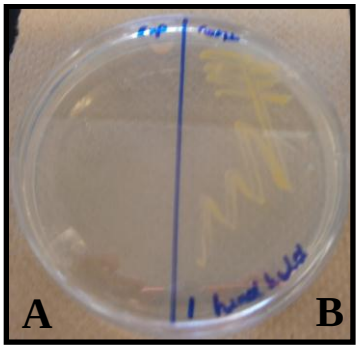

Figure 10: 5 min UV-C exposure to an SD-1 streak plate. A was exposed to UV-C, while B was blocked from UV-C.

3.3.5.2 UV-C Penetration through Borosilicate Glass Cover Slips

All constructed community members were streak plated and subjected to 5 min UV-C exposure through two borosilicate glass cover slips. SD-1 is displayed in Figure 11. Constructed community members were spread plated and subjected to 5 min UV-C exposure through borosilicate glass slips. The results demonstrated that some penetration through the cover slips occurred.

Both streak plates and spread plates were used to help determine the effectiveness of UV-C penetration through the borosilicate slides. Streak plates often possess multiple layers of cells at their peak growth; therefore, inhibition of microbial growth through the borosilicate slides would indicate adequate penetration to apply the UV-C to a biofilm, which develops multiple layers. UV-C was applied immediately after streaking to determine whether UV-C was capable of inhibiting multi-layered cell growth. However, microbial growth was not inhibited on the streak plate, so a single cell-layered spread plate was tested.

Conventional Plexiglass flowcells are traditionally covered with glass cover slips for ease of microbial attachment and performing microscopy (Bester *et al.*, 2010). Upon further investigation it was verified that the specific composition of the glass significantly affects the % transmittance of UV light and subsequent penetration into the desired object. The glass cover slips originally affixed to the flowcell in this study were composed of borosilicate glass. Recognizing that there was incomplete penetration of UV-C onto the streak plate, but adequate penetration through the cover slip to inhibit bacterial growth on the spread plate, a third test was performed by fluence measurements obtained from a radiometer which showed that the % transmittance of the UV-C source employed in the current study through the borosilicate glass cover slips was 0.001%, rendering the borosilicate glass cover slips unfit to achieve the objectives outlined in this study.

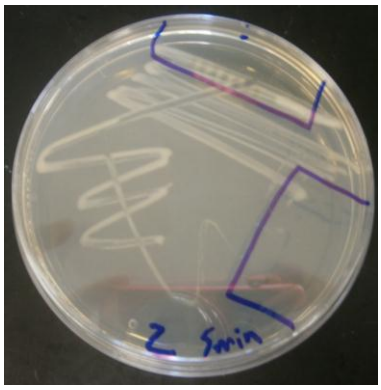

Figure 11: Image of an SD-1 streak plate exposed to UV-C for 5 min. Two borosilicate glass slides were suspended above the plate as drawn onto the plate in blue.

3.3.5.3 UV-C Penetration through Quartz Glass Cover Slips

The ability of UV-C to penetrate quartz glass cover slips was demonstrated by the images in Figure 12, where A was the unexposed plate, and B was the UV-C exposed plate. Quartz glass sleeves are commonly used in wastewater treatment facilities employing UV disinfection as they have been shown to allow 90-95% transmittance of UV-C light (Harris *et al.*, 1987). As such, quartz glass cover slips were tested with the UV-C light source used in the current study to ensure adequate transmittance and penetration of UV-C light occurred. Additionally, the quartz glass cover slips were tested with the radiometer and found to allow >99% transmittance.

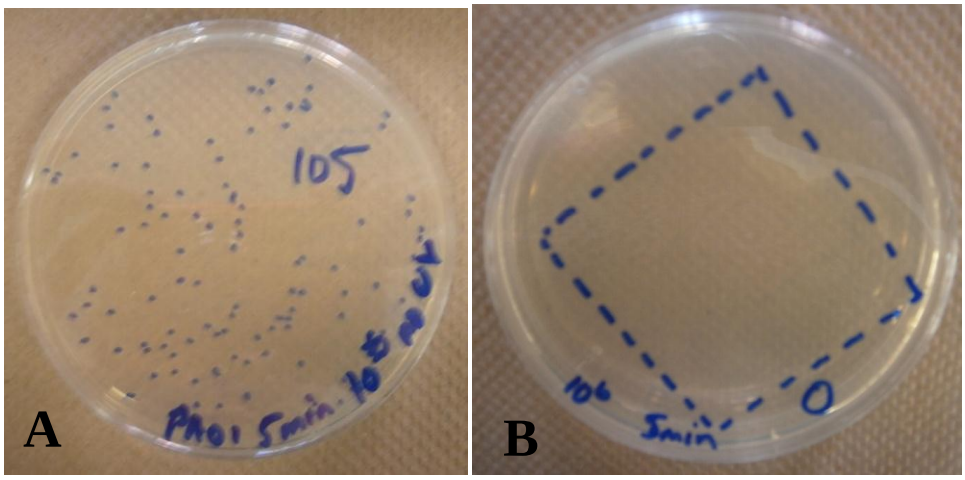

Figure 12: Image of UV-C unexposed (A) and exposed (B) *Pseudomonas aeruginosa* PA01 gfp spread plates. Two side-by-side quartz glass slides were suspended above the agar as shown with a dotted blue line (B).

3.3.5.4 Effect of UV-C on Planktonic Cultures

The effectiveness of UV-C at inactivating planktonic bacteria cultures of *Pseudomonas aeruginosa* PA01 gfp were quantified from biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}) (Fig. 13). The pre-culture possessed an average Log_{10} CFU mL^{-1} of 8.82. From the original culture a dilution of 1:10 (represented as the high concentration), and a 1:1000 (represented as the low concentration) were passed through the flowcell and subjected to UV-C exposure through quartz glass cover slips. Effluent samples were collected at 0.25 h and 1h. Log_{10} CFU mL^{-1} corresponded to biofilm-derived planktonic cell yield numbers originating from pure-culture biofilms (Bester *et al.*, 2011). Residence time in the flowcell channel was determined to be 169 sec.

The UV-C source was successful at inactivating planktonic bacteria of PA01. Inactivation in both planktonic cultures further solidifies the proof that the UV-C lamp is capable of transmittance through the quartz glass cover slips and penetrating cultures within the flowcell channels.

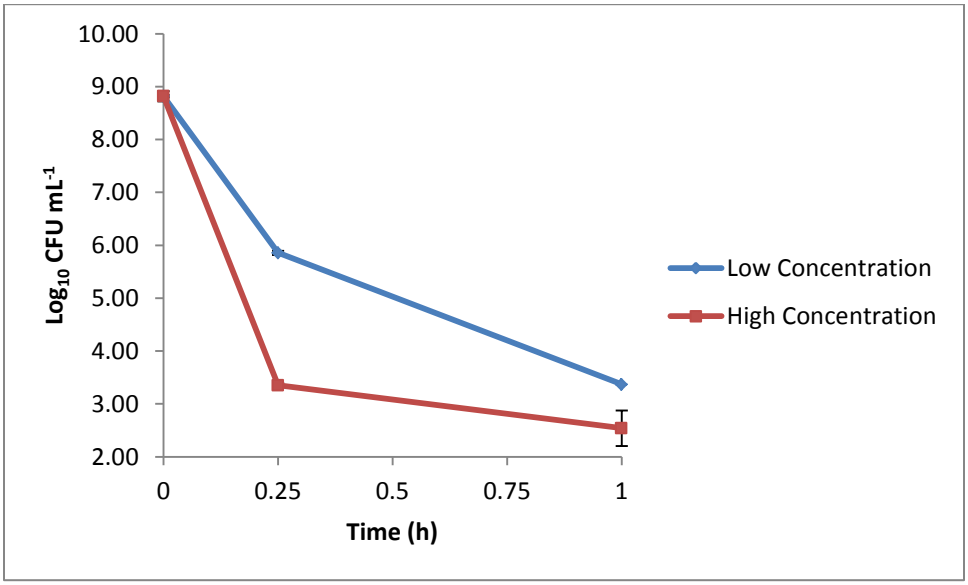

Figure 13: High and low concentrations of *Pseudomonas aeruginosa* PA01 gfp demonstrating average effluent values from duplicate spread plates sampled at 0.25 and 1 h.

3.3.5.5 Determination of UV-C Exposure Time to a Steady-State Pure-Culture Biofilm

The ability of biofilms to better withstand stress when compared to their planktonic counterparts is well documented. With the knowledge that bacterial inactivation in planktonic cultures occurred within 0.25 h of UV-C application, UV-C exposure times of 1.5 h and 2.0 h were tested for their effectiveness on a pure-culture biofilm (Fig. 14), as measured by carbon dioxide production (ppm). Observing less than a 10 ppm change from 1.5 – 2.0 h of UV-C, an application time of 1.5 h for future experiments was decided upon. Because the steady-state biofilms proved to resist UV-C application, it was decided upon to test the response of early stationary phase biofilms.

