Inactivation of Airborne Bacteria by Direct Interaction with Non-Thermal Dielectric Barrier Discharge Plasma:

The Involvement of Reactive Oxygen Species

A Thesis

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Dedications

This thesis is dedicated

to

The loving memory of my father.

and

My mother, who is the greatest source of strength and inspiration to me.

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-- Nachiket

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ABSTRACT

Inactivation of Airborne Bacteria by Direct Interaction with Non-Thermal Dielectric Barrier Discharge plasma: The Involvement of Reactive Oxygen Species
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The present study examined the effect of Dielectric Barrier Discharge (DBD) plasma on bioaerosol particles. Different DBD plasma devices were designed and tested for their efficacy in inactivation of airborne bacteria. Bacterial aerosols were injected in / through the plasma stream and the treated bioaerosols were analyzed. The results indicated a complete inactivation of bioaerosol upon a very short exposure in the range of milliseconds to plasma discharge. A large system was designed to evaluate its efficacy to inactivate bacterial spores.

After preliminary studies, to study the underlying mechanisms of inactivation, a single filament DBD plasma generating probe was developed and used for subsequent studies. In parallel, a near uniform aerosol generator (nebulizer) was optimized, and aerosol particle size characterized. The kinetics of bacterial inactivation produced by this system was investigated, and sub-lethal dose determined. We hypothesized that the prototype bacteria, *Escherichia coli* when present in aerosols and exposed to single filament DBD plasma system, activates intracellular reactive oxygen species (ROS).

The predetermined sub-lethal dose of DBD plasma was used to study the cellular responses of *Escherichia coli* during its inactivation. Cell membrane is more vulnerable when bacteria are present in aerosols, and hence the changes in features, such as cellular respiration and growth, permeation, and depolarization were investigated following exposure to single

filament DBD plasma system. During studies, the catalase mediated defense system was found to be involved predominantly in the management of intracellular ROS pool. Through the use of *E. coli* derivatives of specific gene mutation, we analyzed the involvement of heat stress-responsive genes. Although the plasma is considered non-thermal, localized heating and the generated interactive stress is likely involved in the inactivation of *E. coli* bioaerosol. These findings provide a new dimension in underlying mechanisms of *E. coli* inactivation during DBD plasma exposure.

1. BACKGROUND AND LITERATURE SURVEY

1.1 Plasma Discharge

The word “Plasma” is used to describe the fourth state of matter in Physics. The term was first coined by Irvin Langmuir who noticed the similarities between the biological liquid and the ionized gas [1, 2]. Plasmas have historically been used in applications related to electronics and chemistry, but recently there have been a large number of studies related to the biomedical applications of plasma [3-7]. The plasma state can be described as ionized form of the gas that they are produced in with the total net charge being neutral [8]. Besides charged particles, plasmas also contain neutral atoms and molecules, excited atoms and molecules, radicals and UV photons.

1.1.1 Non-thermal Plasmas

The broadest classification of plasma is done in terms of their temperature profile as ‘Thermal’ and ‘Non-thermal’ Plasmas. Thermal plasmas are characterized by very high temperatures of electrons and heavy particles, both charged and neutral. In non-thermal plasmas, the gas temperature is closer to room temperature while the electron temperature is high [8, 9]. Since the gas temperature of these non-thermal plasmas is low, it can be used in applications that require the treatment of temperature sensitive materials. This characteristic has opened up the possibility to use these non-thermal plasmas for the treatment of such materials as biological matter such as cells and tissues [10]. Non-thermal plasmas are already being used heavily in material processing applications, such as etching, activation and deposition [11-16]. Recently, there have been many studies into the sterilization effect produced by plasma.

1.1.2 Plasma as a Tool for Sterilization:

Electrical Plasma is an emerging technology for sterilization. Plasma has been used as an effective tool in sterilizing surfaces, cleaning water, medical devices etc. However, there has not been a study that delved deeper into the effect of plasma on bacteria and the role that plasma generated species play in microbial death. DBD is a non-thermal discharge is cold to touch. The gas temperature in this discharge is room temperature (about 25°C) [17]. The electron temperature however, is ~1eV. These high energy electrons are helpful in producing various species. These species are ozone, UV radiation (including VUV) and Reactive Oxygen Species (ROS) [18]. This plasma chemistry is very important in sterilization of microbes. The plasma species stated above, both individually and synergistically, inactivate bacteria. The bactericidal properties of ozone, UV and ROS are well known. This study investigates the sterilization effect using non-thermal plasma discharge (DBD).

1.1.3 Dielectric Barrier Discharge

The Dielectric Barrier Discharge was developed over a century ago by Siemens. Its configuration is quite basic. There are two electrodes that are separated by a gap. The gap can have any gas, such as Air, N_2 etc. There are one or more dielectric layers between the two electrodes. A high voltage AC current is applied across the electrodes. Due to the high voltage, the resulting electric field produces ionization in the gap between the two electrodes.

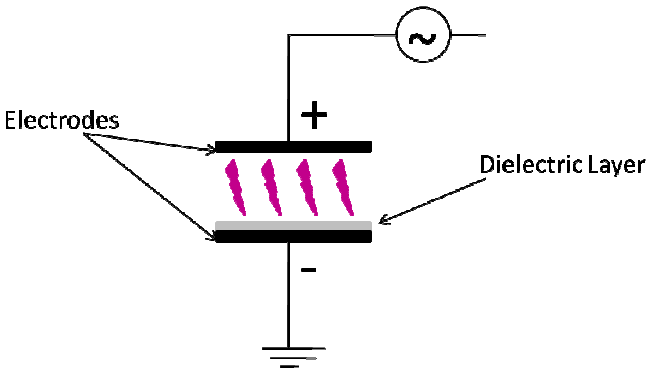


Figure 1: Basic Schematic of a DBD

Conventional sterilization is based on methods such as ozone, UV or Heat Sterilization. These methods, while widely used, all have certain disadvantages and limitations. Plasma discharge produces many highly reactive species and therefore is a great sterilization agent. A reduction in 6 log in the bacterial concentration has been observed for treatment times of 30 seconds or less [19]. This short exposure time makes plasma an exciting alternative to conventional sterilization systems. However, the mechanism of sterilization and bacterial defense against plasma needs to be studied. Plasma is known to produce reactive oxygen species (ROS). Gaunt et.al [20] reviewed the effect of different plasma species on bacteria.

Depending on the gas medium, applied voltage, plasma geometry and electrode gap distance, plasma species vary. The parameters influencing the plasma generation are electron density, gas temperature and the type and amount of reactive species produced and stable species such as ozone. The amount of ROS generated per electron volt is measured by the g-factor. Non-thermal DBD plasma has a g-factor between 0.3-0.5.

1.2 Bio-aerosols

Bioaerosols are viable and/or nonviable biological particles, such as bacteria, virus, fungal spores, and pollen grains and their fragments and by-products (e.g., endotoxins, mycotoxins), that are suspended in the air [21]. These are generated as droplets and dry solid particles, having different aerodynamic diameters that range from 0.5 to 100 μm [22, 23]. The generation of bioaerosol occurs during bubble bursting, where larger droplets of water are broken into smaller droplets and microorganisms (single cells or groups) are usually surrounded by a thin layer of water [24]. Aside from natural activities, land spreading of slurries, pressurized spray irrigation events, and aeration basins at wastewater treatment plants are a few ways microorganisms become aerosolized. The bioaerosols that are generated from dry surfaces (e.g., feedlots, soils, plants) or during the land application of dry manures can be released as individual or groups of cells or associated with inorganic or organic particulate matter [25]. In hospitals and laboratories, bioaerosols are generated by cleaning of wounds, spraying of infected parts, wounds, sample processing and centrifugation.

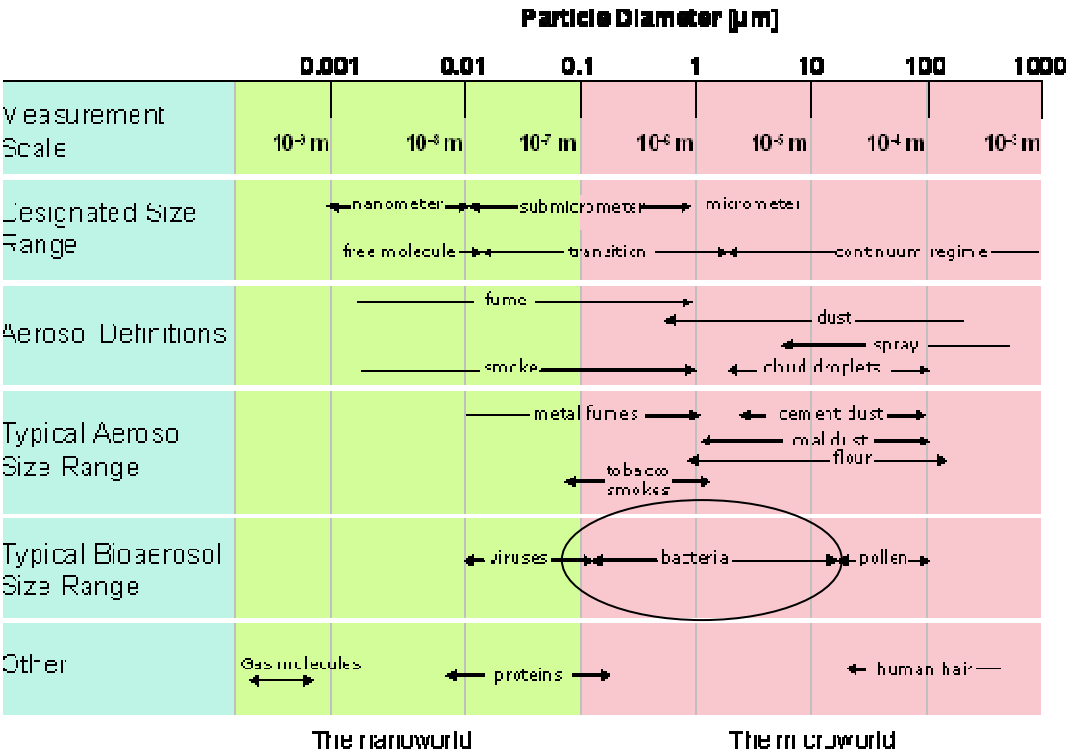


Figure 2: Size Distribution of Various Aerosols and Bioaerosols

The size distribution of aerosols is shown in the figure above. Aerosol particles 1 to 5 µm in diameter are of the greatest concern because they are readily inhaled or swallowed, but the greatest retention in the lung alveoli occurs with the 1- to 2-µm particles [26].

1.2.1 Sources of Bio-aerosols and Adverse Health Effects caused by Bio-aerosols in Indoor Environments

Bio-aerosols can originate from a variety of sources and each source gives rise to a unique composition. HVAC ventilation systems are major culprits of bioaerosol formation and transport. These bioaerosol later deposits on the surfaces inside the rooms connected with the

HVAC systems and produce secondary bioaerosols. There are severe human health effects associated with the accumulation of bioaerosols inside indoor environments.

1.2.1.1 Buildings

Bioaerosols are often said to be responsible for sick building syndrome (SBS) and building related illnesses (BRI). The major sources are building materials; fungal contamination within wall, ceiling, and door cavities by movement of cells, spores, and cell fragments via wall openings and gaps at structural joints. The populations of microorganisms multiply due to the lack of fresh air due to increased insulation of buildings, poorly maintained or operated ventilation systems, and high humidity levels. This is especially true in developing countries where poor design and lack of maintenance can lead to problems.

1.2.1.2 Healthcare Facilities

Health care facilities often house individuals with exposed wounds and respiratory infections. These individuals are a potential source of infection as they shed the infectious agent from the skin, respiratory tract and other contaminated sites. Ventilation causes dilution thus reducing the microbial load but still predisposing patients. Sinks, wash-basins and drains, nebulizers, humidifiers, and cooling towers are potential sources of Gram-negative bacilli, which colonize on the moist surfaces. Hospital environments often house patients with infections of potentially deadly pathogens such as *Acinetobacter baumannii*, MRSA and *Clostridium difficle*. Dressings and bedding also can be the sources of airborne microorganisms [27]. Fungal spores gain entry into the hospital buildings through ventilation ducts. Since exposure levels are high, this may be an issue in the immuno-compromised patients.

1.2.1.3 Infectious Diseases

Infectious diseases that are caused by viruses, bacteria, fungi, protozoa involve the transmission of the causative infectious agent from a reservoir to a susceptible host through direct contact, airborne transmission or vector-borne transmission. Airborne transmission of these disease causing agents is one of the most dangerous methods of transmission. The exposure to bioaerosols containing these pathogens can occur in certain specific professions, for example, health workers (tuberculosis, winter stomach flu, measles), farmers, veterinarians (Q-fever, swine influenza, anthrax). Whenever people are spending large amount of time in confined spaces such as an office or an airplane, there is a greater possibility of disease transmission [28, 29]. (Influenza, winter stomach flu, TB, etc.) *Legionellae* are Gram-negative bacteria that can cause potentially fatal pneumonia, particularly in susceptible subjects (e.g. elderly or immuno-compromised subjects). *Legionellae* become airborne often as a result of active aerosolizing processes. Outbreaks have been reported in varied situations such as hospital bathrooms [30], meat packers [31], workplaces where water mist systems are used (fruit and vegetable stores) [32]. Several diseases have been attributed to fungi that include aspergillosis, histoplasmosis, blastomycosis, coccidioidomycosis and adiaspiromycosis [33, 34]. Thus, high-risk occupations for occupational infectious diseases due to bioaerosol exposure include farmers, veterinarians, health care workers and biomedical workers studying infectious agents.

1.2.1.4 Respiratory Diseases:

Respiratory symptoms and diseases have been widely studied as dust-associated health effects. They can range from acute mild conditions to severe chronic respiratory diseases that require specialist care. The symptoms can include airway inflammation caused by specific exposures to toxins, pro-inflammatory agents or allergens. Non-allergic respiratory symptoms

are caused by non-immune-specific airway inflammation, whereas allergic respiratory symptoms reflect an immune-specific inflammation in which various antibodies (IgE, IgG) can play a major role in the inflammatory response. Irritant –induced asthma is highly prevalent in farmers and farm-related occupations and is in these occupations assumed to be caused by bioaerosol exposures (particularly endotoxin). Although it is clinically characterized as asthma, it has been shown in some populations (e.g. swine farmers) that these symptoms are not only associated with a cross-shift reversible decrease in lung function (asthma) but also with an accelerated chronic decline in lung function (COPD, chronic obstructive pulmonary diseases) [35]. In addition to asthma and COPD, organic dust exposed workers may develop hypersensitivity pneumonitis (HP) and organic dust toxic syndrome [36].

Table 1: Disease Causing Microorganisms that can be transmitted through Aerosols

Microorganisms or diseases	**Infectious doses of microorganisms**	**Inoculation routes**
Adenovirus	>150	Intranasal
Respiratory syncytial virus	> 100-640	Intranasal
Syphilis	57	Intradermal
Malaria	10	Intravenous
Typhoid fever	10^5	Ingestion
E. coli	10^8	Ingestion
Enterohaemorrhagic E. coli O157: H7	10	Ingestion

Bacillus cereus	10^6 or 10^5 per gram	Ingestion
Campylobacter *jejuni*	500 or fewer	Ingestion

http://www.irsst.qc.ca/media/documents/pubirsst/rg-501.pdf

1.2.2 Aerosol Generation and Sampling

For experimentation in the laboratory setting, aerosols and bioaerosols can be generated artificially and sampled using aerosol sampling apparatus. This section details different methodology of aerosol analysis.

1.2.2.1 Nebulizers:

Artificial bioaerosols are generated in the laboratory to simulate naturally occurring bioaerosols, for example to simulate the release of an agent or a bioaerosol created by a biological process such as sneezing. In many bioaerosol studies, the generation of bioaerosols is performed by nebulizers. Collison nebulizers are a prominent type of nebulizers, first described in the scientific literature by Collison in 1935. Nebulization is taken to mean a refinement of fluid atomization. In an atomizer, a gas is used to aspirate the liquid into a sonic velocity gas jet, wherein it is sheared into droplets. In a Nebulizer, the liquid/gas jet is impacted against a barrier (the inside of the jar) to remove the larger fraction of the droplets. The finer droplets thus created, with smaller diameter are then produced as the output. The collision nebulizers are manufactured in 3, 6 or 24 jet form. Because Collison nebulizers are recirculating systems and impose large shear forces, microorganisms in suspension accumulate metabolic damage as a

Collison continues to operate, and may lose viability [37]. The rate of loss of viability is typically not rapid enough to prevent an experiment from being performed.

Other methods of producing bioaerosols include other modes of atomization, such as ultrasonic nozzles that use high-frequency vibrations to produce an aerosol, and electrostatic nebulizers that use electrical forces. Dry powder dispersion techniques are used to produce an aerosol from a powder source, such as dry bacterial spores, and powder scrapers are also used for fungal bioaerosols [38].

Figure 3: A BGI 24-jet Collison Nebulizer

1.2.2.2 AGI-30 Impinger

The All-Glass Impinger, AGI-30 (Ace Glass Inc., Vineland, NJ) is a widely used sampler in bioaerosol experimentation [39, 40]. The AGI-30 has a nominal air sampling flow rate of 12.3–12.6 L/min, which is maintained by drawing a vacuum of at least 410 mm Hg to achieve sonic velocity at the exit plane of a 1.27 mm diameter acceleration nozzle, and typically the initial liquid volume is 20 mL. The jet created by the nozzle causes aerosol particles to impact on the bottom of the glass vessel. Sampling efficiency of an impinger depends on airflow rate, distance between the exit plane of the impingement nozzle and the bottom surface of the liquid reservoir, and the properties of the collection fluid. Liquid evaporation rate in an AGI-30 was measured by Lin et al. [41], who observed a rate of 0.2 mL/min when an AGI- 30 was operated in an environment at a temperature of 25◦C and a relative humidity of 47%.

Figure 4: The AGI-30 Impinger

http://www.opticsplanet.com/ace-glass-laboratory-glassware-and-equipment-impinger-agi-30-complete-7540-10.html

1.2.2.3 SKC BioSamplers

The SKC BioSampler impinger (SKC Inc., Eighty Four, PA), was developed to improve the sampling and retention efficiencies of the AGI-30 [42]. A nominal sampling flow rate of 12.5 L/min is maintained by drawing a vacuum of at least 381 mm Hg across the device. Typically, the initial volume of collection liquid is 20 mL. Instead of the aerosol being accelerated in a single jet and directed normally against an impaction surface (as is the case with the AGI-30), the airflow in the SKC impinger is accelerated through three nozzles that impinge tangentially on the cylindrical wall of the glass vessel.

Figure 5: The SKC BioSampler

http://www.skcinc.com/prod/225-9594.asp

1.3 Existing Methodologies for Control of Bioaerosols

A major motivation of this thesis is the inactivation of microorganisms in bioaerosols. Currently, there are various technologies that address this issue and are detailed in this section. These technologies however have certain limitations that make them not as effective and attractive as we would expect. There are two issues with respect to this challenge.

1) The Precipitation and Capture of Bioaerosols: Certain technologies do not inactivate the microorganisms inside the bioaerosol droplet, but just knock them down through Electro-Static Precipitation or capture on a membrane.
2) Inactivation of Bioaerosols: This thesis details the application of non-thermal plasma technology in inactivating the bacteria inside bioaerosols. In this method, the bacteria are not only out of the airflow but also unable to replicate and grown in culture.

1.3.1 Filters:

While passing through filters, bacteria in bioaerosols are contained by filters such as High Efficiency Particulate Air Filters (HEPA). These filters typically adsorb the infectious microorganisms onto the filter surface and thus removed from the air stream. Since these filters do not inactivate the microorganisms, the microorganisms can survive and even proliferate. There have been efforts to introduce antimicrobial substances onto the surface of the filters to inactivate the microorganisms. Various chemical substances such as Iodine to Silver Nanoparticles have shown to be effective in inactivating bacteria on filter surfaces [43, 44]. Although the drawback of this is that when non-biological dust is deposited on the surface of these filters, the antimicrobial effect of these additive substances are negated. Additionally, filters with tiny wires embedded into them have been used to expose the bioaerosols to thermal energy.

1.3.2 UVGI Radiation:

Ultraviolet irradiation is widely used for controlling bioaerosols. One study demonstrated a 12-fold reduction in bioaerosol inside an operating room when exposed to a high level of UVGI at 290 μW/cm^2 [45]. For 99% inactivation of bioaerosols, the UV doses required were 1.017 to 2.356 μW sec/cm^2 for *E. coli*; 15.949 to 19.345 μW sec/cm^2 for *B. subtilis*; 12.917 to 17.497 μW sec/cm^2 for yeast; and 47.984 to 89.419 μW sec/cm^2 for *P. citrinum* [46]. A UVGI dosage of 289 to 860 μW sec/cm^2 was required to produce a 5 log decrease in the concentration of *Legionella pneumophila* bioaerosols [47]. The advantage of UV radiation is that it consumes less energy than other methods, such as thermal energy. UV lamps are often employed in operating rooms and other healthcare related facilities. UV radiation is also used in conjunction with other modalities to enhance the sterilization effect. TiO_2 particles are used in conjunction with UV to cause a photo catalytic antimicrobial reaction. In a recent test, three 18 W fluorescent visible white-light lamps with a TiO_2 coating of 5.9 g could reduce 9–84% of culturable bacteria bioaerosols and 3–74% of culturable fungal bioaerosols in a 2 m × 2 m × 2m chamber within 30–480 min of irradiation [48].

1.3.3 Thermal Energy:

Thermal energy has been used for a long time to control aerosols. There are two main modalities in which heat can be used; moist heat and dry heat. Moist heat employs pressure with steam whereas dry heat employs very high temperature. Airborne microorganisms have been known to be inactivated with very short exposure to very high temperatures such as 100-140°C. High-temperature exposure at 400°C via simulated combustive explosion was found to inactivate more than 99.99% of *B. subtilis* spores in bioaerosols [49]. It is well known that high

temperature causes the denaturation of the proteins through the breakage of polypeptide bonds and thus alteration in structure [50].

