



The use of photocatalytic thin films to control biofilm growth
by Justin Patrick Gage

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in
Chemical Engineering
Montana State University
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Abstract:

Microbial biofilms form on surfaces exposed to aqueous environments under physiological conditions. Biofilms are often detrimental in industrial and medical environments resulting in reduced heat exchanger and pump efficiencies, enhanced corrosion and medical infections. Methods used to control biofilms such as mechanical cleaning and antimicrobial chemicals are often labor intensive, ineffective and can be detrimental to the environment. A novel method to control biofilms is to generate biocides at the bacteria-substratum interface. The use of photocatalytic thin films as an alternative technology to control *Pseudomonas aeruginosa* (PAOI) biofilm growth was investigated in this study. A dip-coating method was used to deposit TiO₂ thin films with thicknesses of approximately 265 nanometers onto glass slides. UV light, with a wavelength centered around 365 nm, illuminated the photocatalytically active slides with an intensity of 1.0 mW/cm². A drip-flow reactor was used to assess the ability of the TiO₂ thin films to control biofilm growth. Photocatalytic treatment of planktonic PAOI cultures provided a 4 log reduction in the number of viable cells in 3 hours while UV light alone produced less than a 1 log reduction in 3 hours. PAOI biofilms showed initial susceptibility, approximately 1 log reduction, to UV light in the first 24 hours before biofilm growth was observed in the following 48 hours. Compared to UV light alone, the photocatalytic thin films did not enhance killing of the PAOI biofilms. Formic acid and glucose degradation experiments confirm that significant photocatalytic inhibition was caused by components of the *Pseudomonas* basal mineral (PBM) medium used in the biofilm experiments. Results from the present study suggest that mechanisms such as photocatalytic inhibition and/or a biofilm stress response to UV light may protect PAOI biofilms from photocatalytic oxidation.

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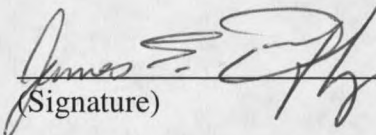
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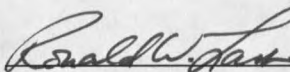
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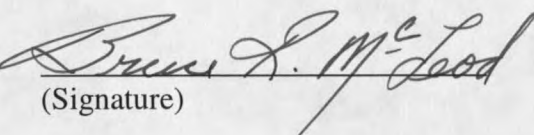
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ABSTRACT

Microbial biofilms form on surfaces exposed to aqueous environments under physiological conditions. Biofilms are often detrimental in industrial and medical environments resulting in reduced heat exchanger and pump efficiencies, enhanced corrosion and medical infections. Methods used to control biofilms such as mechanical cleaning and antimicrobial chemicals are often labor intensive, ineffective and can be detrimental to the environment. A novel method to control biofilms is to generate biocides at the bacteria-substratum interface. The use of photocatalytic thin films as an alternative technology to control *Pseudomonas aeruginosa* (PA01) biofilm growth was investigated in this study. A dip-coating method was used to deposit TiO₂ thin films with thicknesses of approximately 265 nanometers onto glass slides. UV light, with a wavelength centered around 365 nm, illuminated the photocatalytically active slides with an intensity of 1.0 mW/cm². A drip-flow reactor was used to assess the ability of the TiO₂ thin films to control biofilm growth. Photocatalytic treatment of planktonic PA01 cultures provided a 4 log reduction in the number of viable cells in 3 hours while UV light alone produced less than a 1 log reduction in 3 hours. PA01 biofilms showed initial susceptibility, approximately 1 log reduction, to UV light in the first 24 hours before biofilm growth was observed in the following 48 hours. Compared to UV light alone, the photocatalytic thin films did not enhance killing of the PA01 biofilms. Formic acid and glucose degradation experiments confirm that significant photocatalytic inhibition was caused by components of the *Pseudomonas* basal mineral (PBM) medium used in the biofilm experiments. Results from the present study suggest that mechanisms such as photocatalytic inhibition and/or a biofilm stress response to UV light may protect PA01 biofilms from photocatalytic oxidation.

CHAPTER 1

INTRODUCTION

Project Overview

Microbial biofilms form on surfaces exposed to aqueous environments under physiological conditions. The processes occurring during bacterial colonization of solid surfaces can be summarized as the following (1):

- Conditioning of the substratum by organic molecules.
- Transport of microbial cells from the bulk fluid to the solid-liquid interface.
- Adsorption of the cells on the substratum.
- Transformation of reversibly adsorbed cells to irreversibly adsorbed cells.
- Desorption of reversibly adsorbed cells from the substratum into the bulk fluid.
- Growth of irreversibly adsorbed cells.
- Erosion of cells from adsorbed colonies into the bulk fluid.

Technologies used to control biofilms must influence one or more of these processes. These control technologies are employed to combat the negative effects of biofilm formation. For example, biofouling by microbial biofilms adversely affects industrial, municipal and medical systems (2, 3, 4, 5, 6, 7).

The traditional approach for treating biofilms is to introduce doses of antimicrobials into the bulk fluid surrounding the biofilm. However, this approach is often ineffective in eliminating biofilms and often requires the addition of large amounts of chemicals. Technologies that generate oxidizing species without requiring the addition

of chemicals may offer an attractive alternative to current biofilm control methods. Surfaces coated with certain materials are capable of generating reactive oxygen species (ROS) at the biofilm-substratum interface that can subsequently degrade the attached cells. Photocatalysis is a process that utilizes semiconductor materials and UV light to generate ROS. Surfaces coated with photocatalysts are capable of degrading organic compounds, including microorganisms, present in aqueous and vapor phases. This research investigates the use of photocatalytically active surfaces for the control of biofilm growth.

Implications of Biofilms

Microbiology has traditionally only examined bacteria in their planktonic state. However, recently, more attention has been paid to bacteria in their sessile or biofilm state. A bacterial biofilm can be defined as a community of unicellular organisms encased in an exopolysaccharide (EPS) matrix attached to a solid surface (8). Biofilms are responsible for biofouling industrial systems resulting in enhanced corrosion and reduced heat exchanger, membrane and pump efficiencies (5, 7, 9). In the dairy and food processing industries, biofilms can also cause product contamination (4). Biofilms in water distribution systems can harbor coliforms and other microorganisms posing potential health risks (3). Biofilms also cause medical infections associated with catheters, artificial joints and other medical implants (8).

Compared to their planktonic counterparts, bacteria within biofilms can be 10-1000 times more resistant to antimicrobial agents (8). There are many theories to explain

this increased resistance to biocides. The biofilm and its EPS matrix provide a diffusion barrier limiting the penetration of antimicrobials into the biofilm (10). Bacteria in the biofilm state may also transform into a resistant phenotype (10). Nutrient limitation may also induce the biofilm into a state of slow or no growth. Bacteria transitioning from exponential growth to slow or no growth demonstrate an increased resistance to antimicrobials (8). Increased production of enzymes by the biofilm that may degrade antibiotics and sorption of the active biocides into the biofilm matrix may also occur leading to increased biocidal resistance (6). It is likely that multiple mechanisms act in concert resulting in the increased biocide resistance of bacteria in the biofilm state.

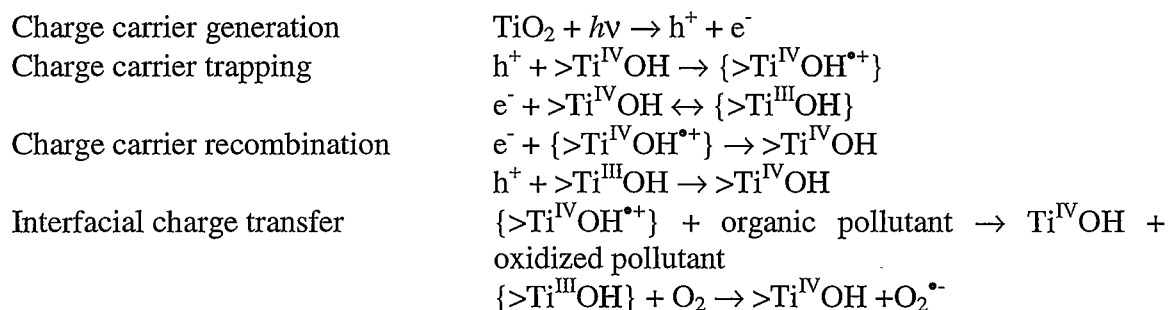
Pseudomonas aeruginosa is a well-studied environmental species that is present in many aqueous and soil environments contaminated with human or animal waste (11). In addition, *Pseudomonas aeruginosa* has been implicated in many medical infections including burn wounds (12), urinary tract infections (13) and respiratory infections in cystic fibrosis (CF) patients (14, 15). It is believed that *P. aeruginosa* exists as biofilm in the lungs of cystic fibrosis patients. Subsequent infections caused by *P. aeruginosa* are thought to be the main factor in CF patient mortality (16).

Overview of Photocatalysis

There is a need for new technologies to remediate hazardous wastes, contaminated groundwater and polluted air. As a result, there has been increased interest and research over the past few decades devoted to advanced oxidation processes (AOP). AOP can loosely be defined as aqueous phase processes based upon the generation of

reactive species such as hydroxyl radicals that are responsible for the degradation of targeted organic compounds (17). Examples of AOPs include ozone, ozone in combination with ultraviolet (UV) light, ozone in combination with hydrogen peroxide, Fenton's reagent, and photocatalysis (18).

Photocatalysis is an AOP that combines common materials and UV light to produce a highly oxidizing environment capable of degrading a wide range of compounds. UV A light, which has wavelengths of 320 to 400 nm (19), is most commonly used in photocatalysis. When a semiconductor (e.g., TiO_2 , ZnO , Fe_2O_3 , CdS , and ZnS) absorbs UV light with energy greater than its band gap, electrons (e^-) are promoted from the valence band to the conduction band simultaneously leaving a positively charged hole (h^+) in the valence band (20). These promoted electrons and positively charged holes can then migrate to the semiconductor surface to participate in oxidation and reduction reactions. The following processes can occur when using TiO_2 as the semiconductor (21).



Redox reactions at the catalyst surface can also produce reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($\text{OH}^\bullet$), superoxide anion (O_2^-) and

perhydroxly radicals ($\text{HOO}^\bullet$) that can also degrade organics present in waters or air streams (22). Studies have indicated that hydroxyl radicals ($\text{OH}^\bullet$) are the reactive species primarily responsible for the degradation of targeted organic compounds (23, 24). Photocatalysis has been used to degrade organics such as acetone, 2-propanol and toluene (25) in air streams and compounds such as salicylic acid (26), monocrotophos (27) and polychlorinated dibenzo-*p*-dioxins (28) in aqueous environments. It has also been used to inactivate biological cells such as bacteria (22, 29, 30, 31, 32, 33, 34, 35, 36), algae (37), coliforms (38) and cancer cells (39, 40, 41). There is some disagreement concerning the bactericidal mechanism of photocatalysis and it likely varies depending on the microorganism studied. Huang et al. (32) have found that the initial oxidative damage occurs on the cell wall, where the TiO_2 catalyst contacts the cells. Following sufficient destruction of the cell wall, oxidative damage focuses on the cytoplasmic membrane. With increased cell permeability, the free efflux of intracellular contents eventually causes cell death. In addition to the effects caused by photocatalysis, UV A light (320 to 400 nm) alone can damage proteins and cell membranes as well as damaging the cell's DNA by producing reactive oxygen compounds such as H_2O_2 and O_2^- (19). Photocatalysis is an attractive technology because it does not require the addition of oxidizing agents and the reaction byproducts are non-toxic. Additionally, the process can operate at ambient temperatures using natural sunlight as the source of UV light.

Titanium Dioxide as a Photocatalyst

Titanium dioxide, TiO_2 , is typically the semiconductor used in photocatalysis due to its low cost, chemical stability and the oxidizing potential of its photogenerated holes (42). TiO_2 can be suspended in aqueous solutions as slurries or immobilized on surfaces as thin films. Many degradation studies have utilized TiO_2 slurries to degrade a wide range of compounds including phenol (43) and formic acid (44) in aqueous environments. Suspensions of TiO_2 powder offer the advantage of increased reaction surface area to volume ratio but usually require separation following photocatalysis to remove the TiO_2 particles (44). Immobilized thin films of TiO_2 require no downstream separation and are often easier to illuminate with UV light than aqueous suspensions (44, 45). TiO_2 thin film preparation commonly uses a TiO_2 solution made via the sol-gel method (46). Thin films of TiO_2 can then be deposited onto glass or other substrates using a dip-coating method (47). Dip-coating solid surfaces with TiO_2 generally involves submersing the solid into an aqueous titania suspension at a controlled rate of speed followed by a firing program to fix the TiO_2 thin film to the substrate. Multiple coatings are applied to obtain the desired thickness of the TiO_2 films.