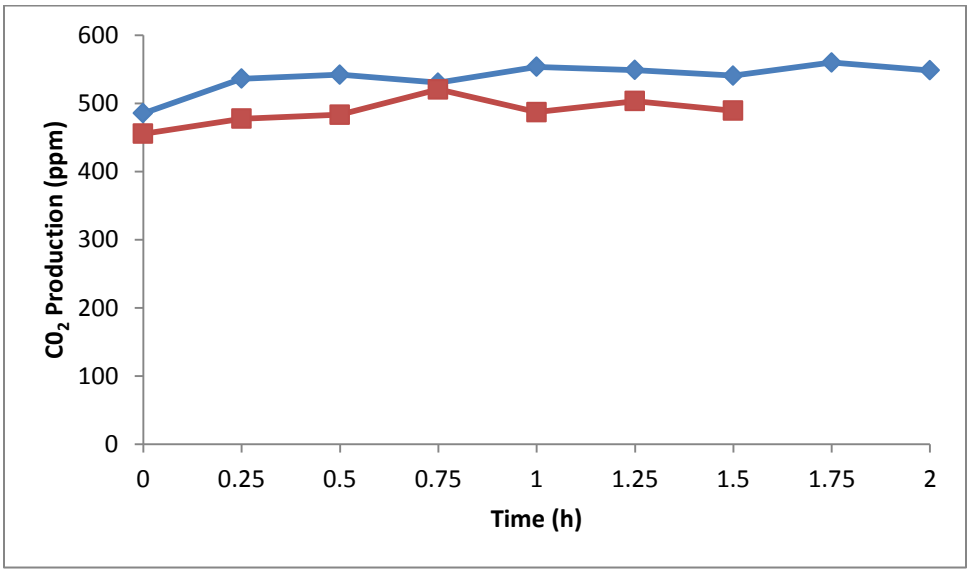

Figure 14: *Pseudomonas aeruginosa* PA01 gfp response to 1.5 h (■), and 2 h (◆) exposure to UV-C as measured by carbon dioxide production (ppm) using a carbon dioxide monitoring reactor.

3.3.5.6 Effect of UV-C to Pure-Culture Biofilm in Early Stationary-phase

The response of a 72 h *Pseudomonas aeruginosa* PA01 gfp (PA01) biofilm to a 1.5 h UV-C exposure at 73 h, was measured by carbon dioxide production (ppm) and biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}) is shown in Fig. 15. Carbon dioxide production remained within a 4 ppm range after UV-C exposure until 96 h, but increased between 96 – 120

h. Biofilm-derived planktonic cell yield increased throughout the 120 h experiment despite UV-C exposure. The PA01 biofilm response to the 1.5 h UV-C exposure is displayed in Fig. 16, as measured by carbon dioxide production (ppm). There was a decrease in carbon dioxide production from 73 – 73.5 h, when the biofilm carbon dioxide production then increased for the remainder of the UV-C exposure. The response of a 72 h SD-1 biofilm to a 1.5 h UV-C exposure at 73 h (Fig. 17), as measured by carbon dioxide production (ppm) and biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}), demonstrated that carbon dioxide production upon UV-C exposure until 96 h remained static with less than a 1 ppm range between 72 – 96 h. Unlike, the PA01 biofilm whose carbon dioxide production increased by nearly 17 ppm from 96 – 120 h, the carbon dioxide production of the SD-1 biofilm remained within 4ppm of the pre-UV-C exposure value (72 h). The carbon dioxide production (ppm) response (Fig.18) of the SD-1 biofilm during UV-C exposure resulted in a decrease of carbon dioxide production from 74 – 74.25h, when it then responded with continued vigour with carbon dioxide production that surpassed all previous recordings. The biofilm-derived planktonic cell yield from the SD-1 biofilm increased from 24 – 96 h, followed by a decrease to 120 h.

The 24 h static carbon dioxide production rates in both pure-culture biofilms upon UV-C exposure suggests that UV-C affects biofilm metabolism for a period of time. With PA01 carbon dioxide production slightly increasing from 96 – 120 h, it seems the impact of UV-C causes only a short-term suppression in biofilm metabolism, whereas the SD-1 biofilm carbon dioxide production is stationary from 72 – 120 h, suggesting that the SD-1 biofilm is more susceptible to the UV-C. The ever increasing biofilm-derived planktonic cell yield from the PA01 biofilm further lends support to the notion that the biofilm is essentially unaffected by the UV-C exposure, with propagation strategies being unaffected by the imposed stress. Conversely, the SD-1 biofilm-derived planktonic cell yield, peaks at 96 h, followed by a decrease to near pre-UV-C exposure quantities, demonstrating a delayed effect from the UV-C exposure. With the observations obtained from monitoring the metabolic and cell yield effects of UV-C on a biofilm transitioning into less rapid growth, and concluding that the UV-C does not deter biofilm metabolism or cell yield, the implications of UV-C applications to systems known to house biofilms of varying maturity levels, which undoubtedly house biofilms more mature than 72 h, must be considered. Moreover, if UV-C is not effective on a relatively young biofilm, what can its efficacy be on mature biofilms?

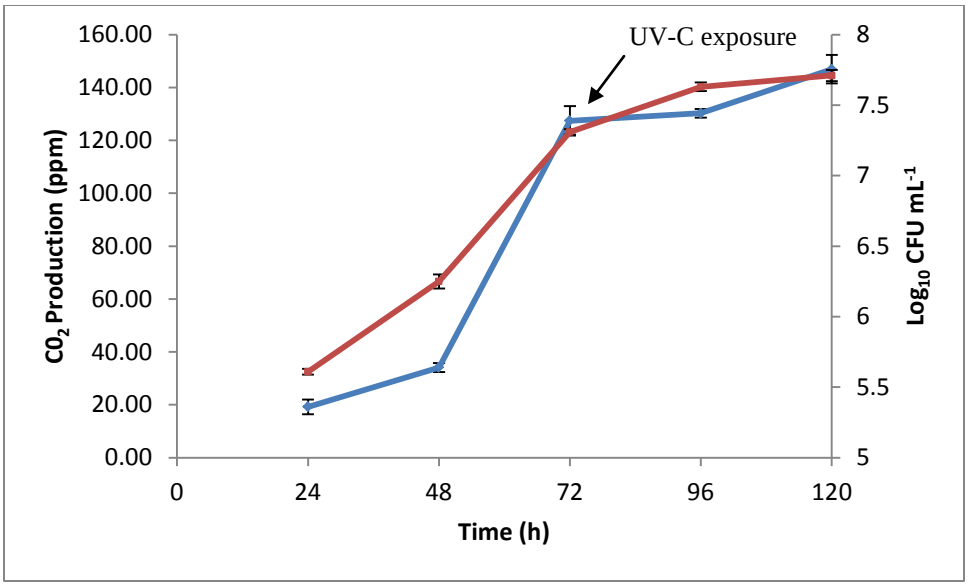

Figure 15: *Pseudomonas aeruginosa* PA01 gfp response to 1.5 h UV-C exposure at 73 h as measured by (◆) carbon dioxide production (ppm) and (■) biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}).

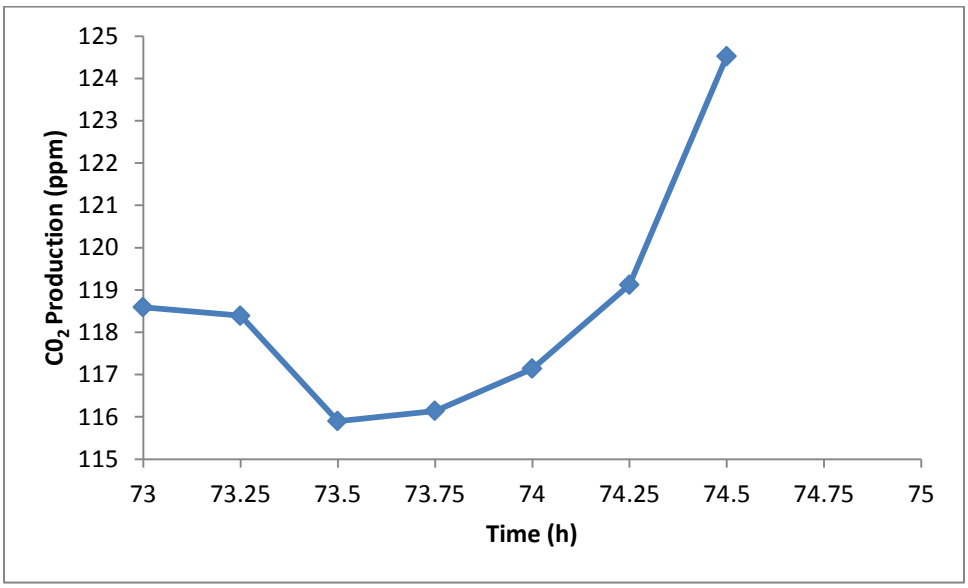

Figure 16: *Pseudomonas aeruginosa* PA01 gfp response to a 1.5 h UV-C application at 73 h, as measured by carbon dioxide production (ppm).

