Elucidation of Levels of Bacterial Viability Post-Non-Equilibrium Dielectric Barrier Discharge Plasma Treatment

A Thesis

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of

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# Table of Contents

Acknowledgements ......................................................................................................................... i

LIST OF TABLES ............................................................................................................................... v

LIST OF FIGURES ............................................................................................................................. vi

Abstract ............................................................................................................................................... xi

CHAPTER 1: BACKGROUND AND LITERATURE SURVEY ................................................................ 1

1.1 Planetary Protection Requirements .......................................................................................... 1

1.2 Criterion Which Define a Plasma: an Introduction ................................................................. 4

1.3 Physics of Plasma Formation ................................................................................................... 7

1.4 Dielectric Barrier Discharge (DBD) ......................................................................................... 10

1.5 Plasma Applications in Industry and Medicine ....................................................................... 15

1.5.1 Plasma in industry ................................................................................................................ 15

1.5.2 Plasma in medicine .............................................................................................................. 15

1.6 Bacteria Selected for the Evaluation of the Antimicrobial Effect of Dielectric Barrier Discharge Plasma on Spacecraft Materials ................................................................. 21

CHAPTER 2: CHARACTERIZATION OF DIELECTRIC BARRIER DISCHARGE PLASMA ............ 29

2.1 Experimental Setup for DBD Plasma Characterization ............................................................ 30

2.2 Sinusoidal, Quasi-sinusoidal, and Micro-pulsed Voltage Waveform Characteristics .......... 32

2.3 Characterization Results for DBD Plasma in Select Gasses .................................................... 34

2.3.1 Argon ..................................................................................................................................... 34

2.3.2 Helium .................................................................................................................................. 35

2.3.3 Oxygen .................................................................................................................................. 36

2.3.4 Nitrogen ................................................................................................................................ 38
CHAPTER 3: PLASMA STERILIZATION EFFICACY .......................................................... 45

3.1 Dielectric Barrier Discharge Operation Parameters Selected for Antimicrobial Experiments ................................................................................................................. 45

3.2 Sterilization Efficiency of Wet versus Dry Samples ............................................................................................................................... 47

3.3 Direct and Indirect Effects of Plasma Exposure on E. coli .............................................................................................................................. 50

3.4 Quantitation of 8-hydroxydeoxyguanosine (8-OHdG) to measure oxidative damage to DNA resulting from plasma treatment ...................................................................... 54

3.5 Evaluation of Sterilization Efficiency Dependence on the Conductivity of Substrate Surface ...................................................................................................................... 63

3.6 Modeling of Bacterial Inactivation by Plasma ................................................................................................................................. 65

CHAPTER 4: VIABLE BUT NON-CULTURABLE (VBNC) AND DORMANCY STATES IN POST-PLASMA-TREATED BACTERIA ...................................................................... 69

4.1 The Classical Definition of “Live” Bacteria Revisited and Revised .............................................................................................................. 69

4.2 The Dormancy State in Bacteria ................................................................................................................................................ 71

4.3 Viable but Non-Culturable (VBNC) Viability State ............................................................................................................................... 72

4.4 Correlation Methodology to Enumerate Viability State ............................................................................................................................... 72

4.4.1 Assay and methods used to assess the bacterial viability state ......................................................................................................................... 73

4.5 Application of the Correlation Methodology to Plasma Treated Bacteria ................................................................................................. 76

4.6 Mechanisms of inducing Viable But Non-Culturable state in Bacteria by Dielectric Barrier Discharge Plasma ......................................................................................... 82

CHAPTER 5: COMPLETE DESTRUCTION OF BACTERIA THROUGH ION ETCHING BY DIELECTRIC BARRIER DISCHARGE PLASMA .................................................... 84

5.1 Scanning Electron Microscopy and Atomic Force Microscopy analysis of morphological changes in bacteria ......................................................................................... 86

5.2 DNA Amplification Protocol ................................................................................................................................................ 93

5.3 NanoDrop Spectrophotometer Instrument and Protocol .............................................................................................................................. 94

5.4 Plasma treatment of dried plasmids to quantify level of destruction ........................................................................................................................ 95
5.5 Plasma treatment of Chromosomal DNA to quantify level of destruction........... 99

5.6 Plasma treatment of B. stratosphericus and B. subtilis to enumerate efficacy and degree of sterilization ............................................................................................................................................. 100

5.7 Plasma treatment of SAFR-032 to enumerate efficacy and degree of sterilization ............................................................................................................................................................................................................. 102

5.7.1 Plasma treatment of SAFR-032 spores protected in Martian soil............. 105

CHAPTER 6: CONCLUDING REMARKS .................................................................................................................. 108

REFERENCES .................................................................................................................................................. 110

APPENDIX A - Capillary DBD Plasma Exposure .............................................................................................. 123

INDEX ......................................................................................................................................................... 126

VITA .............................................................................................................................................................. 128
LIST OF TABLES

Table 1. Proposed Planet/Mission Categories and Range of Requirements [2]. .............. 2

Table 2. Sterilization methods used currently by NASA.[13]................................. 3

Table 3. Typical Parameters of a Microdischarge....................................................... 12

Table 4. Discharge Parameters Used for Viability Experiments...................................... 46

Table 5. Viability measurements of wet D. radiodurans after DBD plasma treatment. .. 49

Table 6. Preparation of 8-OHdG Standard dilutents.................................................... 58

Table 7. Empirical Reaction Rate Constants for modeling of plasma interaction with bacteria [77]............................................................................................................ 66

Table 8. Concentration of Biologically Active Plasma Species [77]. ............................. 66

Table 9. B. stratosphericus respiration and Standard Error Measurement (SEM) post-plasma treatment using XTT technique. ................................................................. 79

Table 10. Environmental and Local Parameters associated with E. coli entering a VBNC State [103]. ................................................................................................................ 83

Table 11. Summary of plasma dose required to induce a particular viability state....... 109
LIST OF FIGURES

Figure 1. Voltage-Current characteristics of plasma discharges [16]. .................................. 7

Figure 2. Inelastic and Elastic Collisions of charged particles in plasma. ......................... 8

Figure 3. Initial Electron Avalanche in Plasma ........................................................................... 9

Figure 4. Filamentary Nature of DBD [17]. ............................................................................. 10

Figure 5. Timeline of microdischarge initiation stage [19]. ..................................................... 11

Figure 6. Simplified electrical schematic of a) electrode itself, b) electrode near the treated object, and c) plasma discharge on the treated object [22]. ......................... 14

Figure 7. Citrated whole blood (control) showing (a) single activated platelet (white arrow) on a red blood cell (black arrow) (b) non-activated platelets (black arrows) and intact red blood cells (white arrows) (c) plasma treated citrated whole blood showing extensive pseudopodia formation (white arrows) and platelet aggregation (d) Citrated whole blood (treated) showing platelet aggregation and fibrin formation (upper white arrow) [39] .................................................................................. 18

Figure 8. Inactivation of CL promastigotes by DBD plasma. [40] ........................................ 19

Figure 9. D. radiodurans wall structure [49] ........................................................................... 22

Figure 10. Resistance of B. pumilus SAFR-032 spores to UV radiation and H2O2. a) Survivability of spores exposed to varying doses of UV254 (100 μW sec⁻¹cm⁻²). Key: B. pumilus SAFR-032, circles; B. subtilis 168, squares; B. licheniformis ME-13-1, triangles. b) Survivability of spores exposed to 5% H2O2 liquid for one hour. [51]. .. 25

Figure 11. Molecular model of the inner and outer membranes of E. coli K-12. Colored ovals and rectangles represent sugar residues, whereas circles represent polar headgroups of lipids: Red, ethanolamine-phosphate; purple, ethanolamine pyrophosphate; yellow, glycerol-phosphate; blue ovals, glucosamine units; gray ovals, N-acetylmuramic acid units. Abbreviation key: Kdo, 3-deoxy-D-manno-octulosonic acid; LPS, lipopolysaccharide [49] ........................................................................................................... 28

Figure 12. Experimental Setup for Plasma Characterization [1] ............................................. 31

Figure 13. Experimental Setup detailing the two gas output ports and the ten gas injection ports [1]. .................................................................................................................. 32
Figure 14. Voltage Waveform characteristics: a) pulsed; b) continuous; and c) sinusoidal [1]........................................................................................................................................................................33

Figure 15. Motion of the filaments with gas flow observed in Argon plasma. .......... 34

Figure 16. Uniform discharge is observed in helium for both pulsed (left) and sinusoidal (right) voltage waveforms in Helium despite surface nonuniformities. Both pictures are taken at ¼ second exposure time. ......................................................................................................................... 36

Figure 17. DBD plasma with sinusoidal waveform in oxygen appears to be uniform at longer exposure times (30 seconds, top). Its filamentary structure is revealed at lower exposure times (0.25 sec, bottom). Both pictures were taken at an oxygen gas flow rate of 1 slpm.................................................................................................................................................. 37

Figure 18. A uniform discharge in nitrogen is observed using a sinusoidal waveform at 1 slpm flow rate, 30 seconds exposure time and f/32 aperture................................. 38

Figure 19. Four regions of a shock in a neutral gas [63].............................................. 39

Figure 20. Typical temperature relaxation processes in a shock [63]......................... 40

Figure 21. Double Layer resulting from diffusion of electrons and ions at shock front [63]......................................................................................................................................................... 41

Figure 22. Nitrogen filaments generated using sinusoidal waveform has no preferential motion direction. Flow Rate: 3 slpm; Exposure: 1/4 sec; and Aperture: f/4.5. ....... 42

Figure 23. Propagation of excitation observed in Nitrogen at low exposures is exclusive to the sinusoidal waveform. Flow Rate: 1slpm; Exposure: 1 ms; and Aperture: f/2.8. ............................................................ ................................................................. 43

Figure 24. Experimental setup for direct treatment of bacterial samples by DBD. ....... 46

Figure 25. Viability measurements of dry D. radiodurans after DBD plasma treatment. 48

Figure 26. Viability measurements of dry D. radiodurans after DBD plasma treatment. 49

Figure 27. Direct versus indirect treatment plasma treatment experimental setup ...... 51

Figure 28. Direct vs. Indirect sterilization of E. coli suspended in water...................... 52

Figure 29. Protective effects of Mn(II) on bacteria exposed to DBD plasma. .............. 54

Figure 30. The formation of 8-OHdG by oxygen radicals [72]........................................ 55
Figure 31. 8-OHdG ELISA Standard Curve which correlates the concentration of 8-OHdG in ng/mL to the optical density measured at 450 nm wavelength. ........................................ 61

Figure 32. 8-OHdG levels increase with plasma treatment dose until a threshold is reached, beyond which DNA is not able to recover. ................................................................. 62

Figure 33. Inactivation efficiency of E. coli does not change significantly when substrate is varied although the kinetics is distinctly different........................................... 65

Figure 34. Modeling of survivability as a function of DBD plasma species is compared with previous experimental modeling results (top) [79] to show that we are able to achieve sterilization on the order of seconds (bottom) [77] which is comparable to the residence time of bacteria in plasma................................................. 68

Figure 35. Relation between transformation frequency for a single marker and DNA concentration. Recipient particles of genotype ab*c* (x) or a*bc* (o) were transformed by denatured DNA of genotype a*b*c*. [82] ................................................................. 70

Figure 36. Percent transformants as a function of DNA concentration. DNA (0.1 ml of each concentration) was added to 5-ml cultures. (donor, Sti; recipient, Stre) [83]. 70

