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Non-Thermal Atmospheric-Pressure Plasma for Sterilization of Surfaces and Biofilms

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NON-THERMAL ATMOSPHERIC-PRESSURE PLASMA FOR

STERILIZATION OF SURFACES AND BIOFILMS

by

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ABSTRACT

NON-THERMAL ATMOSPHERIC-PRESSURE PLASMA FOR STERILIZATION OF SURFACES AND BIOFILMS

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Bacterial resistance to antimicrobial methods is a critical issue in many fields of medicine. This work describes the studies performed to characterize and optimize the bacterial inactivation effects of a non-thermal atmospheric-pressure plasma brush and plasma jet on a laminate surface inoculated with *Acinetobacter baumannii* and *Staphylococcus aureus*, and a cultivated *Enterococcus faecalis* biofilm, respectively. These treatments are pilot studies for eventual application to surface sterilization in hospitals and root canal disinfection. To evaluate bacterial inactivation, after treatment and recovery, the bacterial colony forming units (CFUs) are counted. Several different methods are used to optimize the antimicrobial effect. For the plasma jet, the optimal fraction of oxygen in the helium feed gas is determined, resulting in a 1.5 log (97%) bacterial inactivation. For the plasma brush treatment of a dry laminate environment, the addition of water to the feed gas and surface is investigated. These variations greatly alter the plasma chemistry, which is characterized for the plasma brush by the use of optical emission spectroscopy to detect the presence of excited reactive species. These and other species generated in the plasma plume play a large role in the deleterious effect of plasma on bacterial cells. In a humid discharge, bacterial inactivation is maximized at 0.6 log (75%), whereas adding water to the laminate surface before treatment yields a 4 log (99.99%) reduction in bacterial growth. Thus, while the optimized oxygen or water concentration in a noble feed gas improves

inactivation effect of the plasma, the most significant criterion for maximal bacterial inactivation in these studies is the presence of water at the treatment surface.

This thesis is dedicated to the quest for the improvement of the human condition.

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Figure Page

Page

CHAPTER 1

INTRODUCTION

In recent years, the over-prescription and improper use of antibiotics has led to a rise of drug-resistant bacteria [1]. This has particularly become a problem in hospitals where secondary infections acquired during a treatment can become more problematic than the original reason for the visit. For example in 2015, endoscopes used for the imaging and treatment of gastrointestinal problems resulted in the infection of almost 200 patients with a bacterium that kills 40% of infected persons [2]. The issue resulted from the improper cleaning of the duodenoscopes between usages. The difficulty is that the expensive medical equipment must be reused and is difficult to sterilize. Due to many components being heat-sensitive, wet heat (autoclaving) is often not an option for sterilization. Wiping devices with harsh chemicals may not remove all bacteria in nooks and crannies between mechanical parts. The best option for sterilization is then the use of the toxic ethylene oxide gas [3]. This method is both expensive and difficult to implement in a hospital setting. Due to these challenges for the sterilization of medical equipment and surfaces, non-thermal plasma is being investigated as a more efficient alternative to existing methods. This work seeks to optimize and characterize the bacterial inactivation effect of a plasma brush device for surface sterilization.

Plasma is also being investigated for the inactivation of bacterial biofilms in the root canals of teeth, with advancing research in the field of endodontics [4, 5]. A biofilm is a group of microorganisms that adhere to each other on a surface, composed of the organism cells and an

extracellular polymeric substance, proteins, phospholipids, and other molecules that bind the biofilm together. This results in a heterogeneous matrix with increased resistance to antibiotics, oxidative stress, and starvation, in comparison to planktonic, free-floating bacteria. Bacterial biofilms are notoriously difficult to treat and exhibit increased resistance to many traditional treatment methods such as antibiotics. Root canal biofilms in particular, due to poor accessibility in the narrow cavity, pose a significant problem. Current treatment methods include irrigation with harsh chemicals and antimicrobials which are less effective on biofilms than planktonic bacteria [6].

The typical treatment for root canals, once the majority of the decayed tooth and pulp has been removed via drilling, is irrigation with several different chemicals to remove the remaining necrotic pulp tissue and bacteria. To remove the smear layer, microcrystalline and organic particle debris left by the instrumentation, a 17% solution of ethylene diaminetetraacetic acid or 50% citric acid is often used [6]. Sodium hypochloride at a concentration of up to 6% is used to dissolve necrotic pulp tissue, and an antibiotic, often 0.2% chlorhexidine is added to kill any remaining tissue. However, even though some of these chemicals are highly toxic to the patient, they still do not kill all bacteria present in the root canal, resulting in a failure rate of up to 50% for certain populations [7], where the root canal procedure must be repeated due to continued infection. Non-thermal plasma offers an alternative treatment method to the current irrigants; it has been shown to inactivate bacterial biofilms [4, 8-10]. Presented here is the characterization and optimization of a plasma jet device for inactivation of such biofilms.

1.1 Basic Plasma Principles

A plasma is an ionized gas, and can be described as a collection of freely moving particles with some very interesting characteristics. Each particle will generate electric and magnetic fields which will affect other neighboring particles. If there is a net charge density, the number of positive and negative particles is uneven, and an electric field is generated that tends to expel whichever species is in excess. Thus, plasma is electro-neutral in bulk. Any perturbations to the charge density and potential in a plasma tend to fall off with the Debye length, λ_D. The equation for the potential of a point charge in a plasma as a function of distance from the charge is seen in Equation 1. As opposed to in vacuum, where the potential depends on the inverse of the distance, Equation 2.

$$\Phi(r) = \frac{Q}{4\pi\varepsilon r} e^{-r/\lambda_D} \tag{1}$$

$$\Phi(r) = \frac{Q}{4\pi\varepsilon r} \tag{2}$$

Q is the charge of the particle, r is the distance from the particle, and ε is the electrical permittivity of the medium in which the system is located. Additionally, there is a frequency associated with a plasma due to the harmonic motion of electrons that depends on the square root of the charge density. See Equation 3.

$$\omega = \sqrt{\frac{n_e e^2}{m^* \varepsilon_0}} \tag{3}$$

This frequency is in the megahertz range for the ionosphere, and is essential to over the horizon communications, but is in the gigahertz range for the laboratory generated plasmas investigated here. Also, there is a resistivity associated with a plasma that is due to charged particle collisions with background atoms and molecules. In atmospheric pressure conditions, the collision frequency is on the order of 100 GHz. The behavior of plasma is dominated by these particle collisions as long as the collision frequency is higher than the plasma frequency.

To ignite a plasma, energy is supplied until the free electrons present in any gas acquire sufficient energy to ionize some of the background gas. In man-made plasmas, this energy is often applied via a high electric field which causes the electrons to accelerate, colliding with and ionizing other gas molecules to create an electron avalanche. This is known as a Townsend discharge as seen in Figure 1. In the glow regime, plasma is visible to the human eye and secondary emission at the cathode increases current exponentially. Once the current density is high enough to heat the cathode to incandescence, we enter the arc regime which is a result of thermal emission of electrons from the cathode. In non-thermal applications, arcing is unwanted due to the high temperatures associated with this regime. The current-voltage behavior of plasmas is highly nonlinear as seen in Figure 1 for a DC glow discharge. For the nanosecond pulse-driven plasmas investigated here, the applied voltage drives a displacement current between the ground and high voltage electrodes, and one or more discrete plasma packets are formed for every voltage pulse [11]. After ignition, the non-thermal plasma remains in the glow mode until the end of the voltage pulse.

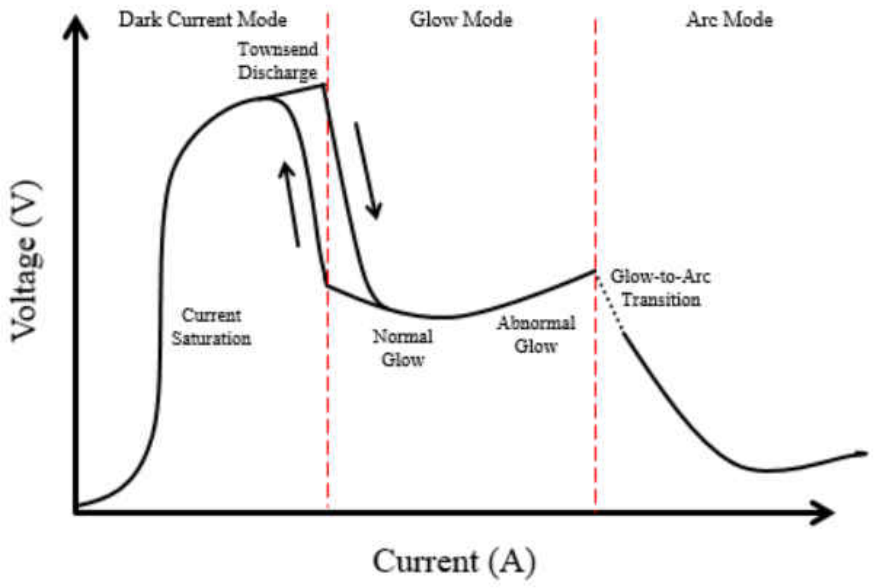


Figure 1. The nonlinear current- voltage relationship for a representative plasma glow discharge. With the application of energy, background free electrons accelerate and collide with ions in the gas, creating more electrons in an electron avalanche (Townsend Discharge). The plasma is then enters the glow mode, in which the current rapidly increases. After cathode heating and secondary electron emissions, the plasma enters the high current arc mode [12].

1.2 Non-Thermal Atmospheric-Pressure Plasma

Plasma is the most abundant state of matter in the universe, comprising stars and nebulae. Plasmas may also be man-made and generated by applying high voltage, either alternating current, direct current, or electrical pulses to a gas via one or more electrodes. See Figure 2.

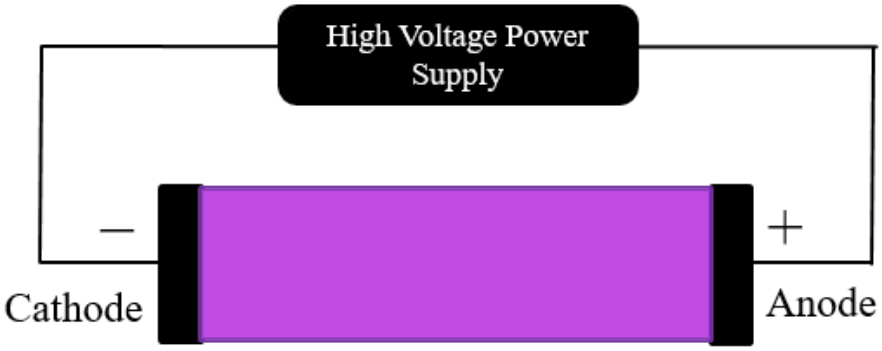


Figure 2. A representative DC glow discharge powered by a high voltage power supply.The gas present between the anode and the cathode will ionize with the application of high voltage, forming a plasma.

If the deposited energy is very high, it is possible to achieve a large degree of ionization, in what is known as a thermal plasma. In this case, the plasma is at thermal equilibrium, with the electrons and ions being at the same temperature. This results in a gas temperature in the range of thousands of kelvin. These types of plasma are used in fusion research and others.

On the other hand, non-thermal plasma (NTP) has a gas temperature of only a few hundred kelvin. The electrons in such a plasma are much hotter than the ions ($T_e >> T_i$) and there is a very low degree of ionization, typically less than one percent. The overall gas temperature is close to the temperature of the ions, and depending on what type of gas is used and the amount of energy deposited, this can be close to room temperature.

Many different types of devices have been used to generate NTP, consisting of, at minimum, a high voltage power supply, a discharge gas, and at least one electrode. Often, a dielectric (insulator) layer is included to separate two electrodes. Several different configurations may be

seen in Figure 3. These are examples of devices that can be used to generate a plasma at atmospheric pressures, whereas other plasma devices require low pressure to function.

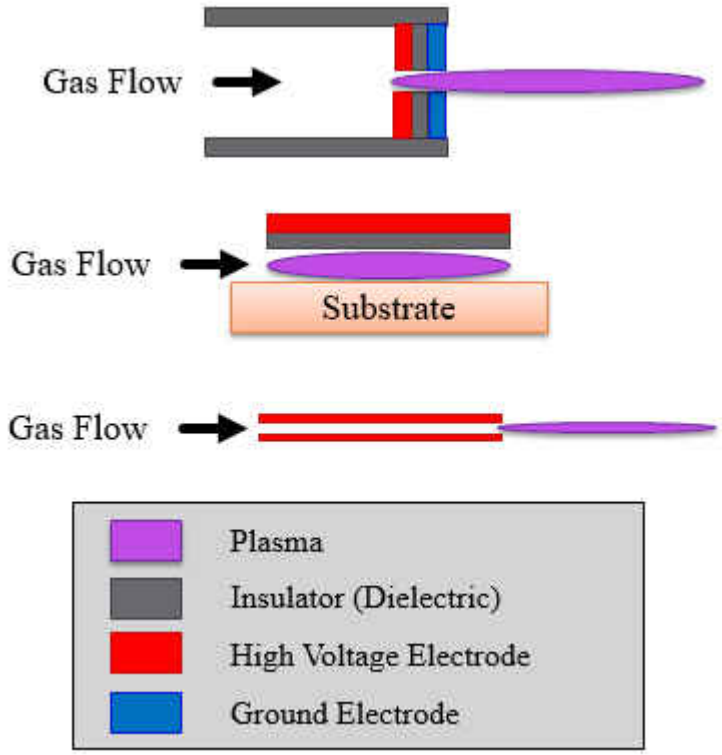


Figure 3. Three examples of plasma devices for biomedical applications. The plasma jet (top) and surface dielectric barrier discharge (middle) are both forms of dielectric barrier discharge. The plasma needle (bottom) is a single-electrode plasma device.