The photocatalytic activity of the TiO_2 thin films depends on many factors including film thickness and TiO_2 crystal structure. The optimal film thickness depends upon the location of the UV light source. If illuminated from the back side by UV light, the photocatalytic efficiency of the TiO_2 thin film will reach a maximum at a certain TiO_2 thin film thickness. The photocatalytic efficiency will then decrease as thicker films will limit the amount of light reaching the surface layers of titania. The photocatalytic

activity using front-side illumination often reaches a maximum and then remains relatively constant with increasing film thickness. Using front-side UV illumination, Tada and Tanaka (48) found the reaction rate increases monotonically until the film thickness is approximately 150 nm and then remains constant for thicker films. Kim et al. (49) report that the amount of formic acid degraded in a four hour experiment increased until the TiO_2 film thickness reached approximately 470 nm and then remained constant with thicker films.

The crystal structure of TiO_2 , primarily the ratio between anatase and rutile forms, is another important factor in obtaining high photocatalytic activity. The firing temperature and length of firing affects surface area, porosity, crystal size and the ratio between anatase and rutile phases (50). Using an appropriate firing program on the TiO_2 -coated slides will produce thin films with anatase structure, which is more photocatalytically active than rutile (51, 52). It has been shown that firing supported TiO_2 films at temperatures up to 500 °C produced films that were 100% anatase (53).

Surface Generated Biocides

Traditionally, the approach for attacking biofilms is with antimicrobials in the bulk fluid. However, the penetration of the biocide into the biofilm is often retarded due to slow penetration, sorption of the antimicrobial to the EPS matrix or degradation of the biocide by enzymes. As a result, it is common that the bottom portion of the biofilm near the substratum remains intact and resistant to these types of treatment. More efficient

treatment may be obtained utilizing materials that facilitate generation of reactive oxygen species at the substratum. Linkous et al. (37) utilized TiO_2 with and without noble metal cocatalysts to control the attachment and growth of *Oedogonium*, an algae, on cement surfaces in an aquarium. Compared to cement surfaces with no catalyst present, the various TiO_2 catalyst mixtures reduced the growth of algae by 70 to 90 percent when illuminated by both black and fluorescent lamps. Wood et al. (54) exposed polymer disks containing *Pseudomonas aeruginosa* biofilms to various concentrations of hydrogen peroxide and potassium monopersulfate. Incorporating copper and cobalt phthalocyanine catalysts into the polymer disks greatly enhanced the biocidal activity of these oxidizing agents. These catalysts enhance the production of active oxygen species from peroxides and persulfates at the biofilm-substratum interface. In general, the copper and cobalt catalysts provided an additional 2 to 3 log reduction in the number of cells attached to the polymer disks. These studies show that technologies that generate active oxygen species at the substratum may be used to help control and prevent growth of various microorganisms.

Goals and Objectives

In this study, biofilms refer to *Pseudomonas aeruginosa* cells that are attached to the glass slides, unable to be removed by rinsing with phosphate buffer water. The overall goal of this study was to determine if photocatalytic oxidation is capable of removing *Pseudomonas aeruginosa* biofilms from glass slides coated with TiO_2 thin films. To achieve this goal, three objectives were completed.

The first objective was to develop a method for producing titanium dioxide-coated slides and to test their photocatalytic activity by degrading different organic compounds.

The second objective of this study was to assess the degree of photocatalytic inhibition caused by salts and minerals present in the biological medium used in subsequent experiments.

The final objective was to test the abilities of the TiO_2 -coated slides to photocatalytically degrade planktonic *Pseudomonas aeruginosa* (PA01) and to also control biofilm formation on the glass slides.

CHAPTER 2

MATERIALS AND METHODS

Bacteria

Pure cultures of *Pseudomonas aeruginosa* PA01, kindly provided by Michael Franklin of the Department of Microbiology at M.S.U. – Bozeman, were used in this study. Frozen stock of the *P. aeruginosa* were prepared and stored at -70°C in a solution of 20% glycerol and 2% peptone.

Media

P. aeruginosa cultures were grown in *Pseudomonas* basal mineral (PBM) medium (55) (Table 1) that contains glucose as the sole carbon source. R2A agar (Difco Laboratories) plates were used for enumeration of viable microorganisms.

Sterilization Methods

The drip-flow reactors, including reactor system tubing and the TiO_2 -coated and uncoated glass slides, were sterilized prior to use by autoclaving at 121°C for 15-20 minutes. All liquid media of 1 L or less, except for glucose solution, were also autoclaved at 121°C for 25-35 minutes to eliminate biological contamination. The 20 L

PBM Medium Component	Concentration (g/L)	Concentration (mM)
C ₆ H ₁₂ O ₆ (Glucose)	14.4	80.0
K ₂ HPO ₄	12.5	71.8
KH ₂ PO ₄	3.8	27.9
(NH ₄) ₂ SO ₄	1.0	7.57
MgSO ₄ ·7H ₂ O	0.1	0.83
H ₃ BO ₃	0.0012	0.019
ZnSO ₄ ·7H ₂ O	0.00087	0.0030
FeSO ₄ (NH ₄) ₂ SO ₄ ·6H ₂ O	0.00058	0.0015
CoSO ₄ ·7H ₂ O	0.00048	0.0017
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.00011	0.000089
CuSO ₄ ·5H ₂ O	0.000040	0.00016
MnSO ₄ ·H ₂ O	0.000030	0.00018

Table 1. List of *Pseudomonas* basal mineral (PBM) medium components. Concentrations of PBM medium components correspond to full strength dilution (1x).

carboys full of PBM medium were autoclaved at 121 °C for 3-4 hours. The glucose solution was filter sterilized prior to use. Both the polycarbonate and acrylic lids used with the drip-flow reactors were disinfected with a 95% ethanol solution prior to use.

Slide Preparation

An aqueous solution of titanium dioxide (TiO₂) , kindly provided by Professor Marc Anderson of the Environmental Chemistry and Technology Program at the University of Wisconsin – Madison, was used in the slide coating procedure. This aqueous TiO₂ suspension was prepared by adding 74.3 mL of Ti(I-OPr)₄ (Aldrich

Chemical) to a solution of 6.5 mL of concentrated nitric acid in 900 mL of water. This solution was then rapidly stirred for three days and finally dialyzed to a pH of about 3.5 (56, 57). This aqueous solution is a suspension of titanium dioxide with a primary particle size of about 4-5 nanometers. Microscope cover glass slides (Fisher Scientific) with dimensions of 22 mm wide, 60 mm long and 1.5 mm thick were used as the support for the titanium dioxide thin film. These slides were fired in an oven before coating using the following program: heating at a constant rate of 3°C/minute up to 450°C, baking at 450°C for 5 hours, cooling to room temperature at 10°C/minute. A dip-coating method was used to coat the slides with TiO₂ from the aqueous titanium dioxide solution. Figure 1 shows the system used for this dip-coat procedure. A reversible motor connected to a switch and batteries was used to control the withdrawal speed. A withdrawal speed of approximately 9 cm/min was obtained when using 2 D-size batteries. The number of coatings applied was varied depending on the thickness of the TiO₂ layer required. The slides were dried in an oven at 60°C after each coating. Following the final coating, the slides were fired in an oven using the same program that was used before coating.

Scanning electron microscopy (SEM) was then utilized to determine the thickness and uniformity of the titanium dioxide coatings. The slides were fractured at various positions by a diamond-tipped tool. The slides were then fixed to an aluminum mount and sputter coated with gold to a thickness of 10 nm. The samples were then loaded into the scanning electron microscope for analysis and photography. All experiments utilized

slides prepared with 4 coatings that resulted in a TiO_2 thickness of approximately 265 nanometers.

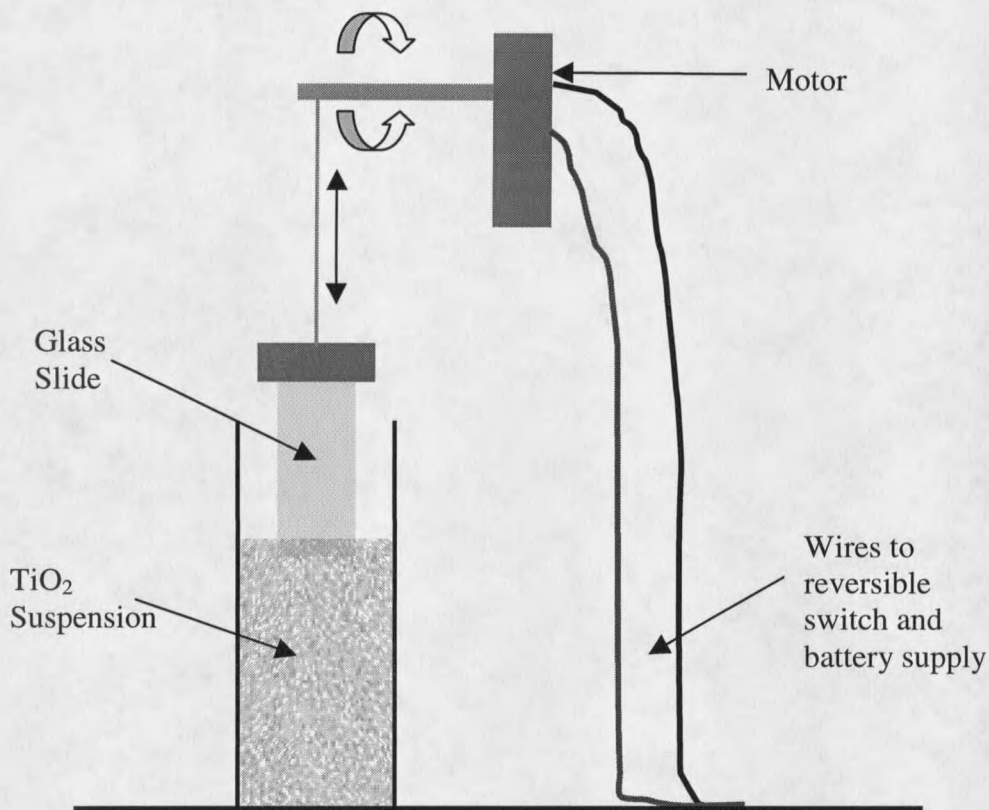


Figure 1. Illustration of system used to dip-coat glass slides with TiO_2 suspension.

Formic Acid Degradation

Formic acid (88% by weight, Fisher Scientific) was used to assess the photocatalytic activity of the coated slides. Formic acid was added to a 1000 mL beaker containing 300 mL of nanopure water to provide a solution that was 10 ppm carbon by weight. Three TiO_2 -coated slides with a total coated area of approximately 30 cm^2 were also placed into this beaker that sat on top of a bank of 5 UV lights (F8T5BL, WKO

Corporation). This batch reactor system (Figure 2) was sparged with oxygen resulting in a dissolved oxygen level of 35 ppm as measured with a DO meter (Hach, model DO175). Solution samples were taken periodically and analyzed with a total organic carbon (TOC) analyzer (Dohrmann, model DC-80). Experiments were also conducted with TiO_2 -coated slides that were first subjected to a continuous flow of PBM medium (1/100x dilution, 1 mL/min for 24 hours) before being placed into the 10 ppm carbon by weight formic acid solution. A control experiment using three uncoated slides was also performed.