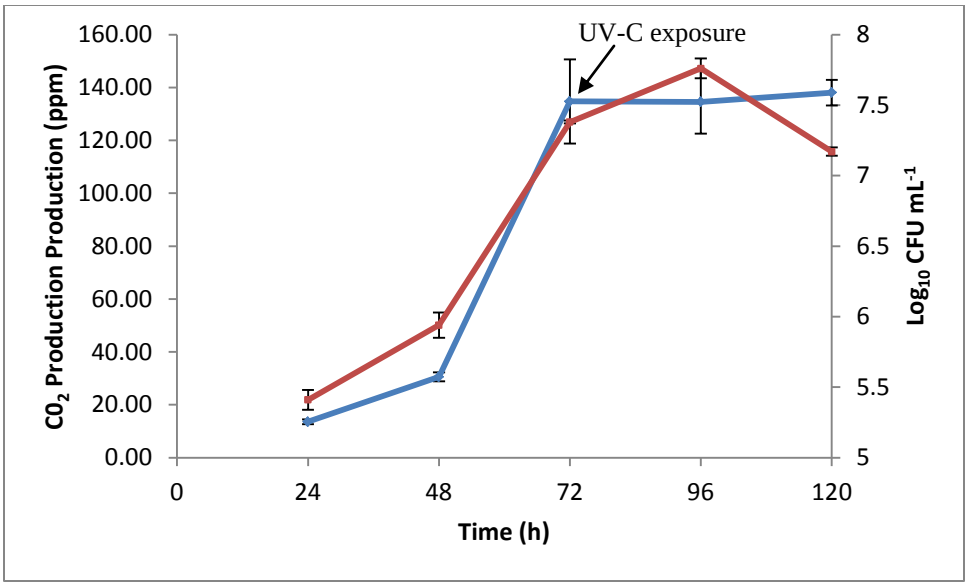

Figure 17: SD-1 biofilm response to a 1.5 h UV-C exposure at 73 h, as measured by (◆) carbon dioxide production (ppm) and (■) biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}).

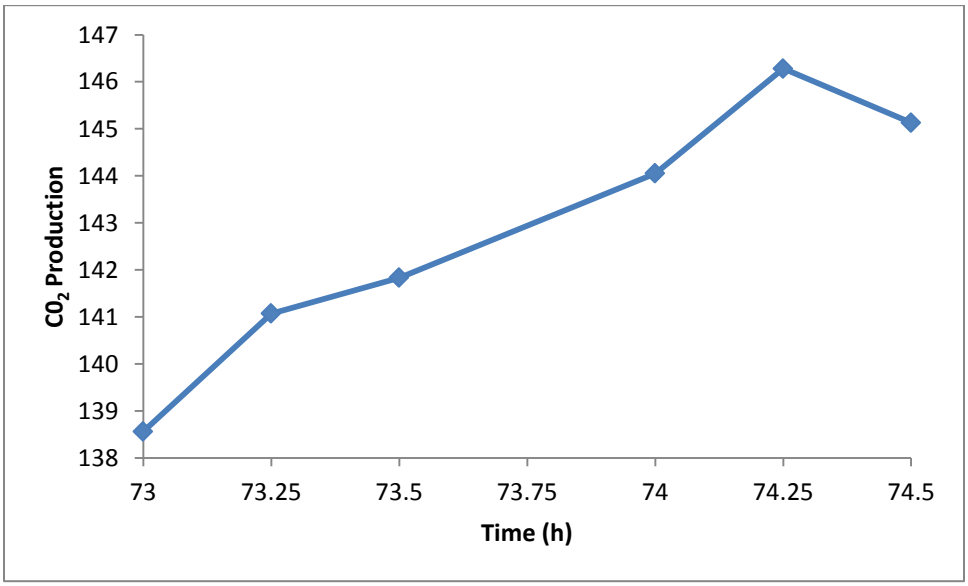

Figure 18: SD-1 biofilm response to 1.5 h UV-C exposure at 73 h, as measured by carbon dioxide production (ppm).

3.3.5.7 Effect of UV-C on Actively Growing Pure-Culture Biofilms

The response of a *Pseudomonas aeruginosa* PA01 gfp (PA01) actively growing biofilm to UV-C exposure at 25 h (Fig. 19) was measured using carbon dioxide production (ppm) and biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}). Fig. 20 displays the PA01 biofilm carbon dioxide production during the 1.5 h UV-C exposure. The responses of a SD-1 actively growing biofilm to a 1.5 h UV-C exposure (Fig. 21) was measured using carbon dioxide production (ppm) and biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}), with the carbon dioxide production response (ppm) to 1.5 h UV-C displayed in Fig. 22.

The effect of UV-C application on actively growing pure-culture biofilms was overall negligible as neither carbon dioxide production nor biofilm-derived planktonic cell yield demonstrated an observable impact from the UV-C application, as measured in their continued increases from 0 – 48 h. Although the 1.5 h UV-C exposure resulted in a decrease of 7 ppm from 25 – 25.5 h in the PA01 biofilm, and the carbon dioxide response of the SD-1 biofilm during UV-C was more dramatic with an immediate increase from 25 – 25.25 h, followed by decreased carbon dioxide production throughout the remaining 1.25 h. Overall, UV-C had no impact on deterring biofilm growth, as both CO_2 and cell yields increased to quantities found in same species biofilms without UV-C, as confirmed from previous experimental data. Moving forward from examining the effects of UV-C on immature and relatively young (72 h) biofilms, determining whether UV-C has an impact on already stressed (carbon-starved) biofilm was the logical next step.

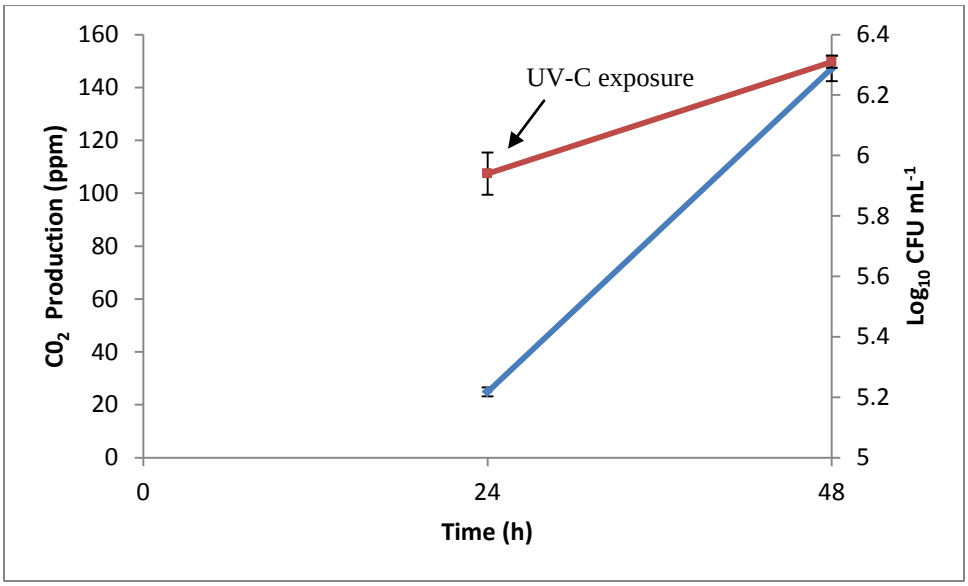

Figure 19: The response of an actively growing *Pseudomonas aeruginosa* PA01 gfp biofilm to 1.5 h UV-C exposure at 25 h, as measured by (◆) carbon dioxide production (ppm) and (■) biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}).

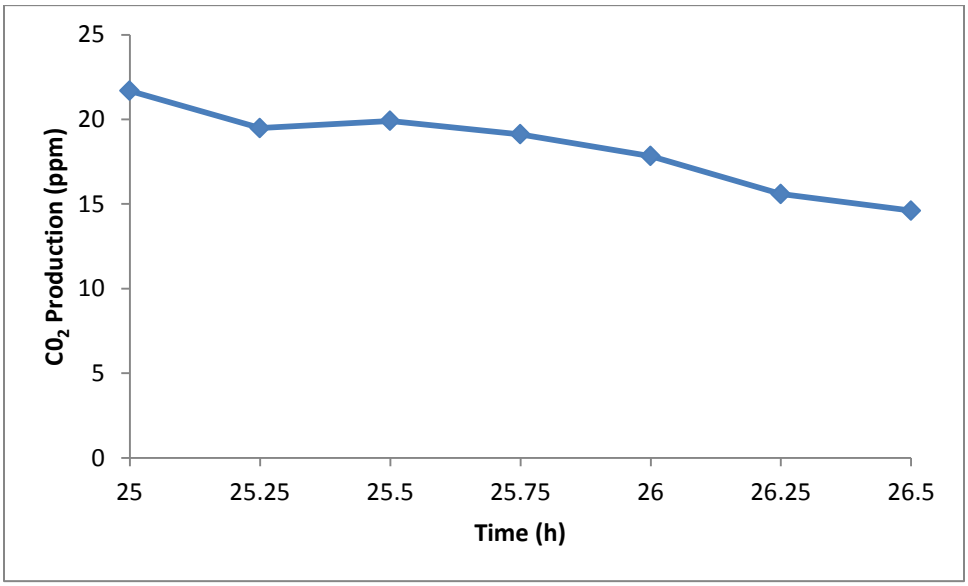

Figure 20: The carbon dioxide response of a *Pseudomonas aeruginosa* PA01 gfp biofilm at 25 h to a 1.5 h UV-C exposure as measured by carbon dioxide production (ppm).