A dielectric barrier discharge (Figure 3 middle) consists of a single planar electrode, covered with an insulator, resulting in microfilaments of plasma discharge. It is a direct current device,

meaning the skin or tissue is the ground electrode, and current passes through the body. However, current densities are low enough that no damage occurs. The radicals, ions, and electrons produced are attracted toward the electrodes of opposite polarity and form a charge layer on the dielectric surface. These charges cancel the charge on the electrodes so that the electric field in the gap falls to zero, the discharge stops, and the current is limited. A ground electrode may be placed below the substrate, or as a wire mesh between the dielectric and the substrate surface. In the latter case, the device will produce an indirect plasma, with only the chemical species generated in the plasma impinging on the treatment surface. In most cases, an indirect treatment results in a decreased plasma effect on cells, such as is the case for bacterial inactivation treatments [13].

A plasma jet is a tube with electrodes placed inside or around it, through which gas flows and ionizes when it is subject to the electrical field between the electrodes. The example given in the top image of Figure 3 is also a form of dielectric barrier discharge, resulting in a plasma plume that can be used as an indirect treatment in a narrow space, such as a root canal. Plasma needles, such as in the bottom image of Figure 3 are often single electrode devices and are smaller than jets. Plasma devices such as the jet or needle, can also be arranged in arrays, either with multiple devices, or multiple openings for gas to pass through. These are useful for treating larger surfaces.

1.3 Plasma for Medical Applications

Different applications of non-thermal plasma include food processing, lighting, thin film deposition, and plasma medicine. Plasma medicine combines the fields of medicine and plasma physics. Non-thermal plasma may be applied to biological tissues and cells for therapeutic applications, or even materials for implant [14]. They may be applied in one of three ways. Direct plasmas use the target tissue or cells as the ground electrode, and current flows through the target such as in the dielectric barrier discharge device in Figure 3. Indirect devices generate the plasma away from the tissue and blow the products onto the target via directed gas flow such as the plasma jet in Figure 3. Finally, a grounded metal mesh may be used to direct the current through itself and onto the target. Both devices discussed in this thesis are indirect methods of plasma treatment.

CHAPTER 2

LITERATURE REVIEW

This chapter consists of a detailed literature review on topics pertaining to the research, including the types of bacteria cultivated and treated for the studies. The bulk of this chapter describes the techniques and mechanisms for bacterial inactivation via NTP.

2.1 Bacterial Strains

Three different bacterial strains are cultivated and treated during the bacterial inactivation studies. *Staphylococcus aureus* and *Acinetobacter baumannii* are used to test the efficacy of the plasma brush for surface decontamination. *Enterococcus faecalis* are cultivated in biofilm form as an analogue to root canal biofilms to test a plasma jet for endodontic application.

2.1.1. Staphylococcus aureus ATCC 25923

This particular strand of *S. aureus* is a control strain commonly used to test effectiveness of antibiotics as well as the quality of commercial products [15]. The strain causes food poisoning as well as infections such as pneumonia and septic arthritis [16]. Many strains are Methicillin-resistant, however, this strain is susceptible to the antibiotic. Additionally, *S. aureus* is gram positive, with a thick peptidoglycan layer. Gram positive bacteria are less susceptible to cell

envelope permeabilization by plasma, though it has not been determined which gram type is more susceptible to plasma inactivation [17].

2.1.2 Acinetobacter baumannii ATCC 19606

A. baumannii is a common nosocomial pathogen that is known to cause tissue infections such as urinary tract and respiratory infections [18]. Many strains are resistant to multiple antibiotics and result in a high percentage (>36%) of combat-related infections in military personnel [19]. Antibacterial treatments are tested on both gram positive and negative strains to assess efficacy. Therefore, the gram negative *A. baumannii* is selected as a second test strain for the plasma brush studies.

2.1.3 Enterococcus faecalis ATCC 29212

E. faecalis is another gram positive nosocomial pathogen that has significant resistance to antibiotics, both intrinsic and acquired. It is commonly found in infected root canals with a prevalence of 30 to 90% [20], and agents used for root canal disinfection, such as sodium hypochlorite and chlorhexidine, are highly ineffective against the bacterium [21]. The ATCC 29212 strain is a quality control strain often used to test the efficacy of antibiotics, making it suitable for plasma inactivation studies [22].

2.2 Plasma Medicine

Plasma medicine is the application of non-thermal plasmas to solve medical problems. The most general categorization of such plasmas is indirect or direct as mentioned in Section 1.3. Additionally, the devices generally operate at atmospheric pressures to avoid the expense and complications associated with a low pressure vacuum system. The feed gases used are typically helium, which results in a low plasma temperature, argon, nitrogen, or ambient air, which eliminates the need for gas cylinders. Impurities such as oxygen, water, or even hydrogen peroxide may be added to the feed gas to alter the plasma chemistry products [23].

Depending on its application and specific characteristics, a plasma may have a therapeutic or deleterious effect. This is largely dosage dependent, as the reactive species generated in a plasma play a role in many metabolic and cell survival processes as well as creating oxidative stress [24]. At low doses, the reactive species aid growth stimulation and pro-inflammatory signaling, while at larger doses, oxidation may result in cell damage. The effects vary widely between different cell types, and the plasma must be tailored to the application to achieve optimal results. Plasma medicine is a relatively new field, but is quickly growing with many devices reaching clinical trial stage [25].

The first plasma device was created by Siemens in 1857 [26]: a simple ring dielectric barrier discharge (DBD) for ozone production. After this, plasma medicine research lay dormant for over a century until forays into low-pressure plasma sterilization in the 1960s and 80s [27]. The 1990s saw the advent of atmospheric pressure discharges, and finally in 1997, the first funded

research for plasma medicine research was performed for NTP sterilization [28]. In the two decades since, plasma medicine research has expanded drastically.

NTP is being investigated for many different applications. Decontamination and sterilization of equipment and surfaces in hospitals and other settings is of particular interest due to the rise of multi drug resistant strains of bacteria. Plasma-treated liquids contain many of the important reactive plasma species, with longer lifetimes than they would otherwise have in air [29]. Direct medical therapies include skin disease treatment, chronic wound healing, selective cancer killing, disinfection of cavities and root canals, and blood coagulation, among others. The possibilities are ever advancing, and many technologies are now only a step away from use in medical offices.

Plasma's effects on cells and tissues are being widely studied, but research into how plasma affects these materials is necessary for an understanding of the underlying processes. Plasma has many different components that affect biological systems differently. Ultraviolet radiation is emitted by most plasma discharges. However, this is only significant at low pressure. At atmospheric pressures, most of the radiation is reabsorbed in the plasma volume [3]. Charged particles and electrons are also present, which may contribute to etching and current flow. The most widely investigated aspect of plasma interaction with cells are reactive oxygen and nitrogen species (RONS).

In a pure helium or argon discharge, RONS are formed when plasma ions or electrons collide with nitrogen and oxygen in atmospheric air [30]. These species include ozone, nitrogen oxides (NO and NO_2), superoxide, hydrogen peroxide, hydroxyl and other ground state and excited

species [31]. These species are very important to many biological and cellular processes. An excess may have detrimental effects, but in smaller quantities, they may have an advantageous therapeutic effect. RONS are also largely responsible for plasma's selective effect on cancerous and diseased tissues while leaving healthy cells unaffected [31].

Various additives can be used to tailor the plasma characteristics and reactive species generation to specific applications. The addition of molecular oxygen results in increased production of O, O_2^* and O_3. A humid feed gas results in increased generation of OH due to electron impact dissociation of H_2O and a lower ozone concentration [32]. However, there is a tradeoff between the generation and recombination processes in the presence of these impurities. At concentrations higher than a few percent, plasma temperature increases, and the effect of the associated reduced plasma and electron density due to attachment offsets the greater production of reactive species, and the effectiveness of the plasma treatment decreases [33].

2.3 Liquid and Gas Phase Chemistry

Most biological cells and tissues exist in a moist environment. There is a thin layer of water surrounding cells to ensure adequate hydration and conditions favorable for survival. When an indirect plasma, such as the plumes generated by the brush and jet devices, comes in contact with this layer, plasma interactions with water must be considered.

Shown below are some of the reactions that take place in the liquid plasma phase. Additional hydrogen peroxide (Equation 4) and superoxide (Equation 3) are formed. Plasma treated water

has also been found to contain high concentrations of hydroxyl radicals [29]. The reactive species then diffuse to the cells where they can cause damage such as peroxidation (Equation 8), or oxidation via the Fenton reaction (Equation 5).

$$e^-_{(H_20)} + O_{2(H_20)} \rightarrow O^-_{2(H_20)} \quad (4)$$

$$2H^+ + 2O_2 \rightarrow H_2O_2 + O_2 \quad (5)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^- \quad (6)$$

$$RH + OH \rightarrow H_2O + R^* \quad (7)$$

$$R^* + O_2 \rightarrow RO_2 \quad (8)$$

$$RO_2 + RH \rightarrow RO_2H + R^* \quad (9)$$

$$RO_2H \rightarrow RO_2^- + H^+ \quad (10)$$

Plasma activated water relies on long lived reactive species for a therapeutic or deleterious effect after the plasma is removed [34-36]. For biological treatment conditions where the environment contains water, liquid phase plasma chemistry must be considered.

2.4 Bacterial Structure

In order to understand the effects of plasma on a bacterial cell, the structure of a bacterium must be understood. Bacteria take many shapes. They may be spherical (coccus), spiral-shaped, rod-like (bacillus), or elongated (filamentous), and can link together in colonies. A typical bacterium

is on the order of 1 μm with a cell volume approximately 1 μm^3 [37]. This small size allows for a large surface-to-volume ratio and rapid uptake and distribution of nutrients.

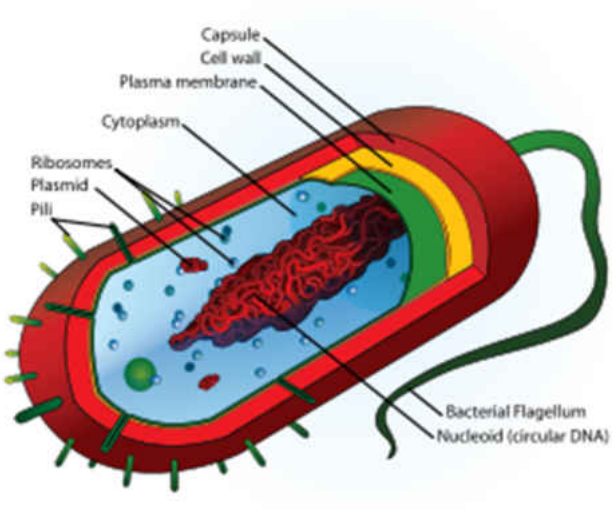


Figure 4. Bacterial structure. The enclosing structure of a bacterial cell consists of several layers, with the plasma capsule on the outside, followed by a cell wall and plasma membrane. Protrusions such as pili and flagella can be present to aid in motion[38]. Photo Credit: Mariana Ruiz Villarreal/Wikimedia Commons/Public Domain

The outer structure of a bacterium consists of a capsule, cell wall, and plasma membrane. These structures are permeable to molecules on the order of a few nanometers. Additional structures such as flagella and pili can be present to aid in locomotion. Internal organelles include ribosomes and free-floating DNA and plasmids. Plasma species can either interact with the outer structures or the internal structures after diffusion or permeabilization.

2.5 Mechanism of Bacterial Inactivation

The mechanisms of bacterial inactivation via plasma are not yet fully understood and research is ongoing. This is partly due to the complex dependence of cellular mechanisms and signaling on RONS. Discussed here are several different possible mechanisms that generally fall into two categories.

Damage to the cell wall, capsule, or plasma membrane occurs either due to restructuring of surface polysaccharides via lipid peroxidation in water or direct etching due to ion bombardment. Once the outer structures are significantly damaged, cell contents vital to cellular function, such as adenosine triphosphate (ATP) leak out of the cell, resulting in cell death [39]. Additionally, if any reactive species diffuse into the interior of the cell, they cause oxidative damage to DNA and proteins [17]. If this damage is significant enough, the cell may no longer be able to function.

There are two theories as to how plasma can disrupt the bacterial capsule. An accumulation of electric charge on the membrane may result in electrostatic disruption, where the outward electrostatic stress exceeds the material tensile strength of the capsule [39]. This mechanism seems to have a greater effect against gram-negative bacteria due to the irregular nature of the capsule and thinner peptidoglycan layer, allowing for greater charge buildup. See Figure 5. Additionally, oxidation of membrane components can occur, in which active radicals diffuse to the membrane and react with the biomacromolecules at the surface, creating structural instabilities.

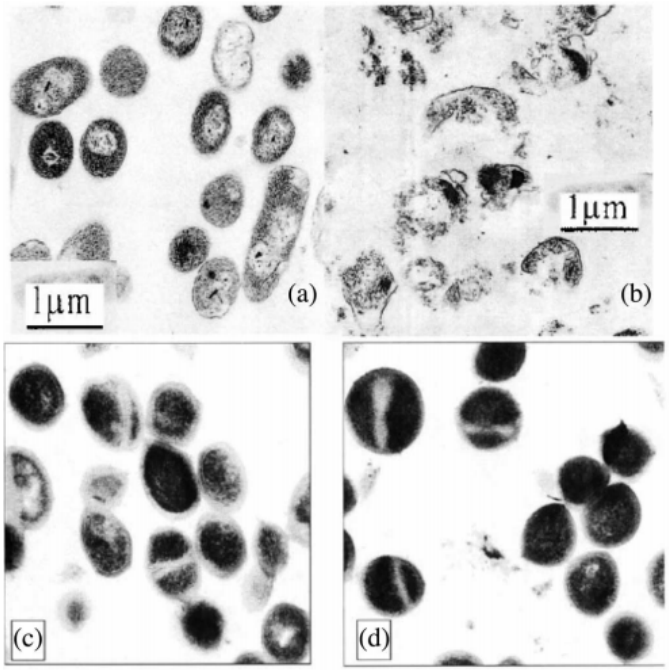


Figure 5. Ruptured cell membranes of E. coli (top) and S. aureus (bottom) after 30 seconds of plasma exposure. The former is gram-negative (top) and the latter is gram positive (bottom). The gram-negative bacteria show more membrane disruption in the atmospheric glow discharge likely due to a thinner outer membrane [39]. © 2000, IEEE

Peroxidation of the phospholipids in the plasma membrane is one such disruption mechanism [17]. This occurs in several steps beginning with radicals abstracting hydrogen from the side chain of an unsaturated fatty acid to produce a lipid-radical and water. Propagation occurs in a chain reaction mechanism, and the process is only terminated when two radicals react with one another or antioxidants scavenge radicals. This results in shorter lipids with an altered ability to rotate within the membrane. The loss of membrane integrity leads to permeabilization and possible lysis [40-42].