1.3.4 Ion Emission:

Ion emission has emerged as a new technology for inactivating bioaerosols in recent years. Various studies have reported that air ions inhibit the growth of bacterial and fungal species [51-53]. Aerosols can be removed through the deposition of ions on the surface of aerosols particles. The same applies to bioaerosols as well, as these particles can be knocked out of the airflow and deposited onto the surface. There is however not much evidence about the exact mechanisms of the action of ions in inactivating bioaerosols. One study [54] has suggested that the air ions cause bactericidal effects through the electro-poration of bacteria in addition to ozone exposure. Kim et al. [55] suggested that electro-poration plays a primary role in the antibacterial effects of air ions. The attachment to and accumulation of air ions on the surfaces of airborne microorganisms induce distortion of the nearby electric fields of cell walls, which then disrupt the transport of electrons and protons inside microorganisms.

2. DEVELOPMENT AND TESTING OF A LABORATORY SCALE SYSTEM FOR THE INACTIVATION OF BACTERIA IN BIOAEROSOLS WITH NON-THERMAL PLASMA

2.1 Inactivation of Airborne Microorganisms

The inactivation of airborne microorganisms is discussed in the first chapter[56]. Here we are attempting to demonstrate the effectiveness of Non-thermal Plasma technology and especially the Dielectric Barrier Discharge (DBD) for the inactivation of airborne microorganisms.

2.1.1 Materials and Methods

2.1.1.1 Dielectric Barrier Grating Discharge

The treatment of a volume of treatment medium such as airflow required the building of a device having specific geometry. The normal one electrode to one electrode planar would not work in this case. Hence this necessitated the development of a new geometry of discharge. Hence a multiple electrode arrangement was used which alternated the electrode and the dielectric covered electrode. The entire assembly had 22 high voltage and 21 ground electrodes. This discharge was called the Dielectric Barrier Grating Discharge (DBGD). The discharge was to be placed perpendicular to the airflow to intercept it. The airflow would flow through the gap between the electrodes of the discharge and be treated with the DBD plasma.

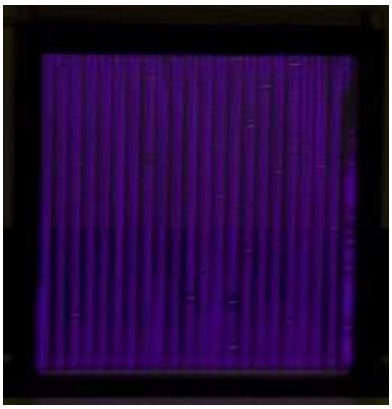


Figure 6: The Dielectric Barrier Grating Discharge (DBGD)

2.1.1.2 Discharge Power Measurements

The DBGD device was operated using a quasi-pulsed power supply that delivers large voltage pulses followed by low voltage oscillations. Oscilloscope measurements indicated that the duration of one pulse period was approximately 600 μs, the maximum peak-to-peak voltage, 28 kV, and the pulsed current was nearly 50 A (peak-to-peak value). The average power of the discharge over one pulse period was approximately 330 W, and considering the discharge area of 91 cm^2, the power density was calculated as 3.6 W/cm^2. Since the majority of power was discharged within the pulse duration (within the duty cycle of the pulse period), it is useful to describe the power in terms of the pulse duration itself since there is essentially no discharge between pulses. Measurements indicated that the pulse duration is 77 μs, nearly an order of magnitude less than the complete pulse period, which gives a duty cycle of 0.1283. The average power in the pulse duration was calculated as 2571 W. Given that the residence time of a bioaerosol particle passing through the discharge area was 730 μs and the pulse period being 600

μs, this meant that each bioaerosol particle that passes through the DBGD area experienced about one pulse of DBD discharge power. The typical concentration of bioaerosol in an experiment was approximately 5×10^5 bacteria per liter of air, which translated to approximately 9×10^3 bacteria within the cross section of discharge area at any given time (in each 2-mm wide cross section of flow passing through the DBGD, assuming plug flow conditions in the DBGD chamber).

2.1.1.3 The PDRF System

To test the efficacy of the treatment, a laboratory scale model of an actual HVAC system was designed. This system was termed the Pathogen Detection and Remediation Facility (PDRF). This consisted of a DBGD and sampling test chamber connected by two pipes to a large volume cylinder. The cylinder contained plate modifiers which increased the residence time inside the system. Two sampling ports were connected on either side of the DBGD, for pre and post plasma treatment sampling.

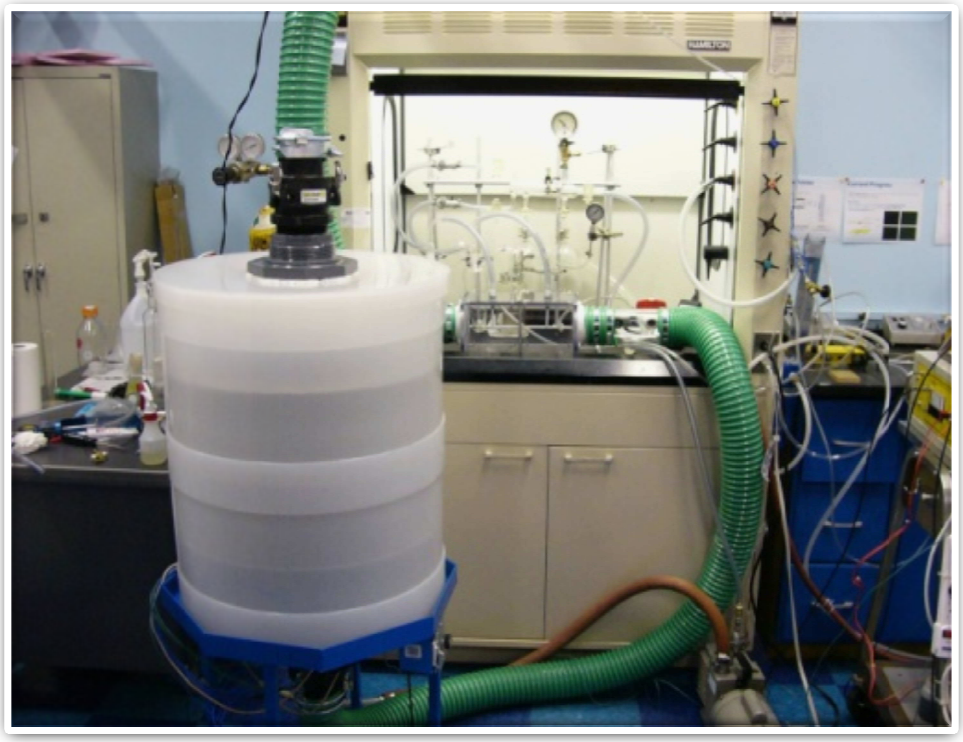


Figure 7: The Pathogen Detection and Remediation Facility

2.1.1.4 Aerosol Injection and Sampling

The PDRF system was initially presterilized using an internal heating system and prehumidified to 70% Relative Humidity. The bacterial culture was placed into a BGI 24-jet Collison nebulizer, which was operated at 40 psi for a period of 45 s (nebulizing rate: 1.1 ml/min). According to the manufacturer's specifications, the Collison nebulizer generates bioaerosol droplets with a median diameter of 2 μm at the operating conditions that are used in experiments (40-psi static pressure of air that drives the nebulizer). The nebulizer had a tapered glass opening into the impingement solution. It was observed that high speed of nebulization was not good for sampling bioaerosols. Therefore, the tip of the nebulizer was modified with an

aluminum assembly. This assembly was connected at the end of the nebulizer and contained radial holes for impingement of bacteria into solution.

2.1.1.5 DBGD Device Operation

The DBGD device was then switched ON for a period of 10s so that the entire volume of bioaerosol in the system gets treated with one pass through the discharge. Subsequent volume treatments were made within a 2-min interval to allow for time to remove used air samplers and replace them with sterile samplers. Air samples were taken in pairs: before and after passing through plasma. Therefore, the decontamination efficiency of the DBGD device was measured on a per pass basis with each set of air samples, and subsequent volume treatments can showed an additive effect of multiple passes through the discharge. Each of the presterilized air samplers was initially filled with 30 ml of sterile phosphate buffered- saline (PBS) solution, and after sampling, each sample solution was serially diluted and plated onto agar plates.

2.2 Results

2.2.1 Testing of *E. coli* survival inside system

To assess the survival of bacteria in flight, control experiments were performed on the system. These experiments were aimed at determining the feasibility of this system for inactivation of *E. coli*. We ran experiments in PDRF system without plasma treatment. The results clearly showed that the *E. coli* survived inside the system and also the sampling process. The concentration of bacteria was reduced by less than 1 $\log_{10}$ 10 minutes after introduction into the system. The experiment run at two different humidity levels displayed no significant difference in the concentration of the bacteria over time and that the concentrations were based

mainly on the initial concentration. As for the sampling efficiency, the expected concentration of bacteria in the air can be calculated as:

Concentration of Bacteria = Total Bacteria Aerosolized/ Total Volume of PDRF

$$= 1x10^{9} / 250 \text{ Liters} = 4x10^{6} \text{ Bacteria/Liter of Air}$$

The control runs indicated that, the first sample taken contained ~10^{5} Bacteria/Liter. This indicates a good sampling efficiency of about 10%.

2.2.2 Inactivation of Airborne *E. coli*

The control experiments show a small reduction in the number of viable *E. coli* inside the system. For plasma treatment experiments, there was reduction in bacterial viability. The samples were taken at the same time points as the control experiments. Due to the restrictions of the system, there was a gap of one minute between each set of pre and post treatment samples. As described earlier, the plasma discharge was kept ON only for 10 seconds. This time of treatment is displayed as the Grey shaded area in Figure 8.

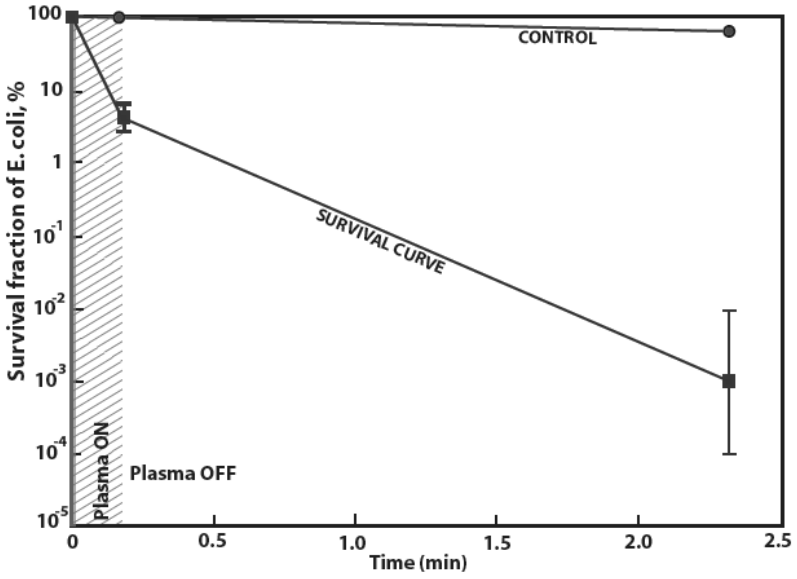


Figure 8: Results of Air Sterilization Experiments inside the PDRF System. The shaded region shows the duration of plasma exposure[56].

The first two samples were analyzed and an approximate reduction of ~1.5logs (97%) was observed between first (pre treatment) and second (post treatment) sample. When the next pre treatment sample i.e. the third sample was taken, there were no viable bacteria observed on the plate. This constitutes a reduction of ~3.5log (99.95%) from the second sample concentration. This rapid reduction took place during the time that plasma was switched OFF. Sample 4 (post treatment) was taken and samples 5 and 6 were taken before and after the third plasma treatment. For samples 4, 5 and 6 there were no culturable bacteria found on plate; therefore they were omitted from Fig.8

2.2.3 Flow Cytometric Analysis of Samples

Flow cytometry is emerging as a real-time technique for detecting bacteria in various environmental samples. This method has been used to detect the number of viable bacteria in

aquatic samples [57, 58] and food samples[59]. In our experiments, flow-cytometric measurements were made using FACS calibur (Becton Dickinson, USA) flow cytometer with 488 nm excitation from an argon ion laser at 15 W. Fluorochromes with a high affinity for nucleic acid SYBR Green I and propidium iodide, PI (Molecular Probes) were used for flow cytometry. The SYBR Green I, a green fluorescent nucleic acid stain, has been shown to stain living and dead Gram-positive and Gram-negative bacteria [57]. PI is a red fluorescent dye that intercalates with dsDNA and only enters permeabilized/disintegrated cytoplasmic membranes [1] The flow cytometer measures the intensities of signal produced in the Green (530nm) as well as Red (670nm) spectrum. Since all bacteria are permeable with the Green dye, the intensities of the signal in the green region can be used to detect the number of bacteria in each sample. The red dye can only enter cells whose membranes have been permeated; hence it can be used as an indicator of such.

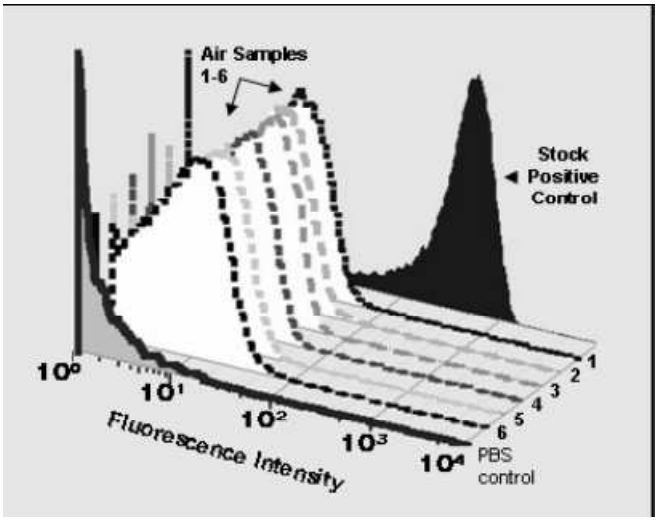


Figure 9: Results of Flow Cytometric Analysis of Air Samples after Plasma Treatment[56].

The results, as shown in the figure above, indicate that the florescent intensity peak for air samples one through six is identical, this means that there are the same number of total bacteria present for each air sample taken during experiments. The stock positive control sample is a pure untreated sample of *E. coli* whose intensity peak was two orders of value greater than the intensity of the air samples. The results Propidium Iodide (not shown here) indicated weak permeation of the dye into the samples. This would indicate that the membrane has not been compromised to the extent that it would cause leakage or lysis. These results are expected as the very short residence time inside the plasma zone is not considered sufficient to completely lyse the membranes of bacteria.

2.3 Inactivation of Spores

This task was a continuation of the air sterilization experiments. Experiments were carried out using *B.Cereus* spores as well as *B. Subtilis* vegetative cells. The *B. Subtilis* vegetative cells can help us see if there is any difference between the degree of inactivation of Gram-negative and Gram-positive bacteria. Experiments with spores are important as they determine if plasma has any effect on a bacterial spore, which has a different structure than the vegetative cell.

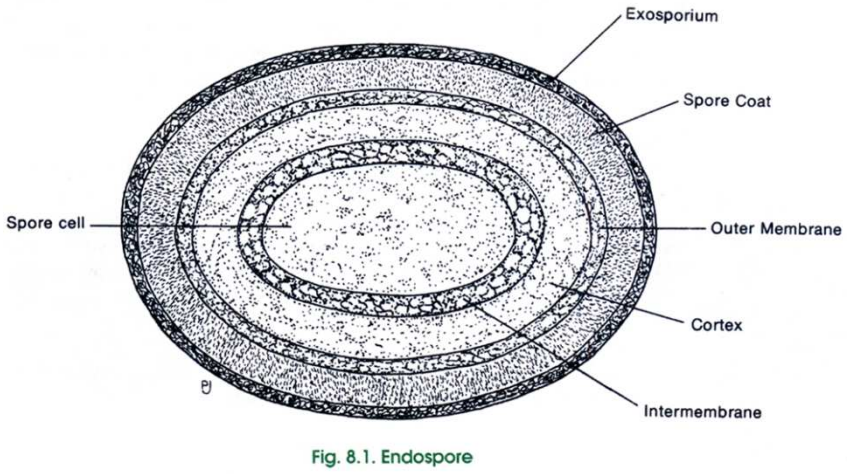


Figure 10: Different layers of an Endospore.

Above illustration shows the structure of a spore cell. The major layers of the endospore are the exosporium, the cortex and the membrane. Each of these layers provides protective coating to the spore cell located in the center. When faced with adverse conditions, spore forming bacteria undergo sporulation and form spores. The water content of these spores is 10~30% less than that of vegetative cells. The enzymatic activity inside the spore is also reduced and the metabolic rate is reduced. This in turn makes the spore more resistant to sterilizing agents. Plasma has been used to treat spores on surface. An electrode array sterilized 10^6 spores in 240 seconds 15W (He), 13W (Air) [60]. Lerouge et al, observed 2 log reduction in the viability of *B. Subtilis* spores after 15 minutes of treatment [61]. The efficiency of sterilization was the least in pure O_2 plasma and the highest in O_2/CF_4 plasma. The main mechanism of sterilization as proposed by them is etching. SEM images showed that spores are significantly etched after 30 min of plasma exposure, but not completely destroyed[62]. The achievement of high efficiency with the addition of CF_4 was attributed by them to high etch rate of organic

solids. In another study of treatment of *B. subtilis* spores on filter [63], for an initial spore density of up to 10^6 per filter, a 3-log reduction was achieved in less than 200s, whereas an initial spore density of 10^9 per filter required a longer plasma treatment of about 360s. With a capillary plasma electrode discharge, reductions in CFUs ranged from 10^4(Helium plasma) to 10^8(air plasma) for plasma exposure times of less than 10 min [64]. Different spore forming bacteria have been used to study their inactivation in comparison. Using a glow discharge plasma, *B. pumulus* spores on paper were inactivated in 2.4 minutes on paper whereas *B. subtilis niger* spores took 4 minutes for the same degree of inactivation i.e. ~4 logs [65]. From these studies it can be inferred that it takes much longer to inactivate the bacterial spore than their counterpart, the vegetative cell. Also, the mechanism of inactivation seems to be more complex than that of bacterial inactivation.

2.3.1 Development of Setup for Inactivation of Spores

The setup consisted of a spore input unit, plasma unit and collection plates. The spores were introduced into the system using nebulization (Producing a mist containing spore cells). A fan on the top of the setup pushed the light bioaerosol downwards to the spores. The spores were collected on agar plates kept at the bottom of the setup. A filter was connected to the exhaust to capture the remaining spores. The entire unit was kept inside the biosafety hood. The geometry of the discharge aerosol interaction was changed in order to better facilitate the treatment and collection of the spore cells.

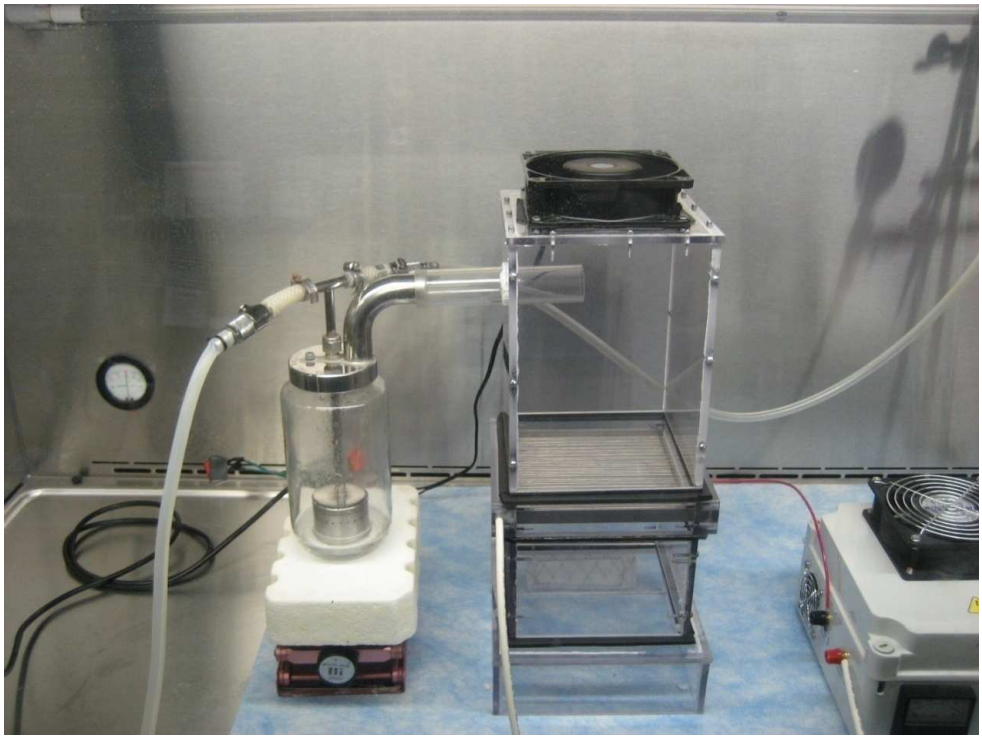


Figure 11: The Setup for the Inactivation of Spores. The aerosolized cells are introduced into the system at the top. Plasma treated samples are collected at the bottom on an agar plate.

2.3.2 The results of bacterial spore inactivation

As a control, *B. Cereus* spores were introduced inside the system for 10 and 20 seconds. The plasma discharge was then initiated and the experiment was repeated for the same time points. For 10 second exposure to plasma, there was ~50% reduction in concentration of spore cells. For longer (20 second) treatment, there was ~90% reduction. The findings are shown in Figure 12. There was a linear decrease in the concentration of the spores.