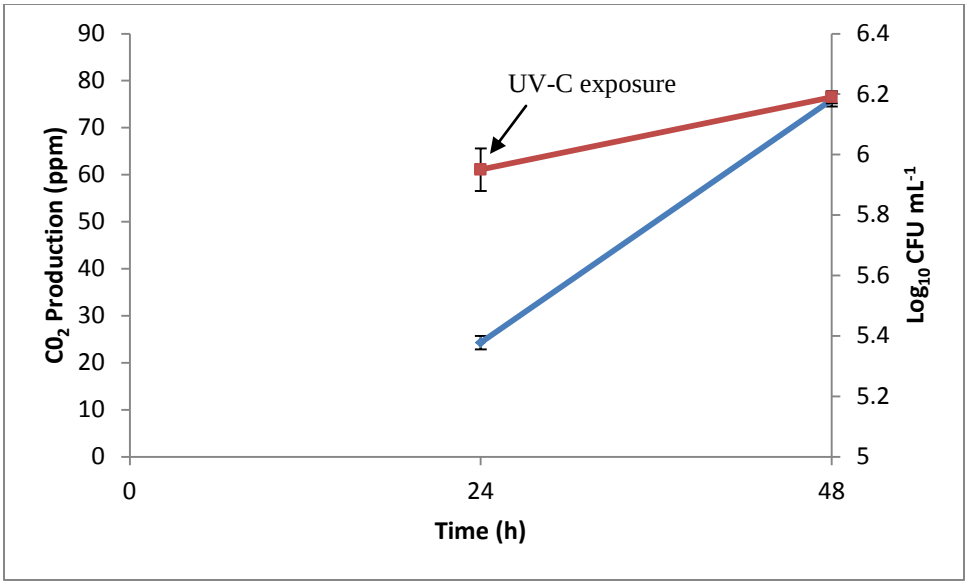

Figure 21: The response of an actively growing SD-1 biofilm to 1.5 h UV-C exposure at 25 h, as measured by (◆) carbon dioxide production (ppm) and (■) biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}).

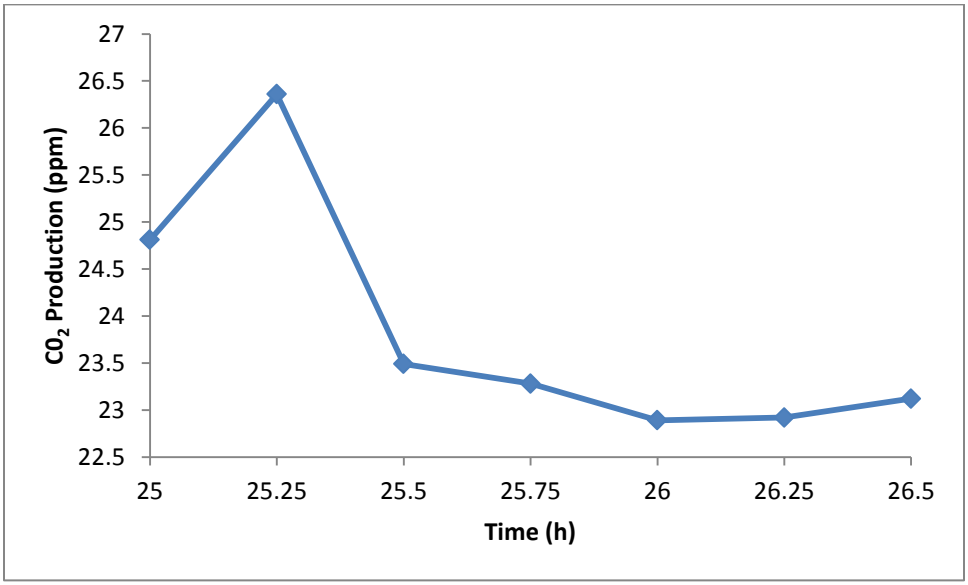

Figure 22: The carbon dioxide response of a SD-1 biofilm to a 1.5 h UV-C exposure at 25 h as measured by carbon dioxide production (ppm).

3.3.5.8 Effect of UV-C on a Carbon-Starved Actively Growing Biofilm

Pseudomonas aeruginosa PA01 gfp (PA01) (Fig. 23) biofilm responses (24 h carbon replete, 24 h carbon-starved, 1.5 h UV-C exposure and carbon reintroduction) were monitored by carbon dioxide production (ppm) and biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}). Carbon dioxide production increased during carbon-starvation (24 – 48 h), while biofilm-derived planktonic cell yield decreased. Each channel was exposed to 1.5 h UV-C at 48-50 h post-inoculation. After exposure, carbon was reintroduced back into the media. The PA01 carbon dioxide production (ppm) during the reintroduction of carbon is displayed in Fig. 24, which demonstrated a decrease in overall carbon dioxide production. The SD-1 biofilm response (24 h carbon replete, 24 h carbon-starved, 1.5 h UV-C exposure and carbon reintroduction) (Fig. 25) were monitored by carbon dioxide production (ppm) and biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}). The carbon dioxide production (ppm) of the SD-1 biofilm during the reintroduction of carbon (Fig. 26) decreased initially from 49 – 49.25 h, when it then remained virtually unchanged until 50 h. The carbon dioxide production (ppm) increased from 24 – 72 h with both pure-culture biofilms, while both pure-culture biofilms resulted in decreased biofilm-derived planktonic cell yield under carbon-starvation conditions (24 – 48 h) with the PA01 cell yields increasing during carbon replete conditions (48 – 72 h). Conversely, the SD-1 biofilm measured a continued decrease in cell yield despite carbon being reintroduced back into the media. Of note, is that unlike previous induced carbon-starvation at times of 72 h, and 120 h, in the current study and performed by Bester *et al.*(2011), carbon-starvation induced at 24 h did not result in the biofilm returning to baseline carbon dioxide production levels. This response is to be further investigated.

Both biofilms were affected by starvation at an early time period (24 h) as shown by nearly static carbon dioxide production (ppm), and decreased planktonic cell yield; however, PA01 rebounded with increased carbon dioxide production and planktonic cell yield, once again demonstrating that a 1.5 h UV-C application did not deter the biofilm with regards to metabolism and cell release. Conversely, the SD-1 biofilm released less cells with the combination of carbon-starvation and cell yield, leading one to postulate that overall SD-1 was more susceptible to the effects of UV-C, than the PA01by these measurement parameters.

The amalgamation of pure-culture biofilm responses to the effectiveness of UV-C exposure beginning with the inhibition of bacterial growth on solid agar, followed by achieving log inactivation in planktonic cultures, concluding with the ineffectiveness of UV-C at deterring biofilm growth in both 24 and 72 h biofilms, the efficiency of UV-C in larger-scale, industrial applications must be considered. UV-C is a physical disinfectant used in an increasing number of water treatment and wastewater decontamination regimes; however, the current study demonstrates that even slight amounts of biofilm growth (not visible to the naked eye) shield the UV-C from inactivating the bacteria, rendering it ineffective as a biofilm control treatment. Of interest is that biofilms in the current study, although constructed of organisms commonly found in DWDS, and / or hospital and domestic sink-drains, were relatively immature compared to biofilms found in the aforementioned environments, especially DWDS. DWDS are known to be colonized by a multitude of microorganisms, and house biofilms to the extent that pipelines and membranes can become blocked and restricted because of biofilm growth. Therefore, the observations obtained from the current study beg the question of how effective UV-C can be in water treatment regimes with regards to combating the effects of mature, well-established biofilms.

To this point, only the SD-1 biofilm upon carbon-starvation and UV-C treatment demonstrated a continued decrease in planktonic cell yield. Biofilm-derived planktonic cell yield is a biofilm propagation strategy (Bester *et al.*, 2011). As such, the perpetual cell yield throughout imposed carbon-starvation demonstrates that biofilm propagation, which may include pathogen dissemination, continues with vigour despite extended periods of nutrient deprivation. Knowing that biofilms are implicated as causative agents in a wide variety of nosocomial and infectious diseases, this proliferation and dissemination strategy clearly solidifies the biofilm stronghold in the role of persistent or chronic disease infections, whether the origin of the biofilm is in hospitals, homes, food processing centers or DWDS.

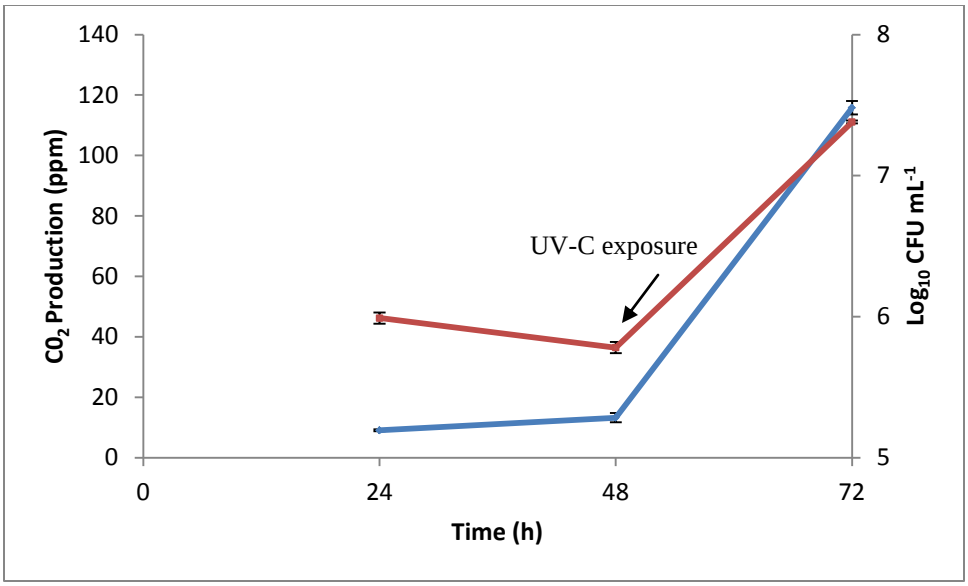

Figure 23: *Pseudomonas aeruginosa* PA01 gfp biofilm response to carbon replete (0 – 24 h), carbon deplete (24 – 48 h), 1.5 h UV-C exposure, and return to carbon replete (50 – 72 h) conditions as measured by (◆) carbon dioxide production (ppm) and ( ) biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}).