2.6 Kinetics of Bacterial Inactivation

Typical antimicrobial methods produce an exponential decrease in bacterial survival with respect to treatment time [43, 44], often graphed as a linear curve on a log scale. Plasma-treated bacteria often display more complex, multi-slope killing curves [45]. This can be explained by different inactivation methods occurring at different times. For example, if initial low inactivation results are seen, followed by an abrupt increase in inactivation rates, the early effects may be due to plasma damage to the cell capsule followed by internal effects [17]. Additionally, rates differ greatly for different species, as will be discussed in Section 5.2.

CHAPTER 3

METHODS AND MEASUREMENTS

The following chapter outlines the bacterial culture methods used for *Staphylococcus aureus*, *Acenetobacter baumannii*, and *Enterococcus facaelis* for both the laminate surface decontamination and biofilm inactivation studies. Additionally, safety measures necessary to minimize infection risk are covered. Finally, the imaging techniques used to detect excited reactive species in the plasma plumes and to quantify the number of live and dead bacteria are detailed.

3.1 Safety

Since *A. baumannii* ATTC 19606, *S. aureus* ATCC 25923, and *E. faecalis* ATCC 29212 are classified as biosafety level 2 organisms, based on U.S. Public Health Service Guidelines, appropriate safety measures are taken to minimize risk of infection and transmission [46].

Protective equipment- gloves and a lab coat- is worn at all times when working with bacteria, and hands are washed before exiting the laboratory. Work surfaces are decontaminated by wiping with ethanol and/or UV exposure. All biohazardous waste is disposed of in the appropriate containers. The laboratory complies with all other BSL-2 regulations.

Due to the lack of a biosafety hood in the laboratory where the plasma devices and gas supply systems are located, all experiments are performed inside a dead air box made of acrylic, which is sanitized with germicide and a 70% ethanol solution before and after each experiment.

3.2 Bacterial Culture

The overnight culture required for all experiments is prepared as follows. One bacterial colony is retrieved from a stock culture plate using a plastic inoculation loop and placed in 4 mL of nutrient broth in a 16 mL plastic centrifuge tube. The loop is agitated within the broth to release the bacterial mass from the loop. The vial is then vortexed for 10 seconds to break up clumps of bacteria and ensure even distribution in the broth. The vial is placed into an incubator at 37°C for 16 hours. Typically, this results in a concentration of 2 to 4 x 10^8 CFU/mL at the end of the incubation period, determined by CFU counts after dilution and plating and the 600 nm optical density (OD600) measurement with an Eppendorf® biophotometer. After determining the OD600 reading and multiplying by the conversion factor for each bacterial strain, dilutions are prepared as needed to achieve the desired inoculum concentration.

3.3 Pulse Generator

A custom pulse generator provided by the University of Southern California was used for all experiments. The pulser is powered by an XHR-600DC power supply from AMETEK® and is

capable of generating a maximum voltage of up to 10 kV. Both the pulse repetition rate and the frequency are adjustable, and the typical pulse width of the generated voltage waveform is 150 ns full width half maximum (FWHM). Power is transmitted to the plasma device via a coaxial cable. For every experiment, the current and voltage waveforms are measured and recorded using a Pearson® current probe (model number 6585), voltage attenuator (Tektronics® model P0015A), and Tektronix® DPO-5204 oscilloscope. Since there is significant attenuation of the voltage pulse in the cable, when possible, both waveforms are measured at the plasma device.

3.4 Plasma Device Setup

Ultra-high purity, 5.0 grade (99.999% pure) helium is used as the feed gas for all experiments, with the possible addition of water vapor or oxygen. Helium and oxygen are stored compressed in metal cylinders, and their release is controlled with a Victor ESS3 gas regulator for inert gases. The gas flow is guided by polyethylene tubing and all joints are fitted with stainless steel or brass Yor-Lok® fittings. The flow rate is controlled by either a MKS® type 146 mass flow controller (MFC) or an Omega® FT-082 rotameter. If impurities are added, this is done through a second channel of the MFC or a second rotameter. The flows are combined via valves and a t-fitting. To ensure the prevention of leaks in the flow system, each time the setup is readjusted, a leak-detecting soap solution is applied to all joints while gas is flowing through the system.

In the case of a humid feed gas, one channel is routed through a bubbler containing deionized water, resulting in a humidity of approximately 2.5% volume by volume (v/v). This flow and a channel for pure helium are recombined, and the humidity is detected using a Vaisala® HMT330

Humidity Probe. See Figure 6. The ratio of humid to dry gas is adjusted to achieve the desired humidity level.

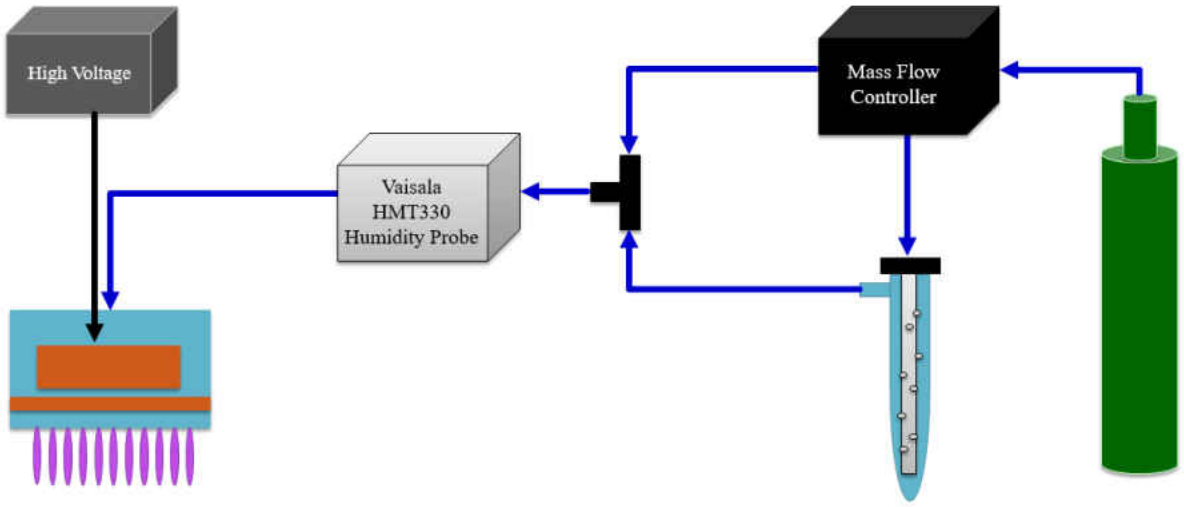


Figure 6. Plasma setup for a humid feed gas. The helium flow is split into two channels, one of which is humidified with a bubbler. The flows are recombined, and humidity is measured before the gas is routed to the plasma brush, where it is ionized and exits the nozzle as a plasma.

3.5 Detection of Reactive Species via Spectroscopy

For the spatially resolved optical emission spectroscopy measurements, the plasma is focused onto the slit of a 0.75 meter Czerny-Turner spectrometer using a set of mirrors to avoid chromatic aberrations. A gated-ICCD camera is used as the spectrometer detector in these experiments. See Figure 7. With the grating tuned to its zero order, the grating acts as a mirror

and an image is produced on the CCD device of the camera for initial alignment of the plasma brush image on the spectrometer slit. By tuning the spectrometer grating, we are able to maintain spatial resolution while dispersing the collected light into its fundamental components.

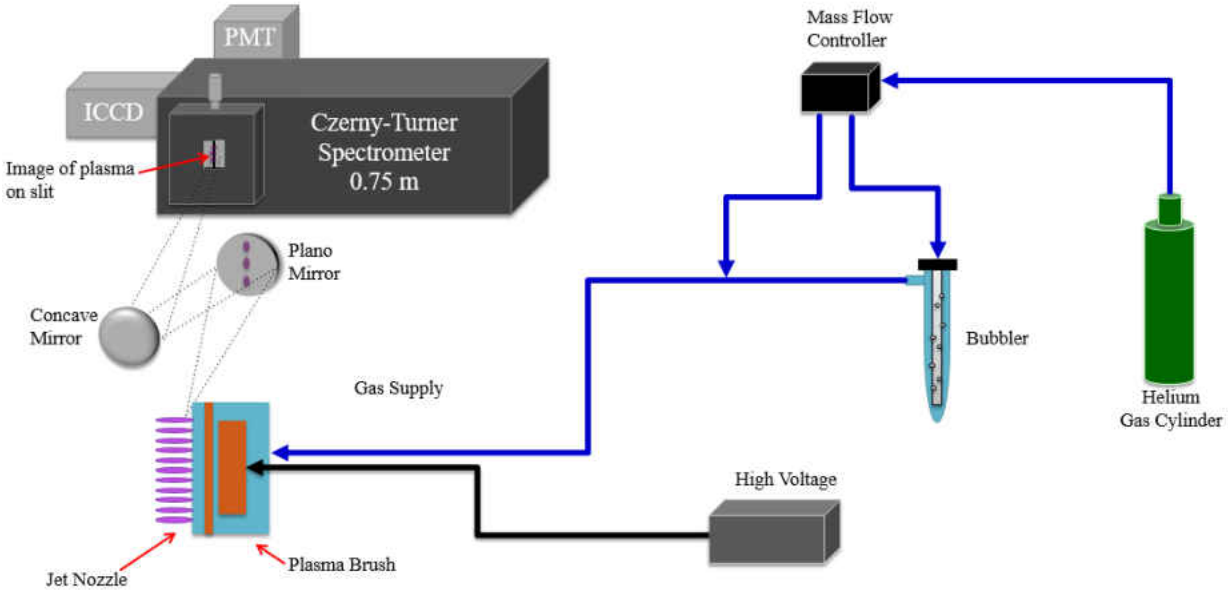


Figure 7. Spectroscopy setup for the plasma brush. The image of the brush is focused on the slit of the spectrometer using two mirrors. An internal grating is then tuned to separate the captured emission wavelengths. A photomultiplier tube (PMT) is used to amplify the emission signal that is captured by the intensified charge-coupled device (ICCD) camera.

Before recording of the optical emission spectrum from the plasma discharge, the instrument response function of the spectrometer and collecting optics is measured using a quartz tungsten halogen lamp to allow our collected data to be correct for relative intensity.

Measurements are typically performed for the full range of the spectrometer from 200 to 850 nm. The grating used is 1800 grooves per millimeter, and the slit width is typically 20 um, resulting in a spatial resolution of approximately 50 μm and a spectral resolution of 0.02 nm. For the plasma brush, emissions are weak, and a large gain of 100 and an 8000 ms gate width are used to maximize the signal. Above 450 nm, an order sorting filter is placed between the concave mirror and the spectrometer slit to eliminate higher frequency harmonics.

Using the Princeton Instruments® Lightfield software, the background and cosmic ray signals are removed. The spectrum was then integrated vertically to give the strongest possible signal for comparison of differing experimental conditions.

3.6 Statistical Analysis

Statistical analysis to assess the significance of the deviation from the control is performed for each treatment, using a two-sample Student's T test for independent samples. First, the mean and standard deviation for each sample group are calculated. The t-value for each treatment condition is then calculated using Equation 10.

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{s_d\sqrt{\frac{1}{n_1}+\frac{1}{n_2}}} = \frac{(\bar{x}_1 - \bar{x}_2)}{s_d\sqrt{\frac{2}{3}}} \tag{11}$$

Where $\bar{x}_2$ is the mean of the control group, $\bar{x}_1$ is the mean of the treatment group, s_d is the pooled variance, and n_1 and n_2 are the sample sizes of the respective groups, typically 3 for the experiments performed.

Since the samples have different standard deviations, a pooled variance, s_d, is used.

$$s_d^2 = \frac{s_1^2(n_1-1)+s_2^2(n_2-1)}{n_1+n_2-2} \tag{12}$$

Where s_1^2 is the variance of the treatment group, and s_2^2 is the variance of the control group. The p- value is determined as follows by evaluating the probability of t for the student's t distribution with degree of freedom: $n_1+ n_2 - 2$.

$$t \leq 0; \quad p = 2\Pr(t < t_{n_1+n_2-2}) \tag{13}$$

$$t > 0; \quad p = 2\left(1 - \Pr(t < t_{n_1+n_2-2})\right) \tag{14}$$

In each chart, a single asterisk represents a p value smaller than 0.05, two asterisks for p less than 0.01, and three for p less than 0.001. The lack of an asterisk denotes no significant deviation from the control group. It can be said with (1-p)*100% confidence that the treatment group shows a different outcome than the control group.

CHAPTER 4

PLASMA JET FOR BIOFILM INACTIVATION

The purpose of this study is to investigate the biofilm inactivation effect of a plasma jet that was previously designed and built for this purpose. As described in Chapter I, biofilms that occur in the root canals of teeth pose a significant disinfection challenge. Due to the small geometry of the cavity and the resilience of the biofilm, bacteria often remain after the tooth is sealed, leading to a high recurrence of root canal infections, up to 20% depending on the treatment method [7, 20, 47, 48]. A plasma jet produces a plume of plasma that can enter the root canal and contains reactive species capable of killing bacteria. The challenge is creating a device that is capable of high rates of inactivation.