Figure 37. Correlation methodology................................................................. 73

Figure 38. Viable and Culturable B. stratosphericus post-plasma wet treatment. ....... 77

Figure 39. Viable B. stratosphericus using LIVE/DEAD fluorescence technique. ....... 78

Figure 40. B. stratosphericus respiration post-plasma treatment using XTT technique. 79

Figure 41. Respiration from few initial survivors (top) increase respiration after 24 hours (bottom) yet remain non-culturable ................................................................. 80

Figure 42. 120 sec of plasma treatment of wet B. stratosphericus shows elongation (white arrow), a morphological state associated with VBNC bacteria. .................. 81

Figure 43. Long-term exposure of PTFE to DBD plasma (90 min) results in topographical changes to the polymer surface on both the large scale (top) and small scale (bottom) ......................................................................................... 85

Figure 44. Flowchart of the SEM visualization procedure of plasma treated bacteria: bacteria are deposited on an aluminum SEM stub and allowed to air dry; the sample is then imaged by the SEM in high vacuum mode; next, it is treated by plasma for the prescribed period of time; lastly, the sample is imaged to determine the level of damage ............................................................................................................ 87
Figure 45. AFM images showing morphological changes before (left column) and after (right column) 10 minutes of DBD treatment. ................................................................. 88

Figure 46. SEM images of Deinococcus radiodurans on blue steel before (a) and after (b) 30 minutes of DBD plasma treatment................................................................. 89

Figure 47. SEM images of D. radiodurans on surgical-grade stainless steel before (a) and after (b) 20 minutes of DBD plasma treatment......................................................... 91

Figure 48. Control experiments on stainless steel reveal only a miniscule amount of drying of the extracellular polysaccharide compounds due to SEM imaging (top) and re-imaging (bottom) in high-vacuum mode......................................................... 92

Figure 49. The NanoDrop ND-1000 micro-volume sample retention system. (A) A sample volume of 1 μl is dispensed onto the lower optical surface. (B) Once the instrument lever arm is lowered, the upper optical surface engages with the sample, forming a liquid column. The sample is assessed at both a 1-mm and 0.2-mm path. [124]..... 95

Figure 50. Degradation of plasmids with increased DBD plasma treatment using gel electrophoresis. Here M is the 100 bp DNA ladder, and 0 sec to 60 sec is the plasma treatment times.................................................................................. 96

Figure 51. Spectrophotometer measurements of plasmids after increased plasma treatment time. .................................................................................................................. 97

Figure 52. Spectrophotometer signal of plasmid concentration nearly zero after only 5 sec plasma treatment...................................................................................... 98

Figure 53. Complete destruction of chromosomal DNA by DBD plasma after 2 sec plasma treatment............................................................................................... 99

Figure 54. Lane spectra of treated chromosomal DNA by plasma shows removal with 2 sec DBD plasma treatment................................................................. 100

Figure 55. Results of the reduction of Nucleic Acid by DBD plasma treatment of dried B. stratosphericus (lanes 3 and 4) and B. subtilis (boxed lanes 5 and 6) exposed to DBD for 0 sec and 60 sec. Here M is the DNA ladder, + is the positive control, and 0 s and 60 s are the plasma treatment times................................................................. 101

Figure 56. SEM images of 120 sec of plasma treatment of dry B. stratosphericus shows etching of bacteria. Etching of the bacterial membrane is clearly visible on the image. Scale bar represents 2 μm......................................................... 102

Figure 57. Degradation of nucleic acid of dry SAFR-032 spores with increased plasma treatment................................................................. 103
Figure 58. Lane spectra of Plasma treated dry SAFR-032 spores showing degradation of nucleic acid. ............................................................. 104

Figure 59. Spectrophotometer measurements verify DNA destruction with plasma treatment................................................................. 105

Figure 60. Plasma sterilization efficiency does not change with the addition of palagonite................................................................. 106

Figure 61. Spectral intensity of DNA signal decreases with increased plasma treatment similarly in palagonite to spores suspended in water.................................................. 107

Figure 62. Capillary DBD Schematic................................................................. 123

Figure 63. Capillary DBD exposure of a "wet" sample of D. radiodurans......................... 124

Figure 64. Sterilization efficiencies of D. radiodurans by capillary DBD in wet and dry environments show two slopes over the evolution from wet to dry. Helium-only exposures show no significant drop in CFU by pure exposure to helium............ 125
Abstract

Elucidation of Levels of Bacterial Viability Post-Non-Equilibrium Dielectric Barrier Discharge Plasma Treatment
Moogega Cooper
Alexander Fridman, Ph.D.

As a solution to chemically and thermally destructive sterilization methods currently used for spacecraft, non-equilibrium atmospheric pressure Dielectric Barrier Discharge (DBD) plasma is proposed to treat surfaces inoculated with everyday and extremophile bacteria. The purpose of this study is to show that non-thermal plasma has the ability to completely destroy bacteria to the DNA level on the surface of spacecraft materials without thermal degradation of the material. This is achieved by a threefold approach: physical, biological, and chemical. The physical approach involves characterizing plasma discharges in varying regimes to understand the properties of the discharge. The biological approach entails gathering evidence of reduction in bacterial load due to dielectric barrier discharge plasma treatment and understanding the sequence of events leading to a microorganism’s death when exposed to plasma. Polymerase Chain Reaction, Gel Electrophoresis, florescent assays and colony counts are among the techniques needed for this facet. The chemical approach adds understanding of sterilization mechanisms via the analysis of chemical reactions caused by UV photons, ions, and other components of plasma. This facet requires, in addition to biological assays, the use of a scanning electron microscope (SEM) to determine the morphological changes of the bacteria with increased plasma dose. This threefold
approach has shown that plasma succeeds in achieving complete disintegration of bacteria and alluded to the possible mechanisms. This will ultimately aide in preventing both forward contamination of planets and moons and reverse contamination of Earth for future NASA space missions.
CHAPTER 1: BACKGROUND AND LITERATURE SURVEY

“To improve life here,
To extend life to there,
To find life beyond.”
-NASA Vision

1.1 Planetary Protection Requirements

There are several scout and sample return missions which are scheduled to be launched through the National Aeronautics and Space Administration (NASA) within the next 5 years. It is our responsibility to preserve and protect the environment on Earth and the environments we are explore; thus, a set of requirements is set in place. The protection of solar system bodies (i.e., planets, moons, comets, and asteroids) from contamination by life on Earth and protection of Earth from possible life forms from other solar system bodies is termed "planetary protection." [2] Internationally, technical aspects of planetary protection are developed through the Committee on Space Research (COSPAR), part of the International Council of Science (ICSU), which consults with the United Nations in this area. The COSPAR Panel on Planetary Protection develops and makes recommendations on planetary protection policy to COSPAR, which may adopt them as part of the official COSPAR Planetary Protection Policy. Under this policy, various category landers were created (Table 1)[2, 3]. Category IV B Landers are the focus of concern for this body of work. Equipped with life-detection experiments, Category IV B landers and probes require the bioburden to be less than 30 bacterial
spores per spacecraft to reduce the probability of contamination to the area being explored [3].

### Table 1. Proposed Planet/Mission Categories and Range of Requirements [2].

<table>
<thead>
<tr>
<th>Category</th>
<th>Type of Mission</th>
<th>Target Planet</th>
<th>Degree of Concern</th>
<th>Range of requirements</th>
<th>Outbound</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Any but Earth Return</td>
<td>Sun, Mercury, Pluto</td>
<td>None</td>
<td>None</td>
<td>Per category of target planet/outbound mission</td>
</tr>
<tr>
<td>II</td>
<td>Any but Earth Return</td>
<td>Any except Mars, Sun, Mercury, Pluto</td>
<td>Flyby: limit on impact probability and contamination control measures</td>
<td>Documentation only (all brief)</td>
<td>Inbound</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Documentation (more involved than Category II)</td>
<td>If not safe for Earth return:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Contamination control</td>
<td>-All of Category IV</td>
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<td></td>
<td></td>
<td></td>
<td>-Organics inventory (as necessary)</td>
<td>-Continual monitoring of project activities</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>-Implementing Procedures such as:</td>
<td>-Preproject advanced studies/research</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>-Trajectory biasing</td>
<td>-If not safe for Earth return:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Orbital design or periapsis raising</td>
<td>-None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-Cleanroom</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>-Bioload reduction (as necessary)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>No direct contact (flyby, orbiter)</td>
<td>Mars</td>
<td>Mars</td>
<td>Detailed documentation (substantially more involved than Category III)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Direct contact (lander, probe)</td>
<td>Mars</td>
<td>Without life detection (Iva):</td>
<td>-Microbial reduction plan</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Limit on probability of non-nominal impact</td>
<td>-Microbial assay plan</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Limit on biodload (active control)</td>
<td>-Organics inventory</td>
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<td></td>
<td></td>
<td>With life detection (Ivb):</td>
<td>-Sterilization plan (with life detection)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Limit on probability of non-nominal impact</td>
<td>-Implementing procedures such as:</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Stringent limit on biodload (active control, reduction)</td>
<td>-Cleanroom</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>Earth return</td>
<td>To be determined</td>
<td>If not safe for Earth return:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• No impact of Earth or Moon</td>
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<td></td>
<td></td>
<td></td>
<td>• Sterilization of returned hardware; and</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Containment of any sample</td>
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</tr>
</tbody>
</table>

-PP plan
-Prelaunch report
-Postlaunch report
-Postencounter report
-End of Mission Report
It is pivotal to the success of life-detection missions that all lifeforms independent of their culturability state are detected before, during, and after assembly and launch processing. The inability to detect non-culturable bacteria will make any successful life-detection mission questionable as to the origin of the microorganism. The methods currently used by NASA to sterilize spacecraft components are listed in Table 2. Each method has several disadvantages. For example, sterilization by wet heat at 120-134 °C for 3 to 20 minutes is harmful to electronics and other sensitive components due to corrosion and water absorption. Similarly, chemically sensitive surfaces cannot be exposed to the hydrogen peroxide and low-pressure plasma treatment option. Furthermore, it is a costly process due to the time and resources needed to create a low-pressure environment. Many scientists outside of NASA use heat [4-7], ethylene oxide [8-10], or low-pressure plasmas [11, 12] for sterilization despite these disadvantages.

Table 2. Sterilization methods used currently by NASA.[13]

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Technique—Problems</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry heat</td>
<td>105-180 °C for 1 to 300 hours - can lead to the failure of electronic components</td>
</tr>
<tr>
<td>Wet heat</td>
<td>120-134 °C for 3 to 20 minutes- corrosion and water absorption</td>
</tr>
<tr>
<td>Alcohol wipes</td>
<td>Swabbing- Interior and encased surfaces (e.g., electronic components) are inaccessible</td>
</tr>
<tr>
<td>Ethylene dioxide</td>
<td>Toxic gas, 40 to 70 °C- The gas can only reach exposed surfaces and it is absorbed by some types of polymers (e.g., rubbers and polyvinyl chloride).</td>
</tr>
<tr>
<td>Gamma radiation</td>
<td>Typically 2.5 Mrad- optical changes in glasses and damage to electronics and solar cells</td>
</tr>
<tr>
<td>Beta radiation</td>
<td>1 to 10 MeV - Limited penetration</td>
</tr>
<tr>
<td>UV</td>
<td>5,000 to 20,000 J/m² - unexposed surfaces remain untreated</td>
</tr>
</tbody>
</table>
Methyl bromide

Toxic gas - Unexposed surfaces remain untreated and the gas catalyzes chemical reactions between metal and other components.