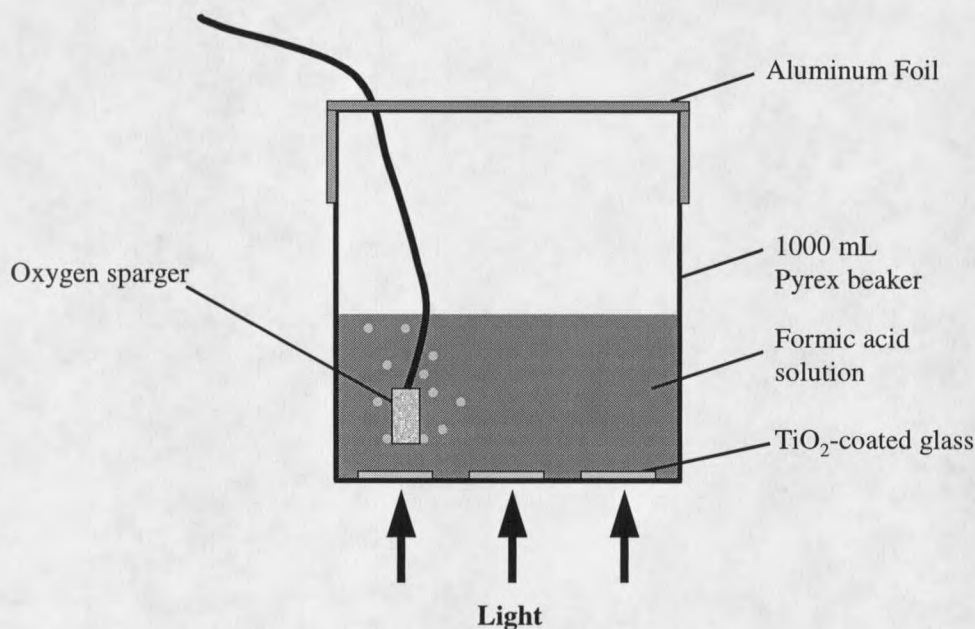


Figure 2. Illustration of experimental system used to assess photocatalytic degradation of formic acid. A 1000 mL Pyrex beaker was filled with 300 mL of a formic acid solution which is 10 ppm carbon by weight.

Glucose Degradation

Degradation experiments using PBM medium were conducted to determine the extent of photocatalytic inhibition caused by components present in the medium. PBM

medium components were added to a 1000 mL beaker containing 300 mL of nanopure water. Glucose (Fischer Scientific) was added to the 1000 mL beaker to obtain a solution that was 10 ppm carbon by weight. Three TiO_2 -coated slides were also placed in the 1000 mL beaker (Figure 3). Liquid samples were taken and analyzed with a Dohrman TOC analyzer to determine the amount of carbon degraded. Several experiments were conducted varying the PBM medium components added as well as medium strength.

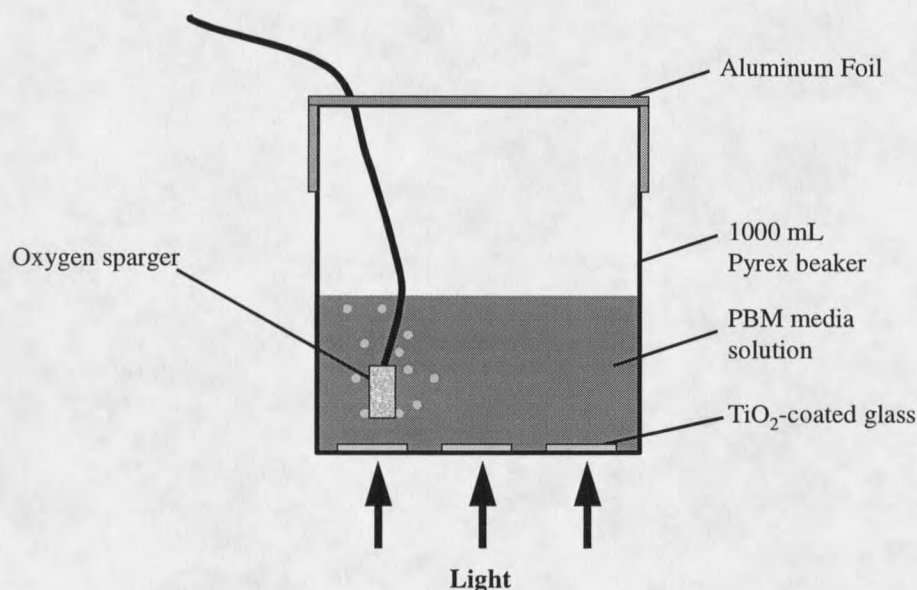


Figure 3. Illustration of experimental system to test photocatalytic inhibition caused by components of the PBM medium.

Planktonic Susceptibility

Planktonic susceptibility experiments started with 25 mL of PBM inoculated with PA01 frozen stock and grown (18-20 hr) at 37°C in an orbital shaker. One mL of this overnight culture was first diluted into 2.0 mL of phosphate buffered water (PBW)

followed by serial dilution into 9.0 mL of PBW to achieve the desired planktonic cell concentration. The glass slides were then inoculated with 100 μ L of the desired dilution of planktonic cells. The slides were placed in a drip-flow reactor that contained PBW to minimize cell dehydration. UV lights were positioned above the drip-flow reactor (Figure 4) such that the UV intensity centered near 365 nm was 1.0 mW/cm² as measured by a photometer (International Light). Polycarbonate drip-flow reactor lids were used in control experiments to eliminate UV light penetration while UV-transparent acrylic lids were used in experiments requiring UV illumination. After UV illumination for a predetermined period of time, the slides were removed, placed into 100 mL beakers containing 20 mL of PBW and vortexed for 45 seconds to remove the cells from the slide surface. A volume of 1.0 mL of this solution was then added to 9.0 mL of PBW. This serial dilution process was continued until the desired dilution factor was achieved. The drop-plate method using 10 μ L volume drops of each dilution was used to determine viable cell counts.

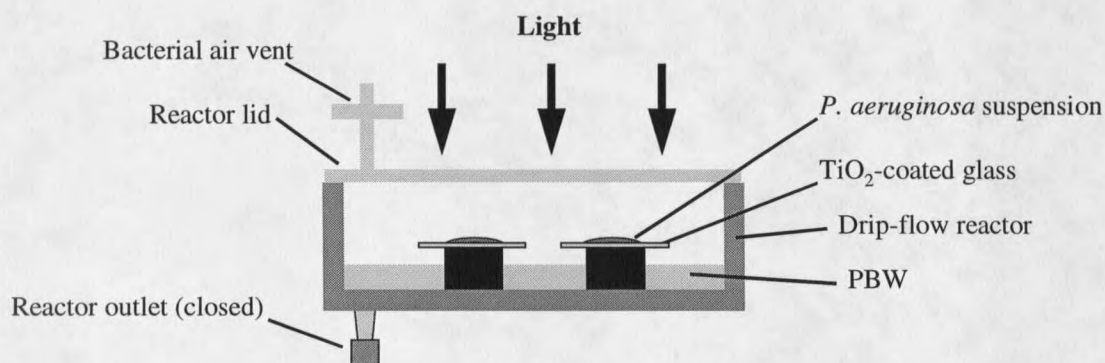


Figure 4. Illustration of the experimental system used to assess planktonic susceptibility to photocatalysis. A suspension of *P. aeruginosa* was placed on top of the TiO₂-coated glass slides that were illuminated from above with UV lights.

Inoculation of Slides for Drip-flow Experiments

PA01 frozen stock was used to inoculate 25 mL of PBM in a shaker flask and allowed to grow for 18-20 hours at 37°C in an orbital shaker. A small volume (1.0 mL) of this overnight culture was then placed into 9.0 mL of PBW and serially diluted to obtain the desired planktonic cell concentration. Drip-flow reactors were used to assess the effects of photocatalysis on biofilm systems. A drip-flow reactor is comprised of 4 segregated flow cells containing both an inlet and outlet. Glass slides were fixed to the bottom of each flow cell with autoclave tape. A volume of 10 µL of planktonic PA01 of the desired dilution was then added to each flow cell of the drip-flow reactor containing 15 mL of full strength PBM medium with 1 g/L carbon as glucose. The reactor was then allowed to sit horizontally for several hours to allow cell attachment and biofilm formation on the glass slide surfaces. The outlets of the reactor were clamped to prevent liquid from exiting the reactor during this initial period (Figure 5).

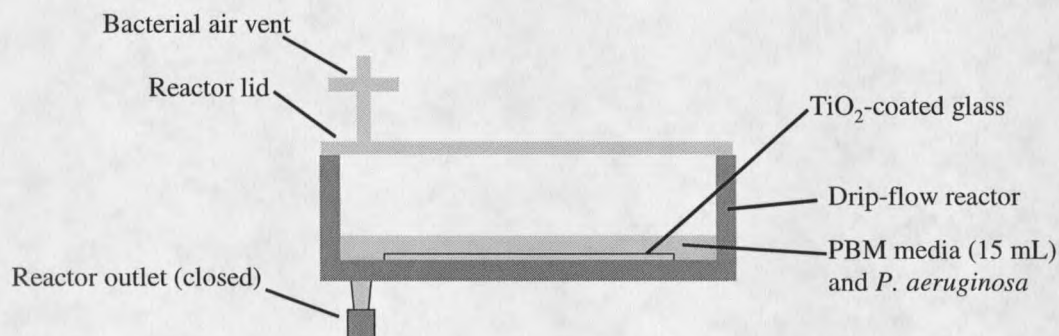


Figure 5. Illustration of drip-flow reactor setup used in initial cell attachment and biofilm formation period. Each flow cell of the drip-flow reactor was inoculated with 15 mL of PBM medium, glucose (1 g/L as carbon) and planktonic *P. aeruginosa* from an overnight culture.

Biofilm Susceptibility

Following this initial cell attachment and biofilm formation period described above, a continuous flow of PBM medium (1/100x dilution) at 1 mL/min was introduced into each drip-flow reactor flow cell (Figure 6). A variable speed pump (Cole-Parmer) was used to deliver the PBM medium from a 20 L carboy to the drip-flow reactor. UV lights were positioned above the reactor so that the light intensity at the slide surface was 1.0 mW/cm^2 as measured with a photometer (International Light). The UV lamps emitted light centered on a wavelength of 365 nm. Polycarbonate lids for the drip-flow reactor were used to eliminate UV light penetration for control experiments while UV-transparent acrylic lids were used to allow full UV light penetration. The UV lights remained on in all experiments to maintain consistent temperatures within the drip-flow chamber.

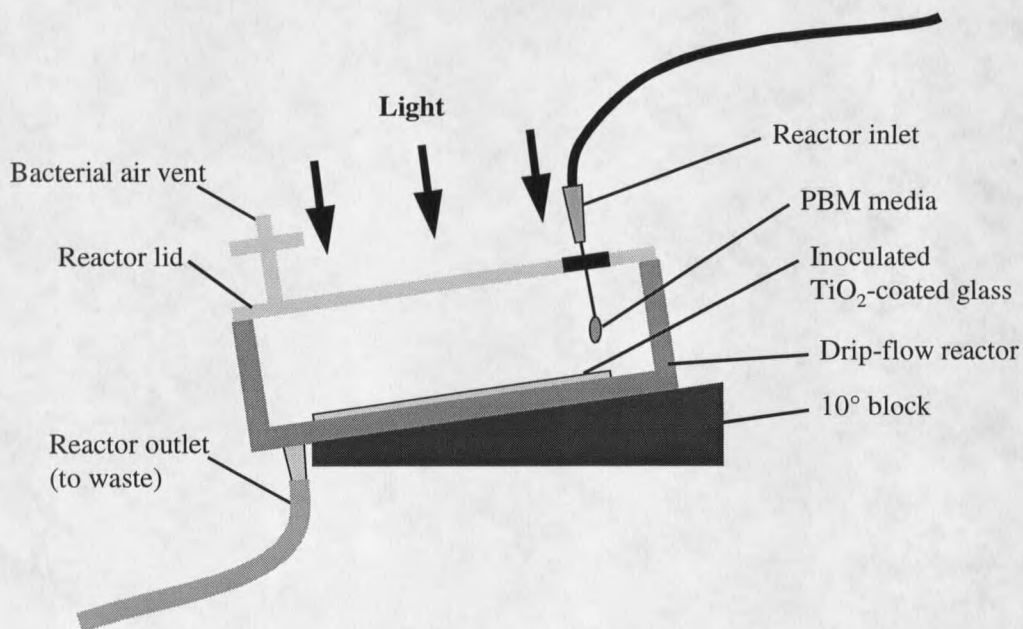


Figure 6. Illustration of angled drip-flow reactor system used to access biofilm susceptibility to photocatalytic oxidation.

Enumeration of Biofilm Cells

The following procedure was used to determine the number of cells attached to the slides. First, the slides were removed from the reactor and rinsed with PBW to remove planktonic and loosely attached cells. The slides were then placed into a petri dish (Fischer Scientific) that contained 20 mL of PBW. The slides were then scraped for 90 seconds with Teflon policemen to remove the cells from the slide surface. The resulting 20 mL of PBW and cells was poured into a 100 mL beaker. This solution was then homogenized at 13,500 rpm for 45 seconds. Finally, the solution was serially diluted and plated on R2A agar plates utilizing the drop plate method (58) to determine viable cell counts.