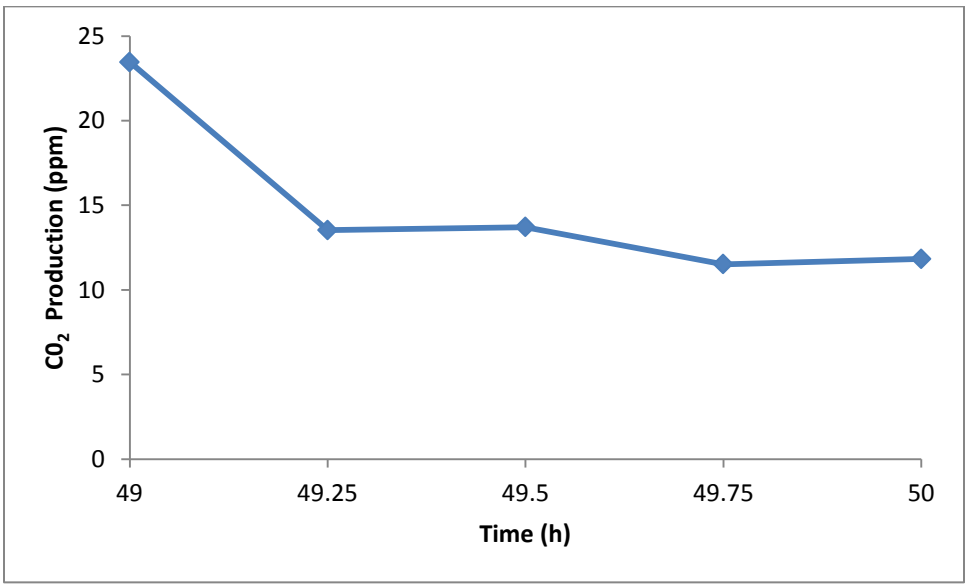

Figure 24: *Pseudomonas aeruginosa* PA01 gfp biofilm response to the reintroduction of carbon at 49 h, after 24 h carbon starvation and a 1.5 h UV-C exposure.

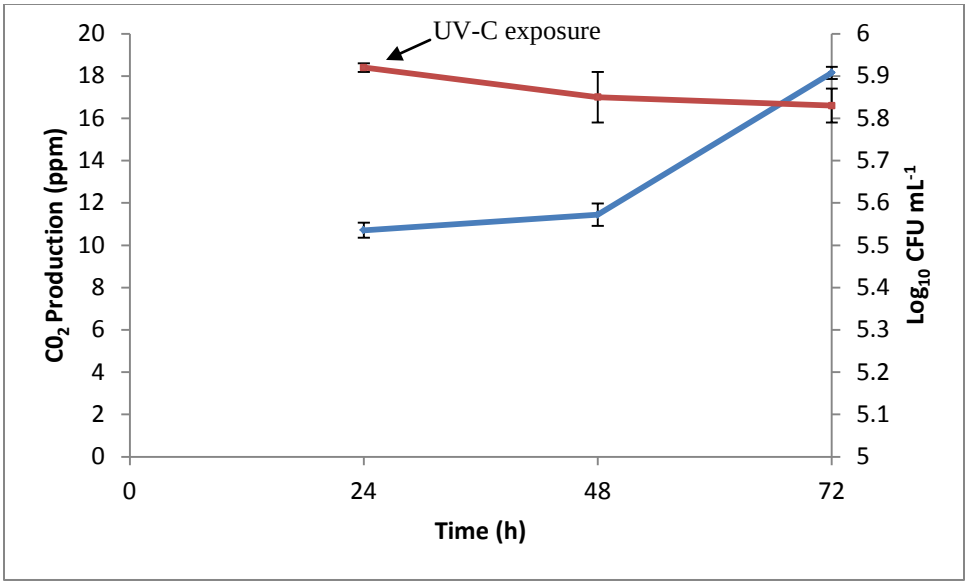

Figure 25: SD-1 biofilm response to carbon replete (0 – 24h), carbon deplete (24 – 48 h), 1.5 h UV-C, and return to carbon replete conditions (50 – 72 h) as measured by (◆) carbon dioxide production (ppm) and (■) biofilm-derived planktonic cell yield (Log_{10} CFU mL^{-1}).

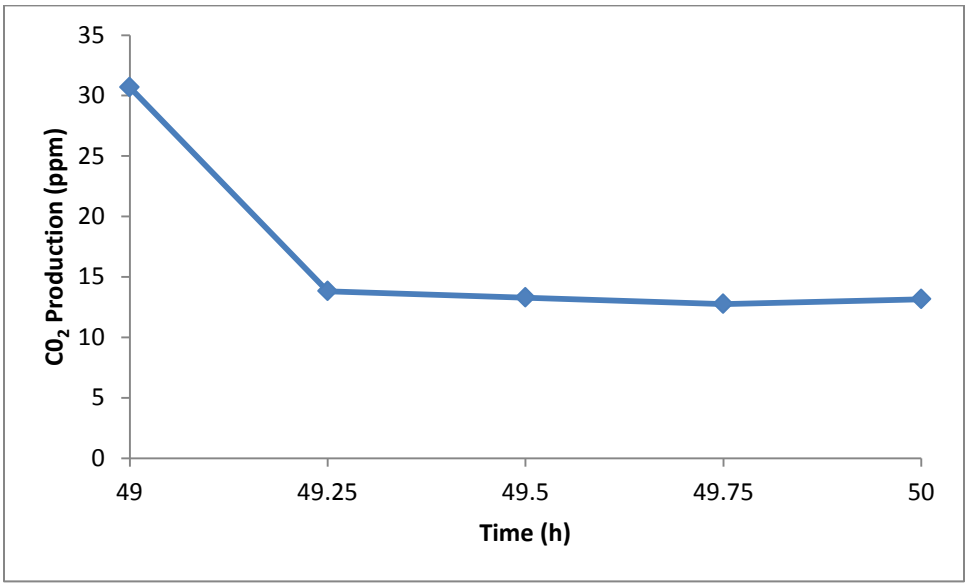

Figure 26: SD-1 biofilm response to the reintroduction of carbon at 49 h, after 24 h carbon starvation and a 1.5 h UV-C exposure.

Chapter 4: Conclusions and Future Directions

This study showed that the metabolic and cell yield responses exhibited by mixed community biofilms to carbon-starvation are similar to those of pure culture *Pseudomonas* sp. CT07::*gfp* and *Pseudomonas aeruginosa* PA01 gfp biofilms, using carbon dioxide production and biofilm-derived planktonic cell yield as measurement parameters. Further, the ability of UV-C to inhibit bacterial growth on solid agar, and result in bacterial inactivation in planktonic cultures was demonstrated. However, UV-C was inept at deterring biofilm growth in 24 h, 72 h, and 24 h-carbon-starved pure-culture biofilms as measured by carbon dioxide production and biofilm-derived planktonic cell yield, despite delivered UV-C doses that exceeded the recommended range as put forward by the USEPA for potable water disinfection.

Future work should include the use of CLSM and image analysis software to monitor the architectural changes of mixed community biofilms under carbon replete and deplete conditions. As the constructed mixed community originated from a sink-drain, subjecting the organisms to stressors other than nutrient deprivation, that are known to occur in sink-drain environments are suggested and include periods of high temperature representing hot water entering the drain, the response to natural cleaning products or disinfectants, and induced periods of desiccation.

Further testing the responses of pure-culture biofilms to induced carbon-starvation is encouraged. Unlike previous experiments with carbon-starvation induced at 72 and 120 h, imposed carbon-starvation at 24 h resulted in a suppression of carbon dioxide production, not the previously observed return to baseline carbon dioxide production values.

Investigating the responses of both pure-culture and mixed community biofilms to nitrogen and phosphorus starvation is suggested. The MSM used in this study was modified specifically to allow for the removal of carbon, nitrogen and phosphorus as discrete components.

Additional recommendations include the application of UV-C under turbulent flow conditions ($Re > 2000$), to test its efficiency at achieving bacterial inactivation, firstly in pure-culture biofilms, and proceeding to mixed community biofilms. Of importance would be the application of UV-C to all constructed community members as pure-culture biofilms to determine whether some members are more susceptible than others to the effects of UV-C. If bacterial inactivation is achieved, the use of denaturing gradient gel electrophoresis (DGGE)

would be useful in tracing which community members are able to withstand the imposed stress and which are not.