Hydrogen peroxide and Plasma

6 mg/l H2O2 concentrated at 58% - Unexposed surfaces remain untreated

Surface sterilization of spacecraft materials with complete disintegration of spores and bacteria without thermal/chemical degradation to the surface is needed. Atmospheric-pressure dielectric barrier discharge plasma has been proposed as a solution to the problems currently encountered and it is the goal of this thesis to provide justifications for this statement.

1.2 Criterion Which Define a Plasma: an Introduction

Plasma is all around us, comprising of 99.999% of observable matter: from stars to lighting and everything in between. The term plasma was first coined by Langmuir in 1927. The discovery is best described by Langmuir’s colleague, Harold M. Mott-Smith:

“So Langmuir began to study mercury vapor discharges. He shortly invented his probe, I did the experimental work and most of the mathematics, and we soon accumulated a lot of data about ion densities and velocity distributions [...]. We noticed the similarity of the discharge structures they revealed. Langmuir pointed out the importance and probable wide bearing of this fact. We struggled to find a name for it. For all members of the team realized that the credit for a discovery goes not to the man who makes it, but to the man who names it. Witness the name of our continent. We tossed around names [...] But one day Langmuir came in triumphantly and said he had it. He pointed out that the ‘equilibrium’
Part of the discharge acted as a sort of sub-stratum carrying particles of special kinds, like high-velocity electrons from thermionic filaments, molecules and ions of gas impurities. This reminds him of the way blood plasma carries around red and white corpuscles and germs. So he proposed to call our ‘uniform discharge’ a ‘plasma’. Of course we all agreed.

But then we were in for it. For a long time we were pestered by requests from medical journals for reprints of our articles. This happens to me this day.

-Yours faithfully, Harold M. Mott-Smith” [14]

Plasma must be carefully defined beyond “an ionized gas” as there is a small degree of ionization in any gas; in 1 cm$^3$ of air, there are about $10^3$ electrons and ions. Plasma is a quasi-neutral gas of charged and neutral particles which exhibits a collective behavior. This collective behavior is seen in plasma as charged particles move around and generate local concentrations of positive or negative charge which give rise to electric fields; the motion of charges generate currents and thus magnetic fields which affect the motions of other charged particles far away.

Jet exhaust is also a weakly ionized gas with collective behavior, but the charged particles collide so frequently with neutral atoms that their motion is controlled by hydrodynamic rather than electromagnetic forces. Thus there must be a second condition: the frequency of plasma oscillations, $\omega$, and the mean time between collisions, $\tau$, must be such that $\omega \tau > 1$ for the gas to behave like plasma rather than a neutral gas [15].
The Debye length, $\lambda_D$, is a measure of the sheath (the cloud of electrons and ions in plasma or the shielding distance) thickness.

$$\lambda_D = \left( \frac{\varepsilon_0 K T_e}{n_e e^2} \right)^{1/2}$$ (1)

where $\varepsilon_0$ is the permittivity of free space, $K$ is the Boltzmann constant, $T_e$ is the electron temperature, $n_e$ is the number density of electrons, and $e$ is the charge of an electron.

Quasi-neutrality is a condition within the definition of plasma. The Debye length, must be greater than the dimensions of the system, $L$, in order for the ionized gas to exhibit the collective behavior as dictated in the definition. If the Debye radius is large, then the electrons and ions move separately and their diffusion should be considered free. Furthermore, for a collective behavior to exist, the number of particles in a Debye sphere, $N_D$, should be much greater than one in order for there to be particles to exhibit the collective behavior. The Debye sphere can be calculated according to Equation 2.

$$N_D = n \frac{4}{3} \pi \lambda_D^3 = 1.38 \times 10^6 \frac{r^{3/2}}{n^{1/2}}$$ (2)

Here, $n$ is the plasma density since quasineutrality allows us to take $n_e \cong n_i \cong n$.

In summary, plasma can be defined as an ionized quasi-neutral gas which fulfills the following criterion:

1. $\lambda_D \ll L$
2. $N_D \gg 1$
3. $\omega \lambda > 1$

Further details regarding how plasmas are generated in the laboratory setting will be discussed in Section 1.3.
1.3 Physics of Plasma Formation

When applying an electric field to a gas, one observes a number of interesting phenomena as current through the gas is increased. This is illustrated in Figure 1 where voltage-current characteristics for a series of plasma discharges are shown [16].

![Voltage-Current Characteristic of the DC Low Pressure Electrical Discharge Tube](image)

Figure 1. Voltage-Current characteristics of plasma discharges [16].

A large series of plasmas can be generated in the laboratory as can be seen from Figure 1. The Townsend regime, for instance, is a regime where only electron avalanches occur. Electron avalanches begin in a region of strong electric field where a neutral atom or molecule is ionized directly by electron collision (Figure 2). Ionization may also occur through stepwise ionization by electron impact, ionization by collision of heavy particles, photo-ionization, and surface ionization (electron emission) [17]. The electric field then separates these particles, reducing their rate of recombination, and accelerating them.
As a result further electron/positive-ion pairs may be created by collision with neutral atoms (Figure 3). These particles then undergo the same separating process creating an electron avalanche. The avalanche travels in the direction of the nondisturbed electric field, $E_o$, and with a velocity equal to the drift velocity which is a function of the electron mobility, $\mu_e$. The mobility is found from the following relation:

$$\mu_e = \frac{\sigma_e}{en_e}$$

Here, $\sigma_e$ is the electron conductivity.

Figure 2. Inelastic and Elastic Collisions of charged particles in plasma.
As the electron avalanche evolves, the lighter electrons move faster than the heavier ions, thus the electron density is highest near the anode. Further evolution beyond an electron avalanche and discussion of the discharge on which this dissertation is focused, Dielectric Barrier Discharge, will be described in more detail in the next section.
1.4 Dielectric Barrier Discharge (DBD)

DBD was first developed in 1857 by Siemens for the purpose of ozone generation from air at atmospheric-pressure [18]. DBD is formed by applying an alternating high voltage across two electrodes where at least one of the electrodes is covered by a dielectric. This dielectric prevents the formation of an arc by accumulating charges on the dielectric surface, thus generating an electric field that opposes the applied field. This limits the current and produces a more controllable discharge.

![Filamentary Nature of DBD](image)

DBD filaments (Figure 4) are formed by a collection of microdischarges which repeatedly strike at the same place even as the polarity of the applied voltage changes. Their process of formation is illustrated in Figure 5. The nondisturbed electric field, $E_0$, in Figure 5 is in the direction of the electric field because it satisfies the Meek criterion of streamer formation in which the electric field becomes comparable with $E_0$ or $\alpha x=18$. 

Process of microdischarge formation [19]:

1. Initiation of an electron avalanche by a free electron produces a streamer.

2. The streamer bridges the gap on the order of a few nanoseconds and forms a conducting channel of weakly ionized plasma.

3. An intensive electron current will flow through this microdischarge until the local electric field is collapsed. The collapse of the local electric field is caused by the charges accumulated on the dielectric surface and ionic space charge (ions are too slow to leave the gap for the duration of this current peak).

Plasma ceases to exist after electron current termination although a high level of vibrational and electronic excitation in the channel volume, charges deposited on the surface, and ionic charges in the volume remain: the microdischarge remnant. This remnant has a lifetime on the order of 1 ms and facilitates in the formation of a new microdischarge in the same location. This phenomenon allows the naked eye to view single filaments. It also results in the ability to, for example, pattern polymer surfaces by repetitive and long-term microdischarge formation in the same location (see Chapter 5).
Only when microdischarges form at a new location will the discharge appear uniform.

Also called the “memory effect” a microdischarge remnant is not fully dissipated before the formation of the next microdischarge [20, 21]. The typical parameters of a microdischarge are listed in Table 3 [17].

<table>
<thead>
<tr>
<th>Table 3. Typical Parameters of a Microdischarge.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lifetime</td>
</tr>
<tr>
<td>Electron avalanche duration</td>
</tr>
<tr>
<td>Cathode-directed streamer duration</td>
</tr>
<tr>
<td>Plasma channel duration</td>
</tr>
<tr>
<td>Microdischarge remnant duration</td>
</tr>
<tr>
<td>Peak Current</td>
</tr>
<tr>
<td>Electron density</td>
</tr>
<tr>
<td>Total transported charge</td>
</tr>
<tr>
<td>Total dissipated Energy</td>
</tr>
</tbody>
</table>

The principal of operation of DBD plasma can be explained with an understanding of capacitance of a parallel plate system. Let the insulated electrode be modeled as a sphere of diameter $D_{el}$, while the object whose surface is being treated is modeled as a sphere of diameter $D_{ob}$. In the absence of the object the electrode capacitance with respect to the far away (located at infinity) ground is given by $C_{el} = 2\pi\varepsilon_o D_{el}$, where $\varepsilon_o$ is permittivity of free space. If the object being treated has a relatively high dielectric constant, i.e. water, it expels most of the electric field from its interior as it approaches the electrode. If the object behaves as a good conductor, its capacitance with respect to
the ground which is far away can also be modeled by \( C_{og} = 2\pi \varepsilon_o D_{ob} \). The region between the object and the electrode can be modeled roughly as a parallel plate capacitor with the value \( C_{gap} = \frac{\pi \varepsilon_o D_{el}^2}{2g} \), (where g is gap distance) if the gap is significantly smaller than the electrode diameter.

In the absence of any conduction current, the electrical models of the electrode by itself, and the electrode near the treated object are well approximated by the circuits in Figure 6a) and b). In Figure 6b), the capacitance across the gap is half of the total capacitance in the circuit. When the electrode is well removed from the ground, the magnitude of the applied voltage \( V \) is insufficient to create electric field strong enough to cause the breakdown and discharge. However, when the object with a high dielectric constant is sufficiently close to the electrode, most of the applied voltage appears across the gap. This is because the capacitance of the object with respect to ground is much larger than the gap capacitance, and the voltage divides across these capacitors proportionally to the inverse of their size. This results in a strong electric field in the gap which can now lead to breakdown and discharge.

The electrical circuit model can be further refined by taking into account non-linear resistance and capacitance of the plasma created in the gap. The resulting circuit refinement is shown in Figure 6c). The refined circuit does not change the main conclusion that most of the applied voltage appears across the plasma gap.
Figure 6. Simplified electrical schematic of a) electrode itself, b) electrode near the treated object, and c) plasma discharge on the treated object [22].

At about 10 kHz, the following circuit parameters typical for our experiments can be estimated assuming that substrate diameter is roughly 1 meter, the electrode diameter is about 25 mm, and the gap is about 1 mm.

\[ C_{\text{gap}} = \frac{\pi \varepsilon_0 D_{el}^2}{4g} \approx 4 \times 10^{-12} \text{F} = 4 \text{pF} \]

\[ \frac{1}{\omega C_{\text{gap}}} \approx 4.2 \text{ M}\Omega \]

\[ R_{\text{plasma}} = \frac{V^2}{\text{Power}} \approx 5 - 10 \text{ M}\Omega \]

\[ C_{og} = 2\pi \varepsilon_0 D_{body} \approx 50 \times 10^{-12} \text{F} = 50 \text{pF} \]

\[ \frac{1}{\omega C_{og}} \approx 0.3 \text{ M}\Omega \]

Electrical safety of the object being treated by plasma is ensured because current the power supply delivers is less than 5 mA. [22]
1.5 Plasma Applications in Industry and Medicine

1.5.1 Plasma in industry

Current applications of atmospheric pressure plasmas include ozone production [23, 24], treatment of gases [25], and industrial surface treatment [26-28]. An example application, plasma immersion ion implantation (PIII) for semiconductor materials and processing is an enormously growing field. PIII is a cluster compatible doping and processing tool offering many advantages over conventional beamline ion implantation [29]. Initially, the technique was used to enhance the surface mechanical properties of metals, and it has recently evolved to applications in areas such as the synthesis of silicon-on-insulator, formation of a shallow junction, large area implantation, trench doping, and conformal deposition [29, 30].