4.1 Materials and Methods

This section details the materials and methods used for the biofilm inactivation studies. Initially, an overnight bacterial culture is grown, which is then seeded to form a biofilm. The biofilms are then treated with a plasma jet device, the specifications of which are given below.

4.1.1 Biofilm Preparation

To grow the biofilm, *E. faecalis* (ATCC 29212), a common biofilm-forming pathogen, is inoculated to hydroxyapatite (HA) disks 5 mm in diameter and 2 mm thick. HA disks are washed with 10% bleach, rinsed 5 times with deionized water, and autoclaved at 121°C before use. Sterile HA disks are placed face-up in 2 mL microfuge tubes. An overnight culture is seeded in Luria-Bertani broth, composed of 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. The overnight culture is diluted to 1.25×10^5 CFU/mL and 1 mL is added to each tube. See Figure 8. Care is taken to ensure that the disks remain level so that the bacteria settle evenly. The disks are incubated at 37°C for 7 days. Every two days, 500 µL of bacterial solution is gently removed and discarded in a liquid waste container containing 100% bleach. A new pipette tip is used to replace the removed solution with 500 µL LB broth, making sure not to disturb the growth on the disk. At the end of the 7 days, the disks are removed from the tubes using flame-sterilized forceps, after which the plasma treatment is performed.

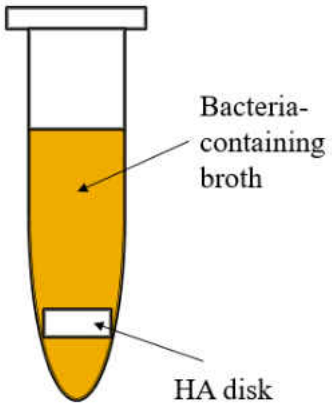


Figure 8. Biofilm cultivation conditions. A 2 mL centrifuge vial contains an HA disk coated with collagen. The bacterial suspension is added, and the bacteria settle and grow on the upper surface of the disk.

During initial biofilm inactivation experiments, bacteria were deposited onto hydroxyapatite disks directly. However, this results in a biofilm that is easily detachable. This results in highly variable results, as some cells slough off during media changes or removal from the vial. Additionally, there is a strong inactivation effect for a gas only treatment, indicating than many of the bacteria are "blown away" off of the surface of the disk. In subsequent experiments, the disks are first coated with bovine type I collagen from calf skin to provide an anchor for the biofilm.

To prepare the stock solution, 50 mg of collagen are added to 50 mL 0.1 M acetic acid, stirring at room temperature for 3 hours until dissolved. This solution is diluted 1:10 with 0.1 M acetic acid to give a 0.01% weight by volume (w/v) collagen solution. Sterile HA disks are coated with 50 $\mu g/cm^2$ of the collagen solution, 10 µL total and allowed to dry. Since the collagen solution is

heat sensitive and not entirely sterile, sterilization is achieved by exposing the coated disks to UV radiation for 30 minutes in the biosafety hood. The bacteria suspension is then added as described previously.

4.1.2 Plasma Jet Device

The plasma jet consists of a stainless steel cylindrical housing, a ceramic tube with a 1 mm outlet hole, and a stainless steel electrode with a 1mm through hole. See Figure 9. The device is connected to the nanosecond pulser via a male-to-female Bayonet Neill-Concelman (BNC) connection in which the ground is connected to the outer housing and the high voltage is connected to the internal electrode. This configuration is that of a dielectric barrier discharge, thus the plasma is generated inside the device, with the plasma plume extending approximately 3 cm from the nozzle opening.

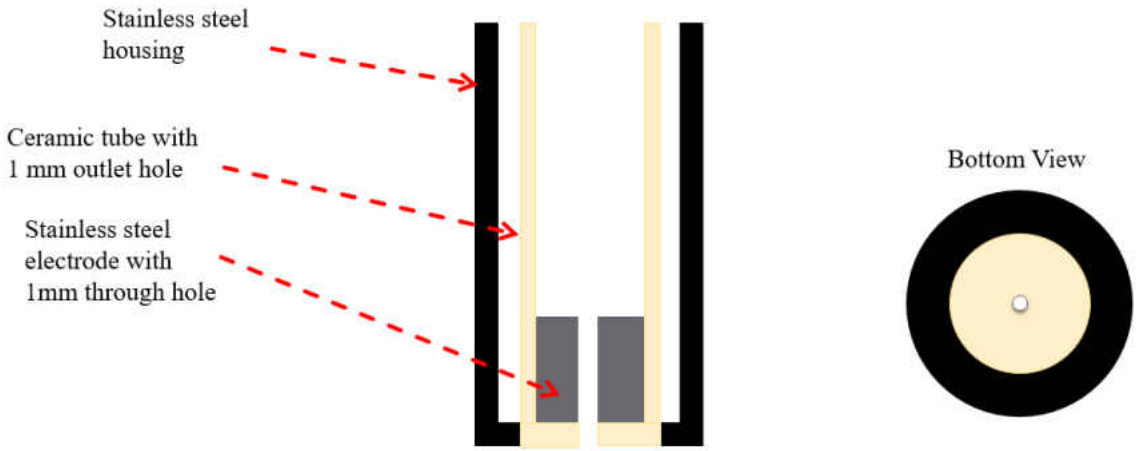


Figure 9. Side and bottom views of the plasma jet. The stainless steel housing contains a ceramic tube with an outlet hole and a stainless steel electrode. The nozzle opening is 1mm in diameter, resulting in a plasma plume of the same dimension.

A pulsed voltage of 6 kV at a frequency of 1.5 kHz is used to generate the plasma. The current and voltage waveforms seen in Figure 10 are typical for the experiments performed, resulting in an energy per pulse of 0.89 mJ. The gas flow rate is 1 standard liter per minute (slpm) helium with the addition of up to 5% oxygen.

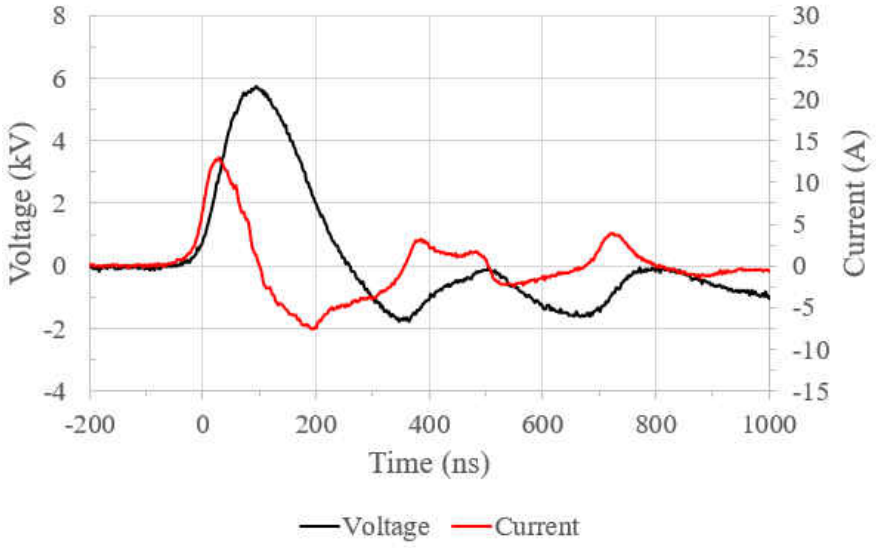


Figure 10. Typical current and voltage waveforms for the plasma jet device

The Reynold's Number of pure helium flowing through and exiting the nozzle of the device is calculated as follows.

$$Re = \frac{D_H v \rho}{\mu} \tag{14}$$

Where D_H is the hydraulic diameter, v is the mean velocity of the fluid, ρ is the density of helium, and μ is the dynamic viscosity of helium. The hydraulic diameter of a circular pipe is the diameter, in this case, 1mm. At 1 slpm, the mean velocity of the fluid is 4.38 mm/s, calculated by dividing the flow rate by the cross sectional area of the channel.

Finally, the density and dynamic viscosity of helium are 0.166 kg/m^3 and 19.6 μPa·s at room temperature (22°C).

This gives a Reynold's Number of 0.037 which is in the laminar range where the fluid dynamics are dominated by viscous forces (Re< 2300).

4.1.3 Plasma Treatment of Biofilms

The general protocol for biofilm treatment using the plasma jet is as follows. For most experiments, three disks are used as a control, receiving no treatment. Three disks are used as a sham treatment, receiving a gas only treatment for the duration of the longest plasma treatment. Each subsequent treatment condition is repeated for another three disks.

The disks are placed so the surface is 5 mm from the nozzle of the plasma jet, centered on the HA disk, and treated at varying conditions. See Figure 11. Plasma treatment time ranges from 30 to 180 seconds and each condition is repeated in triplicate.

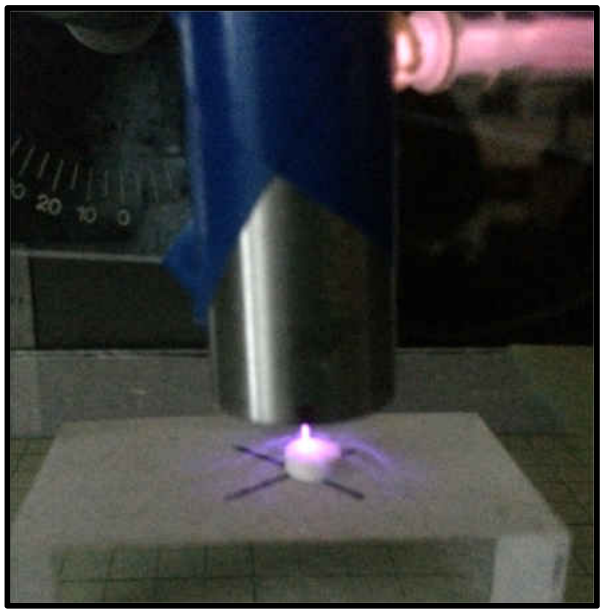


Figure 11. Plasma jet incident upon an HA disk. The plasma plume impinges on the surface of the disk and spreads out in filaments to the side with the gas flow before becoming diffuse.

To recover the bacteria from the disks after treatment, a sterile cotton swab is moistened with the nutrient media or phosphate buffered solution (PBS), with any excess dabbed off on the side of the conical. The treated surface of the disk is are swabbed thoroughly, rolling the swab for best recovery. The swab heads are placed in 2 mL centrifuge tubes containing 1 mL of nutrient broth or PBS, and the handles are cut off to fit in the tube. The tubes are then vortexed for at least 30 seconds to resuspend the bacteria. Serial dilutions are performed as needed. 50 μL of the desired dilution is added to an agar plate and distributed with a spreader to ensure an even dispersal. The plates are then incubated overnight at 37°C, and CFUs are counted manually the next morning to determine inactivation with respect to the control.

4.2 Results

Initial studies are performed on agar to determine the inactivation zone of the plasma jet. Thereafter, studies are performed on biofilms grown on HA disks as described above. Finally, oxygen is added to the feed gas to optimize the bacterial inactivation effect for those parameters.

4.2.1 Agar Studies

For the plasma jet, 10^5, 10^6, and 10^7CFU/cm^2 of *Enterococcus faecalis* ATCC 19606 are inoculated to LB agar and allowed to dry. 3 minutes of plasma treatment with a maximum voltage of 6 kV, pulse repetition rate of 1.5 kHz, and 1 slpm of helium are performed. The results

seen in Figure 12 show an approximately circular inactivation region. For the lowest inoculation density, a region of 12 cm^2 was almost completely free of bacteria. For increasing inoculation density, the plasma proves to be less effective. There might still be a 5 log inactivation, however, remaining bacteria are likely shielded from the plasma effects or the concentration of reactive species is not high enough to inactivate all bacteria.

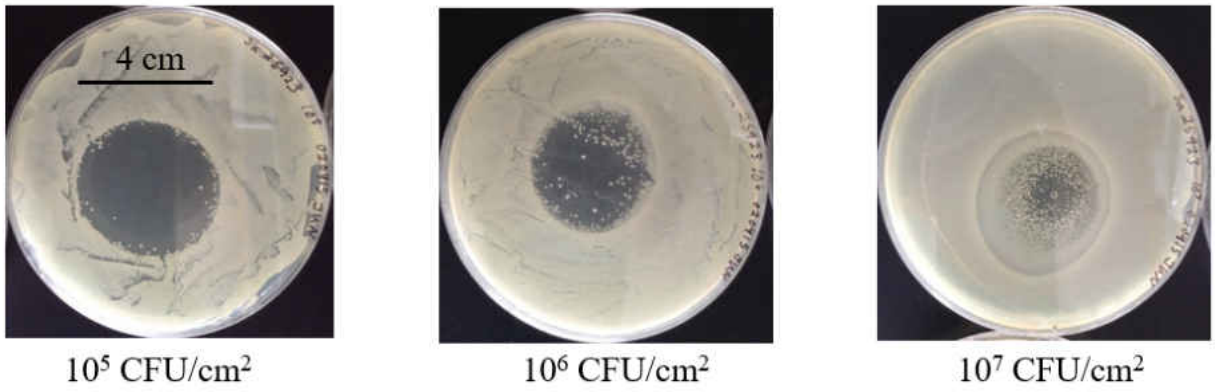


Figure 12. Plasma jet inactivation of differing concentrations of E. faecalis *on agar after 3 minutes of treatment. The plasma jet is incident at the center of the visible inactivation regions. At lower inoculation concentrations, the bacteria are almost completely inactivated inside a circular region with a radius of 2 cm. At higher concentrations, the bacterial inactivation occurs, but is not enough to cause complete inactivation at the center.*

4.2.2 Biofilm Inactivation

To quantify the bacterial inactivation effect of the plasma jet, biofilms are grown on hydroxyapatite (HA) disks as described above. Initial results yielded low bacterial inactivation of

only 0.7 log. Since oxygen is a component of many biologically relevant reactive species generated in a plasma, the effect of added oxygen in the feed gas is investigated. Concentrations of oxygen ranging from 0% to 5% in helium are used as the feed gas. The treatment duration is 3 minutes at the standard treatment conditions.