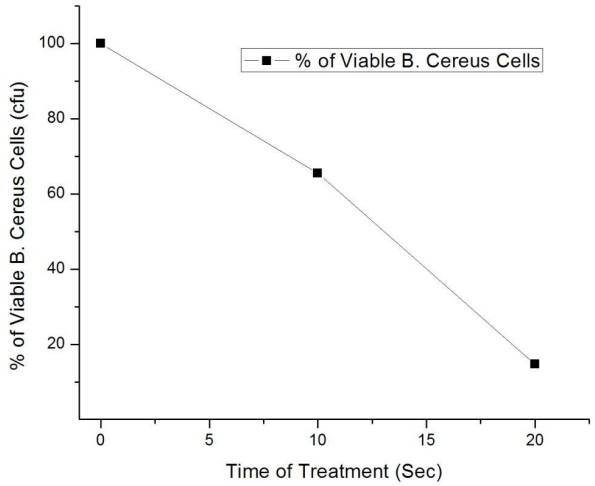


Figure 12: The Results of Inactivation of Spores with the DBGD Device

2.4 Discussion

The PDRF is a bioaerosol decontamination installation that combines DBGD with a filterless ventilation system for the purpose of destroying high concentrations of bacterial bioaerosols from indoor air. The results presented here show that the PDRF system can achieve an ~5 log reduction (99.999%) cfu of *E. coli* in a millisecond of direct exposure in the DBD ventilation grating (DBGD) device without the use of a filter (to trap and treat airborne particles). These results are unique because, in most DBD surface sterilization studies, treatment times are always at least 1000 times longer on the order of seconds and, in some cases, even minutes in duration. This concept of a filterless system has shown that a very short exposure time of bioaerosol to DBGD plasma can cause rapid inactivation of microorganisms. The detection with flow cytometry indicated that the processed air samples indeed contained bacteria that were nebulized in the form of bioaerosol. This in part confirmed our theory that the plasma does not

act as an electrostatic precipitator. Further analysis of the samples with fluorescent dyes indicated that the bacteria were largely intact i.e. their membranes were not compromised by exposure to plasma. This meant that the species produced by plasma enter the bioaerosol droplet and into the bacterial cell and cause cell death.

In additional, the experimental conditions closely mimic the conditions that might exist during a bioterrorist attack, namely, the release of a high concentration of bioaerosol moving at high flow rate inside of a ventilation duct, a possibility for mass casualties. Looking at the rapidity, efficacy advantages of this technology, non-thermal plasma air cleaning technology could be employed in commercial and military buildings for the purpose of mitigating the detrimental effects of a pathogenic bioaerosol. There is a scope in prevention of bioaerosol-related outbreaks in high density civilian population.

3. ANALYSIS OF THE STERILIZATION EFFECT OF PLASMA GEOMETRY AND LONG LIVED SPECIES

3.1 Introduction:

The results detailed in chapter 2 indicated that the DBGD system is effective in sterilizing large volumes of airflow and led to the investigation into the mechanisms of the inactivation and the species mediating it. Plasma produces various short lived and long lived species that have bactericidal effects. The major species produced by plasma is ozone. This study intended to separate the effects ozone from the other species produced. Since ozone itself is used as a sterilizing agent, it was necessary to determine the need to have direct interaction with plasma in the system. The effect of ozone can be separated from the effect of plasma by adding ozone filters, but these filters can only be used post-treatment and there is still certain degree of exposure to ozone that the airborne bacteria see. Hence, it was determined that the degree of exposure to direct discharge can be used to determine how much of an effect plasma filaments themselves have on the bioaerosol.

The previously-described pathogen detection and remediation facility (PDRF) was used to perform air flow sterilization experiments (Chapter 2). In general, the PDRF setup is a closed-loop air circulating system that consisted of a large 250 liter drum connected by pipes to a square box that contains the electrode arrangement of the dielectric barrier grating discharge (DBGD). The drum with internal baffles provides desirable volume of air for experiments and arranges the air flow treatment in the plug flow reactor mode. Air sampling ports on both sides of DBGD were used to sample air from inside the system. The flow inside the system was not interrupted throughout the plasma treatment or the sampling procedure. The flow rate is maintained at 25 liters per second so the entire volume is circulated within 10 seconds; for this reason the plasma

treatment procedure consisted of turning the plasma discharge on for 10 seconds to treat all air in the chamber. The sampling time points were kept constant for all of the experiments. In order to analyze the effect of dosage of plasma and the effect of ozone alone, the geometry of the discharge was changed. This allowed a certain degree of bioaerosol to be not exposed directly to the plasma filaments. All experiments were performed with the same initial conditions and the only parameter that was changed was the type of treatment

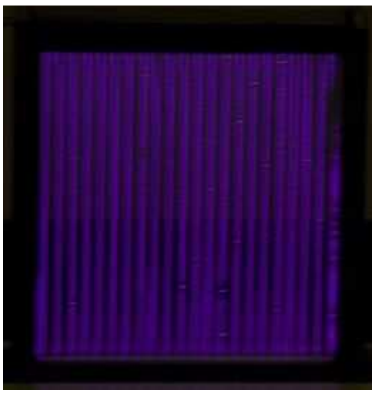
a) direct plasma exposure (same as reported in chapter 2);
b) 75% direct exposure (where 75% of bacteria pass through plasma and 25% do not);
c) Indirect plasma exposure: treatment by ozone (injection of the same amount of ozone as is produced by plasma).

3.2 Materials and Methods

3.2.1 Design of a Variable Geometry DBGD

The plasma discharge setup consisted of 21 high voltage wire electrodes insulated by quartz and 22 grounded wires. When the discharge was initiated, DBD plasma was produced in the air gaps between the electrodes and grounded wires. This createed a screen of plasma that bacteria had to pass through. This arrangement of the plasma discharge is shown in the figure below.

Figure 13: The plasma unit with its multiple-electrode configuration (top). The same plasma unit, with the plasma discharge initiated (bottom). Notice the screen of plasma covering the entire cross-section of air passage[66].



3.2.2 Reducing the Degree of Exposure to DBGD

75% direct exposure: The two ways of investigating the effect of direct exposure to plasma are:

a) Introducing a barrier between the plasma and the sample to be treated, thus removing the influence of the ions and reactive oxygen species produced by plasma: This method is not possible for these experiments as the flow of air is perpendicular to the electrodes and any obstruction will lead to changes in airflow and a pressure drop will be introduced inside the system; and

b) Reducing the total area of direct exposure and letting certain percentage of the sample (in this case, the bioaerosol) is treated indirectly by the long living species such as ozone. In the DBGD setup, this can be achieved by reducing the number of active electrodes, creating gaps in the screen produced by plasma. This way, certain percentage of bacteria escape coming in contact with the plasma and are treated by plasma indirectly.

To understand the influence of direct and indirect plasma exposure of bacteria, every fourth high voltage electrode in the DBGD discharge was removed and the plasma discharge initiated.

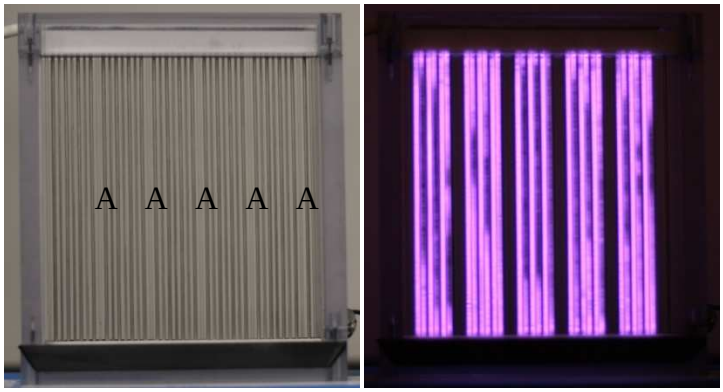


Figure 14: The plasma unit with every fourth high voltage electrode wire removed (left). The quartz tubes are retained in order to maintain the same airflow. This produces zones of indirect exposure (A). The image shown on the bottom is with the plasma[67].

This discharge is shown in Figure 14. This discharge occurs across the air gaps between the wires. Since the area of the discharge is 75% of the total cross sectional area, this can be termed as a 75% direct and 25% indirect exposure. In this case, as the plasma discharge is away from the path of 25% of the bacteria flow, it can be considered indirect treatment for the area

where there is no discharge. The selection of the electrodes to be removed was done in such a way that the indirect treatment is distributed across the cross section of the airflow. The pulsed voltage that is input to the plasma discharge is 28 kV and the current was measured to be 50 A (peak-to-peak value). The total power dissipated in the plasma was 100 W.

3.2.3 Growth and Preparation of Bacterial Strains for Nebulization

The microorganism used in our studies was *Escherichia coli* K12 substr. MG1655. Strains were revived from frozen stocks. The frozen stock was transferred to 10 ml culture tube containing Luria Bertani (LB) media. The culture was grown overnight in an orbital shaker incubator at 37ºC. The culture was then transferred to centrifuge tubes and spun at 3500 rpm for 1 minute. The supernatant was removed and the pellet was again washed with deionized water. The final solution was prepared by adding the bacterial pellet to 30 ml of deionized water.

3.2.4 Injection of Bacteria and Air Sampling

A 24 jet collision nebulizer (BGI Inc., Waltham MA) was connected to the system. Deionized water was added to the nebulizer and the nebulizer was operated at 40 psi input pressure. This injection was performed to increase the humidity inside the system. The humidity was increased to 70% RH and the nebulizer was disconnected. The bacterial solution was then added to the nebulizer and it was connected back to the system. The nebulizer was run again at 40 psi for 45 seconds. The nebulizer was then disconnected and removed. The sampling of the air inside the system was performed using specially modified AGI impingers (Ace Glass Inc., Vineland NJ). A negative air pressure system was used for acquiring the samples from the uninterrupted circular flow system.

3.2.5 Analysis of Surviving cells

The samples were taken in 1X Phosphate Buffered saline (PBS) solution. Each sample was then diluted with PBS using the serial dilution method. The dilutions were then plated on BHI agar plates (BD BBL, Franklin Lakes, NJ). These plates were incubated overnight at 37°C inside a stationary aerobic incubator. The number of colonies growing on the plates was counted on next day to determine the number of bacteria present in the sample.

3.3 Analysis of Long Lived Species

As discussed earlier, the plasma produces highly reactive products that are stable enough to be considered as long lived species. Ultraviolet radiation and ozone are the major products of plasma that are bactericidal. In order to test the influence of these species on the sterilization effect, a separate study of treatments with ozone and UV was performed.

3.3.1 Ozone Production in DBD

The major long-living specie created by DBD plasma in volume is ozone. The dissociation of O_2 molecules in air by energetic electrons is the first reaction in this process. This reaction is followed by a three-body reaction between O, O_2, and M leads to the formation of ozone where M is another molecule or wall:

$$O+O_2+M \rightarrow O_3^* + M \rightarrow O_3 + M \qquad (1)$$

To isolate the effect of plasma-generated ozone on the airborne bacteria we produced ozone elsewhere and injected it into the chamber. For this, ozone concentration generated by DBGD inside the system was measured, total ozone production was calculated, and then the ozone generator (Medozone, Russia) was adjusted to produce the same amount of ozone. The bacteria were introduced into the system through the process of nebulization as usually. Pre-treatment air sample was taken and the ozone generator was switched on for 10 seconds, the same time as

plasma treatment. The ozone was allowed to pass through the experimental system and a post ozone treatment sample was taken. The time of sampling was kept the same as that in plasma experiment. Further samples were taken pre and post ozone exposure.

3.3.2 Measurement of Ozone Produced by DBGD

The concentration of ozone produced by plasma was measured using an ozone meter. The plasma discharge was initiated and kept on for 10 seconds, where the concentration of ozone generated was 28 ppm. As a parallel experiment, a separate ozone generator was employed for producing pure ozone. It had an intake for air and outlet for the generated ozone. It was observed that at 0.5 SLPM, the amount of ozone generated inside the system by the generator was the same as generated by DBD plasma for 10 seconds i.e. 28 ppm. The results are shown in Figure 15 below.

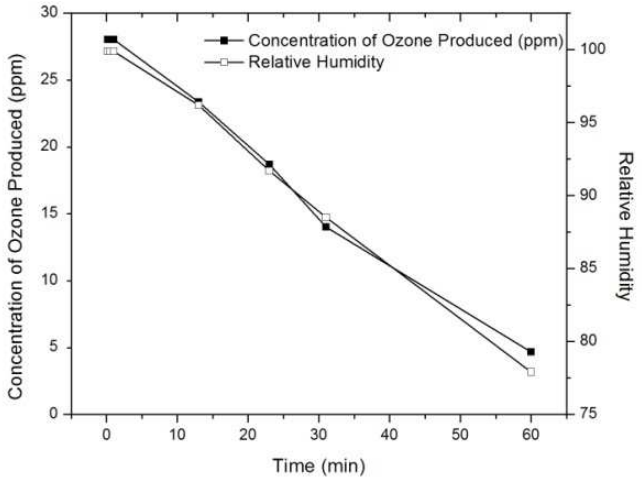


Figure 15: Evolution of ozone generated by DBGD discharge and relative humidity inside the system.

3.3.3 Generation of Pure ozone

The generation of pure ozone was performed using a Medozone ozone generator as mentioned. This device has an inlet port for atmospheric air and an outlet for ozone to be injected into the system. The ozone produced by the device is proportional to the input air pressure. The test was carried out to determine what pressure would produce ozone comparable to the plasma system. The ozone generator was run at four different input pressures and the results measured. As the plasma discharge produces 28 ppm of ozone, the value of input pressure that is closest to that value was identified. The input pressures of 1, 1.5 and 2 SLPM produced ozone at concentrations much greater than what was detected by plasma. For 0.5SLPM input pressure, the output produced is 27ppm for 10 seconds. Around 30 seconds, the concentration of ozone inside increased to 30ppm and higher. Because of this, the ozone generator was only kept ON for the duration of 10 seconds. The injection and sampling of bioaerosol was identical to that of plasma studies.

3.4 Results of *E. coli* Inactivation

Figure 16 below shows the summary of experimental results performed with the PDRF system. 100% direct exposure to plasma led to the greatest degree of inactivation. A 25% drop in the direct exposure led to the inactivation due to plasma exposure to drop to 29% from the 97% observed in 100% direct exposure experiments. Inactivation experiments with pure ozone produced the least degree of inactivation.

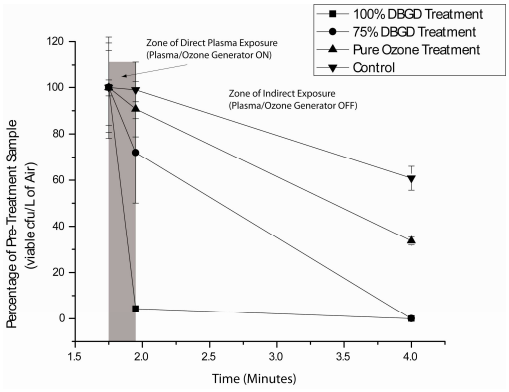


Figure 16: Results of the Experiments. The dark shaded region denotes the first 10 second treatment with 100% plasma (■), 75% plasma (●)/ ozone (▲) as compared to the control runs with no plasma/ozone (▼)[67].

The 10 second exposure to ozone resulted in only 10% inactivation of airborne *E. coli*. Pure ozone failed to produce complete inactivation by the time the next sample was taken. The third sample represents the pre-treatment sample for the 2nd 10 seconds treatment (second pass through plasma). By this time, no viable *E. coli* were detected in either plasma treatments, indicating the clear superiority of plasma exposure over pure ozone.

3.5 Ultraviolet Production in DBD

Ultraviolet radiation is one of the products of DBD plasma discharge. Here we analyzed the UV products produced by DBGD in terms of the spectra and the intensities.

3.5.1 Spectrum of Ultraviolet Radiation Produced by DBGD

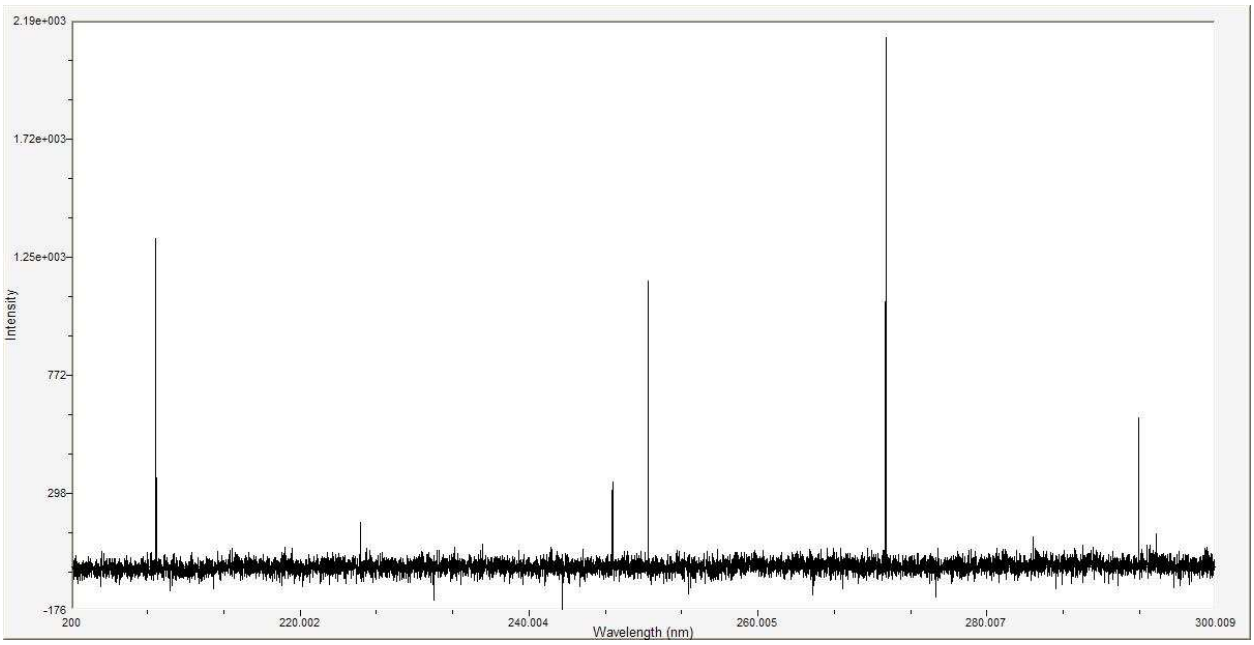


Figure 17: The ultraviolet spectrum of the DBGD Discharge analyzed for the 220nm to 300nm range.

It is well-established that Ultraviolet radiation is produced by plasma discharges, including DBD. The PDRF system was analyzed for UV production and the results are detailed above. The range of the UV wavelengths investigated was in the UVC range. The highest intensities observed were at the 209nm, 254nm and 270nm. The wavelength of greatest interest is 254nm, which is known to be the wavelength producing bacterial inactivation. The intensity of UV at this wavelength was 2.1e3 au. This indicates that UV produced can be involved in the sterilization effect produced by the DBGD. This led us to investigate the intensity of the dosage produce by the DBGD.

3.5.2 Measurement of Ultraviolet Intensities near DBGD

The intensities of the UV produced by plasma were measured. Figure 18 shows the intensities of the UV along the axis of bioaerosol flight, and denotes the maximum intensity

measured was 30μW/cm^2. The intensities gradually decreased further away from the DBGD. This level of intensities for a short period of interaction between bacteria and the aerosols is not enough to produce the amount of inactivation observed in the earlier results.

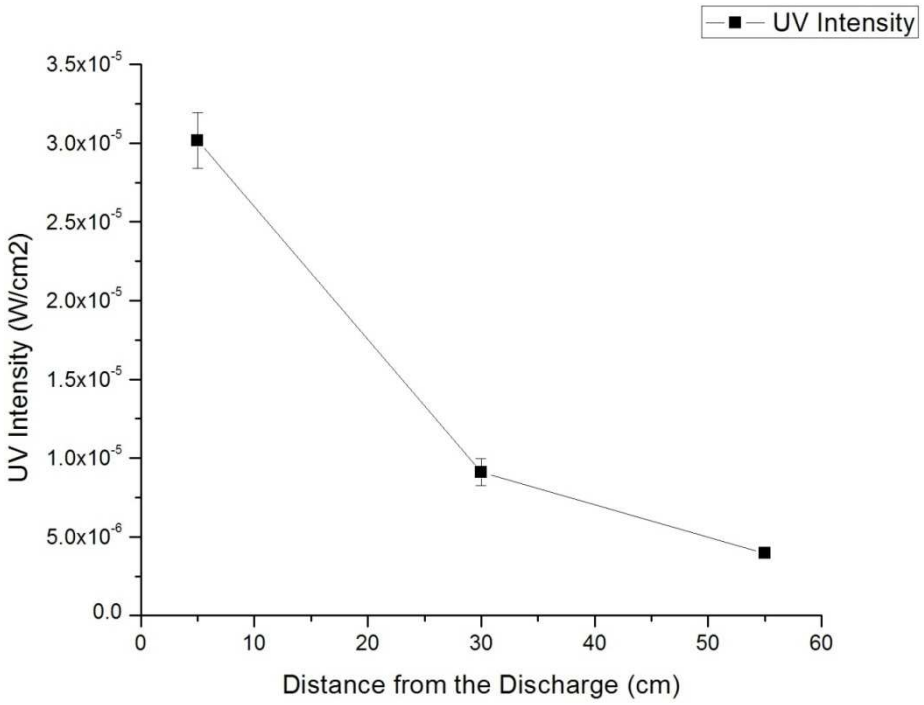


Figure 18: Ultraviolet power densities measured near the DBGD plasma device.