1.5.2 Plasma in medicine

DBD’s biocidal properties make atmospheric pressure DBD potentially a favorable system for medical applications [31-34]. With an evolution in electrical engineering technologies, voltage pulses can be generated at shorter rise-times and with less damage to the substrate being exposed [1, 34]. In addition to ozone, DBD plasma creates reactive oxygen and nitrogen species to include O, OH, NO, and others [35, 36]. A major reason for its use as an antimicrobial technique is the flux of direct charges to the exposed surface which is selective to inactivation of microbes [33, 37, 38].
Blood Coagulation

Regimes exist where non-equilibrium room-temperature plasma is able to rapidly promote the coagulation of blood without heating or desiccation of the blood cells. Evidence was gathered by Kalghatgi et al. [39] to show that plasma treatment does not coagulate blood due to change in pH, as no significant change in pH of blood was observed during the time of treatment. Coagulation proteins may be activated by plasma treatment and is further demonstrated by rapid fibrinogen aggregation of treated buffered solution of human fibrinogen. Non-thermal plasma treatment is demonstrated to be selective, as a similar buffered solution of human serum albumin shows no change even after a longer treatment. DBD treatment of normal whole blood, normal whole blood with sodium citrate anticoagulant and blood from a patient with Hemophilia A is clearly different [32]. Direct conversion of fibrinogen into fibrin may be one of the mechanisms by which non-thermal plasma initiates coagulation (Figure 7) [39]. Plasma treatment may be able to bypass the normal blood coagulation cascade and interfere directly with the later stages of the process, i.e. effect on fibrinogen cleaving to fibrin monomers and their polymerization to fibrin filament matrix, while another possible mechanism involves release of Ca$^{2+}$ ions from bound to free ionic state by plasma [32].
Figure 7. Citrated whole blood (control) showing (a) single activated platelet (white arrow) on a red blood cell (black arrow) (b) non-activated platelets (black arrows) and intact red blood cells (white arrows) (c) plasma treated citrated whole blood showing extensive pseudopodia formation (white arrows) and platelet aggregation (d) Citrated whole blood (treated) showing platelet aggregation and fibrin formation (upper white arrow) [39]
**Treatment of Cutaneous Leishmaniasis**

One example of plasma use in treatment of skin diseases is Cutaneous Leishmaniasis (CL, caused by the Leishmania parasite) which results from the bite of an infected sand fly when it injects the promastigote form of the disease into the host while feeding. There, the parasites are phagocytized by the host macrophages, change into amastigote form, and break the host cell continuing the infection. A series of in-vitro experiments comparing the effect of plasma on human macrophages and on the promastigote form of Leishmania parasite were conducted by [40]. Following a 6 J cm\(^{-2}\) dose of plasma, 20% of macrophages are inactivated while 100% promastigotes appear inactive as observed through a phase contrast microscope with trypan blue exclusion test for macrophages and simple observation for the protozoa (they stop moving the flagellum and begin to disintegrate which takes about 48 h; the organisms do not appear to re-activate following treatment) (Figure 8) [40].

![Figure 8. Inactivation of CL promastigotes by DBD plasma. [40]](image-url)
**Wound Healing and Tissue Regeneration**

The amount of successful and cost-efficient methods targeted to chronic wound healing is quite low. In the case of healing a small and very large venous ulcer, the estimated cost is about $1300 and $5300, respectively[41]. Many modalities explored pre-clinically may provide some solution, but may not be cost effective. For example, a $1600 2-cm$^2$ sheet of bio-engineered skin may not be a cost-effective solution to a venous ulcer if multiple applications are needed over time and greater than one sheet is used per treatment.

Wound healing is a complex and dynamic process of restoring cellular structure and tissue layers in damaged tissue as closely as possible to its normal state. It has 3 general phases: inflammatory, proliferative, and maturation, and is dependent upon the type and extent of damage, the general state of the host’s health, and the ability of the tissue to repair. The role of nitric oxide (NO) as a mediator in wound healing has been elucidated recently. Cold Pin-to-Hole spark Discharge (PHD) plasma was shown to produce nitric oxide (1200 ppm), which is known to have anti-inflammatory effect, and effects of vasodilatation and increasing blood flow on blood vessels [42]. Also, it was shown to have high sterilization effect without tissue damage. Thus it is expected that PHD treatment would stimulate wound healing during the inflammatory stage through effect of NO and through photostimulation. The spark discharge plasma may ultimately be utilized to treat and heal skin wounds including diabetic wounds through local effect of plasma produced NO and other active plasma components [43].
1.6 Bacteria Selected for the Evaluation of the Antimicrobial Effect of Dielectric Barrier Discharge Plasma on Spacecraft Materials

It is important in these studies to choose a form of bacteria which may survive on spacecraft surfaces during launch or realistically return as spacecraft payload. Requirements include a highly resistive nature to unnatural environments, the ability to be culturable with typical incubation and culturing procedures prior to the application of external stressors, and other qualities which may give it semblance to “extraterrestrial” bacteria. The bacteria chosen here fit these requirements and are Deinococcus radiodurans, Bacillus stratosphericus, Bacillus pumilus SAFR-032, and Escherichia coli.

The organisms will now be discussed in further detail below.

*Deinococcus radiodurans*

D. radiodurans is selected for experimentation based on its resistive nature to radiation [44, 45], temperature change [46], reactive oxygenated species [47], and vacuum [45]. It can withstand an instantaneous radiation dose of 5,000 Gray with no loss of viability (60 Gy sterilizes a culture of E. coli); withstand an instantaneous dose of up to 15,000 Gray with 37% viability [44]; and withstand exposure to space vacuum (~10⁻⁶ Pa) for three days with decreased cell survival by four orders of magnitude [45]. It is even hypothesized that its extreme nature is due to the fact that it originated on Mars and migrated to earth on a meteorite [48]. The wall structure of this microorganism assists in the elevated resistive nature of D. radiodurans and is outlined in Figure 9. There are seven distinct components which comprise the wall structure:
1. Cytoplasmic Membrane
2. Periplasmic Area
3. Perforated peptidoglycan (holes 10-11nm in diameter)
4. Intercalating Material
5. Outer Membrane (Backing Layer)
6. Hexagonally Packed Intermediate (HPI) -layer
7. Extracellular polymeric material/polysaccharide compounds outside the cell wall

Figure 9. D. radiodurans wall structure [49].

The resistive nature and robust cellular structure makes D. radiodurans a key choice for plasma exposure.

D. radiodurans was obtained from the American Type Culture Collection, ATCC 13939. For experimental studies, it is grown in 50 ml of tryptone/glucose/yeast (TGY) (0.8% Tryptone, 0.1% glucose, 0.4% Yeast Extract) media at slow shaking speed at 30°C for 2
days. At this time, the optical density is typically 0.6-1.0. The culture was then washed three times in sterile distilled water by centrifugation at 14000 rpm for 10 minutes, and lastly suspended in sterile distilled water for plasma exposure.

*Bacillus stratosphericus*

*Bacillus stratosphericus* (strain 41KF2aT, MTCC 7305T, JCM 13349T) samples were isolated from cryogenic tubes used to collect air samples at altitudes of 24, 28 and 41 km [50]. When grown on nutrient agar, their colonies are white, irregular, raised, and 3-5 mm in diameter. They grow at temperatures from 8 - 37°C, and at pH from 6 - 10. *B. stratosphericus* tolerates up to 17.4% NaCl and is resistant to UV as well as select antibiotics such as penicillin (10 μg), vancomycin (30 μg), erythromycin (15 μg), and colistin (10 μg). It is sensitive to select antibiotics to include streptomycin (25 μg), ampicillin (25 μg) and nalidixic acid (30 μg). It is positive for arginine decarboxylase activity and negative for arginine dihydrolase activity and utilizes a number of sugars, amino acids and other carbon compounds as sole carbon sources. The lipids present are PE, PG, DPG and two unknown phospholipids. The DNA G+C content is 44 mol% [50].

*B. stratosphericus* samples were generously donated to us by the Biotechnology and Planetary Protection Group at the NASA Jet Propulsion Laboratory. Prior to experimentation, samples were grown overnight in Luria Broth (LB) media to mid-logarithmic phase and harvested by centrifugation. Cell pellets were rinsed twice and resuspend in distilled water to a final concentration of approximately $10^7$ cells/mL.
**Bacillus pumilus SAFR-032**

Bacillus pumilus SAFR-032 isolates were recovered aboard the International Space Station from hardware surfaces and air particles, Mars Odyssey spacecraft, and spacecraft assembly facilities as metabolically dormant spores [51]. It is resistant to all UV bandwidths and the total spectrum of UV radiation and concentrations of H₂O₂ that is significantly higher than other Bacillus species [52]. Spores and vegetative cells of SAFR-032, a strain originally recovered from the Jet Propulsion Lab (Pasadena, CA) spacecraft assembly facility, are endowed with UV radiation H₂O₂ resistance capabilities that significantly exceed other Bacillus species and allow survival against standard sterilization practices. Whereas >90% lethality of B. subtilis and B. licheniformis spores is achieved by exposure to 200 Jm⁻² UV₂₅₄, 1500 Jm⁻² are required to kill 90% of B. pumilus SAFR-032 spores (Figure 10a). Twelve percent of B. pumilus SAFR-032 spores survive 5% liquid H₂O₂, which is nearly thrice the survival rate of B. subtilis spores (Figure 10b) [51].
Figure 10. Resistance of B. pumilus SAFR-032 spores to UV radiation and H2O2. a) Survivability of spores exposed to varying doses of UV254 (100 μW sec cm$^{-2}$). Key: B. pumilus SAFR-032, circles; B. subtilis 168, squares; B. licheniformis ME-13-1, triangles. b) Survivability of spores exposed to 5% H2O2 liquid for one hour. [51]

SAFR-032 spores and vegetative bacterial samples were generously donated by the Biotechnology and Planetary Protection Group at the NASA Jet Propulsion Laboratory.
**Bacillus subtilis**

*Bacillus subtilis* is a non-pathogenic soil bacterium. As a result of its relatively large size, *Bacillus subtilis* is one of the best understood prokaryotes. Research on *B. subtilis* has been at the forefront of bacterial molecular biology and cytology, and the organism is a model for differentiation, gene/protein regulation, and cell cycle events in bacteria. A unique trait in which this species of bacterium possess is that it can choose between at least three different genetic programs when nutrients or other resources become scarce, and/or cell density reaches a critical threshold. To survive or adapt to adverse conditions, cells can enter a stationary phase, which is characterized by formation of single motile cells (exponentially growing cells usually grow in chains and are non-motile), differentiate into enduring and metabolically inactive spores, or become competent and take up DNA from the environment for acquisition of new genetic material [53].