Horizontal Drip-Flow Reactor

With the drip-flow reactor at an angle, it was noted that coated and uncoated slides were "wetted" differently by the PBM medium. Therefore, similar experiments were also conducted in which the drip-flow reactor was allowed to remain horizontal without using the 10° angle block (Figure 7). After the initial cell attachment and biofilm formation period, continuous PBM medium (1/100x dilution) was pumped at 1 mL/min into each flow cell of the drip-flow reactor. Small pieces of rubber were inserted into the individual flow cells to allow the medium to completely immerse the slides. The sampling procedure used for these experiments was identical to the one used in the other drip-flow experiments.

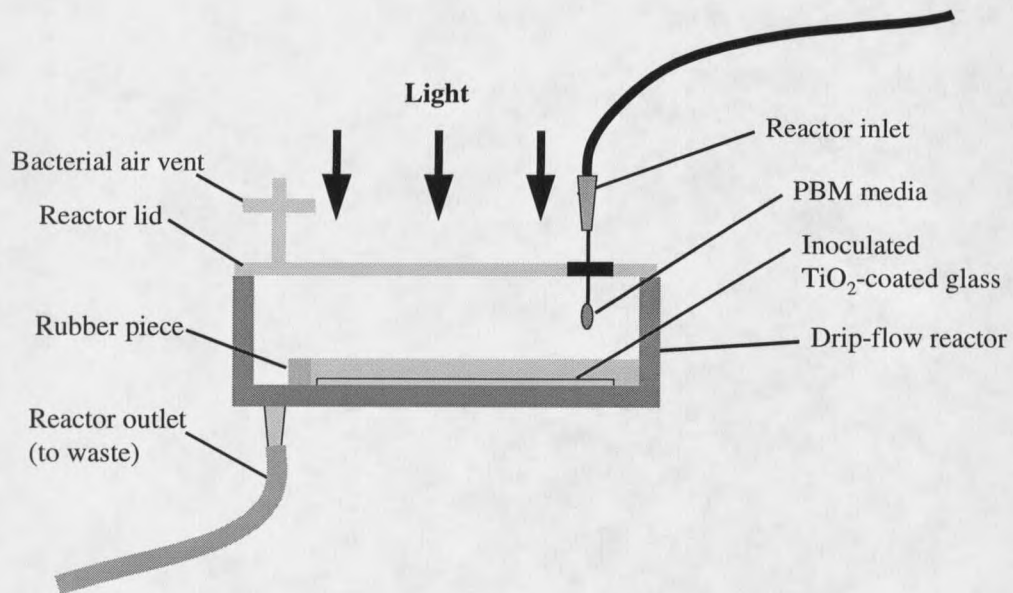


Figure 7. Illustration of horizontal drip-flow reactor system used to access biofilm susceptibility to photocatalytic oxidation.

CHAPTER 3

RESULTS

Slide Preparation

A dip-coating technique was used to fix thin films of titanium dioxide, TiO_2 , to microscope cover glass slides. Scanning electron microscopy (SEM) was used to examine the cross sections of these slides to determine the thickness and uniformity of the TiO_2 layer. Figure 8 below shows the TiO_2 thickness as a function of the number of coatings applied. The TiO_2 layer thickness increased from around 110 nm after two coatings to approximately 420 nm with six coatings applied. This corresponds to approximately 75 nm of thickness added for each coating applied. Figure 9 represents a typical SEM photograph of one of the slides. The apparent size of the TiO_2 agglomerates was approximately 50 nm as estimated from the SEM photographs. The thickness of the TiO_2 layer was found to be very uniform with nearly identical thicknesses found at the top, bottom, and middle of the coated slides.

Formic Acid Degradation

Formic acid degradation experiments were conducted to assess the ability of the TiO_2 -coated slides to photocatalytically degrade a simple organic compound. . The experiments were performed in a batch reactor (Figure 2) and samples were analyzed

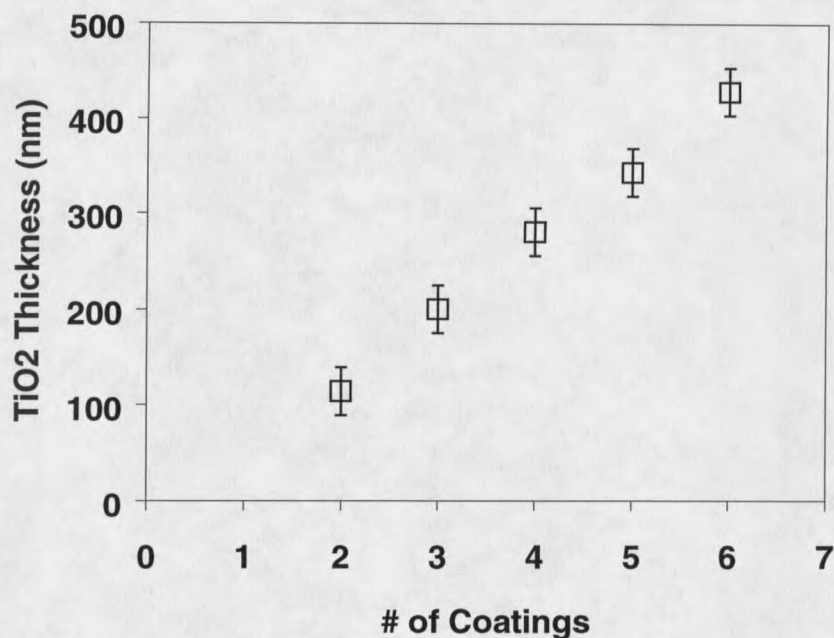


Figure 8. TiO₂ thickness as a function of the number of coatings applied as determined by SEM photography. Error bars represent standard error from duplicate measurements.

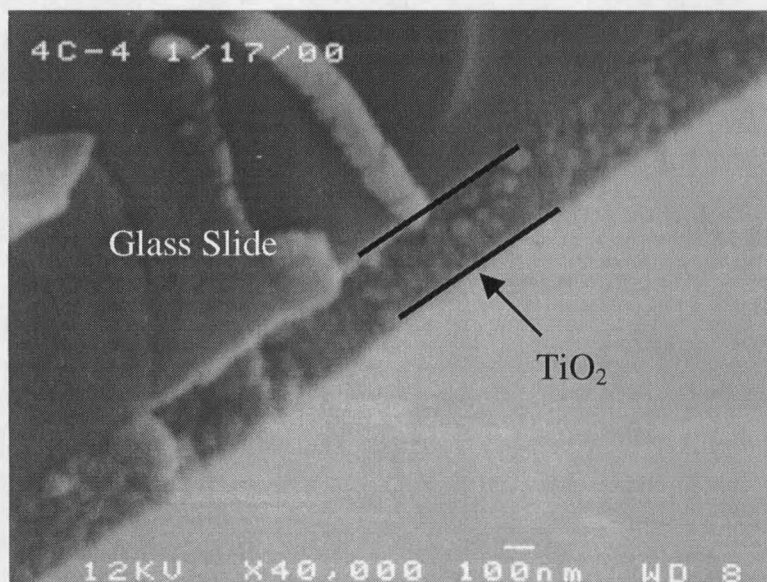


Figure 9. SEM photograph of a cross-section of a TiO₂-coated slide. This slide has been coated 4 times and has a TiO₂ thickness of approximately 250 nanometers. Image and Chemical Analysis Laboratory (ICAL), Montana State University, Bozeman, Montana.

using TOC. Figure 10 shows the results of these experiments. Experiments were also conducted with TiO_2 -coated slides that were first subjected to a continuous flow of PBM medium (1/100x dilution, 1 mL/min for 24 hours) before being placed into the 10 ppm carbon by weight (38.3 ppm formic acid) formic acid solution. The slides subjected to PBM medium and the slides not subjected to PBM medium produced identical degradation of the formic acid after 25 hours. Both resulted in approximately ten percent of total organic carbon remaining in solution after the 25 hours. However, the slides subjected to PBM medium showed much slower initial degradation rates. A control experiment using three uncoated slides was also performed.

Figure 11 shows the results of the first 10 hours of the experiments. In the first four hours, 25.4 ppm formic acid (6.62 ppm carbon) were degraded with photoactive slides present. Considering the TiO_2 catalyst surface area of 30 cm^2 , this corresponds to a degradation rate of $0.063 \frac{\text{mg}}{\text{hr} \cdot \text{cm}^2}$ formic acid ($0.016 \frac{\text{mg}}{\text{hr} \cdot \text{cm}^2}$ carbon). By contrast, 15.2 ppm formic acid (3.97 ppm carbon) were degraded over slides that were pretreated with the PBM medium. This corresponds to a degradation rate of $0.038 \frac{\text{mg}}{\text{hr} \cdot \text{cm}^2}$ formic acid ($0.010 \frac{\text{mg}}{\text{hr} \cdot \text{cm}^2}$ carbon).

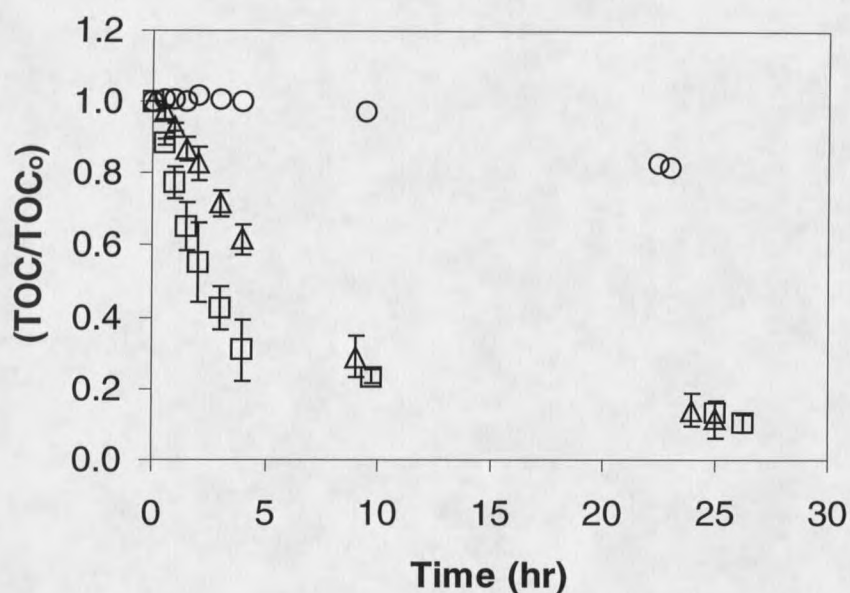


Figure 10. Photocatalytic degradation (25 hour) of a 10 ppm carbon by weight formic acid solution. Data represented by: (□) UV light and TiO_2 -coated slides, (○) UV light only (Δ) UV light and TiO_2 -coated slides after 24 hour PBM medium pretreatment. Error bars represent standard error from duplicate experiments.

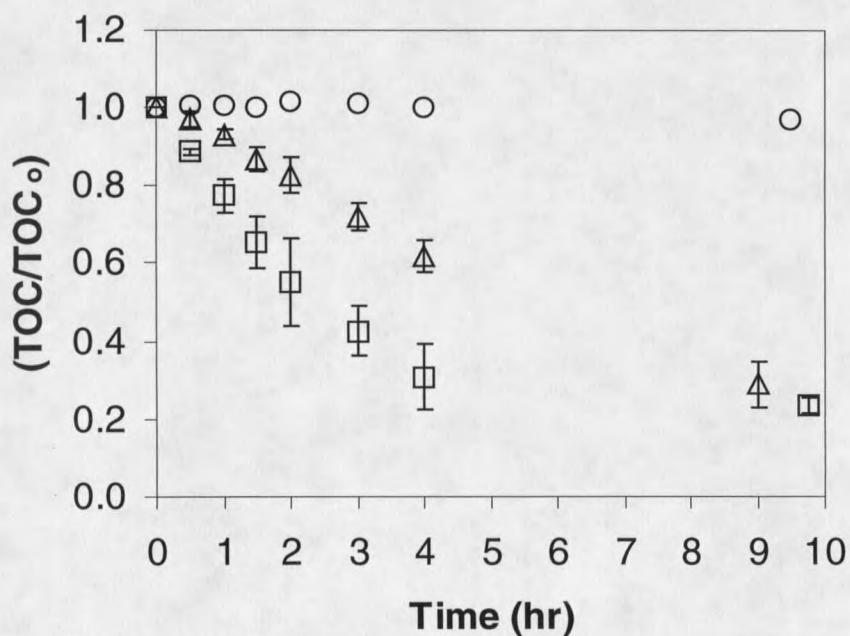


Figure 11. Photocatalytic degradation (10 hour) of a 10 ppm carbon by weight formic acid solution. Data represented by: (□) UV light and TiO_2 -coated slides, (○) UV light only (Δ) UV light and TiO_2 -coated slides after 24 hour PBM medium pretreatment. Error bars represent standard error from duplicate experiments.