3.6 Discussion

In this study, two major factors affecting the plasma-induced sterilization of air using plasma were investigated – direct plasma treatment and ozone treatment. On one hand, it is known that ozone is a relatively slow sterilization agent. On the other hand, experiments shows

significant sterilization effect during the time when plasma is off and ozone is probably the only active agent. If ozone was indeed the major inactivating agent in plasma, the same dosage of pure ozone would produce the same inactivation as seen with plasma discharge. The experiments with ozone injection have confirmed that ozone alone have slow damaging action. To determine the effect of direct exposure and the ions and charges associated with it, the total direct exposure was reduced to 75% and sterilization experiments were performed. The results indicate that the inactivation dropped from 97% to 29%. Hence, a small reduction in the direct exposure results in a much larger reduction in the inactivation immediately after plasma exposure, so the effect of direct exposure has significant non-linearity. The simplest explanation of this non-linearity is synergism between ozone and direct plasma exposure in bacteria inactivation. In addition, our assumption that 25% reduction in plasma area means that 25% of bacteria are not subject of direct plasma exposure is probably oversimplification. After 4 minutes all bacteria were inactive with 75% treatment, and it probably meant that all bacteria received a dosage of direct treatment, e.g. in the form of UV radiation.

The direct exposure to plasma disturbs the bacteria membrane and the charges stick to the membrane. While complete membrane breakdown requires a field of several kV/cm and longer time periods[68], we know that charge absorption leads to pores opening much faster – in millisecond and tens of microseconds time range [69]. Follow-up 2-minute ozone action on bacteria with disturbed membranes provides complete sterilization.

Investigation of the influence of direct exposure shows that there is 3.5 log reduction during the much longer post-plasma exposure. This means that after the initial 97% reduction, remaining bacteria keep flowing through the system, when ozone enters the bacteria and further reacts with the membrane to inactivate them. *Fan et al* [70] observed that there is a synergism

between negative air ions produced by DBD plasma and ozone on bacterial cell death. Bactericidal effect of negative air ions in addition to ozone was found to be far greater than ozone by itself. In their experiments, viability of *Escherichia coli* was reduced to 40% of first sample after 11 hours of negative air ion (NAI) treatment, as compared with 70% in the ozone alone treatment.

The humidity inside the system plays a role in the inactivation. The bacteria in our experiments are in the form of a bioaerosol. This bioaerosol consists of the bacteria enclosed in a fine droplet of water. As the bioaerosol travels inside the system, the droplet shrinks. Dunklin et al [71] show that the shrinkage of the water droplet depends on the relative humidity and that at 50% Relative Humidity (RH), the droplets shrink to one tenth of their size in 4 msec. Our experiments were made at higher RH. As the liquid can act as a protective shield around the bacteria, shrinking of the droplet causes the bacteria to be more vulnerable to the charges and ROS produced by DBGD plasma. Though *Muranyi et al* [72] have recently demonstrated that the fastest inactivation of plasma treated *Aspergillus Niger* spores occurs at high relative humidity (70%), their experiments consisted of treating bacteria placed on surfaces, whereas in this study we discuss the inactivation of airborne bacteria.

Due to the humid air inside our system, OH radical is expected to be formed. It is known that one main path for the generation of the hydroxyl radical in a DBD system is the photo-dissociation of ozone into atomic singlet oxygen and the reaction of this radical with water molecule [73]. This can be another synergetic mechanism that explains non-linearity in direct plasma treatment. With this knowledge and our experimental results, we can conclude that the main cause of inactivation is the synergetic action of short-living plasma agents (charges,

radiation, and radicals such as OH^-) that disturb the membrane and ozone. This synergy creates a toxic environment for the bacteria, ultimately resulting in inactivation.

In this section of the thesis work, we intentionally did not consider potential influence of plasma product on environment in the case of indoor application of the method presented here for several reasons. First, we believe that in the case of bio-terrorist attack, damage caused by plasma products is negligible in comparison with bio-contamination. Secondly, there are known methods of ozone and NO_x absorption and destruction that can be combined with plasma air sterilization.

4. DEVELOPMENT OF A SINGLE FILAMENT DBD BIOAEROSOL INTERACTION SYSTEM AND TESTING ITS EFFICACY AGAINST VARIOUS MICROORGANISMS

The results of chapter 3 have led to the following observations:

- The DBGD plasma discharge is effective in inactivating airborne bacteria in flight, with very short residence time inside the zone of plasma.
- Modified geometry experiments have shown that the direct interaction of bioaerosol particle with the DBD produces significant inactivation.
- The inactivation of the remaining bioaerosol suggest that the species produced by plasma diffuse through the droplet and damage the bacteria so that it is inactivated by the ozone produced in volume by the DBGD.

With these results, it is necessary to determine the effect of the plasma discharge itself on the aerosol, removing completely the effect of long lived plasma species such as ozone. The interaction of the plasma discharge and aerosol droplet would lead us to understand the effect of the short lived species produced by plasma. This chapter details the development of a discharge consisting of only one filament of DBD plasma.

4.1 Filamentary nature of DBD

As discussed in earlier chapters, the Dielectric barrier Discharges are filamentary in nature. The basis construction of a DBD system is two electrodes separated by a dielectric layer. Breakdown of an atmospheric pressure gas or room air with the presence of at least one dielectric barrier in the gap results in multistreamer mode of operation with formation of microdischarges [8] and these filaments are visible to the naked eye. The current passes through these

microdischarges which typically have a diameter of the order of 100 μm. These microdischarges move laterally along the surface of the electrode. This movement has been studied and modeled[74]. This movement gives the impression of the plasma discharge being a volume discharge. For understanding the species produced in one plasma filament, we needed to produce a point to point discharge where the microdischarge is stationery. Such discharge has been studied by *Ayan et.al.* [75].This arrangement is discussed below.

4.2 Single Filament System

4.2.1 The DBD Plasma Producing Device

The setup consists of a DBD-bacterial treatment chamber. It consists of an injection port, a DBD plasma chamber and a sampling system. The entire assembly is shown in Figure 1. One electrode of the system is made of steel and the other is steel covered with quartz. The DBD is initiated between these two electrodes. The DBD behaves as a point to point discharge.

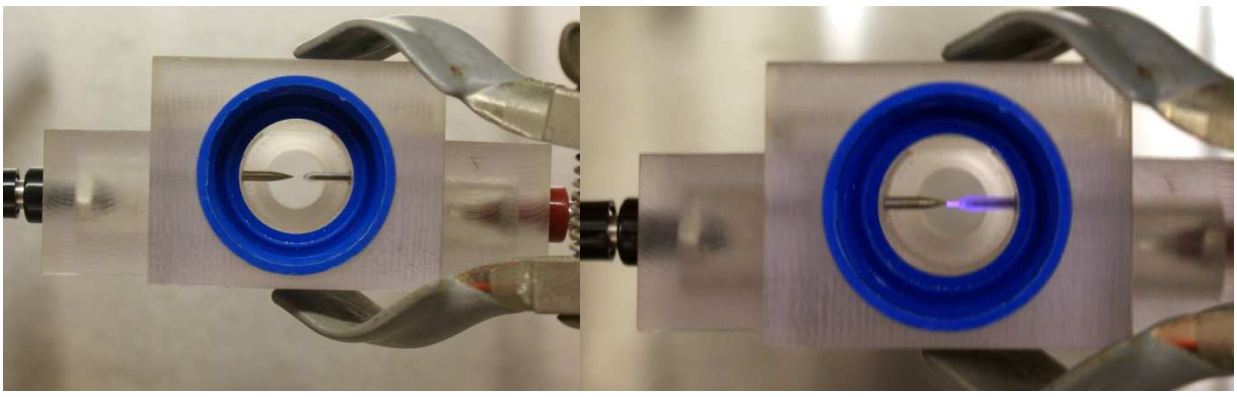


Figure 19: The Single Filament DBD Discharge. The purple discharge is initiated between two electrodes is shown on the right.

4.2.2 Plasma Discharge Geometry

The plasma discharge was initiated between the two electrodes. The geometry of the discharge was designed to be closely mimicking the individual filaments of our original system. In the original system, the discharge was initiated between two cylindrical electrodes, producing plasma discharge across the gap, perpendicular to the length of the electrode wires. Since it is not possible to isolate a single filament with that geometry, the setup and the injection geometry was changed to the new setup. Aerosol injection in the original system was performed into the volume and it was pushed towards the system using fans. This would not be feasible in the new system as the singe filament discharge is much weaker and smaller. Therefore the system was designed for direct injection of the bioaerosol onto the plasma discharge. A modified nebulizer's tip was placed about 2mm from the single filament discharge, perpendicular to its plane. The schematic of the system is shown in Figure 20.

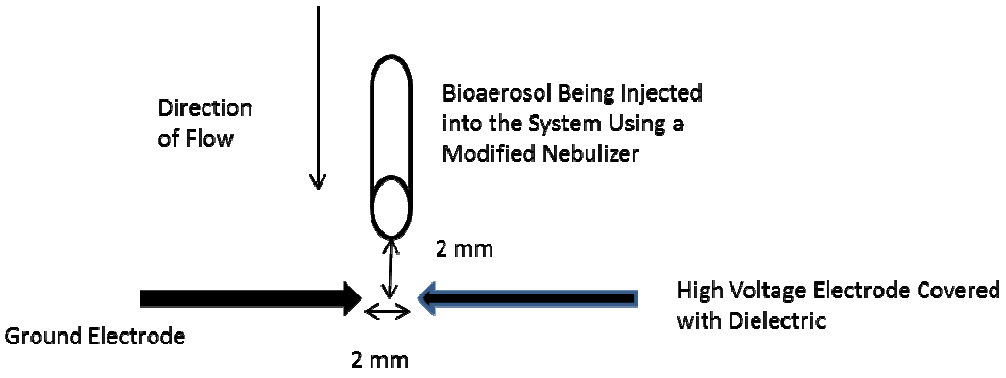


Figure 20: The Schematic of Single Filament DBD Bioaerosol Interaction System

4.2.3 Pulsed AC Plasma Power Supply

The device is operated with an alternating current (AC) pulsed power supply. The power supply consists of a step up transformer that produces a high voltage between the output terminals. The microsecond plasma device produces a sharp discharge peak voltage, whose frequency can be adjusted. As per our earlier results regarding air sterilization technology, the frequency was set at 1.5 kHz. This power supply has a range of 50-3500Hz frequency and 17.8-31.8kV Peak voltage. This translates to 1.4-18.2W Peak power. For the purpose of this study, the two settings chosen were 20kV Peak Voltage@ 1.5kHz which translates to 2.1W peak (Low Power) and 22kV peak Voltage @1.5kHz which translates to 3.2W peak (High Power). These voltage settings chosen were similar to our earlier experimentation [67]. The inactivation efficacy was determined at both power levels for variable loads of bioaerosol.

4.2.4 Bioaerosol Production and Sampling

Injection and Sampling: For this purpose, a high volume Nebulizer (Teleflex Medical, Research Triangle Park, NC) was used. 100ml of the bacterial culture was used as the liquid for nebulization. The output of the nebulizer was modified to inject a fine stream of aerosol. The input to the nebulizer was connected through a filter to a cylinder of medical grade air (Airgas Inc., Radnor Township, PA). Tests were performed to obtain the optimum input pressure and 10 psi was chosen as the input pressure for all tests. After passing through the plasma zone, the bioaerosol was collected at the sampling port. The major methods for collecting bioaerosol particles are impingement and impaction [76-78] Here due to the geometry of the setup, impaction method was used. A 50ml conical micro-centrifuge tube (Fisher Scientific International, Hampton, NH) was connected to the port and the treated bioaerosol was deposited on the inside of this tube. 10ml of deionized water (EMD Millipore, Billerica, MA) was then

added to the tube and mixed thoroughly to collect the bioaerosol particles. This suspension was used as the sample for colony counting.

4.3 Materials and Methods

4.3.1 Modified Nebulizer for Injection of Bioaerosol

The original system consisted of a 24 jet Collison nebulizer as detailed in chapter 1. That nebulizer is very large for this system and could not be incorporated. After extensive research, we isolated a nebulizer that we could connect to the system. The large volume nebulizer is a clinical use nebulizer produced by Teleflex Medical. This nebulizer is not ordinarily used in research studies. It is mostly used to administer drugs in aerosol form. It produces aerosol through a 6 cm diameter outlet. No document exists about its characterization. For getting accurate results through the interaction aerosol with plasma, the stream of aerosol produced needed to be extremely fine. This meant a certain degree of modification of the output of the nebulizer was required. This was performed by designing a nozzle system using a cork and a Pasteur pipette as shown in figure. The modified nozzle had a diameter of 2mm, which is roughly the gap between the two electrodes where plasma is produced. This custom output needed to be analyzed for the properties of aerosols. Therefore, an Optical Particle Counter (OPC) was used to detect the size distribution and number of particles produced.

4.3.2 Collection of Microorganisms from Bioaerosol

The original system as detailed in earlier chapters, employed impingement as the method for collection of bioaerosol. Since the PDRF system is a closed loop system, it can only be sampled in parallel, which is where the impingement process is useful. Here, the single filament system is designed to be a single pass system. For such a system, the impaction method is the

best method for analysis. Impaction is generally used for aerosols that have large particle in them. These large particles, such as micron sized bacteria, are impacted onto a collection surface whereas smaller nanosized particles are evaporated [79]. Some of the most popular air samplers employ multi stage impactors that collect aerosol and bioaerosol particles onto a filter surface [80]. These filters are then removed and analyzed. Since our plasma system is filterless and does not employ filters, we used a conical collection tube to collect the treated bioaerosol particles on the inside surface.

The treated bioaerosol deposited such could be analyzed either by sampling with a swab or adding collection liquid to it. The swab sampling method was considered as less precise and further experimentation could not be performed on the samples. Therefore, for further analysis, the collection tube was then removed from the DBD assembly and collection liquid was added to it. This liquid was the same deionized water that was used to produce the bioaerosol. In cases where there was post treatment addition of certain substances such as scavengers, appropriate collection medium was used. The liquid containing the collected bioaerosol was then used for analysis.

4.3.3 Methods of Analysis

4.3.3.1 Plate Counts

The collected bioaerosol samples were analyzed using standard plate counting methods. Serial dilutions were performed in deionized water and the dilutions were plated out on Tryptic Soy Agar (TSA) plates (BD BBL, Franklin Lakes, NJ). The plates were incubated overnight at 37°C inside a stationary incubator and the numbers of colony forming units were counted at 24 and 48 hours post incubation.

4.3.3.2 Flow Cytometry

Flow cytometry was used to detect the presence of bacteria in collected samples. For this, we employed a bacterial cell counting kit [81](Life Technologies, Grand Island, NY). This kit contains a florescent dye (SYTO 9) along with a polystyrene microsphere standard which is 6µm in size. The microsphere standard is significantly larger than the bacterial particles and is used to differentiate populations grouped on size. SYTO 9 is a cell permeable dye that penetrates the bacterial cells. It is a nucleic acid stain that permeates both gram positive and gram negative bacteria to give a very distinct Green Fluorescence. The Guava Flow Cytometer (EMD Millipore, Millerica, MA) was used for the experimentation.

The control and DBD treated bioaerosol was collected in 10 ml of deionized water. For each sample, 1ml aliquots were made, in order to get data in triplicates. 1µl of SYTO 9 and 10µl of the Microsphere Standard was added to each aliquot. This sample was analyzed using the Guava Easycyte Flow cytometer with the Guava ExpressPlus Assay. In this assay, the green fluorescence produced by the bacterial cells was measured against the forward scatter (FSC). The sizing of the cells was determined using the FSC. Since the 6µm Microsphere standards are considerable larger than the 1µm E. coli cell, two distinct populations were observed. The number of cells fluorescing Green was considered the as the number of cells detected.

4.4 Results

4.4.1 Results of Inactivation Studies with the Single Filament DBD

The Single Filament system was tested by challenging it with various amounts of bioaerosol. Control testing was performed to determine the efficacy of bioaerosol production and collection. The tests indicated that for 30 seconds of nebulization, the concentration of bacteria

collection in the control untreated bioaerosol was about 10^3 cfu/ml. This was considered as the lower end of initial inoculum for the sterilization efficacy experiments.

For testing the efficacy of the single filament system, three power settings were chosen on the adjustable power supply. The power input parameters were kept as close as possible to the original system. Three settings were chosen for the initial experimentation. These were

a) High Power: 22kV Peak-to-Peak Voltage with 1.5kHz Frequency which translates to 3W Power
b) Medium Power: 21.4kV Peak-to-Peak Voltage with 1.5kHz Frequency which translates to 2.5W Power
c) Low Power: 20kV Peak-to-Peak Voltage with 1.5kHz Frequency which translates to 2.1W Power

The results of the DBD treatment are shown in Figure 21. The control bioaerosol contained $2.14x10^3$ viable bacteria. All three treatments resulted in no viable growth on agar plates. The plasma was efficient in completely inactivating all the bacteria.

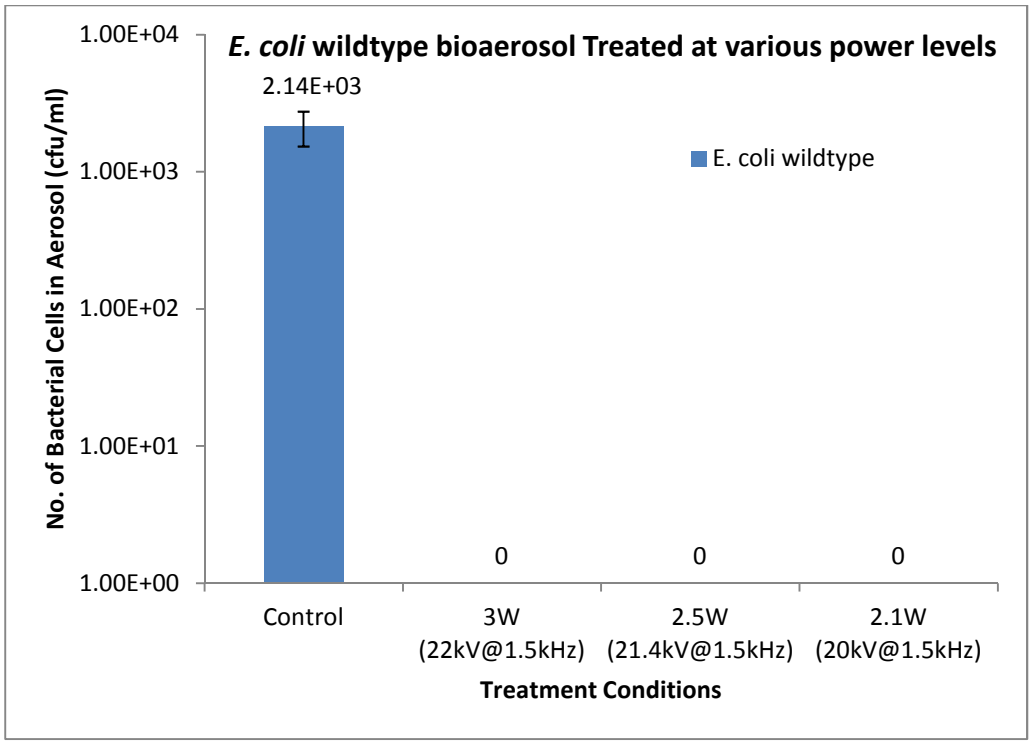


Figure 21: Inactivation of *E. coli* Bioaerosol at Various DBD Power Levels

This held true for all of the strains tested. For further studies, *E. coli*, being the most widely studied bacteria, was chosen as model organism. To understand the mechanism of bacterial cell death, it is essential to subject the bacteria to sub lethal dose of plasma so that the various responses to various pathways that are activated, can be studied. Due to the efficiency of plasma of producing complete inactivation in low amount of bacteria, the initial bacterial load needed to be increased. This was achieved by injecting aerosol into the system for a greater period of time. At larger nebulization times, the plasma is not able to produce complete inactivation and some of the bacterial population survives. For 3min nebulization, the inactivation efficiency dropped. For *E. coli*, it produced 2log reduction from an initial control aerosol of ~10^6 bacteria/ml. For this high inoculum, it was observed that if the power input was

increased, the complete inactivation efficacy was restored. This dependence on the bacterial load and power has been observed in many other plasma studies [82-84] For the treatment samples that produced complete inactivation, the entire sample was added to Tryptic Soy Broth (TSB) (Fisher Scientific, Pittsburgh, PA) and incubated overnight and visually observed for the presence of growth at 24 and 48 hours. No colonies were observed on the agar plates.

4.4.2 Inactivation of Various Microorganisms

The efficacy of the plasma discharge in inactivating airborne *E coli* has been detailed in chapter 1. However, the large volume PDRF system was not used to determine efficacy against other microorganisms. Therefore, it was necessary to use the single filament system to test against bioaerosols of other relevant bacteria. For these studies, we tested some of the most clinically relevant bacteria used in antimicrobial susceptibility testing. This is especially relevant in the clinical setting.

Epidemiological studies on the prevalence of microorganisms in health care facilities have determined that there are certain species of bacteria that are most prevalent and can cause major nosocomial infections. We tested gram-negative strains *E. coli* as well as *Acinetobacter baumannii*. *A.baumannii* is a third most prevalent nosocomial pathogenic species. It has been isolated from various hospital environments and has been shown to colonize patients [85]. It has been identified as the causative agent in many serious medical conditions such as sepsis and nosocomial pneumonia. Since *Acinetobacter* can colonize respirators and propagate respiratory illness, it is especially dangerous in aerosol form.

Staphylococci are also a major cause of hospital-acquired infection (HAI) [86]. Since these organisms are present abundantly on the skin, the principle route of *S. aureus* transmission is from patient to patient via transiently colonized hands of hospital personnel, who acquire the

organism after direct patient contact or after handling contaminated materials [87]. However, recent evidence has suggested that airborne transmission may also be important.