There was a long-held belief that the gram-positive soil bacterium *Bacillus subtilis* is a strict aerobe. But recent studies have shown that *B. subtilis* will grow anaerobically, either by using nitrate or nitrite as a terminal electron acceptor, or by fermentation. *B. subtilis* alters its metabolic activity according to the availability of oxygen and alternative electron acceptors. Nitrate is the preferred terminal electron acceptor when oxygen is absent because of its high midpoint redox potential \((E^{0} = +430 \text{ mV})\). Nitrate respiration and nitrite respiration are the only anaerobic forms of respiration known thus far in *B. subtilis* [54].
B. subtilis samples (ATCC 6051) were generously donated by the Biotechnology and Planetary Protection Group at the NASA Jet Propulsion Laboratory. Samples were prepared by growth in nutrient medium overnight at 37°C and harvested by centrifugation.

**Escherichia coli K-12**

*E. coli* is Gram-negative, anaerobic, and non-sporulating bacterium. Cells are typically rod-shaped and are about 2 μm long and 0.5 μm in diameter, with a cell volume of 0.6 - 0.7 μm³ [55]. The growth rate of *E. coli* is quite rapid, optimally at 37°C and with simple nutritional requirements. In the presented experiments, samples were grown overnight at 37°C in Luria Broth (LB) medium. These qualities of rapid and simple growth have allowed *E. coli* to become a model organism for studying many of life’s essential processes. Several strains of *E. coli* have been sequenced and studied in detail. *E. coli* K-12 was the earliest organism in which whole genome sequencing was performed [56]. It has a single circular chromosome with 4,639,221 base pairs and 4288 protein-coding genes. Of these protein-coding genes, 38% have no attributed function [56]. The molecular model of the inner and outer membranes of *E. coli* K-12 is given in Figure 11.
Figure 11. Molecular model of the inner and outer membranes of E. coli K-12. Colored ovals and rectangles represent sugar residues, whereas circles represent polar headgroups of lipids: Red, ethanolamine-phosphate; purple, ethanolamine pyrophosphate; yellow, glycerol-phosphate; blue ovals, glucosamine units; gray ovals, N-acetylmuramic acid units. Abbreviation key: Kdo, 3-deoxy-D-manno-octulosonic acid; LPS, lipopolysaccharide [49].
CHAPTER 2: CHARACTERIZATION OF DIELECTRIC BARRIER DISCHARGE

PLASMA

It was previously shown [33], and will be discussed further in Chapter 3 of this thesis, that direct contact of charged species from plasma with bacteria is needed for effective sterilization. Thus, uniformity of sterilization depends directly on the discharge spatial uniformity. The intensity and distribution of microdischarges in this plasma must then be characterized. Furthermore, the number of microdischarges depends upon the excitation waveform, power, and the type of gas [34]. A change in any of these three variables may result in a higher filament temperature; as a result, an effective yet non-destructive treatment requires plasma with little or no filamentation. This stemmed the need to observe and characterize plasma in which the excitation waveform, gas type, and flow rate were varied to obtain the most uniform and lowest-power plasma with the highest bactericidal abilities (bacterial studies are presented in the following chapters).

Observations of atmospheric pressure DBD plasma were conducted through the transparent water-filled electrode in Air, Argon, Helium, Nitrogen, and Oxygen gasses at 1 and 3 standard liters per minute (slpm) flow rates through the discharge gap, utilizing three types of excitation waveforms. The three plasma excitation waveforms used for characterization are: a sinusoidal waveform, quasi-sinusoidal continuous waveform, and pulsed excitation. The surface power density was kept at a constant value for each
particular gas, ranging from 0.4 to 2 Watts/cm$^2$ for different gases. Air, Argon, Helium, Nitrogen, and Oxygen were chosen for characterization. The three excitation waveforms chosen here are due to the power supply availability in our lab and their previous use in medical and biological studies (see [57], [33]).

Characterization results reveal three phenomena of DBD plasma: 1) plasma filaments travel with the same speed and direction as the gas flow for all gasses where the filamentary structure is obvious, except in Nitrogen; 2) propagation of excitation is observed in Nitrogen plasma and the filament motion has no directional preference with gas flow direction; and 3) Oxygen, Nitrogen and Helium plasmas were observed to be rather uniform at least over longer time periods.

2.1 Experimental Setup for DBD Plasma Characterization

The experimental setup (Figure 12) features a water-filled electrode 25.4 mm in diameter separated from a brushed stainless steel base by a 2 mm perforated aluminum spacer. The water electrode was comprised of a quartz viewing windows at the upper and lower surfaces. Regular tap water was degasified and used as a conducting medium. The Nikon D70 camera was used for digital photography. Photos of the discharge were taken at decreasing shutter speeds from 30 seconds to 1/6400 seconds to characterize plasma spatial uniformity at different time scales. The upper (transparent) electrode is water cooled by allowing the water contained within the electrode to circulate to an ice-cooled external container (Figure 12).
Due to the nature of the pump and the setup, water recirculation often resulted in bubble formation which is visible in pictures taken at small apertures (less light reaches the camera’s Charge Coupled Device (CCD) sensor) and long shutter speeds (the time during which light is collected on the CCD); therefore, approximately 2-3 mm shadows seen in some of the pictures are artifacts of bubbles just below the upper quartz window; these bubbles do not affect the plasma in any way as the lower plasma-contacting quartz is bubble-free.

The plasma is formed within a two millimeter gap distance between the brushed steel and the lower quartz face of the electrode. Gas is introduced into the setup through ten out of twelve equidistant tubes which are connected to the perforated spacer in the base of the chamber and exit through the remaining two (Figure 13).

![Figure 12. Experimental Setup for Plasma Characterization [1].](image-url)
2.2 Sinusoidal, Quasi-sinusoidal, and Micro-pulsed Voltage Waveform Characteristics

Sinusoidal, quasi-sinusoidal (“continuous”), and micro-pulsed waveforms were applied to the voltage. The pulse duration and characteristics of each waveform are given in Figure 14. The pulse durations shown in Figure 14 are taken to be the full width at half maximum (FWHM). The rise time is time interval for the major pulse to change from 20% to 80% of its maximum amplitude.

The typical pulsed waveform (Figure 14, a) has positive or negative polarity pulses at 0.1 kHz to 1 kHz repetition rate, up to 35 kV peak to peak, 1.7 μs pulse duration and 0.75 μs rise time. The continuous waveform (Figure 14, b) generates up to 35 kV peak to peak at 8.7 kHz with a continuous quasi-sinusoidal bipolar wave signal. The sinusoidal waveform (Figure 14, c) is a varying frequency and voltage system where sinusoidal waves were amplified and stepped up to high voltage. For our experiments, the frequency is fixed at 12 kHz.
Figure 14. Voltage Waveform characteristics: a) pulsed; b) continuous; and c) sinusoidal [1].
2.3 Characterization Results for DBD Plasma in Select Gasses

2.3.1 Argon

When observed at exposure times greater than 0.1 s, Argon plasma is composed of a diffuse background and filaments developing along the direction of flow (Figure 15). At closer observation with low exposure times, it can be seen that filaments travel in the direction of gas flow and at the velocity close to the estimated velocity of gas flow. This effect is observed only in argon and air plasma. This may be due to the memory effect [58] of a filament being bound to the gas and not the surface.

![Figure 15. Motion of the filaments with gas flow observed in Argon plasma. Flow rate: 3slpm; Exposure: 1/100 sec; and Aperture: f/2.8 [1].](image)
A filament travels approximately 3.4 mm in 1/100 sec resulting in a velocity of 3.4 m/s. The gas flow rate for this picture is 3 standard liters per minute (slpm) through the volume of a cylinder with height 2 mm and diameter 25.4 mm, meaning that the average residence time of the gas in this volume is about 0.02 s, and in that case the average linear gas velocity will be on the order of 1 m/s, which is comparable with the filament movement velocity. It means that the memory effect in argon or air DBD [37, 59] is related not only to charge deposition on the surface but also to the pre-ionization (or high concentration of excited molecules) that exists in the gas after DBD polarity change.

2.3.2 Helium

Previous experiments display the uniform nature of DBD plasma at atmospheric pressure in helium [60]. We also observe uniform plasma in helium at least with long exposure time (but for some cases at short exposure also (see results in Nitrogen) and with all three power supplies.

Helium plasma exhibits a uniform background, with some filaments in stationary positions (Figure 16). We believe that these filaments (and their apparent structure) are artifacts of particulate contamination on the bottom steel electrode.
2.3.3 Oxygen

Plasma uniformity was achieved (Figure 17, top), as reported in previous experiments using oxygen plasma [61, 62]. The DBD plasma in oxygen is observed to be diffuse and is visible at a minimum exposure time of 1/30 sec versus 1/4000 sec for Helium at ~2 Watts/cm². As the exposure time decreases, the appearance of diffuse plasma is revealed (Figure 17, bottom).
Figure 17. DBD plasma with sinusoidal waveform in oxygen appears to be uniform at longer exposure times (30 seconds, top). Its filamentary structure is revealed at lower exposure times (0.25 sec, bottom). Both pictures were taken at an oxygen gas flow rate of 1 slpm.
2.3.4 Nitrogen

Nitrogen DBD plasma uniformity is also observed at long exposures and for all waveforms (Figure 18). Interesting occurrences are in Nitrogen using sinusoidal excitation waveform. It is possible to see that if plasma is generated using the sinusoidal waveform, filaments in Nitrogen have no preferential direction of travel (Figure 22), contrary to Argon and Air, where streamer channels flow in the direction of gas and at the velocity comparable to the bulk gas velocity.

Figure 18. A uniform discharge in nitrogen is observed using a sinusoidal waveform at 1 slpm flow rate, 30 seconds exposure time and f/32 aperture.
Shocks in a weakly ionized non-equilibrium plasma

Shocks are formed under certain conditions as a result of satisfying the equations of continuity of mass, momentum, and energy. In a neutral gas, a shock can be divided into 4 regions (Figure 19):

1. Undistributed gas in front of the shock. It is in complete thermodynamic equillibrium
2. Shock front, Rapid changes in temperature, density, pressure , and velocity of flow.
3. The part of the shock in which equillibration of the various internal degrees of freedom takes place
4. Complete thermodynamic equillibrium is re-established.

This can be seen in terms of typical temperature relaxazation processes in Figure 20.
When a shock is present in an ionized gas, large density gradients arise in all plasma constituents at the shock front. As a result of these gradients, there is a diffusion of particles in the upstream direction. Since the mobility if the electrons is much higher than that of either the ions or neutral particles, they will diffuse faster. The resulting charge separation gives rise to an electrical double layer (Figure 21), which ultimately equilibrates the fluxes of ions and electrons. The difference in the charged particle densities leads to an electric field and potential variation at the shock front, both of which are absent form a shock in a neutral gas. Under nonequilibrium conditions, the charged particle shock front thickness is greater than the ion-neutral mean free path by a factor of $T_e/T_n$ which may be as high as 80 or more under typical glow discharge conditions [63].
In nitrogen, an interesting phenomenon related to shock waves is seen in weakly ionized non-equilibrium plasmas. At long exposures (Figure 22) filaments have no preferential direction of motion. The cause of this stochastic movement can be observed at short exposures with the sinusoidal voltage waveform at 1.2 W/cm². The observed effect in Figure 23 can be attributed to either the propagation of non-equilibrium vibrational excitation radially from the filament or to the increase of a reduced electric field, E/n. These probable phenomena results in a cylindrical front of weak breakdown approximately 4 mm from the center of the microdischarge (Figure 23). So, instead of the whole microdischarge current concentrated in one channel that is typical for standard DBD [17, 18], a complex radially symmetrical distribution of the microdischarge current appears, and characteristic radial size of this current is about 4 mm in the particular case presented in Figure 23. Therefore interaction between microdischarges that usually have characteristic distance on the level of 0.5-1 mm now have much larger characteristic distance, and microdischarges form two-dimensional quasi-crystal with
the lattice size of about 5-8 mm. It looks like this crystal structure is rather ‘rigid’ and gas flow cannot move ‘nodes’ of this lattice (in contrast with the argon case, Figure 15). On the other hand, stochastic processes in microdischarge formation [37, 59] case slow random drift of the ‘lattice nodes’ in Figure 23.