Glucose Degradation

Glucose degradation experiments using PBM medium were conducted to determine the extent of photocatalytic inhibition caused by components present in the medium. TiO₂-coated slides were placed in a 500 mL glass beaker containing the various PBM medium solutions. In all cases, glucose was used as the sole carbon source to create a solution which was originally 10 ppm carbon by weight (25 ppm glucose). TOC analysis was used to determine the carbon content of the liquid samples in this batch reactor system. Figure 12 shows the results of these experiments. Without photoactive slides present, the carbon content of the solution remained nearly constant throughout the 24 hour experiment. Approximately 2.8 ppm glucose (1.1 ppm carbon) was degraded in the first 4 hours with the photoactive slides present. This corresponds to a degradation rate of $0.0070 \frac{mg}{hr \cdot cm^2}$ glucose ($0.0028 \frac{mg}{hr \cdot cm^2}$ carbon).

Figure 13 compares the amount of glucose degraded in 24 hours when different components and concentrations of the PBM medium were present. Approximately 22 ppm or 88 percent of the glucose was degraded in 24 hours with no PBM medium components present. Roughly 12.5 ppm or 50 percent of the glucose was degraded when the phosphate components of the PBM medium were added at a concentration of 0.2 mM. With the entire PBM medium present at 1/500x dilution, which contains 0.2 mM phosphate, 12.8 ppm or 51 percent of the glucose was oxidized in the same 24 hour period. With the PBM medium present at 1/500x dilution without the phosphates present, 21.5 ppm or 86 percent of the glucose was degraded in 24 hours. Reducing the

PBM medium strength to 1/5000x dilution resulted in 15.0 ppm or 60 percent of the glucose being degraded. Finally, increasing the PBM medium strength to 1/50x dilution resulted in 10.5 ppm or 42 percent of the glucose being degraded in 24 hours.

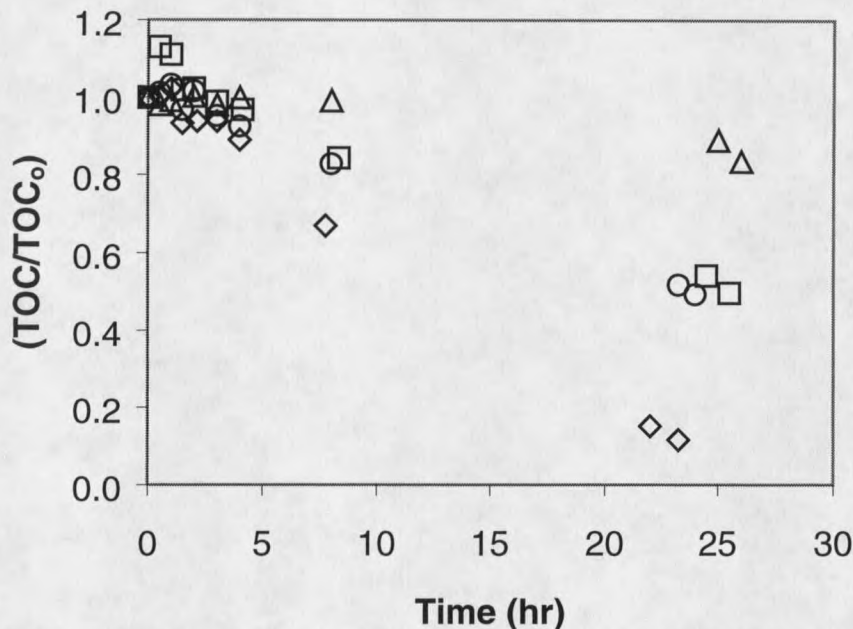


Figure 12. Photocatalytic degradation of glucose with PBM medium components present. Data represented by: (□) Phosphates only, (○) Entire PBM medium, (Δ) UV light only, (◇) No PBM medium components.

Planktonic Susceptibility

Experiments were conducted on planktonic PA01 suspensions to determine the bactericidal effectiveness of the photocatalytic thin films. Two sets of experiments were conducted varying the number of planktonic cells initially present on the slide surfaces. Figure 14 shows the results of experiments in which the slides were inoculated with approximately $10^{3.6}$ cells. The detection limit for these experiments was 10 CFU per

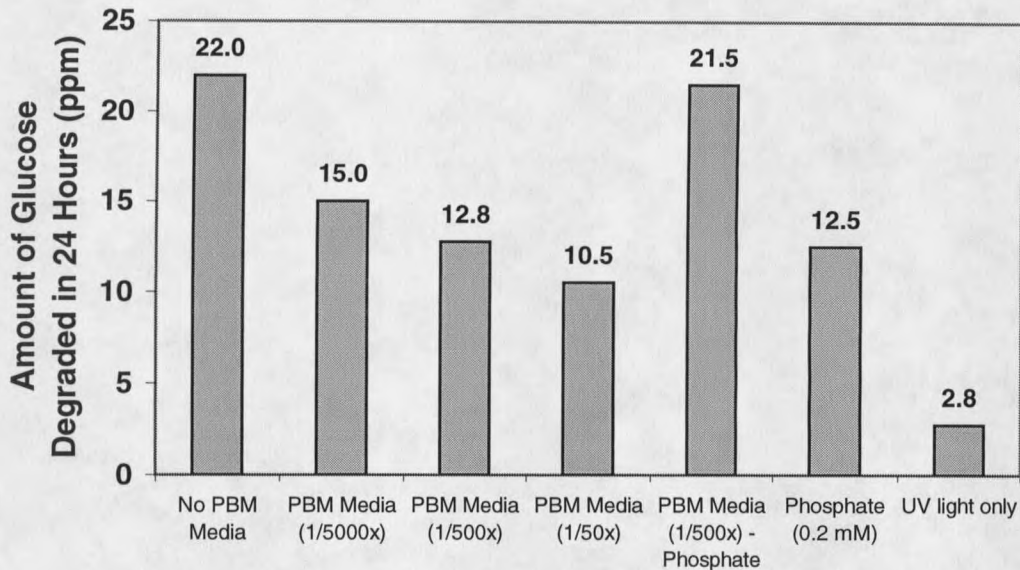


Figure 13. Effects of PBM medium on the photocatalytic degradation of glucose. Initial concentration of glucose was 25 ppm which corresponds to 10 ppm carbon. 1/50x, 1/500x, 1/5000x correspond to PBM medium dilution factor.

slide. The photoactive slides reduced the viable cell counts from $10^{3.6}$ CFU per slide to less than the detection limit in one hour. This corresponds to 99.7% killing (2.6 log reduction) of the PA01 cells in 1 hour. UV light alone reduced the viable cell counts by 16% (0.08 log reduction) in the same 1 hour time period. In 4 hours, UV light reduced the PA01 cell counts to less than 10 CFU/slide.

Figure 15 reports the results of similar experiments conducted with approximately 10^6 cells initially present per slide. The detection limit was 100 CFU per slide for these experiments. The photoactive TiO_2 -coated slides reduced the viable CFU per slide from 10^6 to less than the detection limit in 3 hours. This corresponds to 99.99% killing (4 log reduction) of the planktonic cells in 3 hours. In the first 2 hours, 99.8% (2.8 log reduction) of the cells were inactivated. UV light alone reduced the viable cell counts by 39.7% (0.22 log reduction) in 2 hours and by 93.4% (1.2 log reduction) in 4 hours. In all

planktonic experiments, TiO₂-coated slides without UV light did not reduce the viable cell counts of PA01.

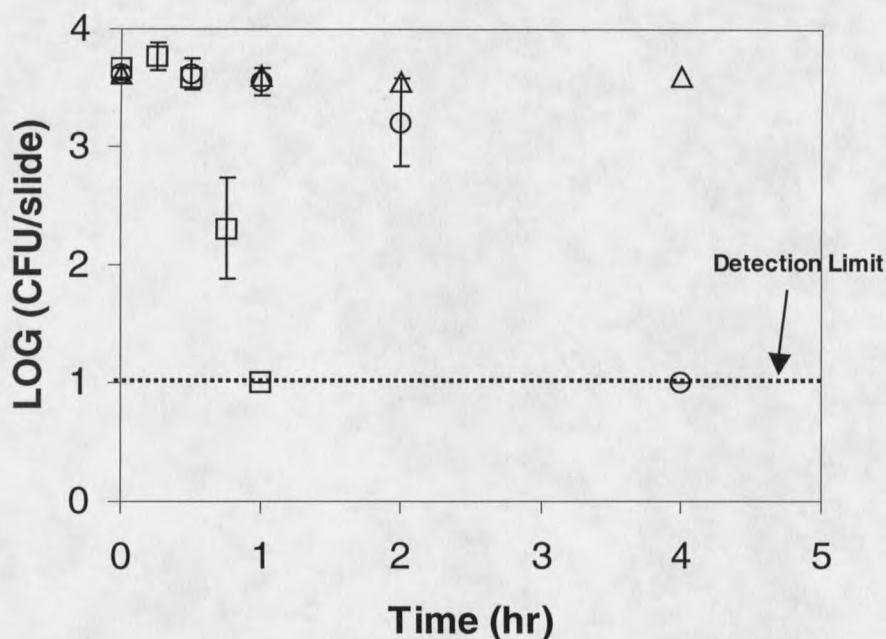


Figure 14. Photocatalytic degradation of planktonic PA01 ($\sim 10^{3.6}$ CFU/slide). Data represented by: ($\square$) UV light and TiO₂-coated slides, ($\circ$) UV light only, (Δ) No UV light. UV light (360 nm) intensity at slide surface was 1.0 mW/cm². Error bars represent standard error from duplicate experiments.

Biofilm Growth Curve

Glass slides were inoculated with diluted planktonic PA01 from an overnight culture as described previously. The amount of planktonic cells added to each flow cell as well as the time allowed for biofilm formation was varied. After several hours, the slides were removed and the bacteria were scraped into 20 mL of PBW. The resulting PBW was then homogenized and serially diluted and plated to determine the number of CFUs present on the slides. Figure 16 shows the results of these studies. Using 1000 μ L

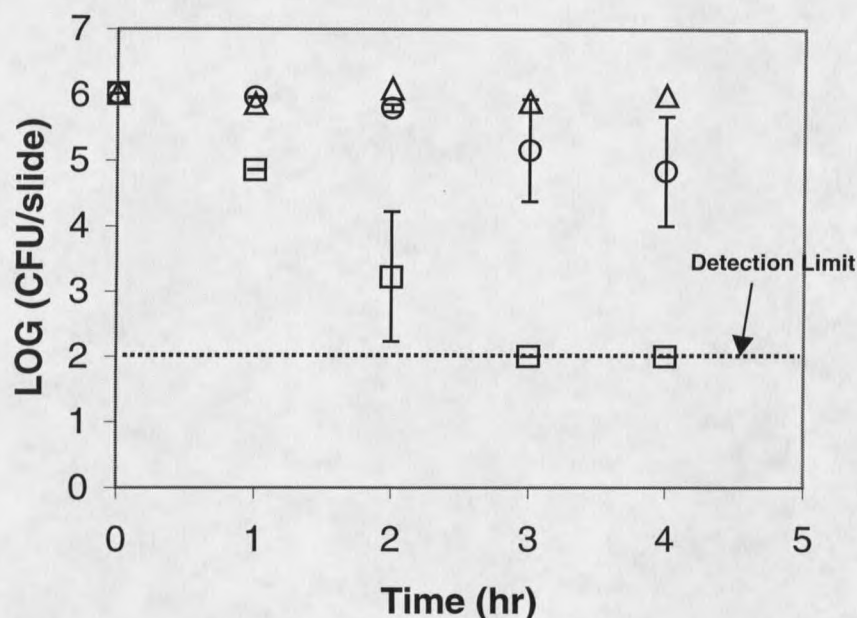


Figure 15. Photocatalytic degradation of planktonic PA01 ($\sim 10^6$ CFU/slide). Data represented by: (□) UV light and TiO₂-coated slides, (○) UV light only, (Δ) No UV light. UV light (360 nm) intensity at slide surface was 1.0 mW/cm². Error bars represent standard error from duplicate experiments.

of overnight PA01 culture resulted in $10^{7.6}$ CFU/slide after 1 hour of seeding time up to $10^{8.9}$ CFU/slide after 24 hours of seeding time. Using 10 μ L of overnight culture resulted in $10^{6.8}$ CFU/slide after 7 hours while using 0.1 μ L of overnight culture produced $10^{3.7}$ CFU/slide after 4 hours of seeding time.