For example, a methicillin-resistant S. *aureus* (MRSA) outbreak originating from the exhaust ducting of an adjacent isolation room ventilation system was terminated once the ventilation system was repaired and an opening in a window sealed [88]. There have been reports of HAI outbreaks due to MRSA contaminated dust in air exhausts [89, 90].

Hence, we investigated the effect of plasma on six (6) strains as detailed in the Figure 22. It can be seen from the results that the plasma was able to inactivate all six strains. The test strains were nebulized for 30s seconds and treated with plasma. The resulting bioaerosol was collected in 10 ml of deionized water. Dilution plating was then employed to detect the number of viable bacteria through colony forming units.

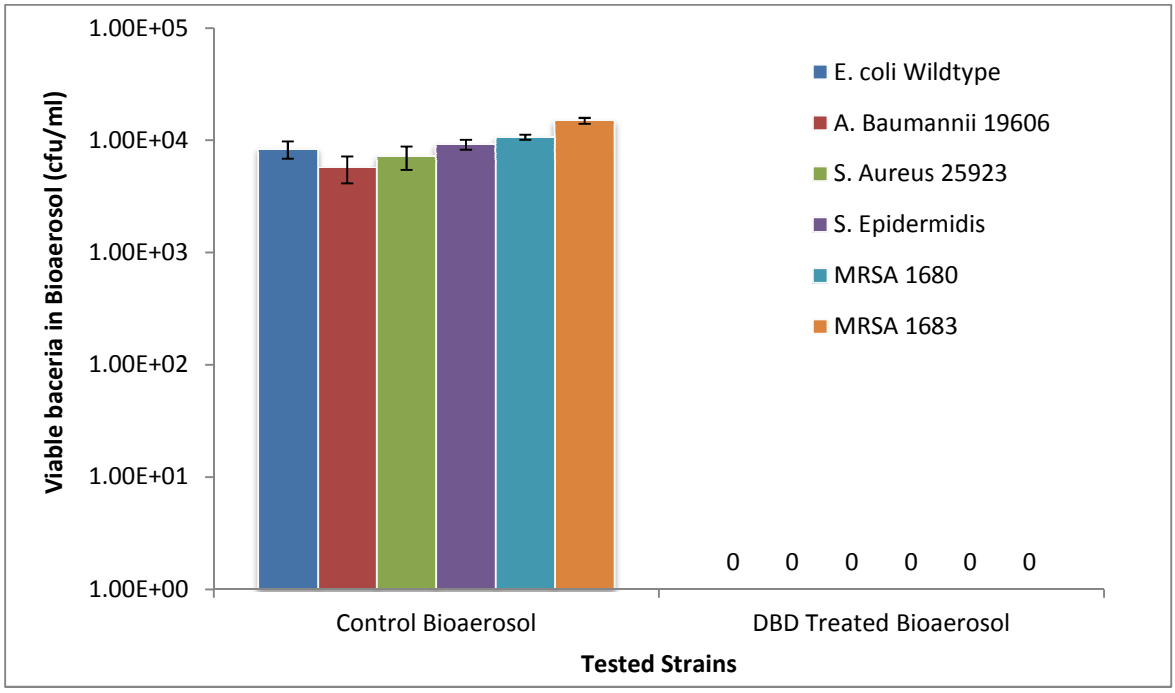


Figure 22: Testing of a Battery of Microorganisms against Single Filament DBD.

These results lead us to believe that the DBD has similar effect on gram positive as well as gram negative bacteria. Also important to note that multi-drug resistant strains are susceptible to this discharge as well. The mechanism of DBD action is thus different from conventional antibiotics.

4.4.3 Power dependency and Identifying the Sub-lethal Dose

One of the major parameters of plasma discharges is the power. There have been major studies detailing the dependence of inactivation on the plasma power [6, 91, 92].

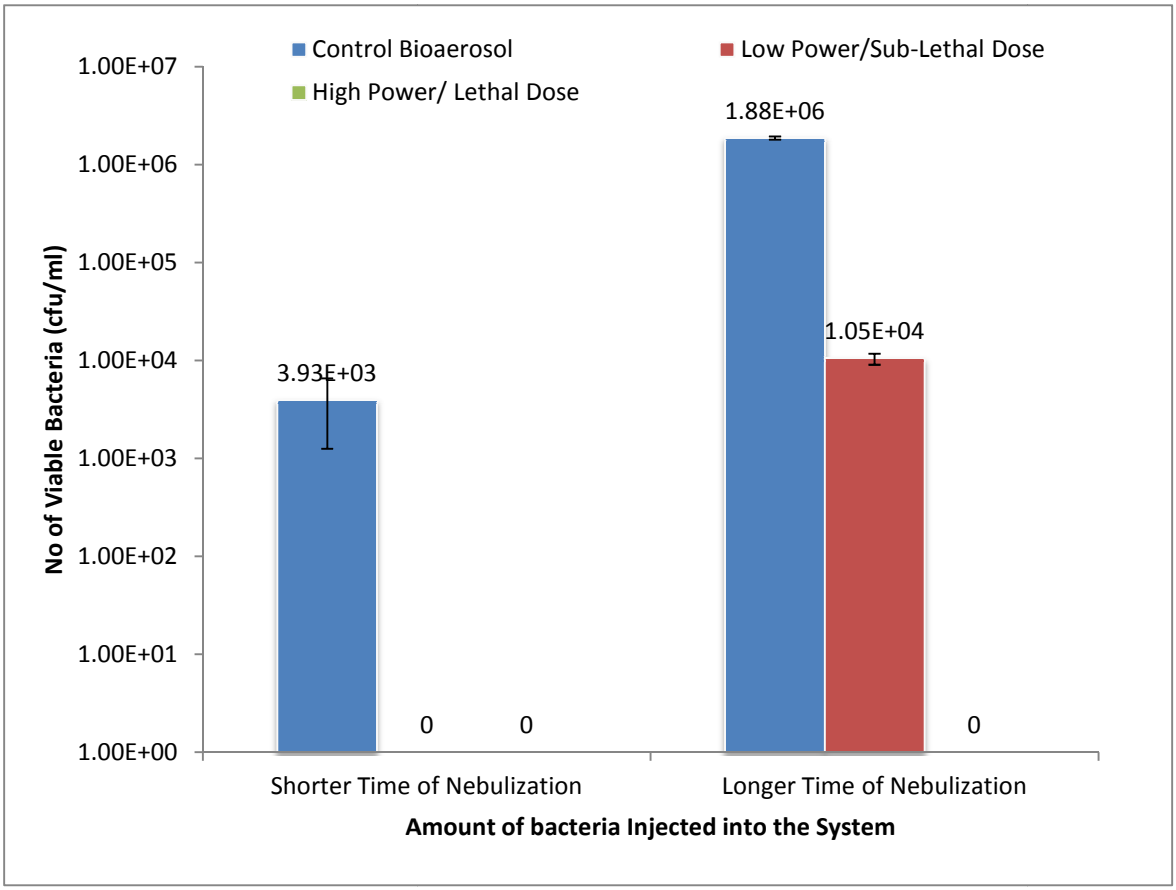


Figure 23: Time of Nebulization affects the Inactivation of Bioaerosol

The results are show in Figure 23. The dependence of the inactivation effect on the initial load and the power was tested. The following power settings used were as discussed earlier in the chapter.

a) High Power: 22kV Peak-to-Peak Voltage with 1.5kHz Frequency which translates to 3W Power
b) Medium Power: 21.4kV Peak-to-Peak Voltage with 1.5kHz Frequency which translates to 2.5W Power
c) Low Power: 20kV Peak-to-Peak Voltage with 1.5kHz Frequency which translates to 2.1W Power

For shorter nebulization time, number of viable *E. coli* captured from bioaerosol was about 10^3 cfu/ml. The DBD plasma was able to completely inactivate bacteria for both treatment power conditions. When the initial inoculum was increased through longer nebulization, there was survival observed. A similar dependency on the bacterial load (cell densities) and power has been reported by other investigators [93, 94].

4.4.4 Analysis of Inactivation through sustained incubation and growth curve

The results of the plasma treatment studies have indicated that the bacteria are inactivated when exposed to lethal dose of plasma. However, the method use for determining this inactivation was the agar dilution method, where the treated samples were plated on agar plates and incubated overnight. A study was required to demonstrate complete inactivation of bacteria in bioaerosols. Plasma is expected to produce stress on the bacterial cell, and when the stress overwhelms the defense mechanism of the bacteria, it leads to bacterial cell death.

There have been studies where antimicrobials agents have demonstrated the ability of bacterial cells to recover from the stress put on them by the treatment [95-97]. This recovery is

generally slower than original growth of the bacterial cell. To confirm the inactivation of microorganism tested in our studies, we incubated the treated samples for longer periods of time. Two sub-lethal doses of plasma and a lethal dose were selected. One ml of control bioaerosol as well as each of the treated samples was added to 5ml of nutrient rich Tryptic Soy Broth. Swabs of the samples were also streaked onto Tryptic Soy Agar plates. Both the plates and the samples cultured in broth were incubated for 24 and 48 hours post treatment. Visual inspection was performed after both incubation periods, to detect growth. The results are shown in Figure 24.

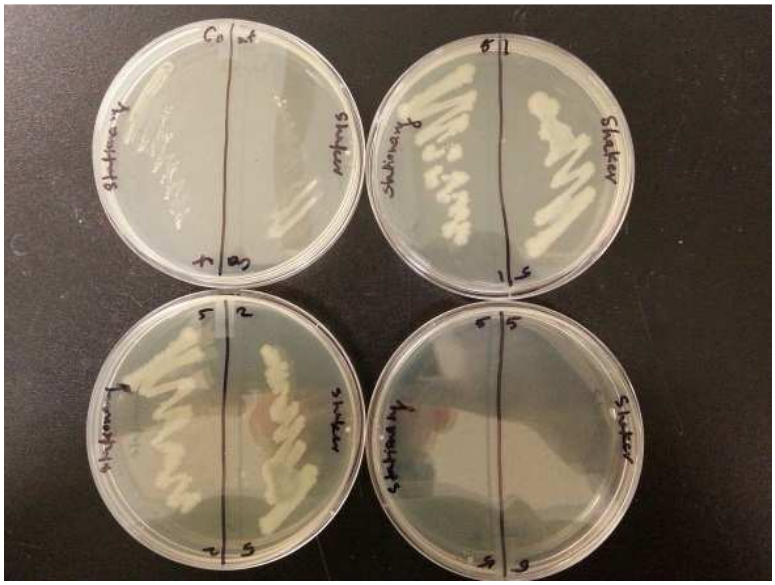


Figure 24: The control and plasma treated bioaerosol inoculated onto agar plates

Figure 25: Treatment Samples from the Lethal and sub-Lethal Doses of DBD, incubated for 24 and 48 hours after treatment. The samples are, from L to R: Control, Sub-lethal dose, Sub-lethal dose and lethal dose

The results of the inactivation are shown in Figure 25. For lethal dose of DBD plasma, no visible colony growth was observed on the agar plates. For 24 and 48 hours of incubation in media, the lethal dose did not produce any visible turbidity. For the control samples, there was growth observed on the agar plates as well in medium. The same was observed for the sub lethal doses tested. For both sub lethal doses, the growth of colonies observed on agar plate was more prominent to the eye than the control sample. This result is interesting since it indicated that the bacteria that were treated with sub lethal doses actually grew faster. To determine the growth patterns of sub-lethal doses of plasma, cell growth measurement and respiration analysis studies were performed.

4.5 Analysis of the Sterilization Effect Produced by Single Filament DBD Discharge

4.5.1 Materials and Methods

4.5.1.1 Optical Density and XTT studies

One of the most widely use methods of analyzing cell growth is the optical density (O.D.) measurement method. When the bacteria grow they absorb light of wavelength of 600 nm. The cell density is proportional to this number. For this, the treated samples were collected in 10 ml dH_2O. The samples, being too dilute, were concentrated using a Vivaspin (Vivaproducts, Inc., Littleton, MA) concentrator to 50µl. 950µl of TSB was added to the samples and samples were incubated at 37°C in a stationery incubator. The optical density of the suspension was measured using the MULTISKAN GO Spectrophotometer (Fisher Scientific, Pittsburgh, PA).

XTT (2, 3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay is a quantification method based on the respiratory metabolic activity of viable cells.
XTT is a tetrazolium salt, which is based on the modification of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active cells [98].
The stock solution of 10 mg/mL XTT was prepared in 10 mL 1X sterile PBS solution. Aliquots of 100µl XTT were stored at -20°C. The working solution was prepared by adding 50 µl stock solution in 1 ml dH2O to yield a final concentration of 0.5 mg/mL, and 1µl of 50 mM Menadione to yield a final concentration of 50 µM. 900µl of this solution was added to 50µl of the treatment samples, along with 50µl of TSB.

A negative growth control containing overnight culture was used. The absorbance of the samples at 492 nm was measured with the MULTISKAN GO Spectrophotometer (Fisher Scientific, Pittsburgh, PA) for 2hr, 4hr, 6hr and 8 hours and overnight after treatment.

4.5.1.2 Flow Cytometric Measurement of Microorganisms in Samples

Three test conditions were used, control, low power plasma treatment and high power plasma treatment. The results were obtained by observing the populations by their fluorescence in the green channel. Sizing beads were added to identify the bacterial populations by correct size. The plot showed two distinct populations. The 6µm beads were observed as a population with higher Fluorescence and the population with smaller green fluorescence was identified as the bacteria in the aerosol sample. The population identified as bacteria were gated and the number of events inside the gated area were measured. Three replicate readings were taken for each bioaerosol sample and averaged. The numbers obtained were compared with the number of viable bacteria observed through colony counting assay.

4.5.1.3 Membrane Permeation

LIVE/DEAD® BacLight Bacterial Viability Kit was purchased Life Technologies (Grand Island, NY). The LIVE/DEAD BacLight Viability Assay Kit contains SYTO® 9 green-fluorescent nucleic acid stain and propidium iodide red-fluorescent nucleic acid stain. SYTO 9 dye can penetrate and stain all bacteria with both intact and damaged membranes. Propidium iodide can only penetrate to cells with damaged membranes. When SYTO 9 and propidium iodide present together, propidium iodide reduces SYTO9 fluorescence. Thus, when bacteria are stained with the mixture of SYTO9 and propidium iodide, cells with intact membrane, whereas stained green and cells with damaged membrane, whereas stained red can be detected. Both dyes are provided as powders in sealed plastic Pasteur pipettes. Both dyes were dissolved in 5 ml of sterile deionized water in order to produce 6 µM SYTO 9 and 30 µM propidium iodide solutions.

4.5.1.4 Membrane Depolarization

The BacLight™ Bacterial Membrane Potential Kit provides solutions of the carbocyanine dye DiOC2(3) (3,3′-diethyloxacarbocyanine iodide) and CCCP (carbonyl cyanide 3-chlorophenylhydrazone), both in DMSO, and a 1X phosphate-buffered saline solution. DiOC2(3) exhibits green fluorescence in all bacterial cells, but the fluorescence shifts toward red emission as the dye molecules self-associate at the higher cytosolic concentrations caused by larger membrane potentials. For the experiment, 10µl of the DiOC2(3) was added to 1ml of the treated samples. The green and red fluorescence was measured with the Guava flow cytometer.

4.5.1.5 Positional Dependence of Sterilization

The results of the inactivation have indicated that the single filament DBD plasma is indeed capable of inactivating bacteria in flight. This study deals with the interaction of microdischarge with the bioaerosol droplet. There have been studies regarding the interaction of particles and droplets interacting with streamer discharges. Whereas low pressure systems are mostly glow discharges having gradients in plasma densities that are small on the scale length of any given aerosol or dust particle. In contrast, the diameter of the streamer in atmospheric pressure discharges can be comparable with the size of the particle. The dynamics of the propagation of streamers that intersect with particles can therefore be significantly perturbed. The intersection of streamers with these aerosols can be expected to change the rate of radical generation by both changing the properties of the streamer and by transferring heat to the droplet, thereby increasing its rate of evaporation. This is evident in the results in chapter 5 relating to

heat stress produced by the streamer on the bioaerosol droplet. The study by *Babaeva et.al* [99] indicates that small particles of moderate permittivity are enveloped by the streamer. Larger particles such as bacterial bioaerosol droplets can intercept the streamer while charging. As plasma envelopes the particle, it provides hot ionizing radiation that can affect the bacterial cell inside the bioaerosol droplet.

4.5.2 Results

4.5.2.1 Optical Density and XTT Studies

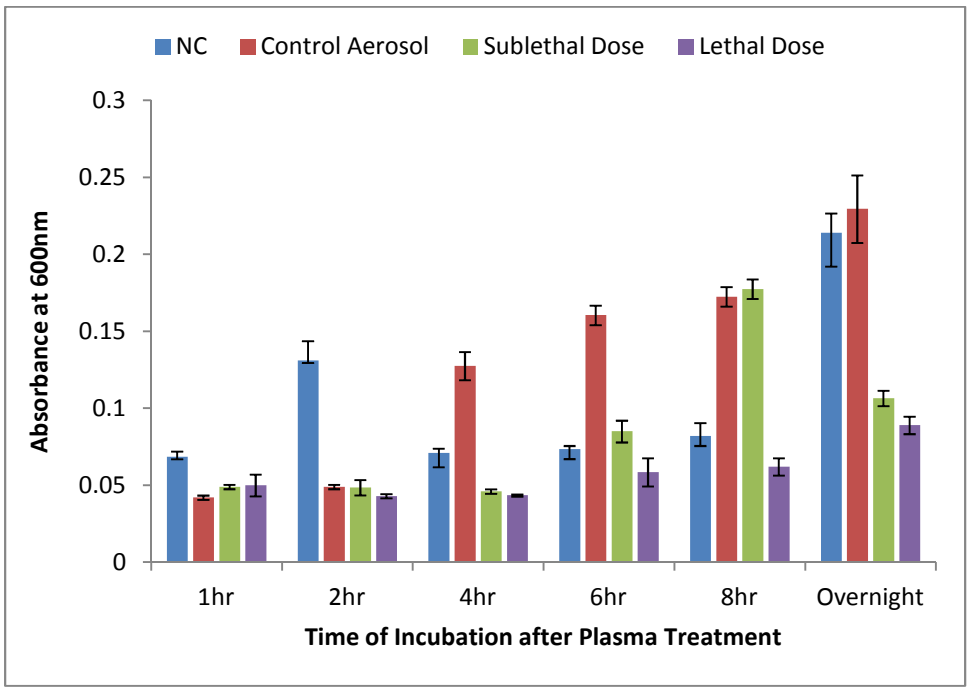


Figure 26: Optical Density Measurements of Samples Post Plasma Treatment

The results of the optical density measurement are shown in Figure 26. Sub-lethal and lethal conditions were tested along with positive and negative control aerosol. The samples were analyzed for 1hr, 2hr, 4hr, 6hr, 8hr and overnight post treatment. Duplicate readings were performed. The control aerosol showed linear growth. The lethal dose of plasma showed insignificant growth for the entire duration of testing. The sub-lethal samples showed dormancy till the 8hr reading, when there was a sharp increase in the concentration. This time point also coincides with beginning of the exponential phase. This indicates the cells damaged by sub-lethal dose of plasma showing recovery effect. This effect was further analyzed with cellular respiration studies.

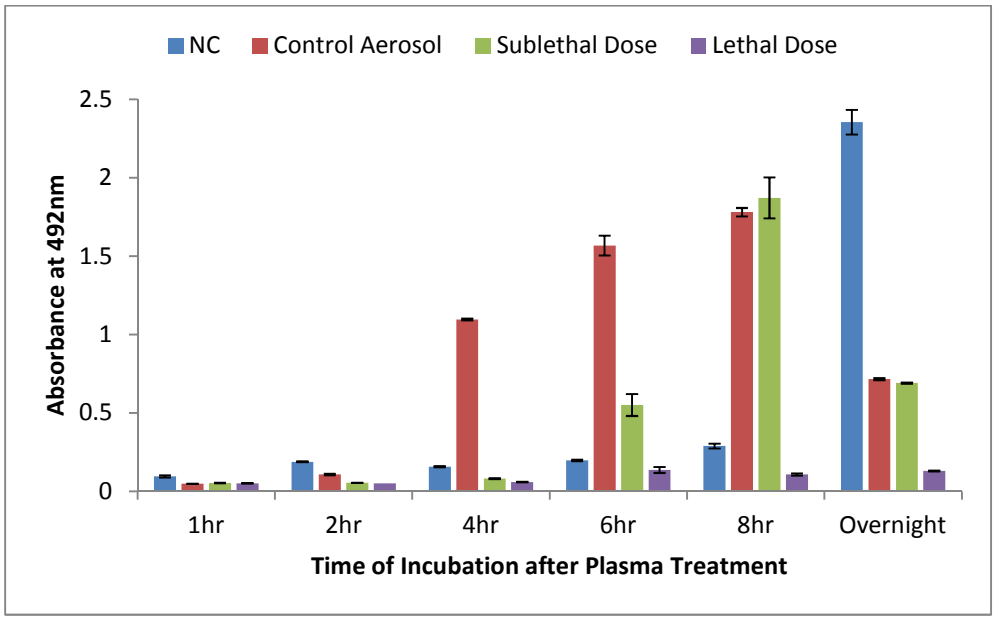


Figure 27: Cellular Respiration Measured with the XTT Assay

The XTT studies are detailed in Figure 27. Sub-lethal and lethal conditions were tested along with control aerosol as well as negative control aerosol. The samples were analyzed for 1hr, 2hr, 4hr, 6hr, 8 hours and overnight post treatment. The readings were taken at 492nm. There was negligible signal for the first two hours post treatment for all samples. The aerosol samples showed greater respiration than the untreated negative control. The growth of the control aerosol was linear for the 4hr, 6hr, 8 hour samples, whereas the sub lethal dose sample showed delayed response. Similar to the optical density measurements, there was increased respiration at the 8 hr. sample for the sub lethal dose. This value was comparable to the control aerosol. However, the respiration in the sub lethal sample significantly decreased overnight. Apart from the negative control, the aerosol samples showed decreased respiration overnight. But this could be due to the cytotoxicity of XTT. This led us to further investigate the effect of plasma on the cell membrane as the respiration is affected by ROS entering the cells and disturbing the respiratory chain.