Figure 22. Nitrogen filaments generated using sinusoidal waveform has no preferential motion direction. Flow Rate: 3 slpm; Exposure: 1/4 sec; and Aperture: f/4.5.
Figure 23. Propagation of excitation observed in Nitrogen at low exposures is exclusive to the sinusoidal waveform. Flow Rate: 1slpm; Exposure: 1 ms; and Aperture: f/2.8.

The propagation phenomenon seen in Figure 23 was not observed in the cases of the pulsed and continuous power waveforms for all gasses. A weaker version of the propagation phenomenon may occur, however was not observed.

Three phenomena were observed in the discussed experiments: memory effects can be bound to the gas or to the surface; a cylindrical front of weak breakdown was observed
in Nitrogen plasma where filaments have no directional preference of motion; and there is great uniformity in both Oxygen and Helium plasma without the use of complex electrode structures or special power supplies. Characterization was performed to understand variation of plasma depending on voltage waveform and gas. Continuous waveform plasma in air was chosen for the biological experiments presented herein in light of its balance of uniformity, power, and low temperature.
CHAPTER 3: PLASMA STERILIZATION EFFICACY

As engineers, it is important to evaluate the possibility of one’s device to achieve the desired effect. In the brief history of studies regarding direct exposure to atmospheric pressure plasmas for antimicrobial effects, it was up to the developers (comprising of primarily physicists and engineers) to test their devices. For this reason, many efficacy studies in the plasma field [33, 64-67] are based solely upon the colony count method, a measure of culturability by observing colony growth on an agar plate. In this chapter, the efficacy of sterilization by DBD plasma will be discussed based solely upon the capability of bacteria to grow after prescribed doses of plasma. Furthermore, the fallacy in such a methodology will be discussed and a new methodology will be proposed and tested in Chapter 4.

3.1 Dielectric Barrier Discharge Operation Parameters Selected for Antimicrobial Experiments

The voltage waveform characteristics and discharge gas were evaluated in Chapter 2, among other parameters, and it was concluded that discharges in air using a continuous waveform was optimum for further experimental use; it is spatially uniform with exposure time on the order of seconds, the power needed for generation is relatively low which results in little to no heating of the substrate. Unlike plasma generated in nitrogen or helium, plasma generated in air does not require a tank source. This
enhances its ability to be adopted easily in future laboratory systems. Plasma parameters used in the discussed experiments are displayed in Table 4.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Voltage</td>
<td>30 kV</td>
</tr>
<tr>
<td>Frequency</td>
<td>10 - 15 kHz continuous wave</td>
</tr>
<tr>
<td>Power Density</td>
<td>1 W/cm²</td>
</tr>
<tr>
<td>Distance</td>
<td>1-2 mm</td>
</tr>
</tbody>
</table>

The bacterial samples are deposited on stainless steel coupons, unless otherwise stated. Variations in the substrate composition occur in studies whose goal is to understand the dependence of sterilization efficiency on surface material. Experiments utilizing the scanning electron microscope are conducted on an aluminum substrate. The configuration of the electrode 2 mm above the surface is shown in Figure 24.

Figure 24. Experimental setup for direct treatment of bacterial samples by DBD.
The plasma discharge forms between the quartz dielectric and the grounded base and encompasses the coupon.

3.2 Sterilization Efficiency of Wet versus Dry Samples

The environment in which DBD plasma may be applied can vary with respect to the level of moisture. It is important to study and compare the efficiency of sterilization in a dry environment to sterilization in the presence of water. It is shown in this section that water in fact has a positive influence on sterilization efficiency.

Samples of *D. radiodurans* were deposited on a stainless steel surface and allowed to dry in room air for 30 minutes. *D. radiodurans* is completely inactivated at 30 minutes of plasma exposure (Figure 25). At t=0, the initial concentration of bacteria is approximately $10^6$ CFU. After 30 minutes of DBD treatment, there is a six-log reduction in the number of culturable *D. radiodurans* cells. Temperature measurements using an infrared non-contact thermometer (Raytek ST60 xB ProPlus) show that during plasma treatment, the average temperature was 26°C, and the sample temperature was no higher than 30°C.
Experiments were also performed on *D. radiodurans* which were not desiccated, but treated immediately after deposition on the substrate and while encapsulated in water. Results show an initial concentration of approximately $10^4$ cfu/ml requires only 15 seconds of DBD treatment to result in complete inactivation (Figure 26 and Table 5). It is interesting to note that the samples treated for longer times of 30, 45 and 60 seconds did not produce colonies after 5 days of incubation.
Figure 26. Viability measurements of dry D. radiodurans after DBD plasma treatment.

Table 5. Viability measurements of wet D. radiodurans after DBD plasma treatment.

<table>
<thead>
<tr>
<th>Plasma Treatment Time (sec)</th>
<th>Viable D. radiodurans (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$2.6 \times 10^4$</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
</tr>
</tbody>
</table>

We hypothesize regarding sterilization mechanisms in water: the species formed by the interaction of plasma with water induces phospholipid peroxidation thus increasing sterilization efficiency. Previous studies show the influence of hydrogen peroxide and superoxides produced in water in synergy with UV to increase sterilization efficiency [68]. There is a mechanism of plasma-induced formation of superoxides in water through following set of chemical reactions:
1. \( e^-(H_2O) + O_2(H_2O) \rightarrow O_2^-(H_2O) \)

2. \( 2H^+ + 2O_2^- \rightarrow H_2O_2 + O_2 \) (Dismutation reaction, catalyzed by superoxide dismutase, SOD)

3. \( Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH + OH^- \) (Fenton reaction)

4. \( RH + OH \rightarrow H_2O + R^- \)

5. \( R^- + O_2 \rightarrow RO_2 \)

6. \( RO_2 + RH \rightarrow RO_2H + R^- \) (Phospholipid peroxidation)

7. \( RO_2H \rightarrow RO_2^- + H^+ \)

Electrons generated by plasma become aqueous at the interface of air and water, which in turn produce hydrogen peroxide and superoxides in the solution. The interaction of these species with bacteria ultimately leads to phospholipid peroxidation, thus aiding in achieving cell death at a higher rate than bacteria which were dried on a surface before treatment [69].

3.3 Direct and Indirect Effects of Plasma Exposure on *E. coli*

Plasma provides a “cocktail” of lethal components to bacteria, including not only various reactive species but also charges (both positive and negative ions, and electrons). Efforts have been focused on understanding the role of ions as a means for sterilization using DBD. Direct plasma treatment was tested by mounting a high-voltage electrode approximately 2 mm above a 2 cm x 2 cm grounded stainless steel coupon which was inoculated with *E. coli*. To test the role of ions in sterilization, the addition of a grounded mesh between the high voltage electrode and the stainless steel coupon was executed...
(Figure 27). This grounded mesh separated the plasma from direct contact with the substrate and prevents ions from penetrating the surface.

![Figure 27. Direct versus indirect treatment plasma treatment experimental setup.](image)

It has been demonstrated that direct plasma treatment is more effective in reducing viable counts of *E coli*, and direct plasma exposure results in an 8-log reduction in CFU while indirect exposure results in a 6.5-log reduction in the same exposure time (Figure 28).
Figure 28. Direct vs. Indirect sterilization of E. coli suspended in water.

It is important to note that there is an apparent difference in treating bacteria in water (as reported in this section) and bacteria dried on agarose gel or on metal. Bacteria on agar, for example, exhibit a 2 to 3 log difference between direct and indirect treatment in favor of direct [33].

Ion bombardment is hypothesized to be the main mechanism for inactivation for treatment of dry bacteria, whereas chemical erosion is the main mechanism for wet treatment of bacteria. Additional evidence of ion bombardment will be presented in Chapter 5. Sterilization efficiency of plasma may be counteracted by the addition of Mn(II), as it will scavenge superoxide and hydrogen peroxide through the following redox cycle [70]:

1. $(\text{H}_2\text{O})_n + \gamma$-IR : Radiolysis of water exposed to ionizing radiation: $\text{H}_2\text{O} \rightarrow \text{HO}^* + \text{H}^+ + e^-$
2. Primary radiolytic reaction: $2\text{HO}^* \rightarrow \text{H}_2\text{O}_2$
3. IR-induced superoxide formation: \( \text{O}_2 + \text{e}^- \rightarrow \text{O}_2\cdot^- \)

4. Fenton reaction: \( \text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{HO}^+ + \text{OH}^- \)

5. Haber-Weiss reaction:
   
   \[
   \text{Fe(III)} + \text{HO}_2^* \rightarrow \text{Fe(II)} + \text{O}_2 + \text{H}^+ \text{ and} \\
   2\text{Fe(III)} + \text{H}_2\text{O}_2 \rightarrow 2 \text{Fe(II)} + \text{O}_2 + 2\text{H}^+
   \]

6. Mn oxidation: \( \text{Mn(II)} + \text{O}_2\cdot^- + 2\text{H}^+ \rightarrow \text{Mn(III)} + \text{H}_2\text{O}_2 \)

7. Mn reduction: \( 2\text{Mn(III)} + \text{H}_2\text{O}_2 \rightarrow 2\text{Mn(II)} + \text{O}_2 + 2\text{H}^+ \)

Reaction 6 is able to occur both with and without a Mn SOD, thus an intracellular and extracellular scavenging of the superoxide anion is possible. Without the presence of an Mn SOD, the reaction rate is \( k_6 = 6 \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \) versus a rate constant of \( k_6 > 10^9 \text{ M}^{-1} \text{ s}^{-1} \) when the Mn SOD is present [71]. The resulting decrease in sterilization efficiency with the addition of MnCl\(_2\) will add evidence to the hypothesis that sterilization occurs through the influence of ions and the ion-induced formation of oxygenated species.

The addition of 1 mM MnCl\(_2\) to the external medium resulted in a small protective effect to plasma treatment (Figure 29). If the amount of MnCl\(_2\) added was not sufficient to scavenge the enormous amount of oxygenated species produced, the resulting short-lived protection is a logical outcome. This result further reflects the suitability of non-thermal plasma discharge for surface sterilization applications.
3.4 Quantitation of 8-hydroxydeoxyguanosine (8-OHdG) to measure oxidative damage to DNA resulting from plasma treatment

Further evidence of bacterial susceptibility to oxidative damage due to plasma exposure is gathered through 8-hydroxydeoxyguanosine (8-OHdG) quantitation. 8-OHdG is a DNA damage by-product among numerous types of oxidative DNA damage. During the repair processes of damaged DNA in vivo by exonucleases, the resulting 8-OHdG is excreted (Figure 30).
Figure 30. The formation of 8-OHdG by oxygen radicals [72].