Biofilm Susceptibility

Following the initial cell attachment and biofilm formation period, a continuous flow of PBM medium (1/100x dilution) at 1 mL/min was introduced into each drip-flow reactor flow cell containing the glass slides. Following the specified time, the slides were removed and the bacteria were scraped into 20 mL of PBW. The resulting mixture was

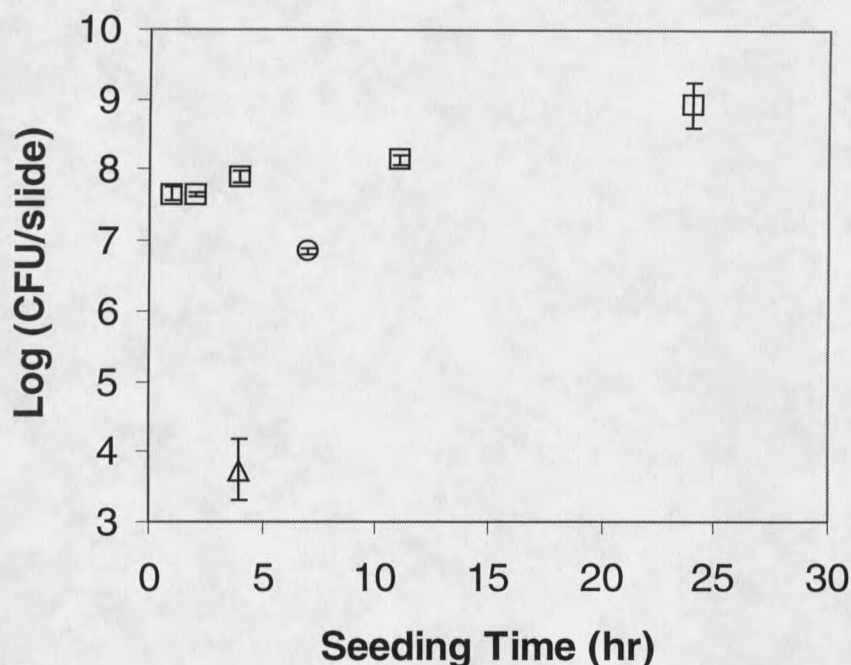


Figure 16. PA01 biofilm growth curve. Data represented by: (□) 1000 µL overnight culture, (○) 10 µL overnight culture, (Δ) 0.1 µL overnight culture. Overnight culture of PA01 was $10^{9.8 \pm 0.05}$ CFU/mL. Error bars represent standard error from triplicate experiments.

then homogenized and serially diluted and plated to determine the number of CFUs present on the slides. Figure 17 shows the results of 72 hour experiments performed in angled drip-flow reactors. TiO_2 -coated slides subjected to UV light reduced the viable CFU per slide from $10^{4.19}$ to $10^{3.43}$ after 24 hours before the CFU per slide increased to $10^{5.71}$ after 72 hours. Uncoated slides subjected to UV light reduced the viable CFU per slide from $10^{4.15}$ to $10^{3.23}$ after 24 hours and similarly increased to $10^{5.68}$ after 72 hours. TiO_2 -coated slides without UV light exposure showed no initial decrease in viable CFU per slide and increased from $10^{4.21}$ to $10^{8.26}$ after 72 hours. Uncoated slides without UV light showed very similar results with the viable CFU per slide increasing from $10^{3.94}$ to $10^{8.12}$ after 72 hours.

Figure 18 shows the results of identical experiments conducted over 24 hours. TiO_2 -coated slides in concert with UV light reduced the viable CFU per slide from $10^{4.19}$ to $10^{2.44}$ after 8 hours before the CFU per slide increased to $10^{3.43}$ after 24 hours. UV light alone decreased the viable CFU per slide from $10^{4.15}$ to $10^{3.23}$ after 24 hours. TiO_2 -coated slides without UV light produced a steady increase in viable CFU per slide from $10^{4.21}$ to $10^{6.36}$ after 24 hours. Likewise, uncoated slides with no UV light exposure allowed the viable CFU per slide to increase from $10^{3.94}$ to $10^{6.03}$ after 24 hours.

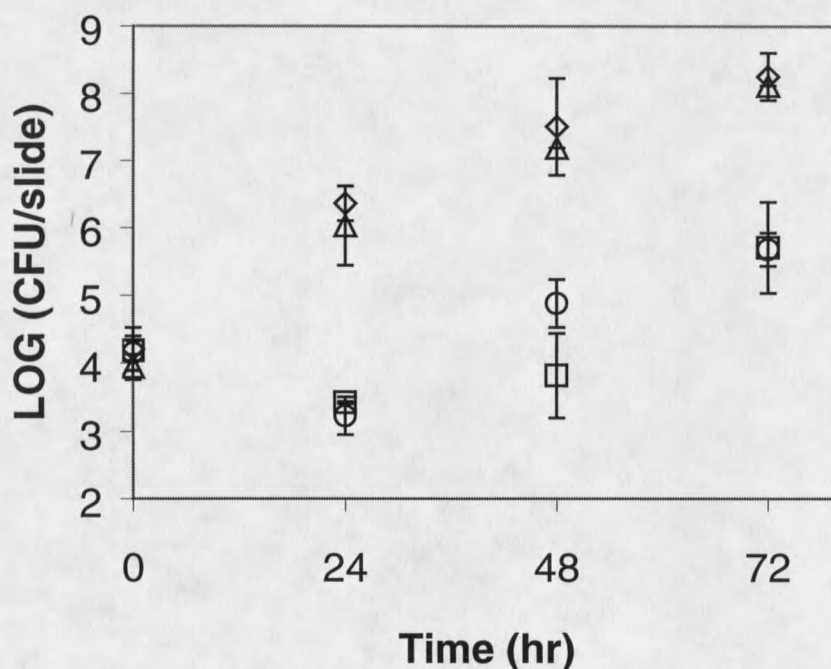


Figure 17. 72 hour angled drip-flow reactor experiment. Data represented by: (□)UV light and TiO_2 -coated slides, (○) UV light only, (Δ) Uncoated slides without UV light, (◇) TiO_2 -coated slides without UV light. Error bars represent standard error from triplicate experiments.

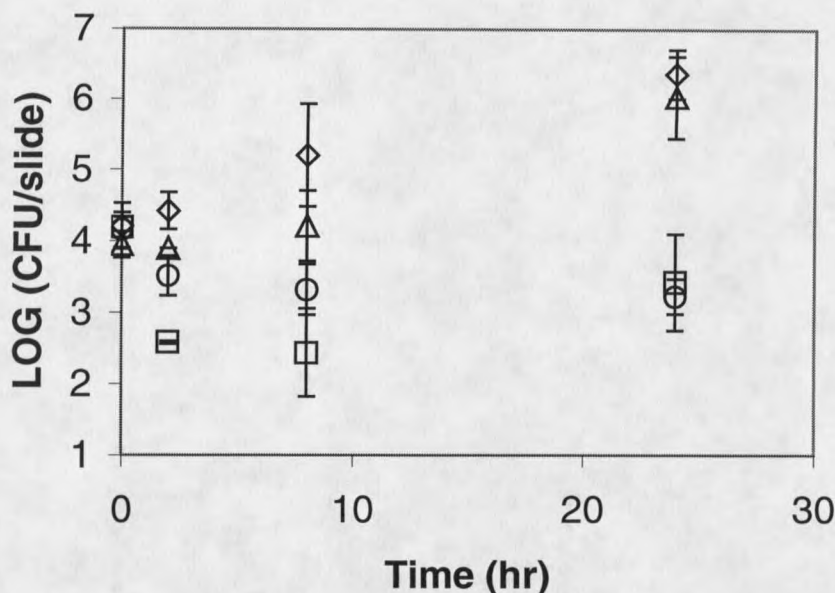


Figure 18. 24 hour angled drip-flow reactor experiment. Data represented by: (□)UV light and TiO₂-coated slides, (○) UV light only, (Δ) Uncoated slides without UV light, (◇) TiO₂-coated slides without UV light. Error bars represent standard error from triplicate experiments.

The angled drip-flow reactor system produced varying flow patterns of the PBM medium across the slide surfaces. The hydrophilic TiO₂-coated slides produced a thin film of PBM medium wetting the entire slide surface as it flowed over the slides. By contrast, the PBM medium flowed in a thin stream that covered only a portion of the uncoated slides. In order to produce identical environments at the slide surfaces, experiments were conducted with a horizontal drip-flow reactor system. Pieces of rubber were placed near the reactor outlet in each drip-flow cell. This produced a system in which the slides were completely immersed by the PBM medium as it flowed through the reactor. An identical sampling procedure was used in these experiments. Figure 19 shows the results of 72 hour experiments with the horizontal drip-flow reactor system. TiO₂-coated slides illuminated with UV light maintained the viable CFU per slide from

$10^{4.09}$ to $10^{4.19}$ after 24 hours before increasing to $10^{6.04}$ after 72 hours. UV light alone reduced the viable CFU per slide from $10^{3.86}$ to $10^{3.53}$ after 24 hours before increasing to $10^{4.73}$ after 72 hours.

Figure 20 shows the results of similar experiments conducted for 24 hours. TiO_2 -coated slides reduced the viable CFU per slide from $10^{4.09}$ to $10^{2.71}$ after 2 hours before increasing to $10^{4.19}$ after 24 hours. Uncoated slides subjected to UV light decreased the viable CFU per slide from $10^{4.09}$ to $10^{2.59}$ after 8 hours before increasing to $10^{3.53}$ after 24 hours. TiO_2 -coated slides without UV illumination allowed the viable cell counts to increase from $10^{3.80}$ to $10^{5.75}$ after 24 hours. Similarly, no reduction in cell counts was observed using uncoated slides without UV illumination. In this case, viable cell counts increased from $10^{4.08}$ to $10^{5.89}$ after 24 hours.

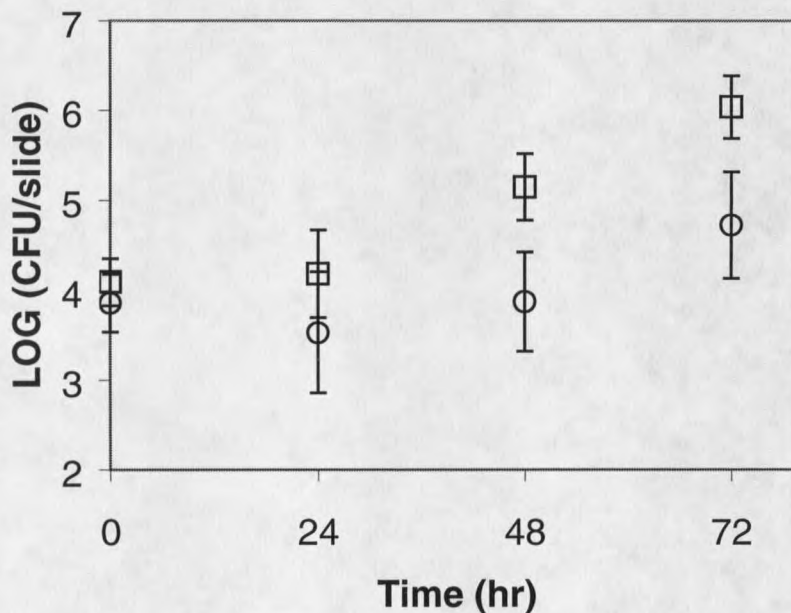


Figure 19. 72 hour horizontal drip-flow reactor experiment. Data represented by: ($\square$) UV light and TiO_2 -coated slides, ($\circ$) UV light only. Error bars represent standard error from triplicate experiments.