4.5.2.2 Flow Cytometric Measurement of Microorganisms in Samples

For the DBGD system, we employed the flow Cytometric method for the rapid detection of bacteria in treated samples. This methodology was again employed for the single filament treatment.

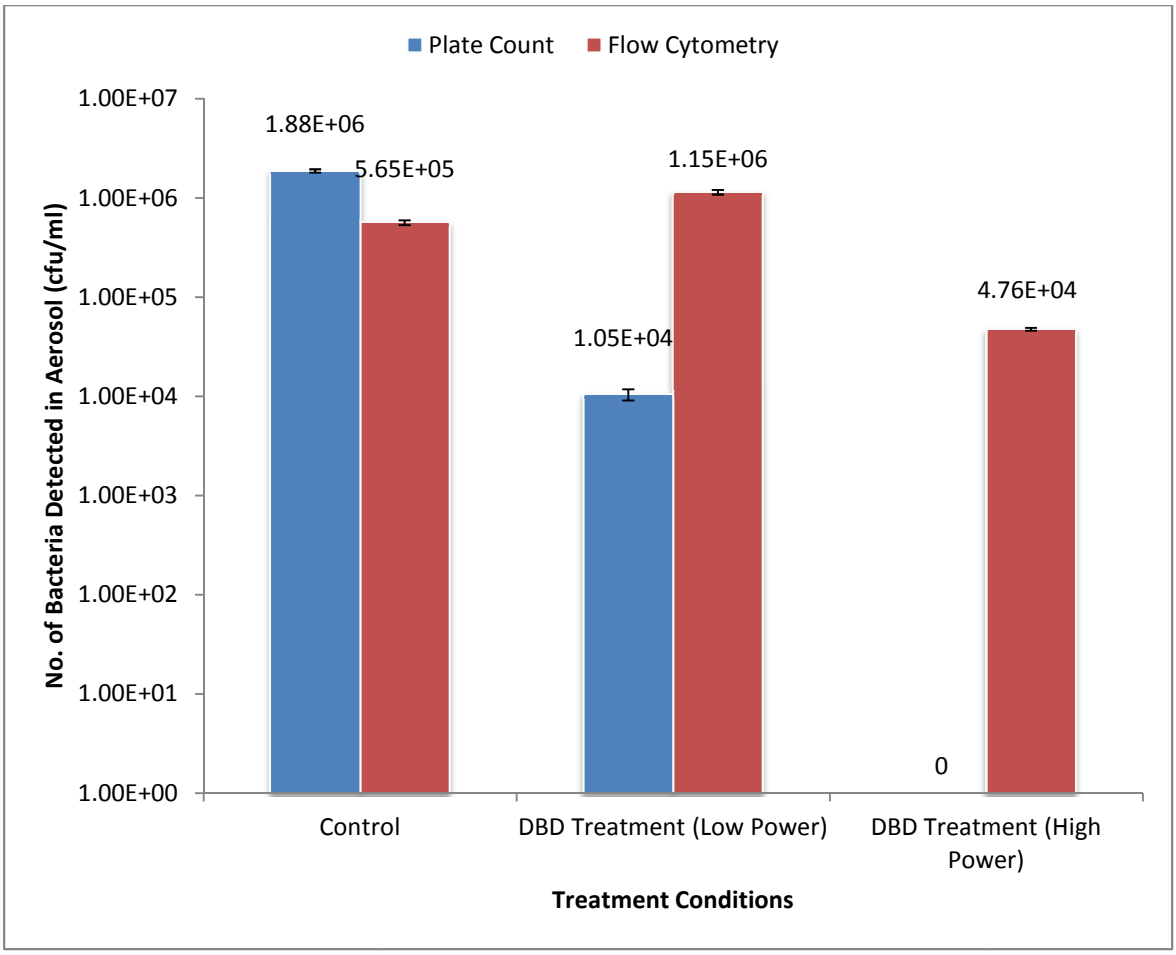


Figure 28: Detection of Bacteria in Treated and Control Aerosol

The results are shown in Figure 28. For low power treatment, there is no significant difference between the bioaerosol particles captured by flow cytometry, however, the viable count decreased by 2 logs. For higher power, it can be seen that flow cytometer detected 90% of the bioaerosol particles whereas no colonies were observed on agar plates. The decrease in the population observed by flow cytometry can be attributed to the fact that higher power of plasma

can lead to membrane rupture and disintegration of bacteria [84]. The membrane effects were thus analyzed with fluorescent dyes.

4.5.2.3 Membrane Permeation

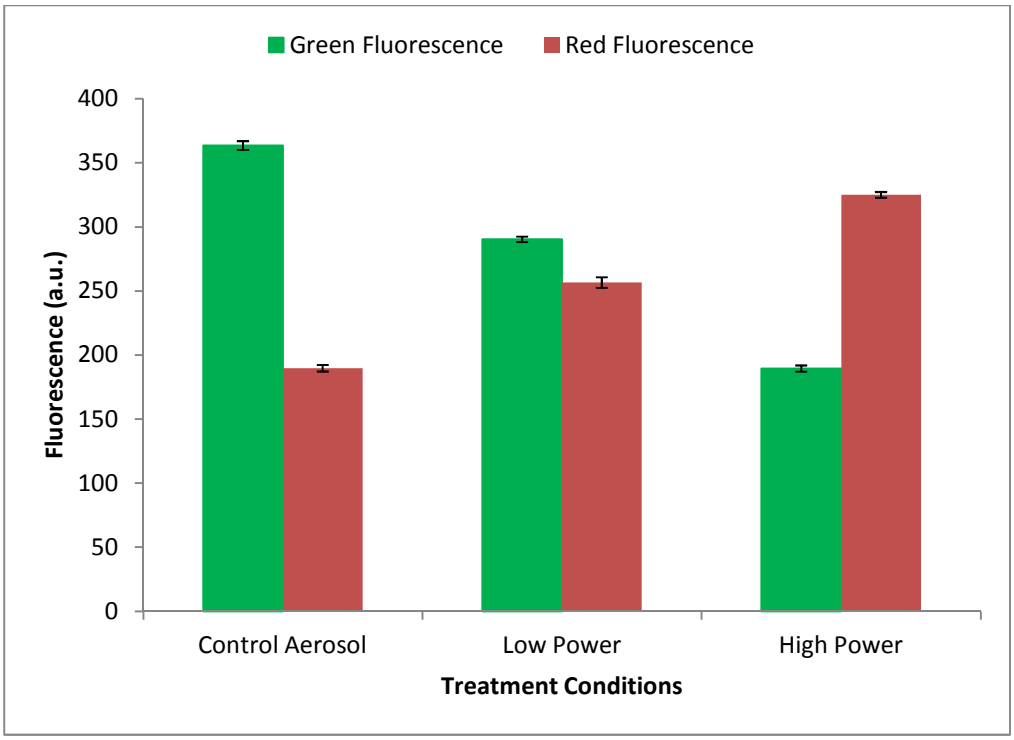


Figure 29: The Red and Green Fluorescence of Plasma Treated Aerosol Samples.

The results of the membrane permeation study are detailed in Figure 29. The fluorescence of the samples was measured in the Green and Red channels of the Guava Flow cytometer. The average fluorescence of the samples was obtained. It can be seen that the control aerosol samples

have a much higher fluorescence in the green spectrum, indicating intact membranes. For the sub lethal and lethal doses, this reduces whereas the red fluorescence increases. The increased permeation is observed according to the lethality of the plasma dosage. This would lead us to believe that there is increasing membrane permeation with plasma exposure.

4.5.2.4 Membrane Depolarization

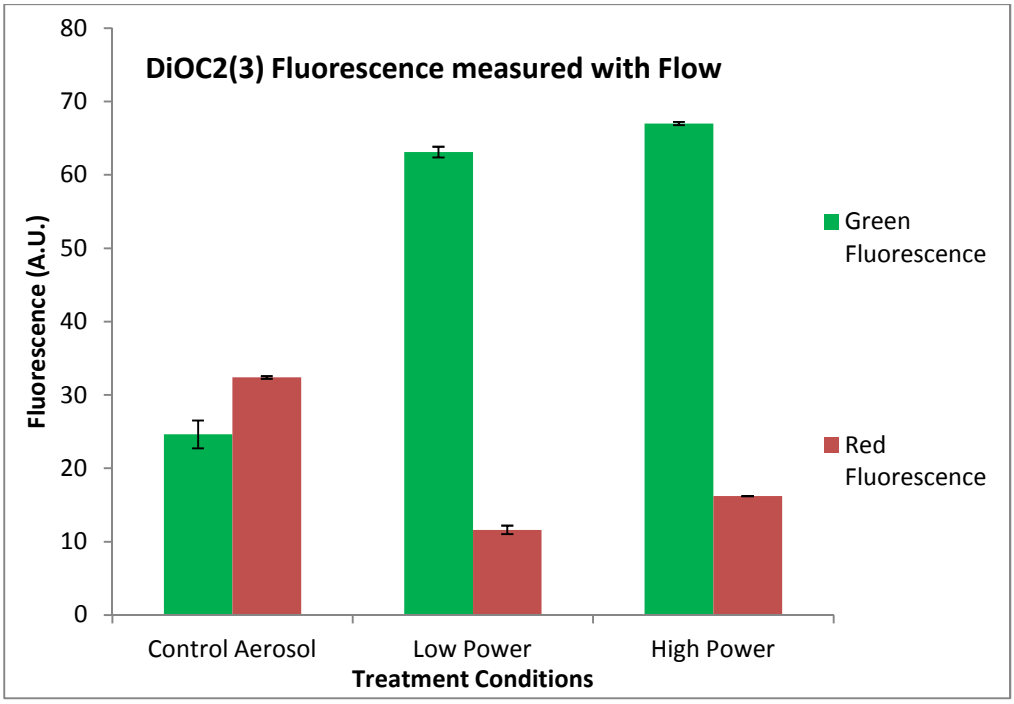
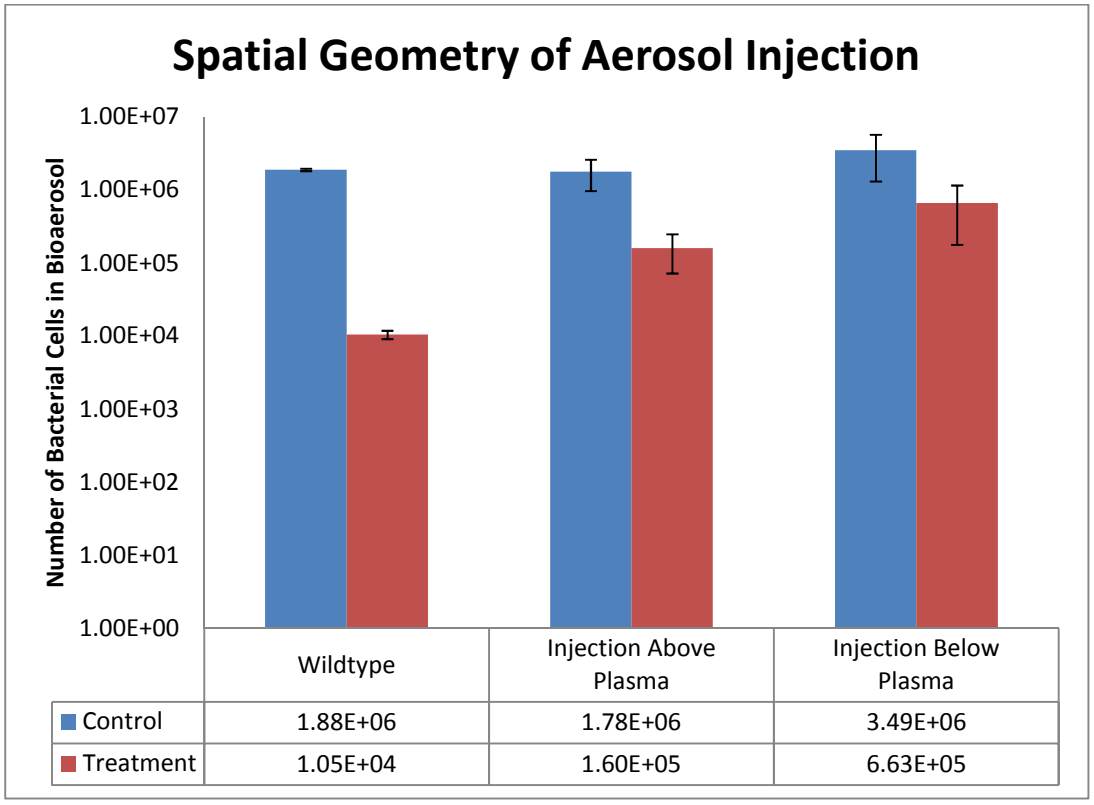


Figure 30: The DiOC2(3) Fluorescence of Plasma Treated Bioaerosol Samples, Measured in the Green and Red Spectrum.

The results are shown in Figure 30. The DiOC2(3) fluorescence was measured with the Guava flow cytometer. The average fluorescence values were obtained for the green and red channels. The depolarization is indicated by the shift in DiOC2(3) fluorescence to the red spectrum. The results indicate that the opposite occurs in the plasma treated aerosol samples. Compared to controls, there is shift in the DiOC2(3) spectrum towards the green spectrum. This is an indication of the hyper-polarization of the bacteria after plasma exposure. The increase in the net negative charge can be attributed to the deposition of charged species on the surface of the bacteria.

4.5.2.5 Positional Dependence of Sterilization



	Wildtype	Injection Above Plasma	Injection Below Plasma
Control	1.88E+06	1.78E+06	3.49E+06
Treatment	1.05E+04	1.60E+05	6.63E+05

Figure 31: Dependence of Sterilization on Position of Aerosol Injection

The spatial geometry of the discharge was analyzed. The dosage used was the sub lethal dose of plasma. Three positions of injection were employed. The injection of the bacteria above plasma discharge produced significant sterilization. However, the survival of the bacteria was significantly higher than the normal injection. When the bacteria were injected below the plasma discharge, there was no significance of inactivation. The effect of inactivation produced when the aerosol is injected above single filament

4.6 Conclusion

Plasma technology is emerging as a new approach to inactivating aerosols. The point to point DBD discharge developed was efficient in inactivating up to 3 logs of bacteria, determined by the flowrate. The efficacy of the discharge was also dependent on the total flow of bioaerosol. Plasma discharge has been used to inactivate certain antibiotic resistant bacteria and this was demonstrated for aerosols of such species in this study. These results have significance in a clinical setting.

Flow Cytometry was useful in determining the presence of bacteria in the post treatment flow. The results indicate that although most of the bacteria in bioaerosol are captured through the impaction process, the viability is decreased greatly by the interaction with DBD discharge. For high power plasma treatment, no viable bacteria were observed through plate counts and almost a log fewer bacteria were observed through flow cytometry. This could be due to the rupture of the cell walls after interaction with plasma, changing the size of the cells.

Membrane effects were analyzed using fluorescent staining method. The results indicate that there is significant membrane permeation after plasma exposure. The results with the depolarization experiments were interesting as there was hyper-polarization, as opposed to depolarization, in the plasma treated bacteria. This would indicate that the species produced by plasma are indeed entering the bacterial cells through permeated membranes and causing inactivation through interfering with cellular processes. The major species produced by plasma are the Reactive Oxygen Species (ROS) and their involvement is studied in the next chapter.

5. ANALYZING THE OXIDATIVE AND PEROXIDATIVE STRESS PRODUCED BY SINGLE FILAMENT DBD

5.1 Reactive Oxygen Species

This chapter deals with the effect of the Reactive Oxygen Species (ROS) produced by the single filament plasma discharge on the bacteria in aerosol. The cascade of ROS production is shown below. In plasma discharges, the free electron is provided by the electric field. The Figure 32 indicates the cascade of species produced by plasma through the reduction of oxygen. The species that are of the greatest interest due to their bactericidal properties are the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). These species are involved in the biological processes of the bacterial cell, and the bacterial cell has defense mechanisms against them. In this chapter, we will explore whether the cellular defenses of the *E. coli* cell that are triggered after exposure to non-thermal plasma.

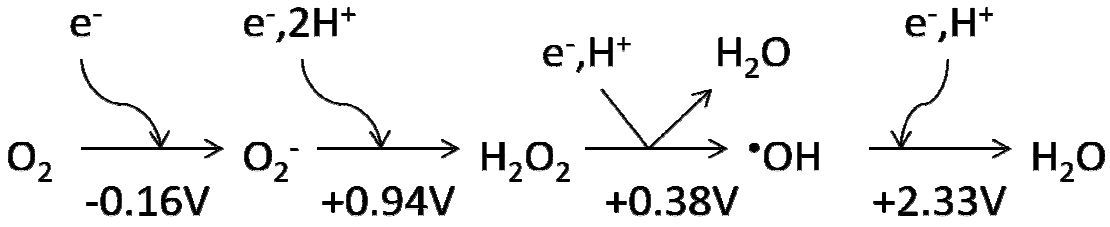


Figure 32: Production of ROS through the reduction of Oxygen [100]

5.1.1 Superoxide Ion

The superoxide radical (O_2^-) is generated within aerobic biological systems during both enzymatic and non-enzymatic oxidations. O_2^- differs from H_2O_2 in that it is a charged species at physiological pH (pKa = 4.8), so it cannot penetrate membranes [101, 102]. However, aqueous O_2^- at low pH forms Hydroperoxyl radical (HO_2^-), which can enter the cell easily. The reaction rate constant is 8 × 104 M-1sec-1, and the reaction proceeds four orders of magnitude faster in the presence of the enzyme superoxide dismutase [103].

E. coli contains three SODs: cytoplasmic iron- and manganese-cofactored enzymes (Fe-SOD and Mn-SOD) and a periplasmic copper–zinc-cofactored enzyme (Cu–Zn-SOD). The two isozymes Fe-SOD and Mn-SOD are coordinately regulated in response to iron levels [104, 105]. The Fe-SOD is the default isozyme and Mn-SOD is synthesized when Fe-SOD cannot be activated. Mn-SOD synthesis is also stimulated whenever O_2^--generating antibiotics are present, with the control being exerted by the *KatG* system [106, 107]. *KatG* is not active during normal aerobiosis, in the absence of these exogenous O_2^- sources.

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

5.1.2 Hydroxyl Ion and Hydrogen Peroxide

Hydrogen peroxide is an important ROS that is produced as a byproduct of cellular respiration. It can also be produced by the dismutation of the superoxide ion. H_2O_2 is a relatively stable and long living species as it has no unpaired electrons. Due to their small size and neutral charge, H_2O_2 molecules freely dissolve in solution and readily diffuse into cells through cell membrane aquaporins [180, 181]. The reaction of hydrogen peroxide with transition metals

imposes on cells an oxidative stress condition that can result in damage to cellular components such as proteins, lipids and DNA, leading to mutagenesis and cell death. H_2O_2 can also produce deleterious effects in the cell by being converted to the Hydroxyl radical OH^-. In the presence of iron or copper ions, H_2O_2 reacts with $O_2^{-}\cdot$ to produce highly reactive ·OH. In non-thermal plasma, hydroxyl radicals combine to form H_2O_2.

$$^{\bullet}OH + {}^{\bullet}OH \longrightarrow H_2O_2$$

The important genetic pathways that provide the defense mechanism against hydrogen peroxide in *E. coli* are OxyR and SoxRS systems, which are elicited by cells to avoid the introduction of oxidative lesions by hydrogen peroxide conditions [108].

The bacterial cell employs multiple pathways to scavenge H_2O_2. There are many enzymes being involved in the defense against H_2O_2. Of these, there are three major ones that have important roles in vivo. These enzymes are: alkyl hydroperoxide reductase (*ahpC*), catalase G (*katG*) and catalase E (*katE*)[109]. *Ahp* is a two-component (*AhpC–AhpF*) thiol-based peroxidase that transfers electrons from NADH to H_2O_2, thereby reducing H_2O_2 to water.

$$NADH + H^+ + H_2O_2 \xrightarrow{Ahp} 2H_2O + NAD^+$$

This peroxidase is the primary scavenging enzyme under regular growth conditions. The evidence suggests that ahpCF-null mutants accumulate enough H_2O_2 to activate the *OxyR* H_2O_2 stress response.

Another system is the catalase system consisting of *KatG* and *KatE*. *KatG* belongs to the catalase–peroxidase family and is normally weakly expressed in exponential cells.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

However, *OxyR* strongly induces both *ahpCF* and *katG* when cells are stressed by exogenous H_2O_2 [110]. *KatE* is strongly expressed in stationary phase cells, as it is induced by the *RpoS* [111]. Figure 33, outlines the metabolice fate of H_2O_2.