The protocol used for treatment, extraction, purification, and analysis of DNA for oxidative damage is as follows:

1. Place 1.01 mL E. coli with initial concentration of approx. $10^9$ CFU/mL in a treatment well.
2. Expose the sample to DBD plasma using an extended flat electrode for 0, 30, 60, 120 and 180 sec.
3. Pipette 1 mL treated solution out of the well and place in a 1.5 mL microcentrifuge tube.
4. Centrifuge at 6,000 rpm for 10 min at 25°C.
5. Remove supernatant and resuspend in 1 mL PBS. Repeat wash twice.
6. Follow Bacteria Lysate Protocol for each sample.

**Bacteria Lysate Protocol (Wizard® Genomic DNA Purification Kit, Promega)**

1. Add 1ml of an overnight culture to a 1.5ml microcentrifuge tube.
2. Centrifuge at $13,000–16,000 \times g$ for 2 minutes to pellet the cells. Remove the supernatant.
3. Add 600μl of Nuclei Lysis Solution. Gently pipette until the cells are resuspended.

4. Incubate at 80°C for 5 minutes to lyse the cells; then cool to room temperature.

5. Add 3μl of RNase Solution to the cell lysate. Invert the tube 2–5 times to mix.

6. Incubate at 37°C for 15–60 minutes. Cool the sample to room temperature.

7. Add 200μl of Protein Precipitation Solution to the RNase-treated cell lysate. Vortex vigorously at high speed for 20 seconds.

8. Incubate the sample on ice for 5 minutes.

9. Centrifuge at 13,000–16,000 × g for 3 minutes.

10. Transfer the supernatant containing the DNA to a clean 1.5ml microcentrifuge tube containing 600μl of room temperature isopropanol.

11. Gently mix by inversion until the thread-like strands of DNA form a visible mass.

12. Centrifuge at 13,000–16,000 × g for 2 minutes.

13. Carefully pour off the supernatant and drain the tube on clean absorbent paper. Add 600μl of room temperature 70% ethanol and gently invert the tube several times to wash the DNA pellet.

14. Centrifuge at 13,000–16,000 × g for 2 minutes. Carefully aspirate the ethanol.

15. Drain the tube on clean absorbent paper and allow the pellet to air-dry for 10–15 minutes.

16. Add 100μl of TAE to the tube and rehydrate the DNA by incubating the solution overnight at 4°C.

17. Divide the sample into two 50μl samples of DNA.

a. Dilute the first sample in TAE for Gel Electrophoresis Analysis
b. Use the second sample for Oxidative Damage Analysis using the OxiSelect™

Oxidative DNA Damage ELISA Kit Protocol.

OxiSelect™ Oxidative DNA Damage ELISA Kit (Catalog # STA-320) (8-OHdG Quantitation) Protocol:

1. Preparation of Reagents
   a. 8-OHdG Coated Plate: Dilute the 8-OHdG Conjugate (1 mg/mL) to 1 μg/mL in 1X PBS.
      Add 100 μL of the 1 μg/mL 8-OHdG Conjugate to each well and incubate overnight at 4ºC. Remove the 8-OHdG coating solution and wash once with dH2O. Blot plate on paper towels to remove excess fluid. Add 200 μL of Assay Diluent to each well and block for 1 hr at room temperature. Transfer the plate to 4ºC and remove the Assay Diluent immediately before use.
   b. 1X Wash Buffer: Dilute the 10X Wash Buffer Concentrate to 1X with deionized water and mix well.

2. Preparation of Standard Curve
   a. Prepare a dilution series of 8-OHdG standards in the concentration range of 0 ng/mL – 20 ng/mL by diluting the 8-OHdG Standard in Assay Diluent Table (Table 6).
Table 6. Preparation of 8-OHdG Standard diluents.

<table>
<thead>
<tr>
<th>Standard Tubes</th>
<th>8-OHdG Standard (μL)</th>
<th>Assay Diluent (μL)</th>
<th>8-OHdG (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>990</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>500 of Tube #1</td>
<td>500</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>500 of Tube #2</td>
<td>500</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>500 of Tube #3</td>
<td>500</td>
<td>2.5</td>
</tr>
<tr>
<td>5</td>
<td>500 of Tube #4</td>
<td>500</td>
<td>1.25</td>
</tr>
<tr>
<td>6</td>
<td>500 of Tube #5</td>
<td>500</td>
<td>0.625</td>
</tr>
<tr>
<td>7</td>
<td>500 of Tube #6</td>
<td>500</td>
<td>0.313</td>
</tr>
<tr>
<td>8</td>
<td>500 of Tube #7</td>
<td>500</td>
<td>0.156</td>
</tr>
<tr>
<td>9</td>
<td>500 of Tube #8</td>
<td>500</td>
<td>0.078</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>500</td>
<td>0</td>
</tr>
</tbody>
</table>

3. Preparation of Samples

a. Convert extracted purified DNA samples to single-stranded DNA by incubating the sample at 95ºC for 5 minutes and rapidly chilling on ice.

b. Digest DNA sample to nucleosides by incubating the denatured DNA with 5-20 units of nuclease P1 for 2 hrs at 37ºC, and following with treatment of 5-10 units of alkaline phosphatase for 1 hr at 37ºC.

c. Centrifuge the sample for 5 minutes at 6000 g and use the supernatant for the 8-OHdG ELISA assay.

4. Assay Protocol

a. Prepare and mix all reagents thoroughly before use. Each 8-OHdG sample including unknown and standard is assayed in duplicate.

b. Add 50 μL of sample or 8-OHdG standard to the wells of the 8-OHdG Conjugate coated plate. Incubate at room temperature for 10 minutes on an orbital shaker.
c. Add 50 μL of the diluted anti-8-OHdG antibody to each well, incubate at room temperature for 1 hour on an orbital shaker.

d. Wash microwell strips 3 times with 250 μL 1X Wash Buffer per well with thorough aspiration between each wash. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.

e. Add 100 μL of the diluted Secondary Antibody-Enzyme Conjugate to all wells.

f. Incubate at room temperature for 1 hour on an orbital shaker.

g. Wash microwell strips 3 times according to step (d) above. Proceed immediately to the next step.

h. Warm Substrate Solution to room temperature. Add 100 μl of Substrate Solution to each well, including the blank wells. Incubate at room temperature on an orbital shaker. Actual incubation time may vary from 2-30 minutes depending on how rapidly the color change occurs.

i. Stop the enzyme reaction by adding 100 μL of Stop Solution into each well, including the blank wells. Results are read immediately (color will fade over time).

j. Read absorbance of each well on a spectrophotometer at 450 nm.

**List of reagents/supplies used:**

1. 1x Tris-acetate-EDTA (TAE)

2. 1.5ml microcentrifuge tubes

3. Water bath, 80°C and 37°C

4. Isopropanol, room temperature
5. 70% ethanol, room temperature

6. 50mM EDTA (pH 8.0)

7. Micropipettes: 0.5 μl to 10 μl, 20 μl to 100 μl, and 200 μl to 1000 μl.

8. 1 L beaker

9. Distilled/de-ionized water

10. Plate reader with capability to read at 450 nm

11. Nuclease P1

12. Alkaline Phosphatase

**List of reagents/supplies supplied by Wizard® Genomic DNA Purification Kit**

1. ESR1 Silencer Select siRNA (cat #4392429, Ambion)

2. Wizard SV minicolumns

3. SV lysis buffer

4. Nuclei lysis solution

5. Collection tubes

6. 0.5 M EDTA

7. Wash solution

8. RNase solution

9. Nuclease-free water

**List of reagents supplied by OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation)**

1. 96-well Protein Binding Plate (Part No. 231001): One strip well 96-well plate.
2. Anti-8-OHdG Antibody (Part No. 232002): One 15 μL vial of anti-8-OHdG.


4. Assay Diluent (Part No. 310804): One 50 mL bottle.

5. 10X Wash Buffer (Part No. 310806): One 100 mL bottle.

6. Substrate Solution (Part No. 310807): One 12 mL amber bottle.

7. Stop Solution (Part. No. 310808): One 12 mL bottle.

8. 8-OHdG Standard (Part No. 232003): One 100 μL vial of 2 μg/mL 8-OHdG in 1X PBS, 0.1% BSA.

The quantity of 8-OHdG is determined by comparing its absorbance with a known standard curve. As stated in the protocol, this curve is determined in series with the treated samples where standard dilutions of known concentrations of 8-OHdG are added to the wells; thus, a direct correlation with OD at 450 nm and concentration can be determined (Figure 31).

![8-OHdG Elisa Standard Curve](image)

Figure 31. 8-OHdG ELISA Standard Curve which correlates the concentration of 8-OHdG in ng/mL to the optical density measured at 450 nm wavelength.
The sensitivity of detection is increased through the successive binding of antibodies: the sample is first added to an 8-OHdG conjugate preabsorbed 96-well protein binding plate, then an anti-8-OHdG monoclonal antibody is added, followed by an HRP conjugated secondary antibody. Interestingly, the level of oxidative damage to *E. coli* increases proportionally with plasma treatment until a dose of 37.2 J/cm², where the large error bar symbolizes the threshold in the maximum level of damage achieved by plasma treatment and the cessation of DNA repair by oxidative damage (Figure 32). This cessation alludes to the fact that the DNA damage level is beyond the point of repair, and thus the signal completely reduces to zero at longer treatment periods.

**8-OHdG Level in Plasma Treated *E. coli***

![Graph showing 8-OHdG levels](image)

*Figure 32. 8-OHdG levels increase with plasma treatment dose until a threshold is reached, beyond which DNA is not able to recover.*
In summary, direct application of DBD effectively inactivates bacteria at a higher rate than indirect application. An 8-log reduction of *E. coli* after direct DBD treatment for 30 sec occurs as a result of direct treatment. Direct plasma treatment of *E. coli* in liquid is shown to have increase of sterilization capability of almost 2-log versus indirect plasma treatment, thus ions play a significant role in sterilization. The addition of MnCl₂ also adds a protective feature to *E. coli* by scavenging superoxides and hydrogen peroxide, but saturation of these products is reached quickly such that the final sterilization efficiency is the same with or without the presence of scavengers. Further evidence of inactivation through oxidative damage to DNA was gathered by 8-OHdG quantitation, showing a threshold of oxidative damage occurs at a plasma treatment dose of 37.2 J/cm². Beyond this value, there is no oxidative damage to be reported, as the level of destruction appears to be beyond the repair capability of bacteria.

### 3.5 Evaluation of Sterilization Efficiency Dependence on the Conductivity of Substrate Surface

Non-equilibrium atmospheric pressure plasma may become an ideal solution for sterilizing spacecraft before flight and upon return to Earth without thermal or chemical degradation to the surface. Spacecrafts are composed of a variety of conductive and non-conductive components. For this reason, it is important to study sterilization efficacy on varying substrates which are conductive, non-conductive, grounded, and un-grounded. The results will allow NASA researchers to better design spacecraft for higher sterilization efficiency.
Materials chosen for this experiment are stainless steel, glass, polyethylene, and gold-coated glass slides. These are all materials pertinent to building spacecraft. Samples were treated as described in section 3.1, and in the case of un-grounded samples, two glass slides 2 mm in height were placed between the sample and the grounded surface. Thus samples were not truly “un-grounded” and were merely separated from ground by a distance equal to the distance between the electrode and the sample.