Bacterial inactivation increases with increasing oxygen concentration up to a point, after which the bacterial inactivation drastically decreases. See Figure 13. The highest reduction of 1.5 log was seen for 1% O_2, whereas 5% oxygen was the least effective with only 0.35 log reduction. There is a tradeoff between generation and recombination of reactive species, which will be detailed in the discussion section.

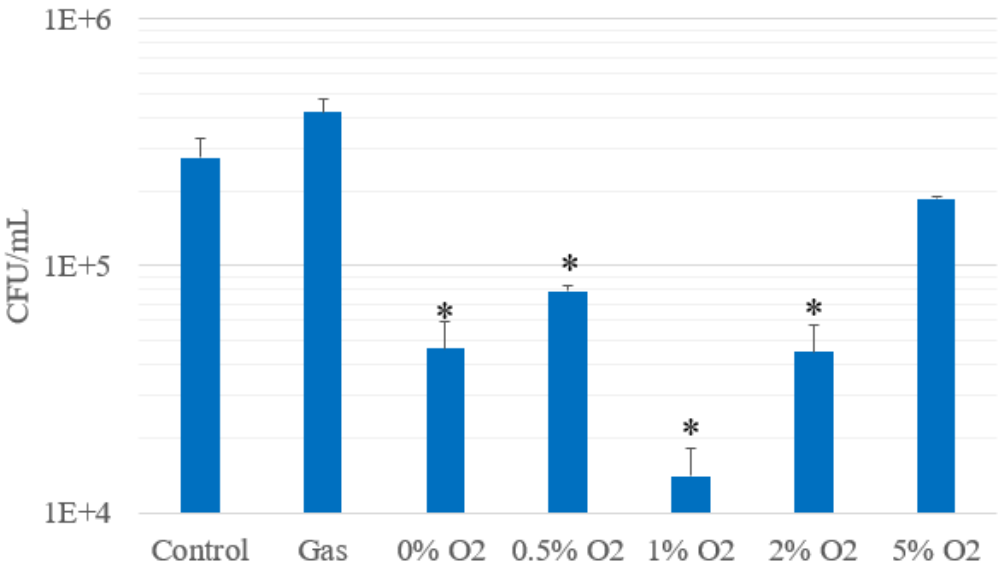


Figure 13. Bacterial biofilm inactivation dependence on oxygen concentration. The inactivation effect is maximized at 1% oxygen, but decreases at higher concentrations.

4.3 Discussion

The plasma jet has a high effectiveness against bacteria inoculated to agar. However, at higher bacterial densities, starting at 10^7 CFU/cm^2, not all bacteria are inactivated. This effect was not quantified, but may partially account for the decreased efficacy of the plasma jet against bacterial biofilms, where the CFU recovery is 10^6CFU/cm^{2}for the control. Note that this is likely less than the true bacterial density in the biofilm, as the recovery method does not recover all bacteria from the surface. Also, diffusion of reactive species in the bulk of the biofilm may be slower that diffusion in agar or solution. This effect is seen for antibiotic treatments, though the molecules involved are much larger than those present in the plasma plume.

When oxygen is added to the feed gas, at low percentages, there is an increased bactericidal effect. This is due to the increased production of reactive oxygen molecules such as O, $\bullet O_2$, and O_3. At higher percentages of oxygen in the feed gas, this effect is offset by recombination and attachment processes, and bacterial inactivation is greatly reduced.

4.4 Conclusion

A non-thermal atmospheric-pressure plasma jet is tested for bacterial inactivation of *E. faecalis* biofilms. On agar, up to a 10^6 CFU/cm^2 reduction in bacterial growth is observed for a 3 minute treatment time. For the same conditions, only a 0.7 log reduction in biofilm growth is recorded. The decreased effect shows the increased resistance of a bacterial biofilm to plasma similar to

other antimicrobial agents. However, the addition of 1% oxygen to the helium feed gas improves the bacterial inactivation by more than a factor of 20.

Future work should include biofilm inactivation studies in a geometry more similar to that of a root canal, as well as optical emission spectroscopy to determine the excited reactive species present in the plasma plume. Additional modifications in the treatment protocol should be performed to increase the efficacy of bacterial inactivation.

CHAPTER 5

PLASMA BRUSH FOR SURFACE STERILIZATION

The purpose of this study is to investigate the surface bacterial inactivation effect of a plasma brush device. In hospitals, drug-resistant strains of bacteria on medical devices and surfaces result in secondary infections that can be fatal. The problem lies with the difficulty of adequately sterilizing heat-sensitive materials such as plastic. Existing methods are expensive and cumbersome, and plasma devices like the one being investigated may provide a suitable alternative. The reactive species in the plasma plume of the brush are incident upon a larger surface area, making it ideal for this application.

5.1 Materials and Methods

This section details the materials and methods used for the surface bacterial inactivation studies. Initially, an overnight bacterial culture is grown, which is then inoculated to an approximately 6 cm square piece of wood laminate. The inoculated regions are then treated with the plasma brush device, the specifications of which are given below.

5.1.1 Laminate Preparation

To quantify the bacterial inactivation effect of the plasma brush on a hard surface, bacteria are inoculated to a laminate surface, treated, and recovered, and CFUs are counted. Two different strains of bacteria, *Staphylococcus aureus* ATCC 25923 and *Acinetobacter baumannii* ATCC 19606, are subjected to the treatment and analyzed. *S. aureus* is used for preliminary experiments, and data for *A. baumannii* is added for comparison. The two strains are selected as representatives from gram-positive and gram-negative bacterial strains and are both common nosocomial pathogens, many strains of which have become drug resistant.

Before inoculation, the rigid sides of the laminate pieces are cleaned by wiping with 70% Ethanol (EtOH) and allowed to dry completely. Then, 25 or 50 µL of the inoculum spread with a pipette tip to cover a central region on the laminate of 1 or 2 centimeters in width, and 4 cm in length. See Figure 14. Typically, 125,000 or 1,250,000 CFU are inoculated to each laminate piece for *S. aureus* and *A. baumannii*, respectively. The latter requires a higher inoculation concentration due to a recovery rate that is almost one order of magnitude lower than for *S. aureus*. The pieces are allowed to dry in the hood for approximately 30 minutes, or until no liquid is evident upon inspection.

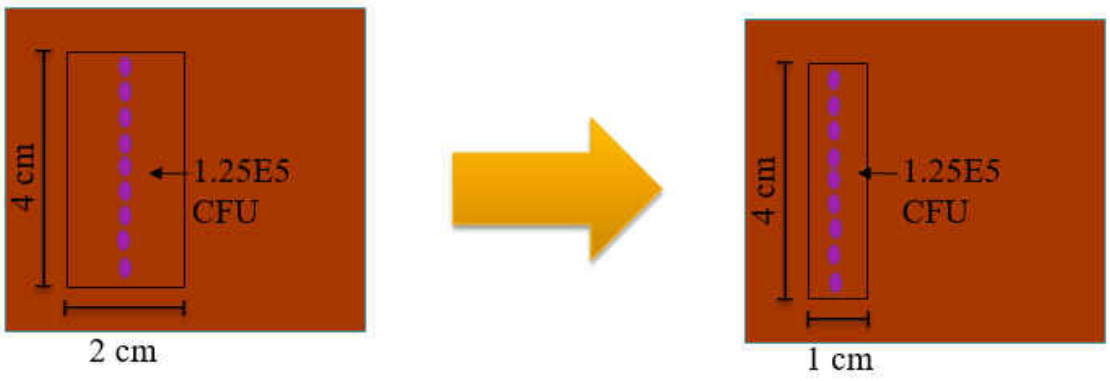
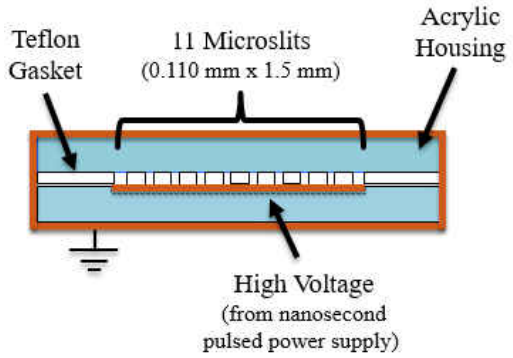


Figure 14. Inoculation area for plasma brush treatment for S. aureus. The bacterial suspension of the dilute overnight culture is inoculated to either a 8 cm^2 or 4 cm^2 region. The number of bacteria inoculated remains the same, doubling the inoculation density. The inoculation region widths are marked on the edge of the laminate, and plasma treatment is performed along the centerline.

Preparation of the laminate and plasma exposure are performed in different lab spaces for convenience. To prevent contamination, the laminate pieces are put into a plastic box with a latching lid during transport. The box is sprayed with germicide, left to sit for 10 minutes, and wiped down with ethanol prior to use. The same procedure is used at the end of the experiment to decontaminate the box. Alternately, the container is exposed to UV radiation for 30 minutes in the biosafety hood.

5.1.2 Plasma Brush

The plasma brush device consists of an external copper ground electrode, wrapped around the acrylic housing and an internal HV electrode powered by a nanosecond pulsed power source. A helium feed gas flows over the HV electrode, and the gas is then guided into 11 flow channels by a Teflon gasket. The nozzle of the device is a series of microslits, 110 um thick and 1.5 mm wide with a 4 mm spacing in between. See Figure 15.



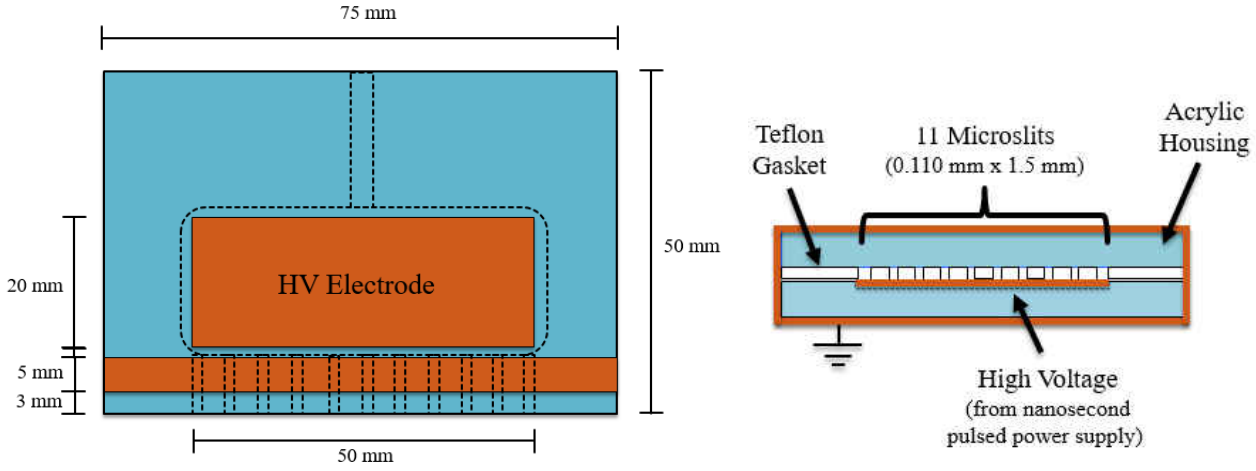


Figure 15. Plasma brush device configuration from the front (left) and the bottom (right). A copper ground electrode is wrapped around an acrylic housing containing the high voltage electrode. A Teflon gasket creates 11 flow channels. After passing over the high voltage electrode, ionized gas exits the microslits, forming 11 distinct plasma plumes.

A pulsed voltage of 9.5 kV at a frequency of 2 kHz is used to generate the plasma. The current and voltage waveforms seen in Figure 16 are typical for the experiments performed, resulting in an energy per pulse of 0.6 mJ. The gas flow rate is 1 slpm helium with the addition of up to 3% water vapour.

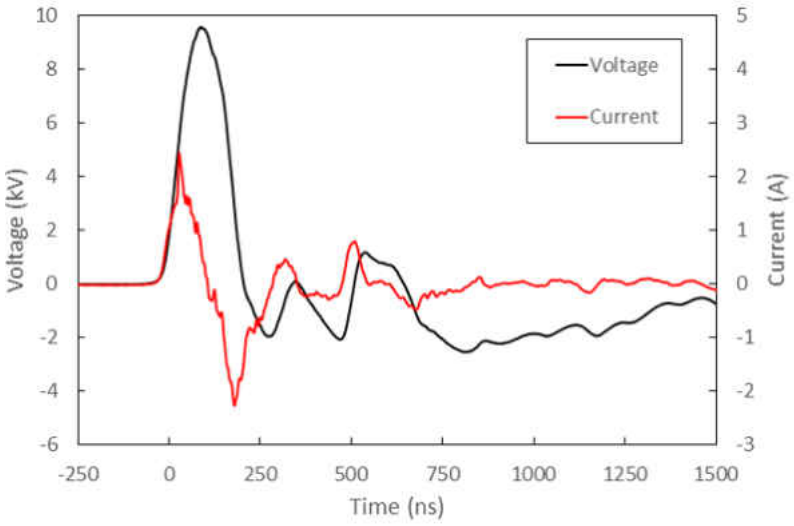


Figure 16. Typical current and voltage waveforms for the plasma brush

The Reynold's Number of pure helium flowing through the each individual microchannel, assuming the gas flow is distributed evenly across all 11 channels, is calculated using Equation . The hydraulic diameter for a rectangular pipe is four times the ratio of area to perimeter.

$$D_H = \frac{A \cdot 4}{P} = \frac{l \cdot w \cdot 4}{2l+2w} = 12.8\ \mu m \quad (16)$$

At 1 slpm, the mean velocity of the fluid is 9.18 mm/s, calculated by dividing the flow rate by the cross sectional area of the channel. This gives a Reynold's Number of 9.95×10^{-4} which is in the laminar range, indicating the fluid dynamics are dominated by viscous forces (Re< 2300).