Of importance is demonstrating the direct effects of UV-C to a planktonic culture without being transmitted through a quartz glass cover slip. A pre –culture could be vortexed briefly prior to use to ensure cells are planktonic and viable. Following this, a 96-well plate can be seeded with planktonic cells and exposed to varying times of UV-C. The fluorescence imparted by GFP could be measured with a plate reader to indicate the impact of UV-C on biomass. The background signal of MSM medium would need to be measured in addition to the half-life of the GFP protein to verify that the observed fluorescence is emitted from viable cells. If the test organism is not GFP-labelled, OD_{600} could be used to estimate bacterial density. In another approach, an overnight pre-culture could be inoculated into small Petri dishes (without lids), constantly agitated by sterile magnetic stirrers, treated with UV-C exposure of various times, and cell counts could be determined using serial dilutions and plating.

Furthermore, development of a method for inducing carbon-starvation and measuring UV-C exposure is imperative for the analysis of biofilms at solid-air interfaces.

Appendices

Appendix 1: Determination of the Reynolds number for the flowcell

For flow in a channel the Reynolds number is determined by Equation (E2)

$$R_e = \frac{uD}{v} \qquad (E2)$$

where *Re* is Reynolds number, *u* is average velocity of the fluid, *D* is equivalent diameter of the channel, and *v* is kinematic viscosity. A kinematic viscosity for water at 20°C is 1.004×10^{-6} m^2 s^{-1}.

The velocity of the fluid was obtained from:

$$u = \frac{Q}{A} \qquad (E3)$$

From Equation (E4) we obtained

$$D = \frac{4(w * h)}{2\,(w + h)} = \frac{4(4mm * 5mm)}{2\,(4mm + 5mm)} \qquad (E4)$$

Since flow rate *(Q)* value is given by the system configuration, the Reynolds number can be obtained as follows:

$$D = 4.4\ mm \times \frac{1\ m}{1000\ mm} = 4.4 \times 10^{-3} m$$

$$A = w * h = 20.00 mm^2 \times \frac{1\ m^2}{10^6 mm^2} = 2.0 \times 10^{-5}\ m^2$$

$$Q = 17\frac{mL}{h} \times \frac{1L}{1000\ mL} \times \frac{1m^3}{1000\ L} \times \frac{1\ h}{60\ min} \times \frac{1\ min}{60\ s} = 4.72 \times 10^{-9}\ \frac{m^3}{s}$$

$$u = \frac{4.72 \times 10^{-9}\ \frac{m^3}{s}}{2.0 \times 10^{-5}\ m^2} = 2.36\ \times 10^{-4}\frac{m}{s}$$

Thus, the Reynolds number at 20°C is

$$R_e = \frac{2.36\ \times 10^{-4}\ \frac{m}{s}\ (4.4 \times 10^{-5}\text{m})}{1.004 \times 10^{-6}\frac{m^2}{s}} = \ \ 1.03$$

Re = 1.03 which is a Laminar flow

Appendix 2: Determination of the Critical Dilution Rate for the flowcell

The critical dilution rate (D) within the flowcell is calculated as the flow rate of medium (F) divided by the channel volume (V).

$$D = \frac{F}{V} = \frac{17mL/h}{0.8\ cm\text{^}3} \qquad (E5)$$

Appendix 3: Determination of UV-C Fluence (Dose)

y= 114976x where x is the actual radiometer recorded value, and y is mW/cm^2

Radiometer Reading (1 cm)

x = 1.5 x 10-2

Therefore, y = 114976 (1.5×10^{-2}) = 1724.64 $\mu W/cm^2$ = 1.725 x 10-3 W/cm^2 = 1.725 mW/cm^2

$Dose = I * t$ = (1.725 mW/cm^2) * (169.4s) = 291.53 **= 292 mJ/cm^2**

where I is the intensity (mW/cm^2), and t is the exposure time (s).

Appendix 4: Confocal Laser Scanning Microscopy

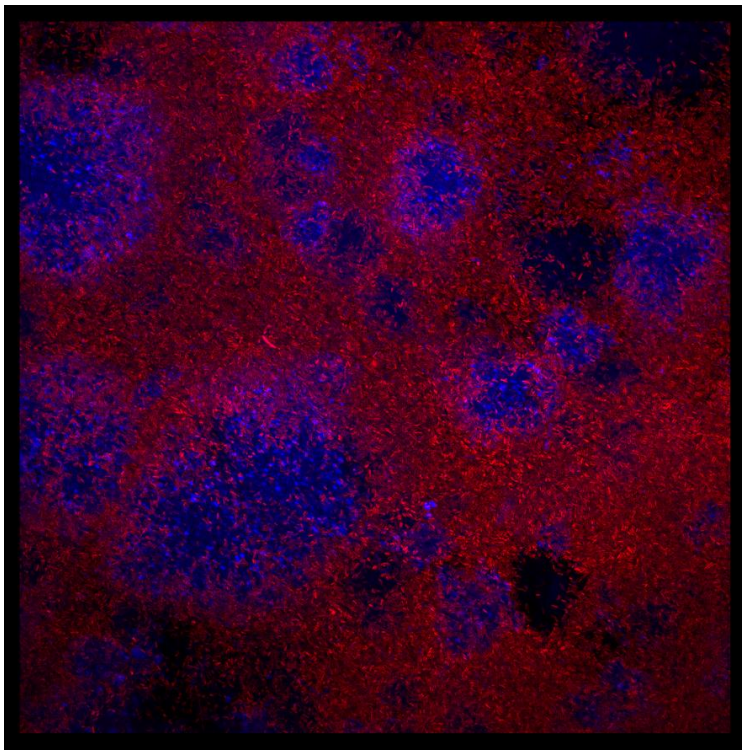

Figure 27: CLSM image of top-view, 168 h mixed community biofilm using a 60 x 1.4 oil immersion objective lens (Nikon Eclipse 89i). Cells stained with ethidium bromide.

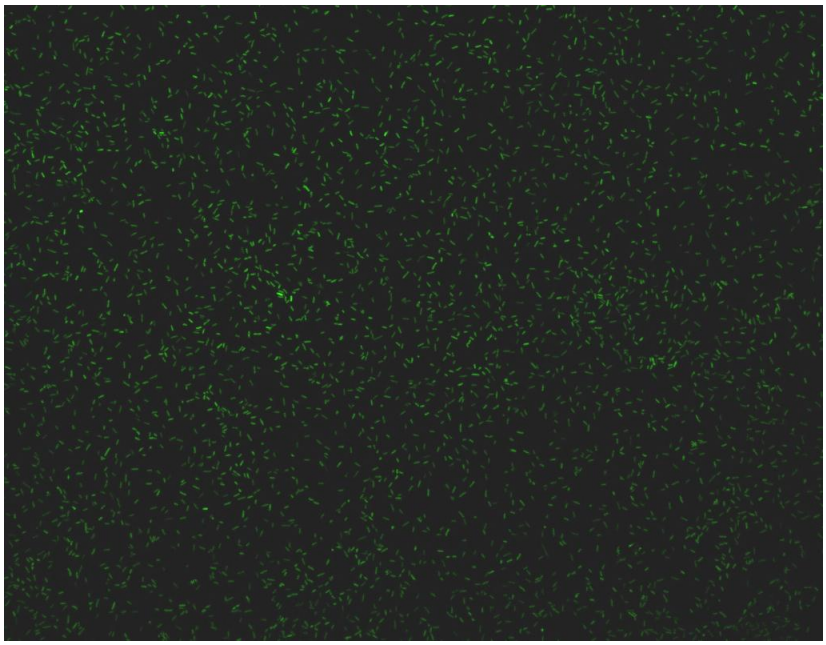

Figure 28: CLSM image of top-view, 168 h *Pseudomonas aeruginosa* PA01 gfp biofilm using a 60 x 1.4 oil immersion objective lens (Nikon Eclipse 89i).

Appendix 5: Determination of Maximum Specific Growth Rate (μmax) and Doubling Time for Community Members

5.1*: Pseudomonas aeruginosa* PA01 gfp

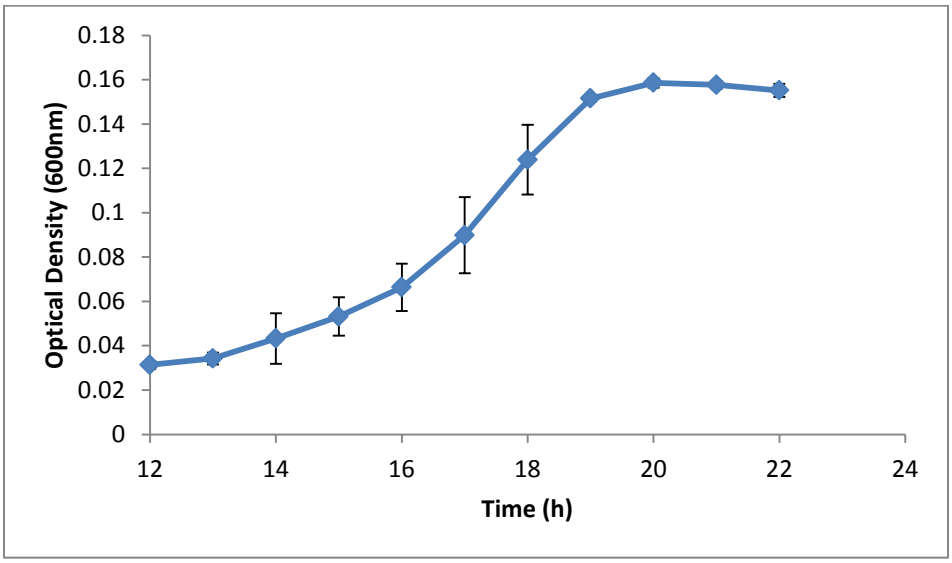

Figure 29: Growth curve of *Pseudomonas aeruginosa* PA01 gfp as measured by optical density at 600 nm; n=6.