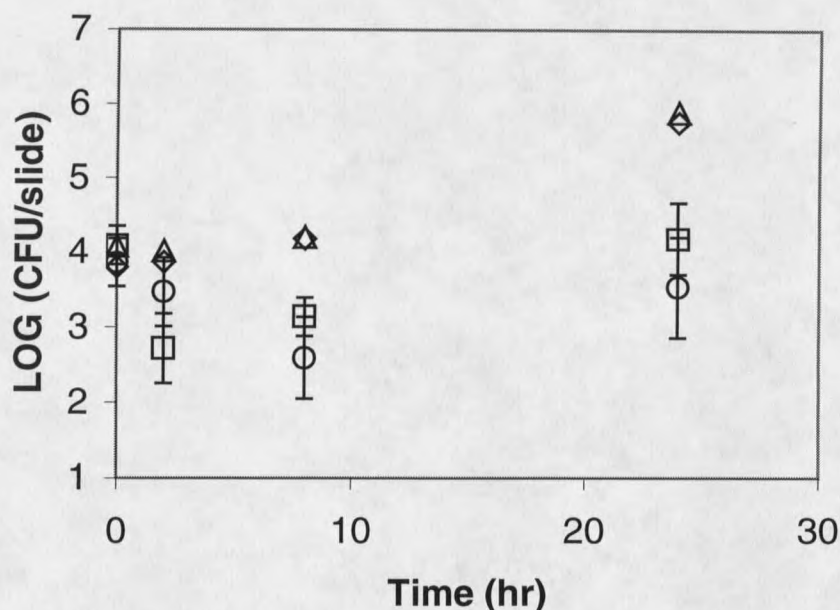


Figure 20. 24 hour horizontal drip-flow reactor experiment. Data represented by: (□)UV light and TiO₂-coated slides, (○) UV light only, (Δ) Uncoated slides without UV light, (◇) TiO₂-coated slides without UV light. Error bars represent standard error from triplicate experiments.

In order to investigate a stress response mechanism, experiments were conducted with the horizontal drip-flow reactor system in which the exposure to UV light was cycled over the course of 72 hours. Uncoated slides were used in these experiments. The procedures for initiating biofilm formation and the slide sampling were identical to those used previously. UV transparent acrylic lids were used in the first 24 hours of the experiments. In one set of experiments, UV blocking polycarbonate lids were used for the remaining 48 hours. In the other experiments, the polycarbonate lids were used from 24 to 48 hours followed by acrylic lids from 48 to 72 hours. Figure 21 shows the results of these experiments. As expected, the two sets of experiments showed very similar results in the first 48 hours. In the experiments where polycarbonate lids were used from

24 to 72 hours, the viable CFU per slide were reduced from $10^{4.09}$ to $10^{2.45}$ after 24 hours before increasing to $10^{5.31}$ after 48 hours and $10^{6.67}$ after 72 hours. In the experiments in which the acrylic lids were used from 48 to 72 hours, the viable CFU per slide were reduced from $10^{3.94}$ to $10^{2.66}$ after 24 hours before increasing to $10^{5.15}$ after 48 hours and $10^{5.51}$ after 72 hours.

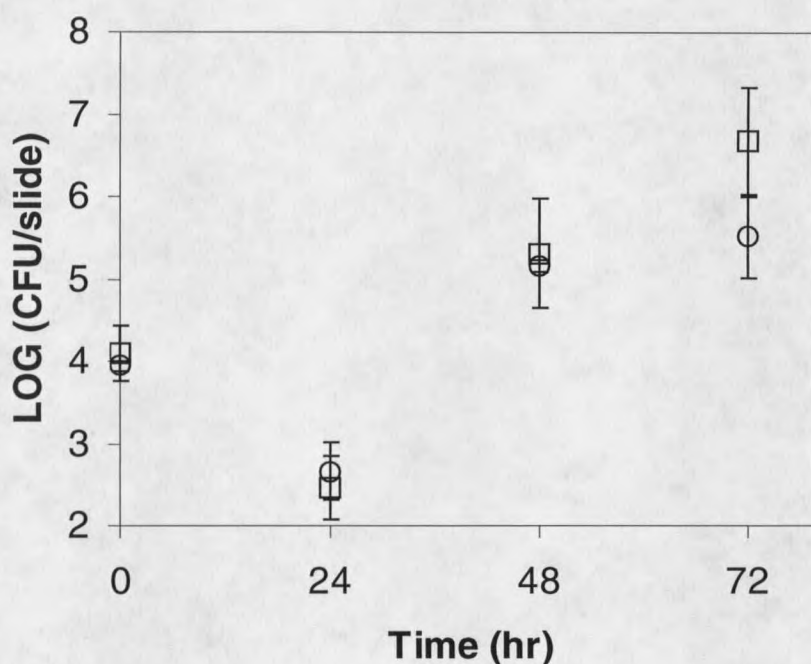


Figure 21. 72 hour horizontal drip-flow reactor experiment with UV light cycling. Data represented by: ($\square$) UV light on 0-24 hours, off 24-72 hours, ($\circ$) UV light on 0-24 hours, off 24-48 hours, on 48-72 hours. Error bars represent standard error from triplicate experiments.

CHAPTER 4

DISCUSSION

Slide Preparation

Thin films of TiO_2 were deposited on glass slides by use of a dip-coating procedure (9 cm/min withdrawal speed) with an aqueous solution of titanium dioxide. Scanning electron microscopy (SEM) revealed that each coating added approximately 75 nanometers to the overall TiO_2 film thickness (Figure 1). Previous studies using slightly different procedures obtained similar results. Tada and Tanaka (48), using a withdrawal speed of 2.6 cm/min, found that the thickness of the first coating was approximately 100 nm and each subsequent coating added around 40 nm to the overall TiO_2 thin film thickness. Kato et al. (47) produced thin films that increased by approximately 30 nm thickness with each coating applied while using a withdrawal speed of 10 cm/min. Following this dip-coating procedure, the slides were fired in an oven to 450 °C at a rate of 3 °C per minute and held at 450 °C for five hours before being allowed to cool to room temperature at a rate of 10 °C per minute. It has been shown that firing supported TiO_2 films at temperatures up to 500 °C produced films that were 100% anatase (53). The firing procedure as well as the TiO_2 thickness used is consistent with highly active TiO_2 thin films with anatase crystal structure used in previous studies (47, 48, 53).

Photocatalytic Oxidation of Organic Compounds

Formic acid degradation experiments with initial formic acid concentrations of 38.3 ppm (10 ppm carbon) were conducted. Glucose degradation experiments were also performed in the same batch reactor system with initial glucose concentrations of 25 ppm (10 ppm carbon). The photocatalytic degradation rates of glucose are expected to be considerably slower than those of formic acid due to its larger and more complex chemical structure. The degradation of glucose involves a complicated pathway consisting of several intermediate species before glucose is completely mineralized to carbon dioxide and water. In addition, the titania surface is positively charged in acidic environments. Therefore, species such as HCOO^- , the conjugate base of formic acid, will more readily adsorb to the positively charged TiO_2 surface leading to faster degradation rates. Figure 22 compares the results of photocatalytic degradation of formic acid and glucose in terms of the amount of carbon degraded after 4 and 24 hours. Considering the first four hours, the degradation rate of carbon using formic acid was $0.017 \frac{\text{mg}}{\text{hr} \cdot \text{cm}^2}$ carbon. The corresponding rate using glucose was $0.0028 \frac{\text{mg}}{\text{hr} \cdot \text{cm}^2}$ carbon. The photocatalytic degradation rate for formic acid was 6 times greater than that for glucose in this initial 4 hour period.

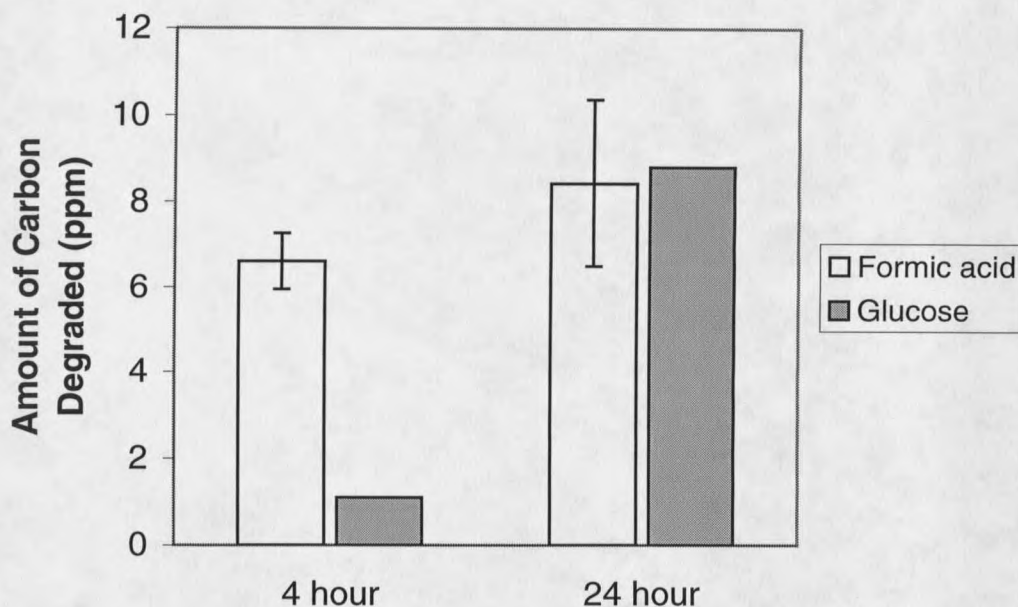


Figure 22. Comparison of the amount of carbon photocatalytically degraded when using formic acid and glucose. Initial concentration of carbon in both experiments was 10 ppm. Error bars represent standard error from duplicate experiments.

A previous formic acid degradation study using a similar reactor system produced very similar results. Kim and Anderson (49) obtain a degradation rate of $0.014 \frac{\text{mg}}{\text{hr} \cdot \text{cm}^2}$ carbon. The similarity of the results of these experiments with previously published studies suggests that the TiO_2 -coated slides produced were indeed photocatalytically active and capable of degrading organic compounds. In addition, the lack of degradation that occurred without TiO_2 -coated slides present indicates that the oxidation of these organic compounds is due to the combination of UV light and photocatalytic slides.

Photocatalytic Inhibition Studies

Pseudomonas basal mineral (PBM) medium (55) is an aqueous solution used for the growth of *Pseudomonas* microorganisms and consists of salts, minerals, and pH buffers with glucose as the organic carbon source. PBM medium contains compounds such as phosphate (PO_4^{3-}) and sulfate (SO_4^{2-}) which are known to inhibit photocatalytic rates when present in small concentrations (59). These species can reduce the photocatalytic oxidation rates by competing for oxidizing radicals and/or by occupying the active sites on the TiO_2 catalyst surface. Abdullah et al. (60) investigated the inhibitory effects of several inorganic anions on the photocatalytic oxidation of several organic compounds. Perchlorates and nitrates were found to have little influence on the overall degradation rates of salicylic acid, aniline and ethanol. However, chloride, sulfate and phosphate ions had a significant negative effect on the oxidation rates. Phosphate was found to have the largest effect and reduced rates by as much as sixty five percent when present in concentrations as low as 1.0 mM. Two sets of experiments were conducted to assess photocatalytic inhibition caused by the PBM medium used in subsequent biological experiments.

In the first set of experiments, TiO_2 -coated slides were first placed into a drip-flow reactor and PBM medium (1/100x dilution) was flowed over the slides for 24 hours at a rate of 1 mL/min/slide. The slides were then transferred to the batch reactor system used in the formic acid degradation experiments. The formic acid degradation rates were found to be forty percent lower than corresponding rates obtained without subjecting the TiO_2 -coated slides to the PBM medium. These results suggest that minerals and salts can

foul the TiO_2 for extended periods of time when transferred to an environment free of minerals and salts.

The second set of PBM medium inhibition experiments used the same batch reactor system used for the formic acid degradation studies. Several experiments were conducted varying the components and concentration of the PBM medium present while using glucose (10 ppm carbon) as the organic compound to be degraded. Glucose degradation rates were reduced by 42 percent in the presence of a 1/500x dilution of the PBM medium, which contains 0.2 mM phosphate in addition to many other salts. Nearly identical results were obtained when only the phosphate components of the PBM medium were present at 0.2 mM. This result suggests that the phosphate ions are primarily responsible for any photocatalytic inhibition. Even when present in very small concentrations as low as 0.02 mM, as in the 1/5000x dilution of the PBM medium, phosphates caused approximately 32 percent reduction in the photocatalytic oxidation rates of glucose.

Results from the present studies agree well with previously published findings (59, 60). Subsequent biological experiments using PBM medium at 1/100x dilution (1.0 mM phosphate) are expected to experience significantly decreased photocatalytic rates primarily due to phosphates present in the medium.