5.1.3 Plasma Treatment of Laminate

The general protocol for laminate treatment using the plasma brush is as follows. For most experiments, three laminate pieces are used as a control, receiving no treatment. Three pieces are used as a sham treatment, receiving a gas only treatment for the duration of the longest plasma treatment.

The laminate pieces are placed so the surface is 5 mm from the nozzle array of the plasma brush, centered along the long axis of the inoculated area, and are treated at varying conditions. See Figure 17. Plasma treatment time ranges from 1 to 5 minutes and each tested condition is repeated in triplicate.

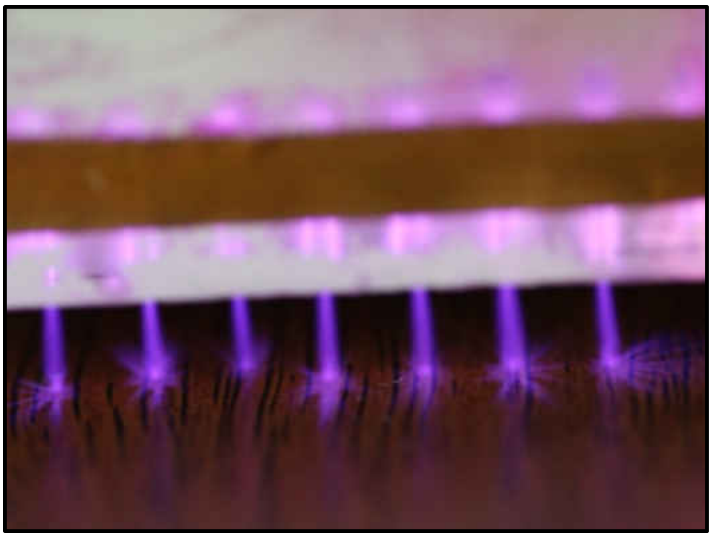


Figure 17. Plasma brush incident on laminate surface. The individual plasma plumes impinge on the surface and spread, creating a star-like pattern.

To recover the bacteria from the laminate after treatment, a sterile cotton swab is moistened with the nutrient media or phosphate buffered solution (PBS), with any excess dabbed off on the side of the conical. The inoculation area and surrounding few millimeters in every direction are swabbed thoroughly, rolling the swab for best recovery. The swab heads are placed in 2 mL centrifuge tubes containing 1 mL of nutrient broth or PBS, and the handles are cut off to fit in the tube. The tubes are then vortexed for at least 30 seconds to resuspend the bacteria. Serial dilutions are performed as needed. For each of two dilutions per laminate piece, 50 µL is added to an agar plate and distributed with a spreader to ensure an even dispersal. The plates are then incubated overnight at 37°C, and CFUs are counted manually the next morning to determine inactivation with respect to the control.

5.2 Results

Before any experiments are performed on laminate, inactivation studies are performed on agar to characterize the inactivation patterns of the plasma brush. Thereafter, the bacterial inactivation of the plasma brush on laminate is optimized by adjusting various parameters, including electrode configuration, feed gas humidity, and inoculation area. Finally, water is added to the laminate surface to characterize the effects of liquid phase plasma in comparison to gas phase.

5.2.1 Agar Studies

10^7 CFU/cm^2 of *Staphylococcus aureus* ATCC 25923 are inoculated to TSB agar and allowed to dry. Treatment conditions are a 5 minute gas control treatment and 1, 3, and 5 minutes of plasma treatment at a distance of 1 cm from the nozzle exit to the agar surface with a maximum voltage of 9.5 kV, pulse repetition rate of 2 kHz, and 1 slpm of helium. The results show a roughly oval inactivation region, increasing in area with treatment time. See Figure 18. The area of inactivation is somewhat irregular. This may be attributed to slight variations in the geometry of the microslit nozzles, as the acrylic housing and Teflon gasket of the device are manufactured by hand. The plasma plumes then do not exit the nozzle at precisely 90°, but at a slight angle. Some plumes even exhibit a split stream shape. Additionally, some slits might receive less of the gas flow and become more turbulent.

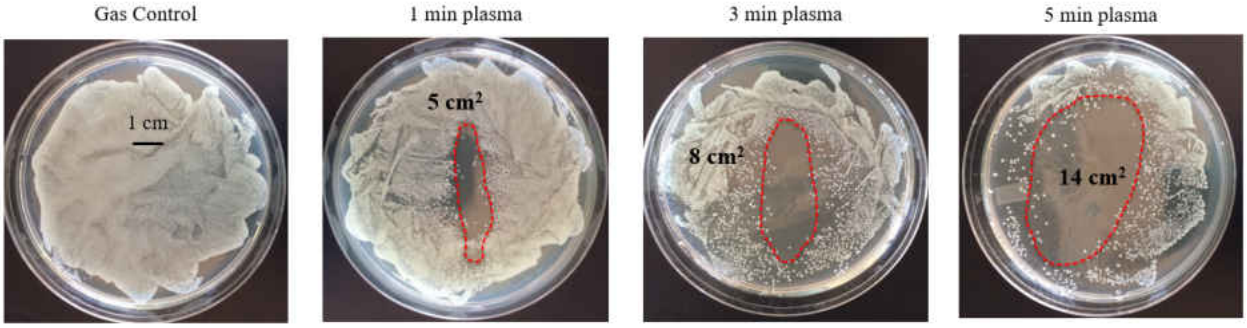


Figure 18. Plasma brush inactivation of S. aureus *on agar. For an inoculation density of 10^7 CFU/cm^2, the inactivation region increases from about 5 cm^2 with 1 minute of plasma treatment to nearly 14 cm^2 at 5 minutes.*

For the 5 minute plasma treatment, inactivation appears to be skewed to the left side of the image. This is explained by the use of the dead air box for the experiments. A person walking by or the air conditioning turning results in air disturbances that push the plasma toward the rear of the box and result in the skewness seen in the image.

5.2.2 Plasma Optimization

Initial bacterial inactivation experiments on laminate were performed with the plasma brush and resulted in 0.4 log (60%) reduction of bacterial viability for *S. aureus*. Sterilization is the complete inactivation of all microorganisms, and typical anti-bacterial household agents kill

upwards of 99.9% (3 log) of bacteria on a surface. To increase bacterial inactivation, several modifications are made to the plasma device and operating protocol.

Initially, pulse voltage is limited by arcing that occurs between the ground and high voltage electrodes voltages above 6 kV. To increase the supply voltage and power deposited into the plasma, the ground electrode is moved 3 mm from the edge of the brush. This allows for use of the full range of the pulse generator, up to 10 kV.

However, for certain flow rates, arcing still occurs even with the adjusted electrode. Using flow rates above 5 slpm and below 1.5 slpm alleviates this issue, likely due to decreased buildup of plasma around the nozzle and ground electrode. Bacterial inactivation studies are performed for the integer flow rates between 1 and 5, and the highest and lowest flow rates exhibited similar bacterial inactivation rates. See Figure 19. All further experiments use a flow rate of 1 slpm to conserve gas.

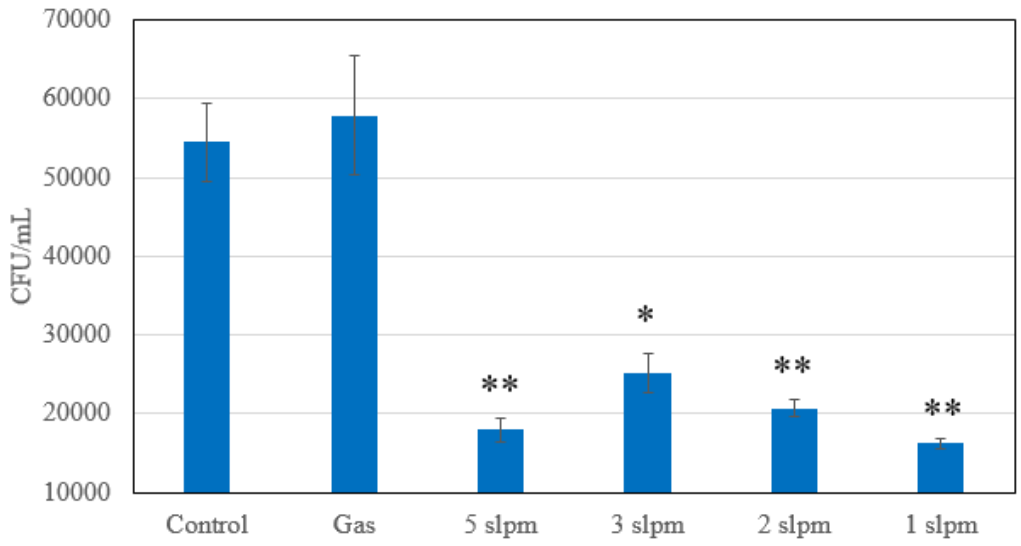


Figure 19. Bacterial inactivation dependence on feed gas flow rate. Bacterial inactivation is lower at 2 and 3 slpm. These flow rates correspond to increased arcing, likely indicating that less energy is deposited into the plasma that interacts with the surface.

With the altered electrodes, increased voltage, and optimized flow rates, bacterial inactivation increases slightly to 0.5. While an improvement, this was still not satisfactory. On agar, the plasma brush is capable of a 7 log reduction at the incidence region. One large difference between agar and laminate, aside from surface characteristics, is the presence of water. Thus, the effect of adding humidity to the feed gas is investigated.

Humidity is added to the feed gas as detailed in Section 3.4. Maintaining a flow rate of 1 slpm, the ratio of the dry and wet gas is adjusted and allowed to stabilize for 10 minutes to give the desired humidity level.

The bactericidal effect of the humid plasma is maximized at intermediate humidity values, between 0.7% and 1.4% v/v (7000 and 14000 ppm). See Figure 20. This once again increases bacterial inactivation slightly to 0.6 log.

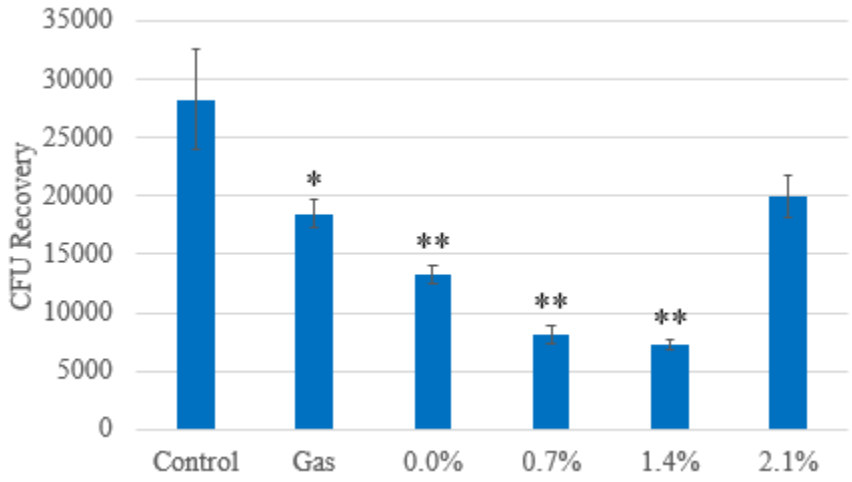


Figure 20. Bacterial inactivation dependence on feed gas humidity. As with oxygen in Figure 13, bacterial inactivation is maximized at around 1% water in the helium feed gas.

Finally, when comparing results on agar to results on laminate, it is noted that the bactericidal effect is much greater on agar, with an inoculation concentration of 10^7 CFU/cm^2 being almost completely inactivated for an area of 14 cm^2 after 5 minutes of plasma treatment. See Figure 18. This might still be in part to the water concentration of the agar. However, another consideration is the porosity of the agar, which may allow reactive species to diffuse within the medium instead of being reflected off of the surface as is likely with the laminate. This would result in a

much smaller inactivation region on laminate than on agar. Thus, to detect the range of the reactive species generated in the plasma plume, the inoculation area is reduced from 2 by 4 cm to 1 by 4 cm. The number of bacteria inoculated remains the same, doubling the inoculation density for the smaller region. A 0.5 by 4 cm region is also tested but yielded highly variable results due to the inaccuracy of inoculating by hand and aligning laminate pieces of different sizes with the nozzle of the plasma brush.

With the reduction of inoculation region, bacterial log inactivation almost doubles to 1.1 log for *S. aureus* under the same treatment conditions of 9.5 kV, 2 kHz, 1 slpm, and 50% RH. See Figure 21. This shows that for the larger inoculation region, a large portion of the recovered bacteria has not been affected by the brush, and only bacteria at the central region are negatively affected by the plasma. The maximum bacterial inactivation effect is seen at 9.5 kV, 2 kHz, 1slpm He/1.4% O_2, 5 mm gap, and 5 minute treatment time.