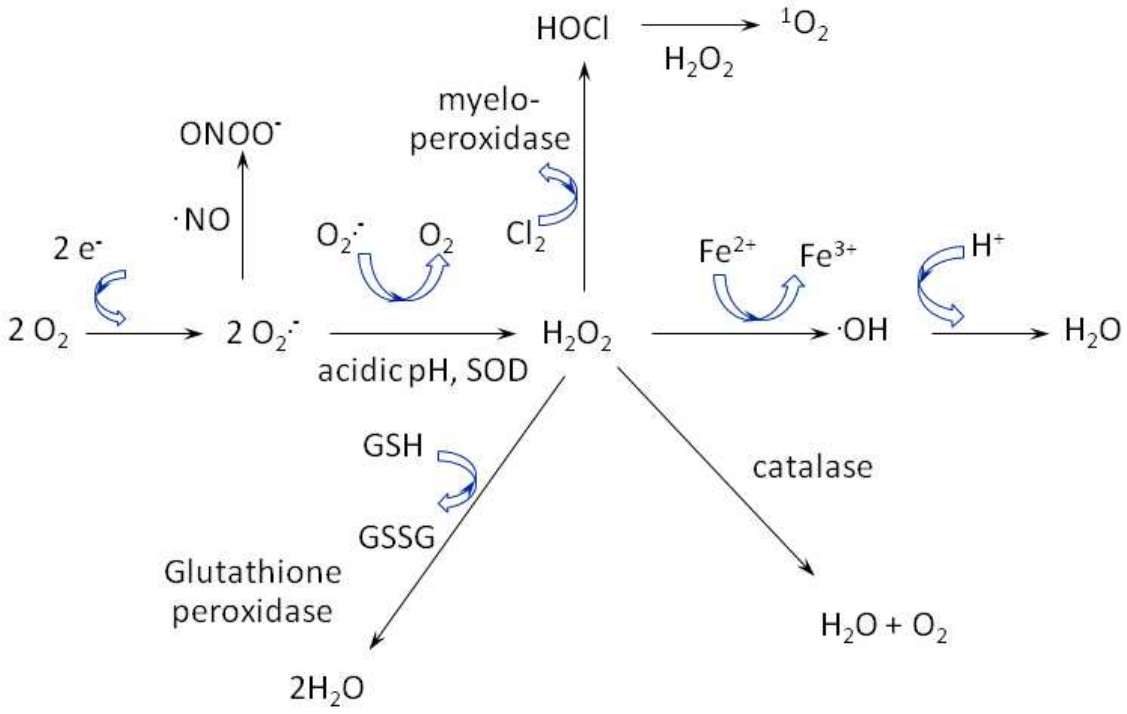


Figure 33:The transition between Reactive Oxygen Species [112]

5.1.3 The Oxidative Stress Regulon *oxyR*

OxyR, is a 34kDa protein [113] , is a homolog of the LysR family of transcriptional regulators in E. coli [110, 114], and characteristic of this protein family [115], *OxyR* controls the regulon system consisting of about 40 genes, which protect the cell from hydrogen peroxide toxicity. Hence, *oxyR* mutants have been shown to be hypersensitive to H_2O_2, and constitutive expression of the *OxyR* regulon due to dominant mutations in *oxyR*, such as found in mutants *oxyR*1 of *S. enterica* and *oxyR*2 of *E. coli*, has been shown to increase the resistance of the cell to H_2O_2 [116]. *OxyR* also has a role in protecting against heat stress [116], near-UV [117], singlet oxygen [118] and lipid peroxidation-mediated cell damage [119].

5.2 Materials and Methods

5.2.1 Bacterial Strains

The bacterial strains were obtained from multiple sources. The table denotes their sources.

Table2: Gene deletion mutants used to study the inactivation produced by single filament

Strain no.	Relevant genotype	Source
BW25113	Wild type	CGSC #7636
3144	BW25113 *ΔsodA*	Dr. Xilin Zhao
3145	BW25113 *ΔsodB*	Dr. Xilin Zhao
3156	BW25113 *ΔsodAΔsodB*	Dr. Xilin Zhao
3157	BW25113 *ΔkatG*	Dr. Xilin Zhao

3202	BW2511*3 ΔkatE*	Dr. Xilin Zhao
3201	BW25113 *ΔkatGΔkatE*	Dr. Xilin Zhao
3200	BW25113 *ΔahpC*	Dr. Xilin Zhao
TA4110	*oxyR2 oxyR Constitutive*	Dr. Gisela Storz
TA4112	*oxyRΔ3 oxyR Deletion*	Dr. Gisela Storz
JW4103-1	groEL: groL768(del)::kan	CGSC#: 10954
SX1398	groES: groS791-YFP(::cat)	CGSC#: 12953
MF634	DnaJ: dnaJ259(ts)	CGSC#: 5828
GR756	DnaK: dnaK756(ts)	CGSC#: 5829
DA16	grpE: grpE280	CGSC#: 7795
JW0462-1	HtpG: htpG757(del)::kan	CGSC#: 8616
JW0866-1	clpA: clpA783(del)::kan	CGSC#: 8898
JW2573-6	clpB: clpB757(del)::kan	CGSC#: 11763
JW0428-1	clpX: clpX724(del)::kan	CGSC#: 8591

The strains were grown in Tryptic Soy Broth and subcultured onto agar plates, and stored at 4°C. For experimental purposes, colonies were picked from the plate and inoculated into 20ml of Tryptic Soy Broth and incubated at 37°C overnight in a shaker incubator at 200 r.p.m.

For strains containing antibiotic resistance gene, LB medium was used for growth. 50µg/ml Kanamycin was added to the culture. The strain of interest was added to the selective culture and incubated at 37°C overnight in a shaker incubator operating at 200r.p.m. The overnight culture was then added to 80 ml of deionized water (Millipore, MA) to obtain the working solution for nebulization purposes. This cell suspension was then added to the nebulizer.

5.2.2 Experimental Conditions

The nebulizer input pressure was maintained at 10 p.s.i. The samples were obtained from running the test conditions. Deionized water was used as the collection liquid for the samples.

10ml of deionized water was added to the collection tubes and the collected bioaerosol was collected in the liquid through thorough mixing. This sample was then used for further analysis.

5.2.3 Colony Count Assay

Standard plates count assay was performed to assess the survival of each strain. Each sample was plated in triplicate. Experiments were run in duplicates. Standard t-test was employed to ascertain the statistical significance.

5.2.4 D-Mannitol and Thiourea

The D-Mannitol was obtained in powder form (Sigma Aldrich, St Louis, MO). Aliquots were made by dissolving required amount of the powder in distilled water. Thiourea was obtained in powder form and aliquot in similar way. The working solutions used in the experiments were prepared fresh for each experiment.

5.2.5 Catalase

The catalase was obtained in lyophilized powder form. Phosphate Buffer (50 mM Potassium Phosphate Buffer, pH 7.0 at 25 °C) (Sigma) was prepared and a stock solution of 10 mg/ml was prepared in this Phosphate Buffer. The assay to determine the enzymatic activity was performed according to protocol. Solutions of the experimental concentrations were prepared fresh before each experiment.

5.2.6 Mediation of Heat Stress

For the experiments involving the mediation of heat stress through temperature, the overnight cultures to be nebulized were suspended in water that was maintained at 4°C. The collection of treated samples was also processed in water and collected at 4°C.

5.2.7 Detection of H_2O_2 by DCFH-DA Fluorescence

The DCFH-DA was obtained from Cayman Chemical (Ann Arbor, MI). A 10mg/ml solution was made in DMSO. From this, 48.7µl was added to 100ml of H_2O to get 10µM working solution. The Mannitol solution was prepared by adding 728mg of mannitol powder to 40 ml dH_2O to make 100mM working solution. Six samples were analyzed for three treatment conditions. The overnight culture to be nebulized was incubated for 30 mins at 37°C in a solution of PBS containing the DCFH-DA. The samples of the control and treated bioaerosol were collected in PBS or the Mannitol solution prepared. The samples were analyzed by flow cytometry for their relative fluorescence in the green spectrum.

5.2.8 Statistical Analysis

Data sets were analyzed using Microsoft Excel and verified using GraphPad Prism 4 software (GraphPad, San Diego, CA). The *P* values are derived against corresponding untreated conditions, unless and otherwise stated, and a *P* value of <0.05 is considered significant. All experiments were repeated minimum three times unless stated, and data are means ± standard error.

5.3 Results

5.3.1 Inactivation of Superoxide Dismutase Deficient Mutants

The findings of the inactivation of *sod* deficient mutants are shown in the Figure 34. The single and double knockouts were tested and compared to the controls.

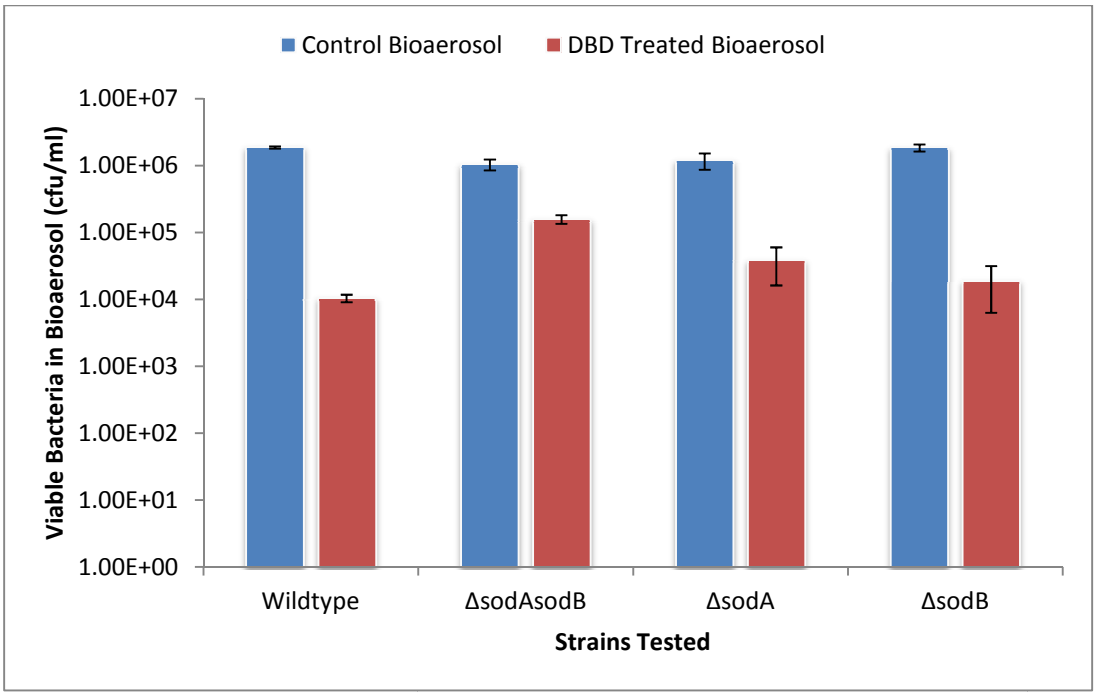


Figure 34: Inactivation of *sod* deletion strains compared with wildtype

The inactivation pattern of the single knockouts was not significantly different than the wildtype (p=0.0943 for *ΔsodA*, p=0.3088 for *ΔsodB*). The double knockout strain *ΔsodAΔsodB* actually showed significantly greater survival as compared to the wildtype (p= 0.0004). The greatest susceptibility was displayed by the mutant of *sodB*. These results are interesting in the sense that they indicate that the defense against the superoxide radical is not activated after plasma exposure. This would lead us to believe that O_2^- is not directly impacting the bacterial cells. Next the focus was turned towards the peroxide defense genes.

5.3.2 Inactivation of Catalase Deficient Mutants

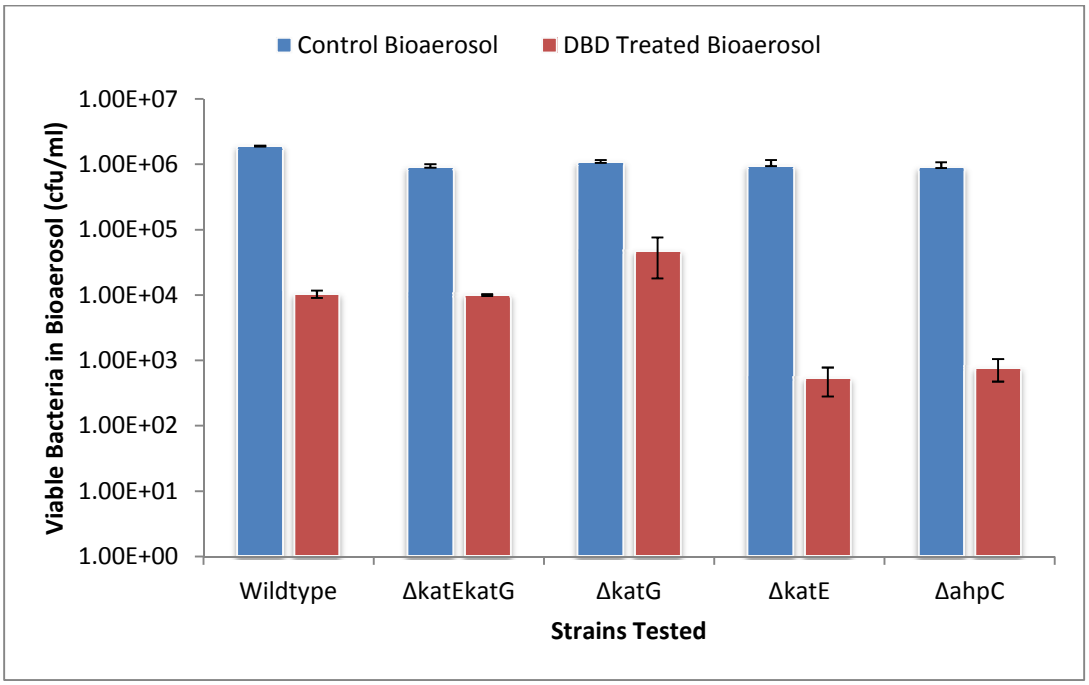


Figure 35: Inactivation of catalase/peroxidase deficient mutants

The single and double knockouts of *kat* were tested and the results are shown in Figure 35. For the double knockout *ΔkatEΔkatG*, there no significant difference in inactivation when compared to wildtype (p=0.7017). *ΔkatE* also showed no significant difference. *ΔkatE* however, was inactivated to a significantly greater amount (p=0.0002). The inactivation observed was 1.5 logs greater than in wildtype. This was the first indication of the involvement of Peroxidative stress. The *ahpC* gene is part of the *oxyR* regulated system. The alkyl hydroperoxidase is another system involved in the defense against peroxidative stress and *ΔahpC* was tested with plasma. The inactivation of this strain was similar to the *ΔkatE* and significantly greater than wildtype (p=0.0003). Hence, two strains that are deficient in defending against peroxidative stress were shown to be susceptible to plasma discharge.

5.3.3 Oxidative Stress Regulon oxyR

The susceptibility of the catalase and hydroperoxidase mutants led us to further investigate the involvement of the oxidative stress pathways.

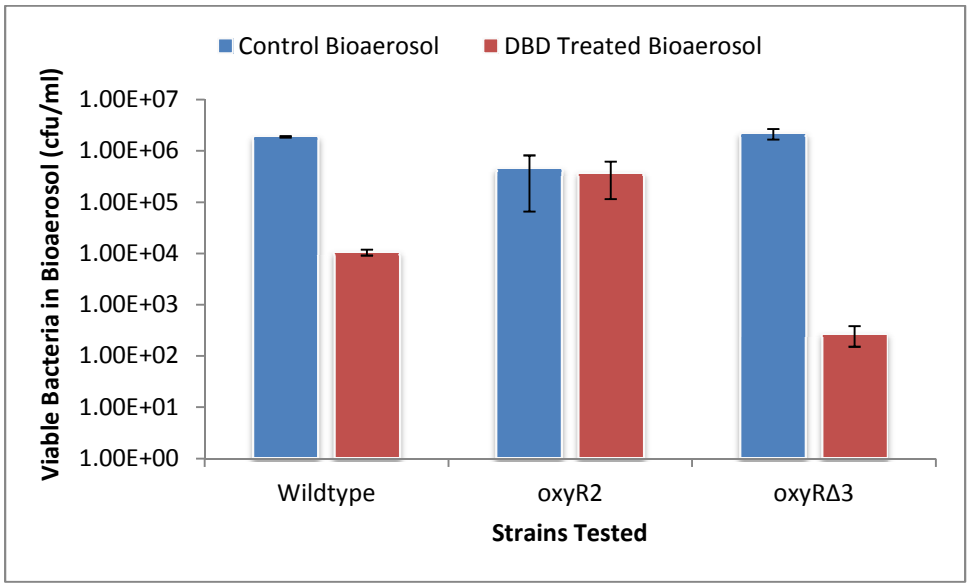


Figure 36: Inactivation of *oxyR* mutant strains.

The system was thus tested at the regulon level with the deletion and overexpression mutants of *oxyR* (*oxyRΔ3* and *oxyR2*). The results are shown in Figure 36. The over-expressor of the *oxyR* was protected better than the wildtype. (p=0.707). The deletion mutant was found to be susceptible as with *ΔkatE* and *ΔahpC* mutants (p=0.002). This indicates the overall involvement of the *oxyR* system in the defense against oxidative stress produced by plasma.

5.3.4 DCFH-DA Fluorescent Detection of H_2O_2

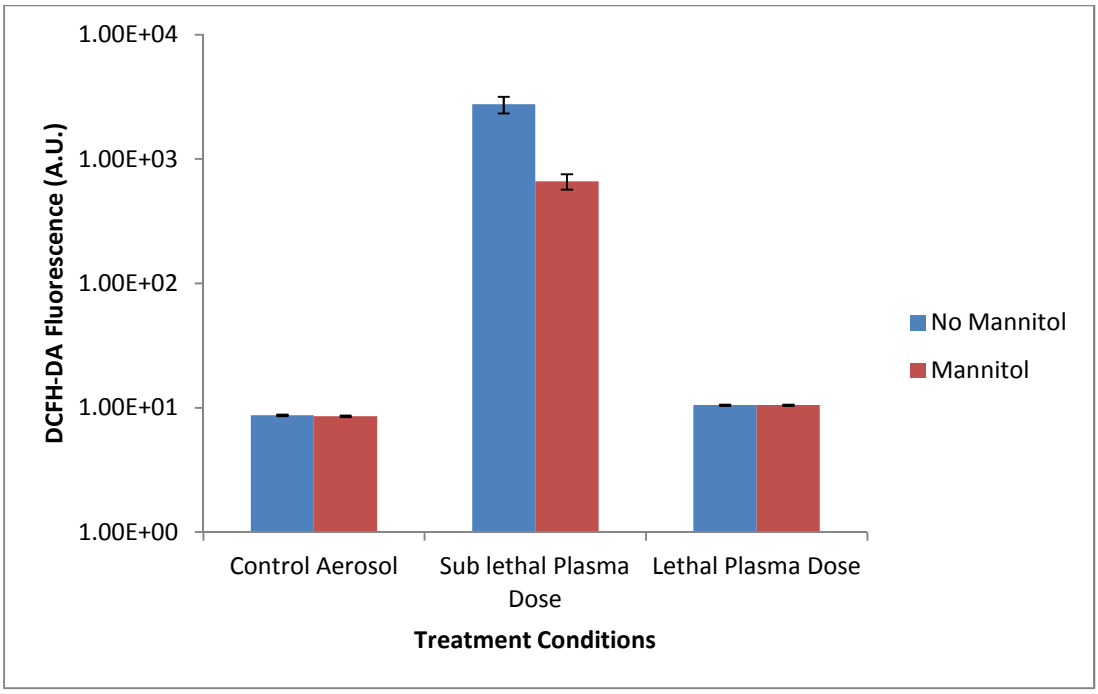


Figure 37: DCFH-DA Fluorescence of Treated Samples.

Flow cytometry was employed to detect the production of H_2O_2 inside bacterial cells. The results are shown in above figure. The highest levels of H_2O_2 were detected in the sub-lethal dose treatment. Both the control and lethal doses produced low fluorescence. The addition of Mannitol produced reduction in the fluorescence, indicating the scavenging effect of Mannitol.

5.4 Scavenging of Oxidative Stress Effect

5.4.1 Protective Effect of Catalase

Testing of the gene knockout mutant strains has indicated the involvement of peroxidative stress produced by plasma. The addition of exogenous catalase has been shown to protect bacteria from exogenous peroxidative stress [120]. Of the catalase gene knockouts, *ΔkatE* was chosen to analyze the effect of external catalase, since it has been shown to have

significantly higher susceptibility than the wildtype. Various concentrations of the catalase were tested as a pre and post treatment protective mechanism. Addition of the catalase prior to nebulization and treatment produced no significant change in the inactivation. Post-treatment addition consisted of collection of the treated aerosol in a solution containing catalase.