Photocatalytic Bactericidal Effects

Experiments were conducted in a closed system (Figure 4) to assess the ability of the TiO_2 thin films to photocatalytically deactivate planktonic *Pseudomonas aeruginosa*

(PA01) cells. In experiments conducted with $10^{3.6}$ cells initially present, the TiO_2 -coated slides in combination with UV light reduced the viable cell counts by 99.7% (2.6 log reduction) in 1 hour while UV light alone produced only a 16% (0.08 log reduction) decrease. With 10^6 PA01 cells initially present, active TiO_2 slides illuminated with UV light reduced the viable cell counts by 99.8% (2.8 log reduction) in 2 hours while UV light resulted in a 39.7% (0.22 log reduction) in the same time. No reduction in viable cell counts was observed in either set of experiments when TiO_2 -coated slides were used in the absence of UV light. The results of these studies suggest that TiO_2 thin films can readily and quickly inactivate planktonic cultures of *Pseudomonas aeruginosa*. A previous study conducted on planktonic cultures of *E. coli* shows similar results (22). It is also clear that photocatalysis is much more efficient in killing planktonic bacteria than UV A (320-400 nm) light alone. Finally, it is seen that the TiO_2 -coated slides alone were not toxic to the bacteria.

Experiments were conducted in angled drip-flow reactors (Figure 6) to assess the ability of photocatalytic thin films to remove PA01 biofilms from glass surfaces. Non-illuminated slides, both with and without the TiO_2 coating, produced nearly identical results. In these cases, the PA01 biofilms grew over the entire 72 hour experiments without any decrease in viable cell counts. Illuminated slides, both with and without the TiO_2 coating, produced similar results to each other. These treatments both reduced the PA01 biofilms for an initial period of time followed by biofilm growth. The minimum biofilm surface coverage was found to exist in the first 10 hours of treatment. While these treatments were unsuccessful in eradicating the PA01 biofilms, the treatments did

provide a 2-3 log reduction (99.0-99.9%) in biofilm surface coverage over the course of 72 hours when compared to the untreated slides.

It was observed that different flow patterns of the PBM medium existed over the surface of the glass slides when using angled drip-flow reactors. The flow of the PBM medium over the uncoated slides was in a narrow stream and did not cover the entire slide surface. The hydrophilic surface of the TiO_2 -coated slides allowed the PBM medium to completely wet the entire slide surface with a thin film. Therefore, in order to provide identical conditions at the TiO_2 -coated and uncoated slide surfaces, horizontal drip-flow reactors (Figure 7) were used in which the slides were completely immersed in the PBM medium. Similar results to the angled drip-flow reactor experiments were obtained.

The inability to differentiate between PA01 biofilm results using UV light alone and UV light in combination with TiO_2 -coated slides suggests that the bactericidal effect is primarily produced by UV light alone. One or more of the necessary components for photocatalysis to function may not have been present at the interface between the attached cell and the TiO_2 thin film. Oxygen or another electron acceptor is required at the TiO_2 surface to maintain charge transfer. In these experiments, the PBM medium was found to contain approximately 6 ppm of dissolved oxygen, which is sufficient for photocatalysis to proceed. In addition, bacterial air vents on the lids of the drip-flow reactor ensure the exchange of air to the system. Due to the low surface coverage of the PA01 biofilms, it is assumed that the biofilm thicknesses would not provide a significant diffusion barrier for oxygen to reach the catalyst surface. However, it is unclear as to whether the oxygen can diffuse into the region between the attached cell and the TiO_2

surface. In addition to oxygen, light of the proper wavelength is required for photocatalysis. In these experiments, UV light centered at 365 nm reached the catalyst surface with an intensity of 1.0 mW/cm^2 . Measurements of the amount of UV light penetrating the biofilms and underlying TiO_2 layer as well as the glass slide suggest that sufficient light is present at the slide surface. In addition, an experiment was conducted with UV light incident from the bottom of the drip-flow reactor. In this scenario, UV light is guaranteed to reach the TiO_2 surface as it passes only through the glass reactor bottom and glass slide before reaching the catalyst surface. As seen in Figure 23, the results of this experiment are similar to experiments conducted with illumination from above the drip-flow reactor. These results eliminate insufficient UV light at the TiO_2 surface as an explanation to poor photocatalytic killing of the PA01 biofilms. Finally, the active sites of the TiO_2 catalyst must be available in order to maintain the photocatalytic processes. It was found that the PBM medium caused significant reduction in photocatalytic rates. Therefore, it is possible that components, such as phosphates, of the PBM medium are inhibiting photocatalysis by blocking the active sites on the TiO_2 -coated slides. A rough calculation was performed to estimate the accumulation rate of carbon on the slide surface due solely to PA01 cell growth. The following assumptions concerning the PA01 cells were made: the cells are approximately 12 percent carbon by weight (61), the density of the cell is equivalent to water, the cell has a surface area of $4 \text{ } \mu\text{m}^2$ and a volume of $4 \text{ } \mu\text{m}^3$. This accumulation rate was calculated using the increase in PA01 cells from 24 hours to 48 hours on the illuminated slides. The local accumulation rate of carbon due solely to the increase in the number of PA01 cells was found to be

approximately $2 \cdot 10^{-3} \frac{\text{mg}}{\text{hr} \cdot \text{cm}^2}$ carbon. It is seen that this carbon accumulation rate is similar to the carbon degradation rate obtained using glucose ($2.8 \cdot 10^{-3} \frac{\text{mg}}{\text{hr} \cdot \text{cm}^2}$ carbon). This suggests that the slides may not be capable of photocatalytically degrading the carbon due to cell growth.

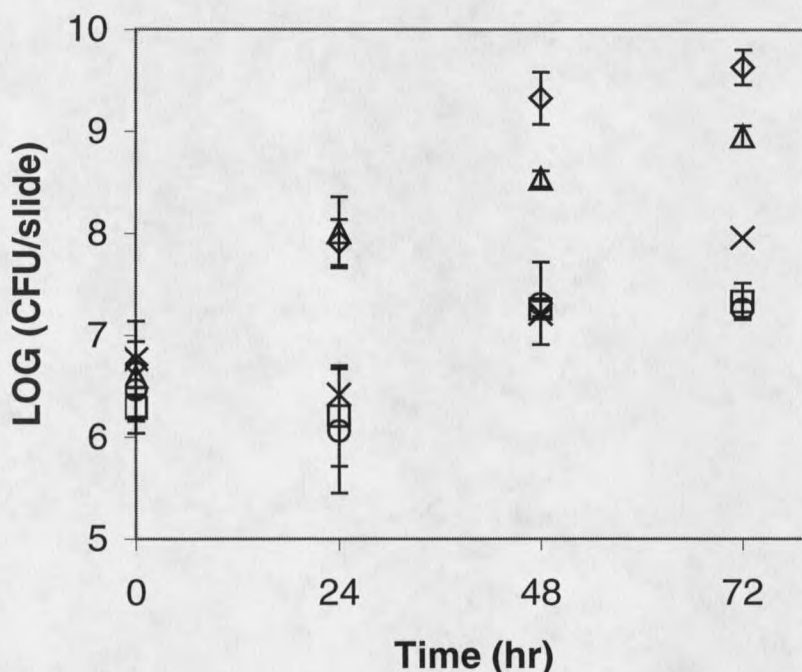


Figure 23. 72 hour angled drip-flow reactor experiment conducted with different UV light source locations. Data represented by: (□) UV light (top illumination) and TiO₂-coated slides, (○) UV light (top illumination) only, (Δ) Uncoated slides without UV light, (◇) TiO₂-coated slides without UV light, (×) UV light (bottom illumination) and TiO₂-coated slides. Error bars represent standard error from triplicate experiments.

Biofilm UV Stress Response

In the present studies, PA01 biofilms showed enhanced resistance to degradation by both photocatalysis and UV light alone when compared to PA01 in the planktonic

state. The PA01 biofilms were initially susceptible to these treatments but quickly recovered and continued to accumulate at approximately the same growth rate as biofilms without treatment.

The doubling time for the PA01 biofilm cells was calculated using data from the angled drip-flow reactor experiments. For the illuminated slides, the doubling time was calculated using data following the initial 24 hour period in which the cell numbers decreased. For non-illuminated slides, the doubling time was calculated over the entire 72 hours of the experiments. Biofilms on UV illuminated slides, both coated and uncoated, had an average doubling time of 6.1 hours compared to a 5.3 hour doubling time for the non-illuminated slides. The similarity of the biofilm growth rates following initial susceptibility suggests that the cells within the biofilm, to resist further killing, invoked an oxidative stress response mechanism.

Reactive oxygen species such as H_2O_2 , $\text{OH}^\bullet$, $\text{HOO}^\bullet$ and O_2^- can be produced by photocatalysis and UV A light alone. It is well known that bacteria can respond to environments containing oxidizing species by inducing a stress response mechanism (9, 18, 62, 63, 64). For example, *Pseudomonas aeruginosa* biofilms exposed to H_2O_2 increase production of catalase, an enzyme capable of degrading H_2O_2 , in order to protect against further oxidative damage (9). Sanderson and Stewart (63) investigated the response of *Pseudomonas aeruginosa* biofilms to repeated doses of monochloramine, a common antimicrobial agent. In their work, the *P. aeruginosa* biofilms were highly susceptible to initial doses of monochloramine and less susceptible to subsequent doses. This increased resistance to prolonged monochloramine exposure is evidence of an

adaptive stress response allowing the biofilms to neutralize the antibiotic minimizing further damage. Kidambi et al. (64) have shown that planktonic cultures of *P. aeruginosa* exposed to UV A radiation induce expression of the *recA* gene. *recA* is a gene which, when expressed, increases production of the protein RecA, which plays a crucial role in DNA repair. This is another example of how bacteria can respond to oxidative stress such that further damage and death can be minimized.

In order to investigate the UV stress response mechanism by the PA01 biofilms, experiments were conducted in which exposure to the UV light source was discontinued after 24 hours before being continued again after 48 hours. Limiting UV exposure for 24 hours should allow sufficient time for cells within the biofilm to suppress any stress response that may have been induced by the initial exposure to UV light. Therefore, reintroducing exposure to the UV light should produce a reduction in the viable cells when compared to biofilms that were not exposed again to the UV light source. In the present experiments, biofilms were reduced from average values of $10^{4.02}$ to $10^{2.56}$ CFU/slide in the initial 24 hours followed by growth to $10^{5.23}$ CFU/slide in the next 24 hours without UV illumination. This corresponds to a log increase of approximately 2.67 in the number of viable cells in the second 24 hours. This 24 hour increase is significantly greater than any 24 hour increase seen in the previous drip-flow experiments. This may suggest that the PA01 bacteria are able to repair injured and non-viable cells when the UV illumination is removed. Biofilms reintroduced to the UV light after 48 hours increased by $10^{0.36}$ CFU/slide from $10^{5.15}$ to $10^{5.51}$ CFU/slide after 24 additional hours. Biofilms not re-exposed to UV light after 48 hours increased by $10^{1.36}$ CFU/slide from $10^{5.31}$ to $10^{6.67}$ CFU/slide in the same 24 hour period. This increase in

the number of biofilm cells was 25 times higher than the case in which UV light exposure was reintroduced. These results suggest that biofilm susceptibility to UV light exposure can be reintroduced following periods without light in which stress response mechanisms are relaxed.

Future Work

Future work would focus on confirming what mechanisms allow *Pseudomonas aeruginosa* biofilms to be resistant to both photocatalysis and UV light. Work should quantify the degree of photocatalytic inhibition caused simultaneously by the PBM medium and PA01 biofilms. In addition, experiments could be conducted utilizing medium with alternatives to phosphate buffers. Future studies should identify the existence of a stress response mechanism invoked by the biofilm when treated with photocatalysis or UV light alone. Several techniques may be used for confirmation of a stress response mechanism. Two-dimensional gel electrophoresis approaches can identify proteins that are produced by the biofilms before and after the oxidative stress is introduced. By comparing these results, it is possible to identify proteins that are only present in oxidative stress environments. Another possible approach is the use of different strains of *Pseudomonas aeruginosa* that lack the ability to produce enzymes capable of breaking down reactive oxygen species. Comparing the susceptibility of different *P. aeruginosa* strains can provide valuable insight into the reactive oxygen species responsible for the bactericidal effects of photocatalysis and UV light.

Conclusions

The following conclusions can be drawn from the present studies:

1. The TiO_2 thin films are photoactive and capable of degrading compounds such as formic acid and glucose.
2. TiO_2 -coated slides are efficient in photocatalytically degrading planktonic cultures of *Pseudomonas aeruginosa* (PA01) cells.
3. Certain components, particularly phosphates, of the *Pseudomonas* basal mineral (PBM) medium cause a significant reduction in photocatalytic degradation rates.
4. PA01 biofilms are susceptible to UV A light for an initial period of time before expressing enhanced resistance to this treatment.
5. Photocatalytic oxidation appears to have no effect on biofilm formation.
6. Mechanism(s) such as oxidative stress response and/or TiO_2 catalyst fouling may exist to provide the PA01 biofilm resistance to UV light and photocatalytic oxidation.

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