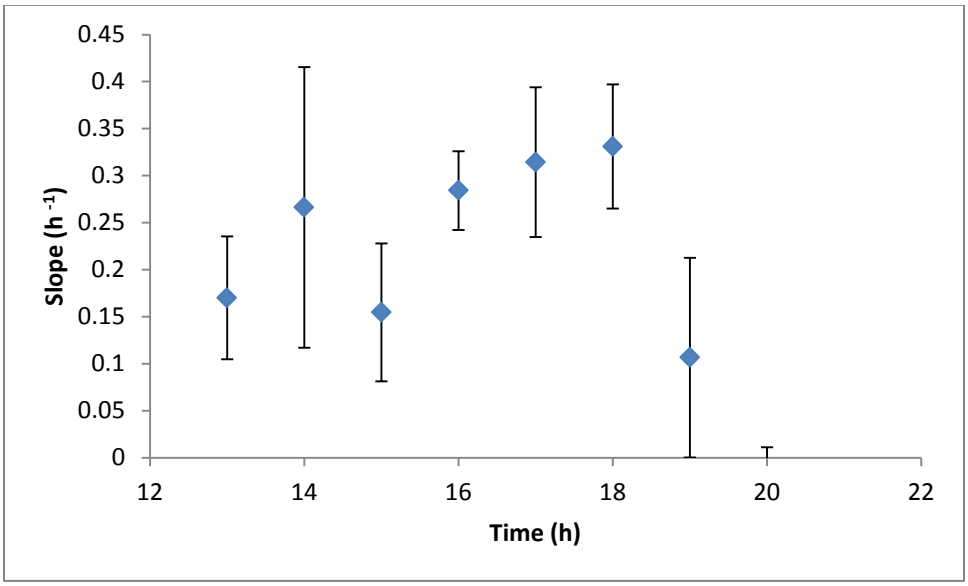

Figure 30: Natural logarithm (ln) of growth curve optical density 600 nm values of *Pseudomonas aeruginosa* PA01 gfp.

5.2: SD-1

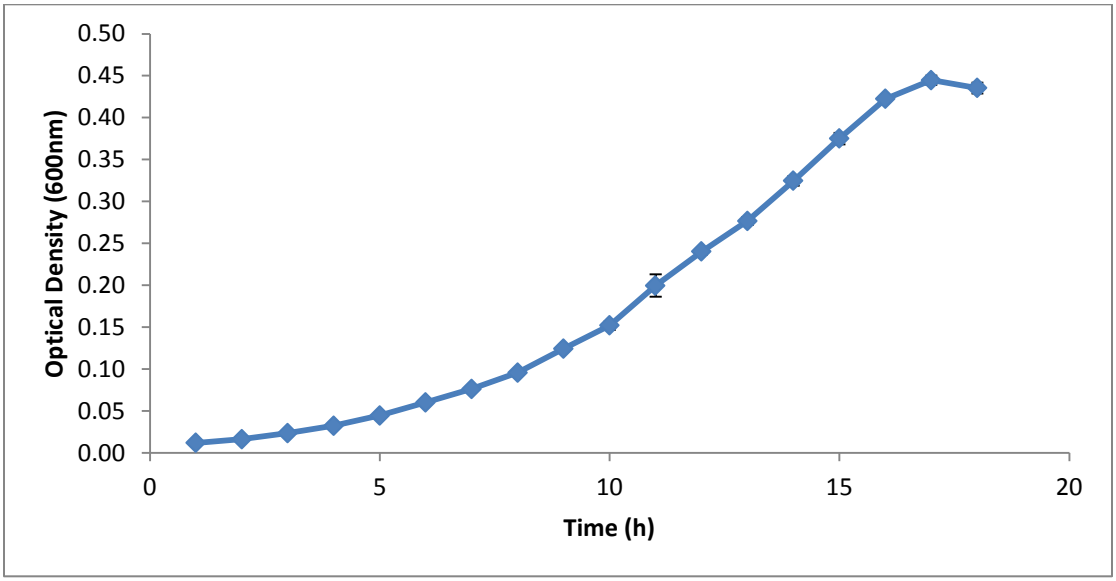

Figure 31: Growth curve of SD-1 as measured by optical density at 600 nm; n=3.

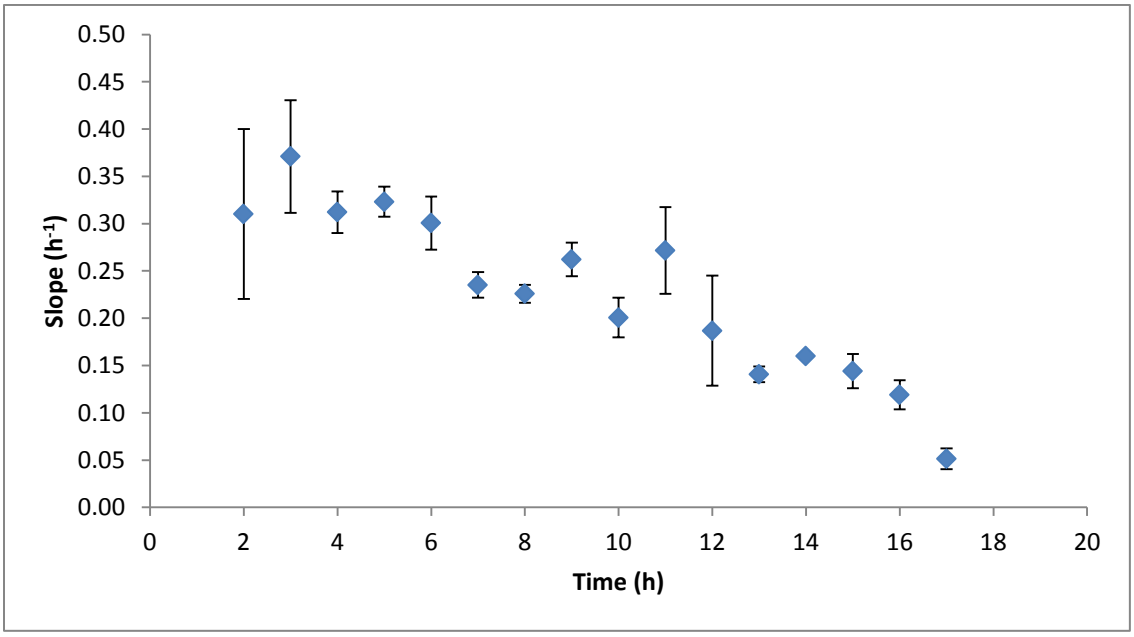

Figure 32: Natural logarithm (ln) of growth curve optical density 600 nm values of SD-1.

5.3 SD-4

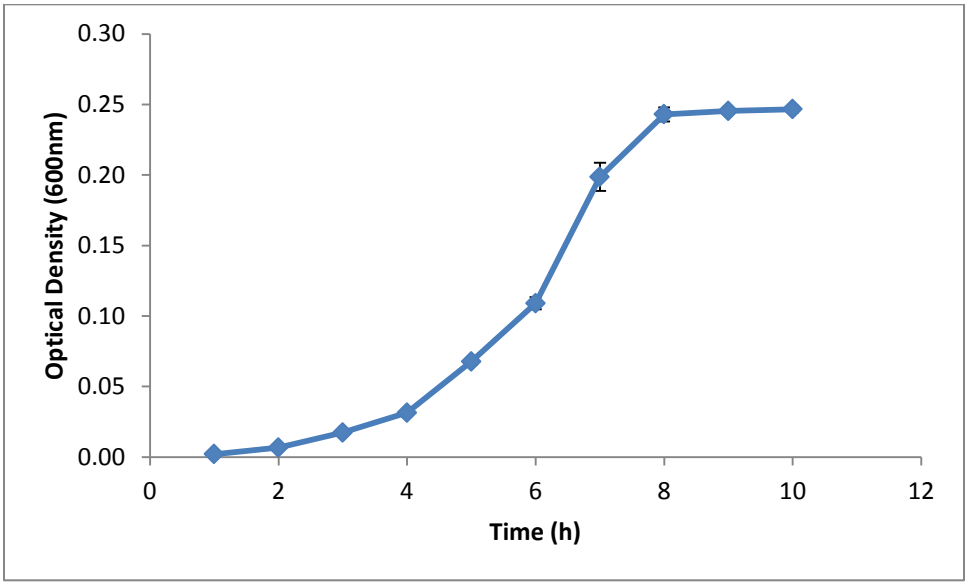

Figure 33: Growth curve of SD-4 as measured by optical density at 600 nm; n=6.