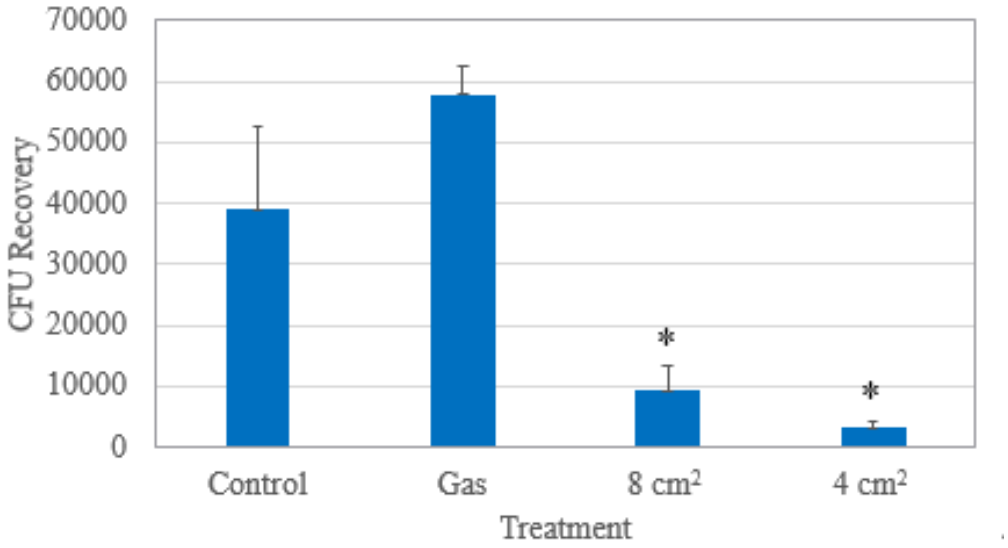


Figure 21. Inoculation area dependence of bacterial inactivation. Bacterial inactivation increases from 0.6 log to 1.1 log for a smaller inoculation region.

Adding water to the feed gas improves bacterial inactivation up to a point. However, there is a tradeoff between the production of additional reactive species due to the presence of hydrogen and oxygen and recombination processes. Thus a different method of adding water to the system is investigated.

5.2.3 Plasma over Wet Laminate

On agar, bacteria are covered by a thin film of water, resulting in a moist environment and the liquid phase of plasma when subjected to plasma treatment. See Section 2.3. Plasma treated

liquids have been found to retain reactive species for therapeutic use without direct plasma treatment [34-36]. On the dry laminate, reactive species remain in contact with the surface and bacteria for a relatively short period of time. Thus instead of simply adding moisture to the feed gas, a thin layer of water is added to the laminate before the plasma treatment.

After the laminate is inoculated and allowed to dry and just before treatment, 100 μL of deionized water is spread in a thin layer over a region covering the inoculation region and approximately 2 additional millimeters in each direction. Wet laminate is also used for the control and gas treatment groups. Plasma treatment with a helium only feed gas is then performed on dry and wet laminate. It is noted that some of the water evaporates during treatment and some of the inoculated region becomes dry during treatment. The results show

greater than a 2 log reduction for the plasma over wet laminate and no statistically significant reduction for the dry laminate. See Figure 22.

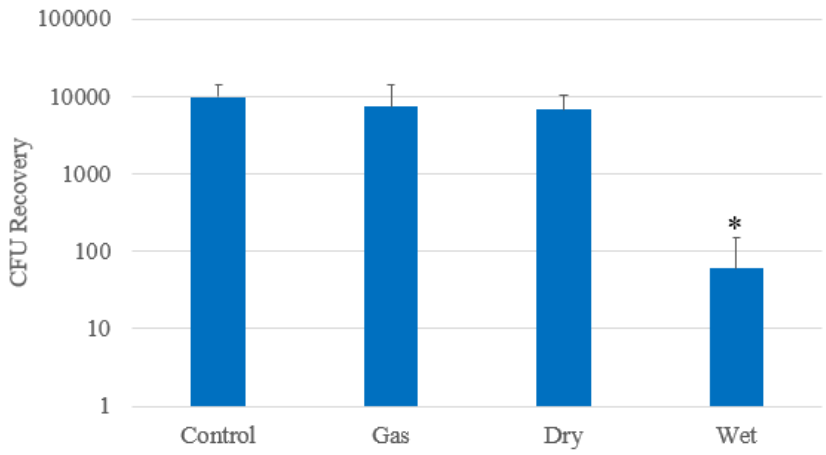


Figure 22. Comparison between bacterial inactivation of the plasma brush over dry laminate and laminate covered with 100 µL of water. No significant reduction is seen for the dry laminate, but for wet laminate, a reduction of bacterial growth of 99% is achieved.

With the addition of 150 µL of water, the entire laminate surface remains covered, and bacterial inactivation reaches 4 log for a 3 minute treatment time; zero bacteria are detected using the recovery method. However, it is possible that there are still bacteria present on the surface. Using this protocol, killing curves for *S. aureus* and *A. baumannii* are determined.

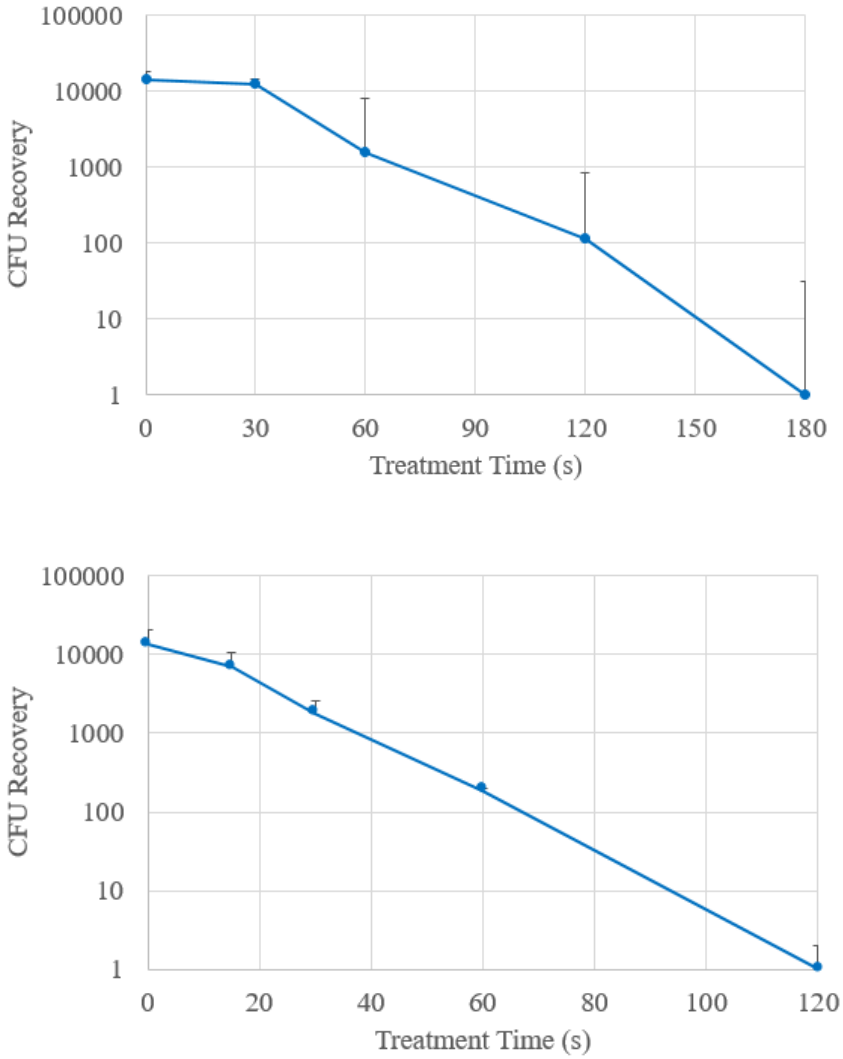


Figure 23. Killing curves for S. aureus (top) and S. aureus (bottom). S. aureus exhibits a two-phase bacterial inactivation. Due to it being a gram-positive bacterium, it is likely that the initial slow inactivation phase is due to the protection of a thicker outer layer. Once this barrier is permeabilized, the inactivation rate increases. A. baumannii, on the other hand, exhibits a single-slope killing curve.

S. aureus exhibits a two-phase killing curve, indicating different modes of plasma inactivation as described in Section 2.6. Between the start of treatment and 30 seconds, there is a very low

inactivation rate of 0.1 log/minute, after which it increases to 1.4 log/minute. One explanation is that during the initial phase of treatment, the reactive species act on the bacterial envelope. Once these membranes are permeabilized, RONS enter the cell and oxidize proteins and genetic material, leading to rapid cell death.

A. baumannii, on the other hand, has a single-phase killing curve as seen in Figure 23 bottom, with an inactivation rate of approximately 2 log per minute. The difference may be due to structural dissimilarities of the bacteria; *A. baumannii* is gram negative while *S. aureus* is gram positive. The thicker peptidoglycan layer of the latter may be more resistant to permeabilization by plasma.

5.3 Optical Emission Spectroscopy

Spectroscopy is performed for a humid discharge as well as over water. The plasma brush emissions are measured to determine what reactive species are present that may affect the bactericidal effect of the plasma. Time- and space- integrated spectroscopy provides no temporally or spatially resolved information, but instead enables comparison of relative intensity between several different plasma conditions. For a humid feed gas, the emission spectra are recorded for five different water concentrations, up to 1.4%. In a separate experiment, the plasma conditions recorded are all combinations of dry and humid (1.4% H_2O) feed gas, and dry and wet laminate.

5.3.1 Humid Feed Gas

Spectra for 0, 0.2, 0.3, 0.7, and 1.4% water in helium feed gas are obtained at the standard pulse conditions: 9.5 kV, 2 kHz, 1 slpm, and 5 mm nozzle-to-surface distance. The excited reactive species detected include OH, N_2 (2+), N_2^+ (1-), He, and atomic oxygen. See Figure 24 for a typical spectrum of the pure helium discharge.

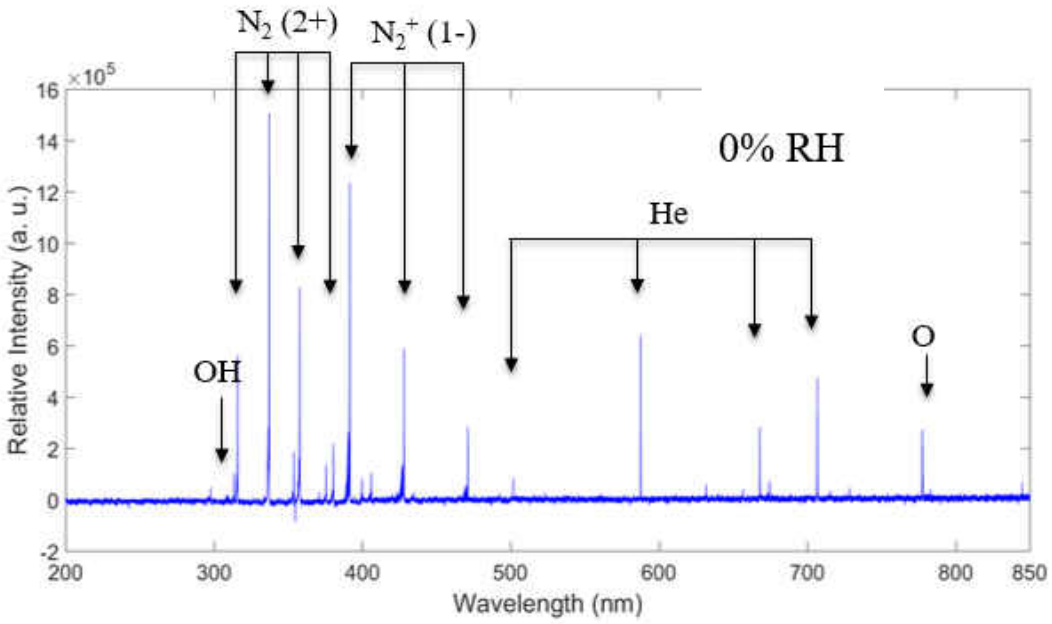


Figure 24. Emission spectrum of dry gas for pure helium. Several different excited reactive species are detected in the plasma plume.

Comparing the relative emission intensities of several characteristic excited species reveals a very interesting result. Emission intensities decrease for increasing water concentration for all excited species across the spectrum. See Figure 25 for the helium 706 emission line, a

representative example of what is seen across the spectrum. This is the inverse of the bactericidal effect, which increases with humidity. Thus the plasma affects the bacteria through some different mechanism, most likely other, ground-state reactive species such as ozone or NO_x.

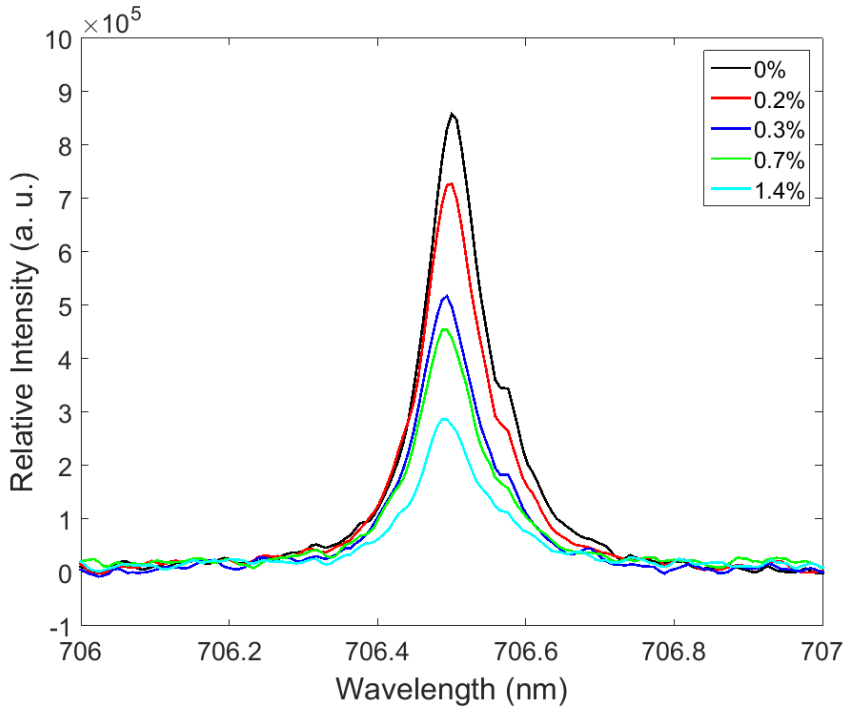


Figure 25. Comparison between the excited helium emission lines at 706 nm for plasma generated in feed gases of varying humidity. The emissions are strongest for low concentrations of water and decrease as humidity increases.