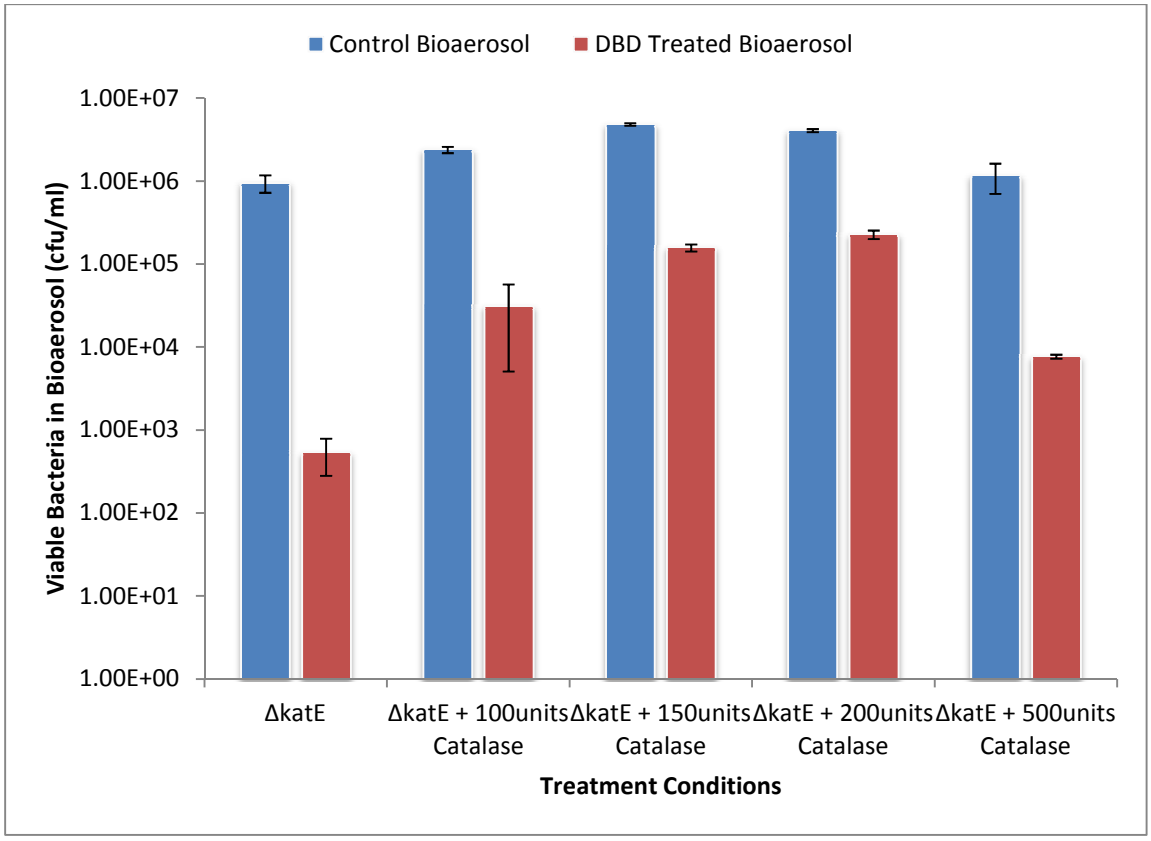


Figure 38: External Catalase Scavenging the Effect of Plasma Exposure

The results, shown above in Figure 38, indicate that for all of the concentrations of catalase tested, significant protection compared to wildtype treatment was observed. For

increasing values of catalase, the protective effect increased, but complete protection was not achieved. 200 units of catalase addition provided the greatest protection ($p=0.0001$). For higher values such as 500 units, the level of protection deceased but was still significant ($p=0.001$). This validates our earlier statement about the involvement of peroxide in the inactivation of *E. coli* during plasma exposure. Complete protection was not observed and indicates the involvement of other species produced by plasma.

5.4.2 Mannitol and Thiourea as ROS Scavengers

Two non-enzymatic scavengers of ROS, D-mannitol and Thiourea were also used. D-mannitol is a sugar that has been shown to defend against peroxidative stress [121]. Thiourea is an organospulphur compound that has been used as an OH- scavenger [122]. OH^- is one of the major ROS that is deleterious to bacteria. The two scavengers are not endogenous to bacterial cell. Therefore, the wildtype was also tested to determine their efficiency in protection. Both scavengers were added to the test strains pre and post treatment. Pretreatment in this case provided no significant protection.

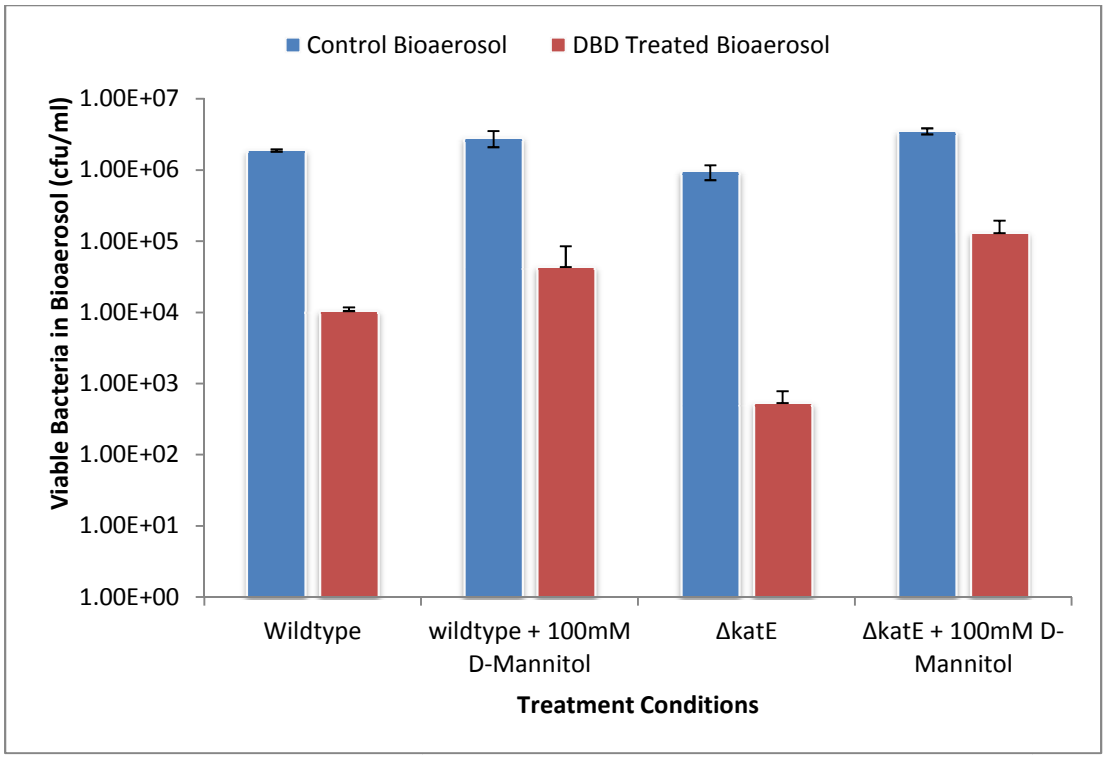


Figure 39: Protective Effect of Mannitol Addition

For post treatment, the Addition of Mannitol protected the catalase deficient strain to an extent even greater than wildtype. Of the various concentrations of the scavenger added, the greatest protection was observed for 100 mM concentration ($p=0.001$).

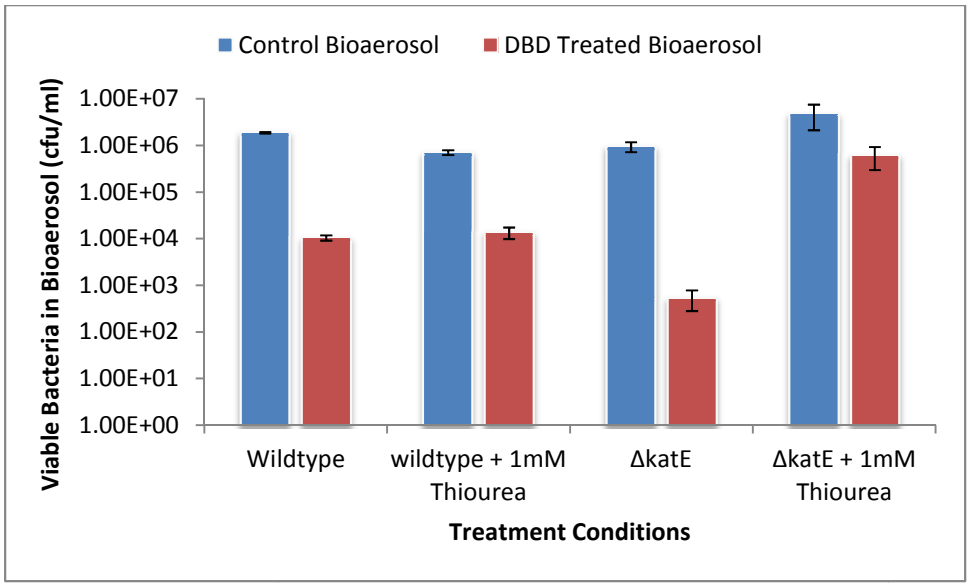


Figure 40: Protective Effect of Thiourea Addition

The results with thiourea showed also followed similar trend (Figure 40). 1 mM of thiourea provided significant protection to the *ΔkatE* strain (p=0.0028). The protective effect for wildtype was not significant. The protective effect decreased for higher concentration of thiourea. For 100 mM thiourea addition, there was 3 log reduction in untreated as well as treated bioaerosol. This effect might be attributed to the fact that thiourea has been shown to be toxic to cells at high concentrations [122].

5.5 Heat Stress in DBD

The plasma density in the microdischarges is much higher than in the surrounding space. Therefore, these microdischarges can be considered as the sole active locations of the DBD volume. It can be considered that all the energy of the plasma is dissipated in the microdischarge volume and eventually the relative temperature of the gas and the surface treated in increased.

Energy dissipation is minute but highly localized and can be very important when the surface to be treated is temperature sensitive, such as biological materials.

5.5.1 Heat Stress Mutants

The results from the oxidative mutant studies indicate that there is involvement of *katE* gene in the defense against stress generated by plasma. It has been observed that *katE* is involved in defense against multiple types of stresses, including heat shock and osmotic stress[123]. Therefore other genes involved in heat and general stress mechanism needed to be investigated. Two families of hsp, hsp60 and hsp70 (60- and 70-kDa hsp) have been implicated in protein folding and assembly [124]. These are the groES/groEL system and the dnaJ/dnaK/grpE system, respectively. These systems constitute 15–20% of the total protein content of *E. coli* cells heat stressed at 46°C [124].

5.5.1.1 The dnaJ/dnaK/grpE system:

DnaK, the Hsp70 homolog of *E. coli*, is controlled by the two co-chaperones DnaJ (41 kDa) and GrpE (22 kDa), which if acting together increase the weak ATPase activity of DnaK by 2 orders of magnitude [125-127]. DnaJ accelerates the rate of γ-phosphate cleavage of DnaK-bound ATP [126, 128], whereas GrpE promotes the release of ADP. Several model cycles for the DnaK/DnaJ/GrpE molecular chaperone machinery and their interaction with target polypeptides have been proposed. In two of those models [129, 130], dnaJ interacts with substrate polypeptide first and then through the DnaJ-induced hydrolysis of DnaK·ATP to DnaK·ADP·Pi, a ternary DnaJ·substrate·DnaK·ADP·Pi complex is formed. Thiscomplex is responsible for the chaperone effect by sequestering the substrate protein and thereby preventing it from aggregation [129]. Another model of the system states that [126], the role of DnaJ is to convert the low-affinity DnaK·ATP form to the high-affinity DnaK·ADP·Pi form, thereby locking the chaperone onto the

target polypeptide. In this model, DnaJ may act in a catalytic manner and fulfill its action without forming a stable ternary complex with peptide·DnaK·ATP. This way, dnaJ has a catalytic effect on the binding of the ς32 heat shock transcription factor to DnaK; DnaJ promotes the binding without becoming itself part of the DnaK·ς32 complex [128]

5.5.1.2 groES/groEL system

The groES and groEL genes of *E. coli* constitute the groE operon. The products of these genes are required for bacterial cell growth at high temperature (42°C). These genes are members of the heat shock regulon. The groES and groEL genes code for 10,368- and 57,259-Mr acidic polypeptides, respectively, found at high intracellular levels (about 2% of total cell proteins at 37°C) [131-133]. Furthermore, as members of the heat shock regulon, the intracellular levels of their products increase with temperature through a positive transcriptional control exerted by the *rpoH* (U32) gene product [134].

5.5.1.3 htpG system

Hsp90 of *E. coli*, encoded by the htpG gene, is an abundant protein that is further induced during heat stress. Although not essential for viability, deletion of htpG results in slower growth at higher temperatures [135], and a slight increase in protein aggregation in heat-stressed cells. Thomas and Baneyx [136] showed that at 42 ◦C, the absence of either *clpB* or *htpG* led to increased aggregation of preS2--galactosidase, a fusion protein whose folding depends on DnaK–DnaJ–GrpE, but not GroEL–GroES. Recently, *Genest et al.* [137] demonstrated that htpG promotes reactivation of heat-inactivated luciferase in a reaction that requires the prokaryotic Hsp70 chaperone (DnaK) system. It was suggested by them that the DnaK–DnaJ–GrpE system is the first to act on the client protein, and then htpG and DnaK act in a synergistic way to complete

the protein remodeling. The in vitro and in vivo interaction of htpG and DnaK of *E. coli* is additional evidence that htpG functions together with the Hsp70 system.

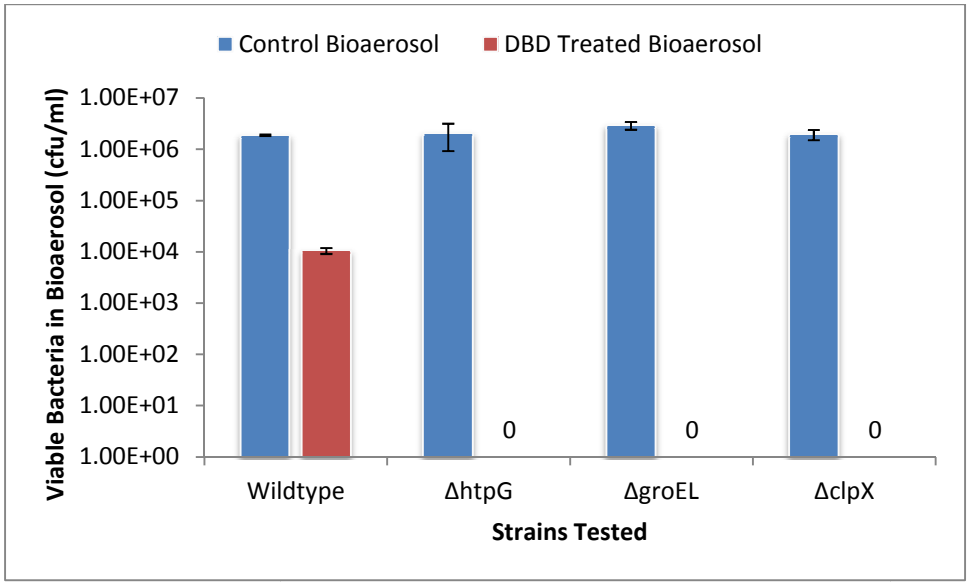


Figure 41: Deletion Mutants of Genes Involved in Heat Stress

The results of inactivation of heat stress mutants are shown in Figure 41. All three of the strains tested showed complete inactivation after exposure to plasma. The same dose that produced only a 2 log reduction in wildtype produced complete inactivation in the mutants. This is a clear indication of the involvement of heat stress in the

5.5.2 Mediation of Heat Stress

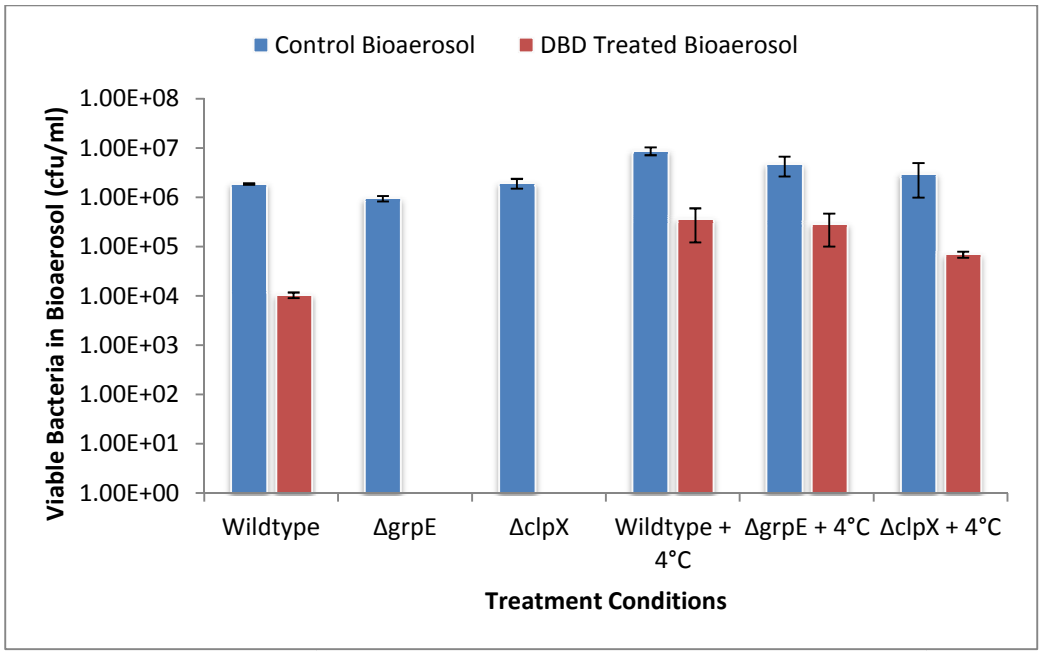


Figure 42: Mediating Heat Stress through Low Temperature Experimentation

The results are shown in Figure 42. The ΔgrpE and the ΔclpX mutants showed significant survival when treated at 4°C than at room temperature. The wildtype also was protected for 1 log. However, there was not completer protection for any of the strains tested. This would lead us to believe that heat stress is not the only stress imparted on the cell by plasma discharge, and there is involvement of other pathways as well.

5.6 Discussion

Gene deletion experiments provide an indication as to which markers in the pathways are involved in response to external stimuli. The results indicate that plasma produces oxidative stress on the bacteria. *katE* gene was found to to be significantly involved in oxidative management. The ΔkatE mutant was inactivated significantly compared to wildtype. The double

knockout of *kat* genes and the *katG* knockout showed no significant difference from wildtype. For the superoxide dismutase mutants, no significant change was observed, as compared to wildtype. The double knockout of the *sod* genes was protected better than the wildtype. This maybe be partly due to the ability of O_2^- to permeate the bacterial membrane poorly as compared to H_2O_2 [138]. The superoxide anion might also be getting converted to the more stable H_2O_2 within the water in the bioaerosol droplet. On the regulon level, the *oxyR* over-expressor was stronger than all the other strains, including wildtype. The involvement of *oxyR* in the defense against oxidative stress has been studied before [119]. The mutant deficient in alkyl hydroperoxide reductase (*ahpC*) also showed greater sensitivity. *ahpC* has been shown to be the major contributor to the defense against ROS [109, 139]. Both *ahpC* and *katE* are controlled by *oxyR* and *katE* is also induced by *RpoS* activation [123]. Therefore the susceptibility shown by the *katE* and the *ahpC* mutants indicates the involvement of both *oxyR* and *rpoS* pathways in the defense against plasma. The interesting part is that *katG* mutant did not show any significant change in inactivation from wildtype. This might be due to the fact that both *oxyR* and *rpoS* pathways are functional, even if you take out *katG*. The loss of either *katE* or *ahpC* seems to have overwhelmed the defenses of the bacteria, causing greater cell death. The reduction in the number of bacteria detected in the bioaerosol with Flow Cytometry also points to physical damage imparted upon the bacterial cell by the discharge. Plasma discharge is known to destroy the membranes of cells. There is localized heating inside the zone of the plasma and bioaerosol that interacts with this zone is bound to be affected by it. The importance of *katE* to bacterial survival is an indication of great stress as *katE* has been shown to be involved in heat stress as well as osmotic and weak acid stress [116, 123]. This was investigated with heat shock susceptible mutants. Genes responsible for the production of various Hsp were investigated.

There was susceptibility observed in all of the heat stress mutants tested. Mediation of this heat stress by temperature control did protect the bacteria significantly but there was not a complete protection afforded by this method. This indicated potential involvement of heat stress in conjunction with the oxidative stress.

6. CONCLUDING REMARKS AND FUTURE DIRECTIONS

6.1 Summary

This thesis investigates the efficacy of DBD plasma for the inactivation of airborne bacteria. The investigation began with the development of a prototype system for air sterilization. HVAC systems account for the greatest amount of interaction we have with aerosols. A lab scale model of an HVAC system was developed for testing. The treatment of airflow necessitated the development of a specific geometry of discharge. A unique Dielectric Barrier Grating Discharge (DBGD) was developed. The efficacy of this discharge was investigated through the production and introduction of artificial bioaerosols of *E. coli* with a nebulizer. The results measuring survival of the bacteria indicated complete sterilization for a very short period of interaction between bioaerosol particle containing the bacteria and plasma. This unexpectedly rapid sterilization effect led us to analyze further the mechanisms underlying this rapid inactivation.

Plasma discharge produces a large number of stable and unstable reactive species in the medium that it is ignited. The most prevalent species produced by plasma is ozone. DBD in particular has been used to produce ozone in large quantities for industrial purposes. Therefore, we investigated the effect of the ozone produced by our DBGD system. It was observed that the ozone produced by plasma was not the major contributor to the inactivation effect, and indicated that it is involved in the inactivation. Since most of the energy in the plasma discharge is concentrated in the zone of the plasma, the direct interaction of the plasma with the bacteria in the aerosol droplet is important to the sterilization effect. This was demonstrated in experiments conducted to analyze the influence of plasma geometry on the sterilization of airflow.

These results led to further investigation into direct interaction of plasma microdischarge with bioaerosol droplet. We further hypothesized that lived reactive oxygen species are another major contributors to the sterilization effect. Experiments were designed to test this hypothesis with unique single filament DBD discharge developed for this purpose. The efficacy of this discharge in inactivation of bacteria was determined. To understand the pathway involvement, a sub lethal dose of plasma was determined. Systems that are involved in the defense against oxidative stress were analyzed using mutants. Out of the mutants tested against plasma, the catalase mutants were found to be significantly more susceptible. The genes *katE* and *ahpC* were the most susceptible. ROS scavengers were employed to mediate the ROS produce by plasma.

The involvement of *katE* in multiple bacterial stress defense mechanisms led us to investigate another closely related system i.e. the heat stress system. The work was performed with heat stress deficient mutants. The results indicate involvement of localized heat stress produce by the DBD single filament affecting the bioaerosol. The observations lead us to believe that the hydrogen peroxide stress, combined with heat stress and membrane changes cause the bacterial inactivation.

6.2 Future Work

The effectiveness of plasma technology in inactivating bioaerosols rapidly has opened a whole new area of plasma sterilization. There is scope to further investigate the sterilization effect through transcriptional analysis. The genes that are indicated to be involved in the defense can be studied for their expression.

The development of larger prototypes was successfully performed in this study. There is scalability to the technology and it can be applied to various modalities where bioaerosols are a problem, such as hospital ventilators and other respiratory apparatus.

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