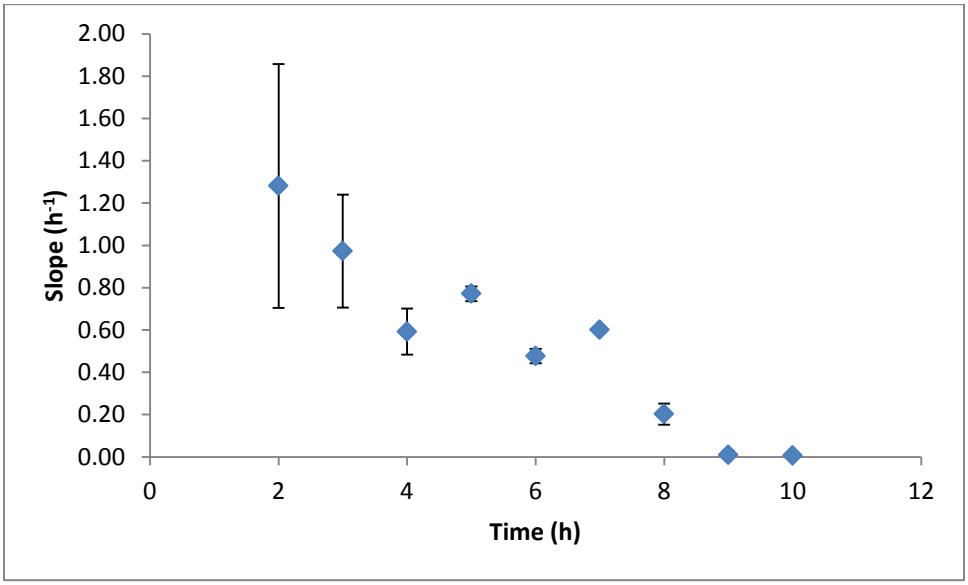

Figure 34: Natural logarithm (ln) of growth curve optical density 600 nm values of SD-4.

5.4: SD-5

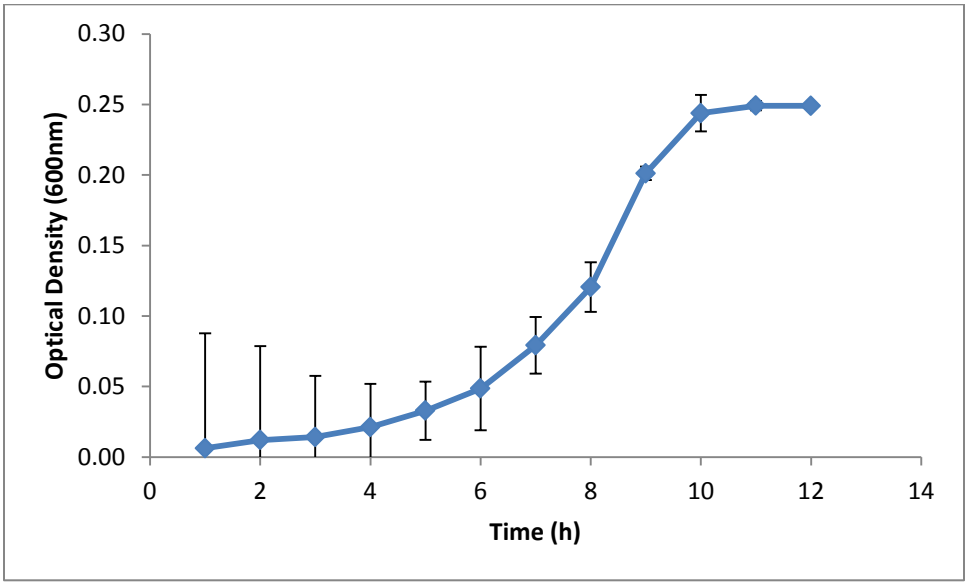

Figure 35: Growth curve of SD-5 as measured by optical density at 600 nm; n=6.

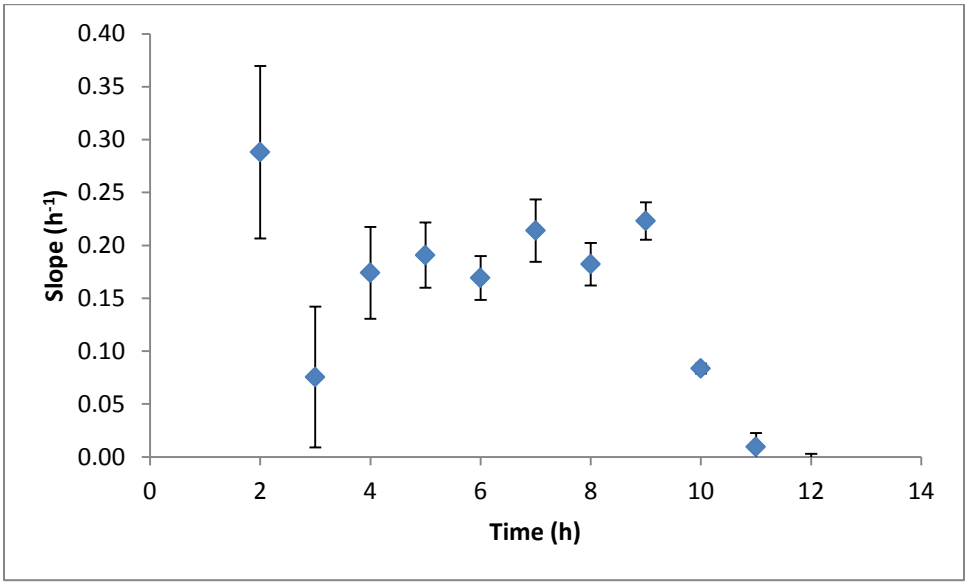

Figure 36: Natural logarithm (ln) of growth curve optical density 600 nm values of SD-5.

5.5: SD-6

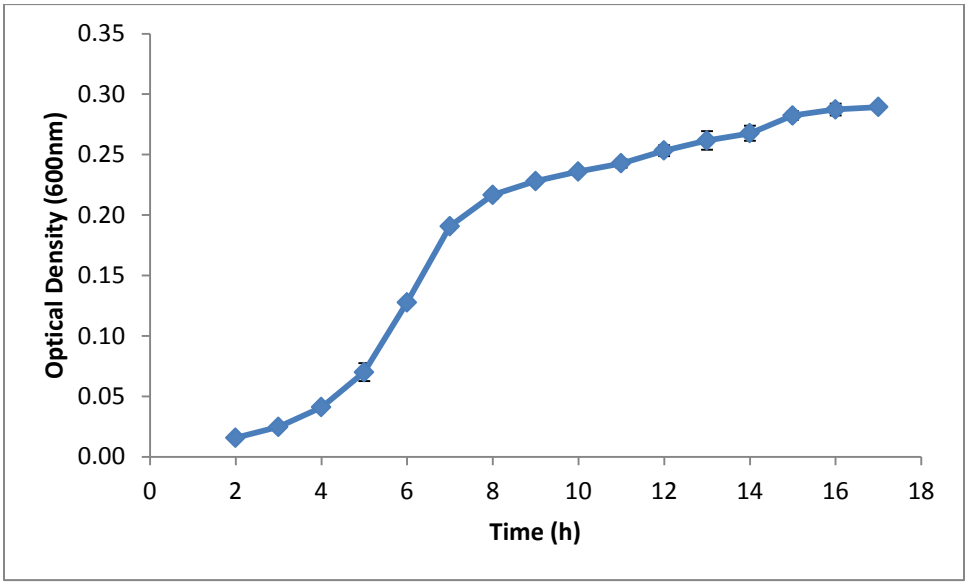

Figure 37: Growth curve of SD-6 as measured by optical density at 600 nm; n=3.

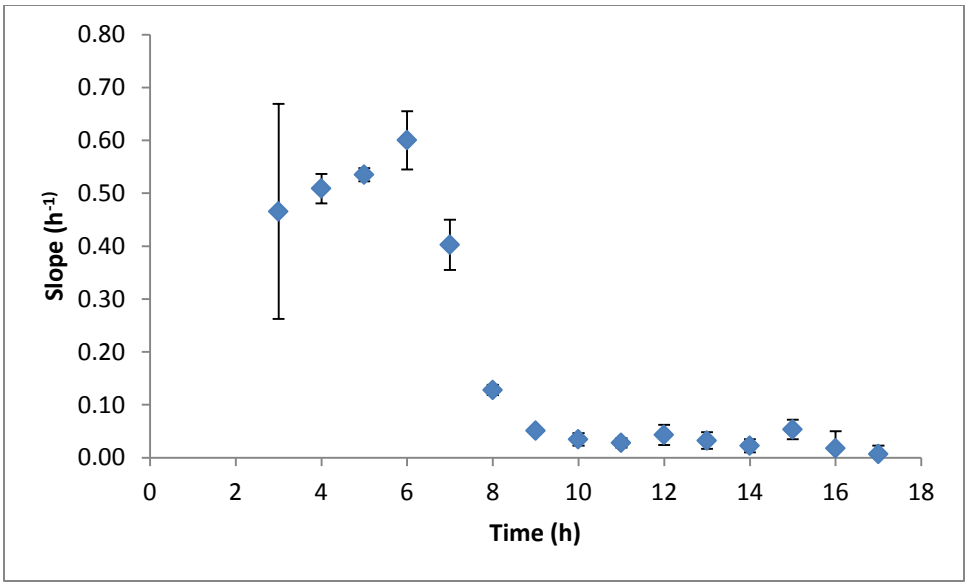

Figure 38: Natural logarithm (ln) of growth curve optical density 600 nm values of SD-6.

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