To determine the presence of these reactive species, specifically ozone (O_3) and NO_x, a pump system is set up to funnel the plasma gas to ozone and NO_x detectors. Both detectors have a detection limit of one part per million (ppm), and both fail to detect any ozone or NO_x. This does

not eliminate the possibility that these species are present, particularly since the smell of ozone is very strong when the plasma is running, but instead signals that the concentration is below the detection limit of one ppm. The limit of smell detection of ozone in humans is 0.02-0.05 ppm [49]. Further diagnostics to determine the presence of these species is not performed.

5.3.2 Dry and Wet Plasma Over Water and Laminate

Spectra for a dry plasma over water, dry plasma over laminate, humid plasma over water, and humid plasma over laminate were obtained at the standard pulse conditions. The excited reactive species detected include OH, N_2 (2+), N_2^+ (1-), He, and atomic oxygen. See Figure 26 for the overlaid spectra for all parameters.

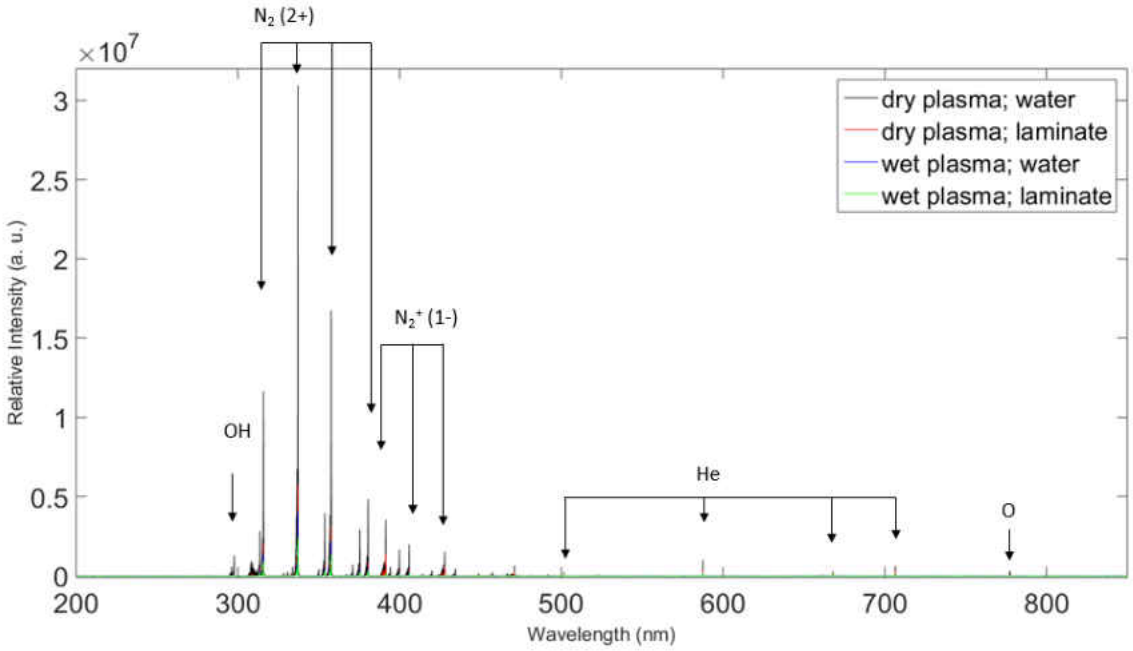


Figure 26. Emission spectra for the plasma brush device for all combinations of dry and humid feed gases over water and over laminate. Several different reactive species are detected in the plasma plume.

When comparing the emission intensity for the different plasma conditions, several different emission lines are examined and similar trends are seen. The highest emission levels occur for a dry plasma over water. The second highest emissions are generated by the dry plasma over laminate. The same trend follows for the humid plasma over water and over laminate, albeit at far smaller emission intensities. Figure 27 shows the emission lines for the hydroxyl radical, centered at 310 nm, and one of the N_2 lines at 337 nm.

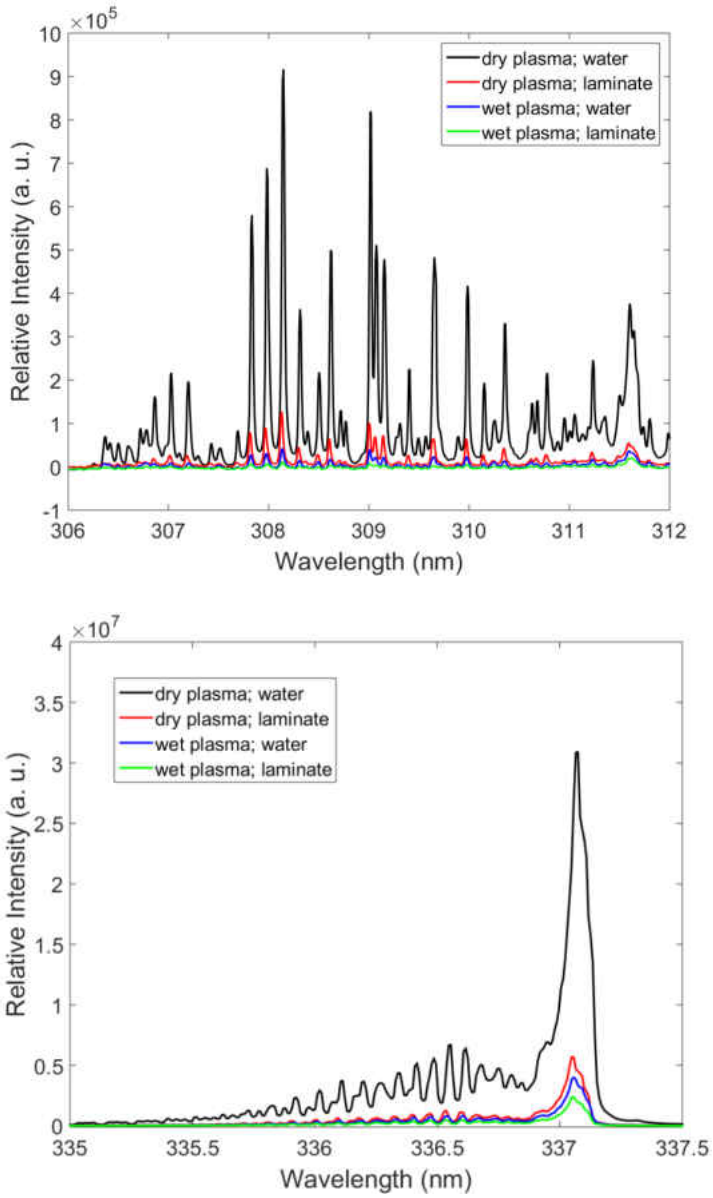


Figure 27. Comparison of the emission lines for OH centered at 308 nm (top) and for N_2 at 337 nm (bottom) for different plasma conditions.

The lower emission intensities for a humid discharge are discussed in Section 5.3.1. Looking at dry plasma over water and over laminate, for each emission line, there is at least a six-fold

increase over water. This is due to secondary reactions that take place at the water surface, as discussed in Section 2.3.

5.3 Discussion

The plasma brush has a high effectiveness against bacteria inoculated to agar. Initial results with dry helium on dry laminate showed a much smaller bacterial inactivation effect. The addition of water to the feed gas incrementally increases the inactivation effect of the brush. This method of increasing efficacy is limited by the tradeoff between the presence of water generating more hydroxyl radicals due to electron-impact dissociation and recombination and quenching events.

In a humid discharge, more hydroxyl radicals are formed. See Equations 10 and 11, where M^+ is any ion with ionization energy above the H_2O ionization threshold.

$$M^+ + H_2O \rightarrow M + H_2O^+ \quad (17)$$

$$H_2O^+ + H_2O \rightarrow H_3O^+ + OH \quad (18)$$

Hydroxyl radicals can then react with nearby organics, leading to chain oxidation and thus damage of cellular membranes and other cell components (here R is any organic molecule).

$$OH + RH \rightarrow R\cdot + H_2O \quad (19)$$

$$R\cdot + O_2 \rightarrow RO_2 \quad (20)$$

$$RO_2 + RH \rightarrow RO_2H + R\cdot \quad (21)$$

However, at the low humidity at which bacterial inactivation is maximized, this effect is not much larger than that imparted by the ambient humidity.

Over dry laminate, plasma is only present in the gas phase. The addition of water to the treatment surface adds the chemistry of the liquid phase to the equation. See Section 2.3. Additionally, reactive species enter the liquid and are essentially trapped in proximity to the bacterial cells.

5.4 Conclusion

A non-thermal atmospheric-pressure plasma brush was tested for bacterial inactivation of *S. aureus* and *A. baumannii* on laminate surfaces. On agar, up to a 14 cm^2 inactivation zone was observed for a 5 minute treatment time. For the same conditions, using a 2x4 cm inoculation region, resulting in only a 0.5 log reduction in bacterial viability. With the addition of 1.4% water to the feed gas, a 0.6 log reduction was achieved. Decreasing the inoculation area to 4 cm^2 nearly doubled the effects to a 1.1 log reduction. The greatest improvement in efficacy was seen with the addition of water to the treatment surface, resulting in at least a 4 log reduction in bacterial viability in 3 minutes for *S. aureus* and 2 minutes for *A. baumannii*.

Optical emission spectroscopy was also performed for varying humidity in the feed gas and over water. This resulted in a decrease in emission intensity with increased humidity, likely indicating

the increased presence of ground state and electronegative species. Water at the surface greatly increased emissions due to the addition of liquid phase plasma chemistry.

Future work should include further studies to analyze the mechanism for bacterial inactivation, including analysis of the plasma-treated liquid, live-dead staining and imaging, or polymerase chain reaction.

CHAPTER 6

SUMMARY AND PERSPECTIVE

Antimicrobial resistance is a significant problem in many aspects of the medical field. Many bacterial strains are becoming resistant to multiple drugs and are particularly prevalent in hospitals. Medical equipment may be sensitive to heat and difficult to sterilize, and reuse without proper disinfection has caused illness and death in many patients. Additionally, in biofilm form, such as is found in the root canals of teeth, bacterial are more resistant to antimicrobial chemicals and treatments. Non-thermal atmospheric-pressure plasma is being investigated as an alternative method for sterilization under these circumstances.

In summary, a plasma jet and a plasma brush device were investigated for their bactericidal effect when incident on a biofilm and contaminated surface, respectively. Initially, decontamination of surfaces and biofilms proved to be challenging with an indirect plasma source. However, it is possible to increase the bacterial inactivation effect with adjustment of the plasma chemistry with the addition of oxygen or water in the feed gas or water at the treatment surface. Biofilm inactivation is maximized for the plasma jet device at a 1% concentration of oxygen in the helium feed gas. For the plasma brush, while the addition of humidity to the feed gas slightly improves results, the addition of water to the treatment surface results in a 4 log inactivation effect. The addition of the liquid phase plasma is critical for the maximization of the antibacterial effect.

The reactive species present in the plasma plume of the brush at different treatment conditions are also characterized. The excited species OH, N_2, N_2^+, He, and O are detected. Emissions decrease with increasing humidity and over dry laminate compared with over water. These trends combined with the bacterial inactivation results provide some insight into the mechanism for bacterial inactivation.

Future studies should incorporate additional spectroscopy and imaging to further clarify the inactivation mechanism, and the technology should advance to eventual clinical trials.

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APPENDIX A

PERMISSIONS

Title: An overview of research using the one atmosphere uniform glow discharge plasma (OAUGDP) for sterilization of surfaces and materials

Author: T. C. Montie; K. Kelly-Wintenberg; J. R. Roth

Publication: Plasma Science, IEEE Transactions on

Publisher: IEEE

Date: Feb 2000

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VITA

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Researched the effects of combining low temperature plasma and pulsed electric fields. Mastered microbiological laboratory techniques, including culturing and handling bacteria. Independently designed experimental protocols to test the bacterial inactivationof a surface dielectric barrier discharge (sDBD) plasma in combination with pulsed electric fields.

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The University of Texas Women in Engineering Program

PUBLICATIONS

Conference Presentations and Manuscripts

1) (**Oral Presentation at the 57th Annual Meeting of the APS Sivision of Plasma Physics**) J. Neuber, M.A. Malik, S. Song, C.Jiang, "A Nanosecond Pulsed Plasma Brush for Surface Decontamination", Proc. of the 57th DPP Meeting, November 16-20, 2015 in Savannah, Georgia
2) (**Live Demonstration at the 2015 Biomedical Circuits and Systems Conference**) J. Neuber, S. Song, C. Jiang, "Cold Microplasma Brush and Needle for Surface Disinfection", Proc. of the 2015 BioCAS Conference, October 22-24, 2015 in Atlanta, Georgia
3) (**Oral Presentation at the 2015 International Conference on Plasma Science)** J. Lane, S. Song, J. Neuber, C. Jiang, "Enhanced efficiency of atomic oxygen generation in a single electrode, 5 ns pulsed microplasma jet", Proc. of the 2015 ICOPS, May 24-28, 2015 in Antalya, Turkey
4) (**Oral Presentation at the 2015 International Conference on Plasma Science)** S. Song, J. Lane, J. Neuber, C. Jiang, "Spatiotemporal evolution of a single-electrode nanosecond pulsed microplasma jet over water", Proc. of the 2015 ICOPS, May 24-28, 2015 in Antalya, Turkey