Results show that there is no statistically significant difference in sterilization efficiency when sample materials are varied (Figure 33). A variance, however, is seen in the kinetics of inactivation. In the case of gold, there is an initial dramatic decrease in bacterial CFU and a plateau in later time points whereas in the case of un-grounded stainless steel, the decrease in CFU does not occur until the later time points. There is apparently some dependence on the surface which contributes to the kinetics; Nonetheless, the effect remains the same: all cultures are inactivated equally within 2-logs independent of surface.
3.6 Modeling of Bacterial Inactivation by Plasma

A simplified model of bacterial inactivation was developed taking into account primary species known to reside in plasma: atomic oxygen, ozone, hydroxyl radical, ultraviolet radiation, hydrogen peroxide, $N_2^+$, and nitric oxide. The model was developed using CHEMKIN, a chemical kinetics software which analyzes how a given chemical kinetic model will work in a controlled environment and was developed specifically for solving stiff systems of many non-linear equations. This program allows the user to design the model diagram; choose a chemistry set; pre-process the raw data; set inlet, reactor, and solver conditions; run the model; and post-process the results. Kinetic modeling of
bacterial inactivation requires reaction rate constants which are often derived from experimental data. For example, in the case of ozone, the change of bacteria over time is modeled by the following differential equation: \( \frac{d[B]}{dt} = -k_{O_3}[B][O_3] \). The rate constants used in Table 7 were derived from [73-77]. Species concentrations were also derived from literature [77-79] and are listed in Table 8.

### Table 7. Empirical Reaction Rate Constants for modeling of plasma interaction with bacteria [77]

<table>
<thead>
<tr>
<th>Reaction Rate Constant</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_D )</td>
<td>( 9 \times 10^{-14} \text{ cm}^3 \text{ sec}^{-1} )</td>
</tr>
<tr>
<td>( k_{H_2O_2} )</td>
<td>( 3 \times 10^{-22} \text{ cm}^3 \text{ sec}^{-1} )</td>
</tr>
<tr>
<td>( k_{O_3} )</td>
<td>( 1.5 \times 10^{-16} \text{ cm}^3 \text{ sec}^{-1} )</td>
</tr>
<tr>
<td>( k_{NO} )</td>
<td>( 1.7 \times 10^{-24} \text{ cm}^3 \text{ sec}^{-1} )</td>
</tr>
<tr>
<td>( k_{OH} )</td>
<td>( 3.6 \times 10^{-13} \text{ cm}^3 \text{ sec}^{-1} )</td>
</tr>
<tr>
<td>( k_{N_2^+_2} )</td>
<td>( 6.5 \times 10^{-7} \text{ cm}^3 \text{ sec}^{-1} )</td>
</tr>
<tr>
<td>( k_{UV} )</td>
<td>( 3.8 \times 10^{-4} \text{ cm}^3 \text{ sec}^{-1} )</td>
</tr>
</tbody>
</table>

### Table 8. Concentration of Biologically Active Plasma Species [77].

<table>
<thead>
<tr>
<th>Species</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>( [O] )</td>
<td>( 1 \times 10^{15} \text{ molecules cm}^{-3} )</td>
</tr>
<tr>
<td>( [H_2O_2] )</td>
<td>( x \text{ molecules cm}^{-3} )</td>
</tr>
<tr>
<td>( [O_3] )</td>
<td>( 1 \times 10^{15} \text{ molecules cm}^{-3} )</td>
</tr>
<tr>
<td>( [NO] )</td>
<td>( 30 \text{ molecules cm}^{-3} )</td>
</tr>
<tr>
<td>( [OH] )</td>
<td>( 4 \times 10^{14} \text{ molecules cm}^{-3} )</td>
</tr>
<tr>
<td>( [N_2^+] )</td>
<td>( 30 \text{ molecules cm}^{-3} )</td>
</tr>
<tr>
<td>( [UV] )</td>
<td>( 50 \mu W \text{ cm}^{-3} )</td>
</tr>
</tbody>
</table>

The model consists of a partial differential equation modeling the change of bacteria as a function of the biologically active species:

\[
\frac{d[B]}{dt} = -k_O[B][O] - k_{O_3}[B][O_3] - k_{OH}[B][OH] - k_{UV}[B][UV] - k_{H_2O_2}[B][H_2O_2] \\
- k_{NO}[B][NO] - k_{N_2^+}[B][N_2^+] 
\]
where \([B]\) is the bacterial concentration, \([O]\) is the concentration of atomic oxygen, \([O_3]\) is the concentration of ozone, \([OH]\) is the concentration of hydroxyl, \([UV]\) is the UV intensity, \([H_2O_2]\) is the concentration of hydrogen peroxide, \([NO]\) is the concentration of nitric oxide, \([N_2^+]\) is the concentration of \(N_2^+\), \(k\) is the reaction rate constant for each respective specie, and \(t\) is time. It can be seen that this can closely model bacterial inactivation when compared to sterilization experiments obtained by [79] (Figure 34). In these experiments, bacteria were inactivated in the first pass (on the order of seconds), although the next time point was taken minutes later. This makes the modeling results comparable since the level of inactivation is comparable in the time bacteria actually resided in the plasma discharge.
Figure 34. Modeling of survivability as a function of DBD plasma species is compared with previous experimental modeling results (top) [79] to show that we are able to achieve sterilization on the order of seconds (bottom) [77] which is comparable to the residence time of bacteria in plasma.
CHAPTER 4: Viable but Non-Culturable (VBNC) and Dormancy States in Post-Plasma-Treated Bacteria

In this chapter, a correlation methodology to assess bacterial viability and the need for such methodology will be introduced. It will be shown that plasma has the ability to induce a viable but non-culturable (VBNC) state within bacteria; nevertheless it will be shown in Chapter 5 that plasma can inactivate bacteria completely without inducing intermediate viability states.

4.1 The Classical Definition of “Live” Bacteria Revisited and Revised

A microorganism is traditionally deemed “dead” when it does not exhibit all the following: homeostasis; response to stimuli; metabolism; growth; and reproduction [80]. From this list, it is obvious that using plate counts as the sole method for assessing viability only tests a microorganism’s ability to reproduce and thus neglects bacteria in other states of existence.

Conjugation and transformation permits the persistence of genetic material in the environment even if the organism has lost its capability to propagate. Conjugation is when genetic information is transferred from one metabolically active bacterial cell to another. Transformation is the ability of a bacterium to uptake naked DNA (DNA without associated cells or proteins) from the surrounding environment. Plasmids are capable of autonomous replication within a suitable host and at least 40 bacterial species are known to be naturally transformable [81]. The probability of transformation
is drastically reduced when the concentration of DNA is on the order of ng/μl, as indicated by Figure 35 [82] and Figure 36 [83].

**Figure 35.** Relation between transformation frequency for a single marker and DNA concentration. Recipient particles of genotype ab*c* (x) or a*bc* (o) were transformed by denatured DNA of genotype a*b*c*. [82]

**Figure 36.** Percent transformants as a function of DNA concentration. DNA (0.1 ml of each concentration) was added to 5-mi cultures. (donor, Sti; recipient, Stre) [83].
The threat of transformation and conjugation is high when bacteria in all viability states are neglected to be detected. Furthermore, their influence on the environment remains, as toxins may be maintained or even produced in cells long after the ability for proliferation has been lost. The following sections will expand on the types of viability states a bacterium may assume and a methodology is proposed for comprehensive detection.

4.2 The Dormancy State in Bacteria

Dormancy is a reversible state of dramatic metabolic reduction/cessation [84]. This state can be indirectly measured through a lack of culturable bacteria and little to no metabolic activity for a brief to an extended period of time. Similar to spores, dormant bacteria can be non-culturable until they receive a specific stimulus which is unique to all spectrums of microorganisms; however, dormant bacteria are not in spore form and many cases look indistinguishable from healthy growing bacteria under the microscope with the exception of complete lack of or very low levels of cellular respiration, for example. Non-sporulating vegetative cells have been reported to remain dormant for a number of days after the initial lag of the transplant: cells of _Bact. coli_ were found to remain dormant for 16 days, 85% of the cells developed in 48 hours [85]. Spores, on the other hand, may remain dormant for months even when under favorable growth conditions. Spores of _B. subtilis_ were found to lie dormant for 39 days and of _B. megatherium_ for 90 days [85]. It is pertinent to account for both sporulating and non-sporulating bacteria, as its prolonged dormancy can be a potential source of future infection and latent toxicity to the host as well as potential for latent contamination.
4.3 Viable but Non-Culturable (VBNC) Viability State

Viable but non-culturable (VBNC) bacteria are metabolically active, while being incapable of undergoing the cellular division required for growth in or on a medium normally supporting growth of that cell [86]. VBNC bacteria retain the ability to perform functions such as respiratory activity [87-89], cellular elongation[90], and/or incorporation of radio-labeled substrates [91].

The VBNC state is dangerous for pathogenic bacteria as stressed bacteria are more virulent than well-fed bacteria [92]. Further discussion on how this state is induced and its relation to plasma will be given in section 4.6.

There are opponents to the nomenclature “VBNC” bacteria. It has been “proposed to apply the term ‘viable’ only to recoverable cells, the term ‘dormant’ only for recoverable cells with low activity, and to use the term ‘non-culturable’ in the strict sense, for cells that cannot be recovered under any conditions” [93]. In order to reduce confusion, the term “active but non-culturable” (“ABNC”) was developed to describe microorganisms that are non-recoverable and thus non-viable. Since it is difficult to discern the difference for each experiment, some of the cells hitherto addressed as “VBNC” may instead just be in a state of injury, where viability (and limited culturability) is retained for limited periods.

4.4 Correlation Methodology to Enumerate Viability State

It is important to develop a methodology to correlate various assays to determine whether bacteria are alive, dormant/VBNC, or dead. The motivation behind choosing three assays for correlation to enumerate the viability state is illustrated in Figure 37. If
a bacterium has an intact membrane, is respiring at normal levels, and is able to reproduce, it is considered alive. On the other hand, it can exist in additional viability states. If there is an intact membrane, no respiration, and no reproduction, it is dormant. Furthermore, if there is an intact membrane, minimal to normal level of respiration, and no reproduction, it is considered to be in the VBNC state. The level of respiration, membrane integrity, and ability to reproduce is accounted for by the XTT assay, Live/Dead assay, and plate count method respectively.

4.4.1 Assay and methods used to assess the bacterial viability state

Here we will discuss methods used to differentiate living, dormant, and VBNC bacteria.
LIVE/DEAD® BacLight™ Bacterial Viability Kit

Membrane integrity was determined using the LIVE/DEAD BacLight Bacterial Viability Kits from Molecular Probes. It was first introduced in the 1990s, and consists of SYTO® 9 green-fluorescent nucleic acid stain, and the membrane-impermeant stain propidium iodide (PI) which fluoresces red. Both are added at the same time and the red dye (PI) quenches the green emission by the other dye [94]. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for PI. The background is essentially non-fluorescent. It is important to be aware of the concentration when using PI, as pointed out by Williams et. al [95]. A well tested concentration is used in the LIVE/DEAD BacLight Bacterial Viability Kits. With higher concentrations, cells can become leaky [96].

A 2X stock solution was created by mixing the reagents in 5 mL dH₂O. Equal volumes (100 μl) of bacterial samples and stock solution were mixed for analysis and incubated in the dark at room temperature for 15 minutes. Fluorescence measurements were acquired centered at 485 nm for live and dead bacteria at 530 nm and 630 nm respectively. BioTek Synergy 4 Hybrid Multi-Mode Microplate reader and Gen5 (1.06) software were used to acquire the images.

**XTT** (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)

Tetrazolium salts are used to assay cell proliferation, viability, and/or cytotoxicity. Cleavage of tetrazolium salts by enzymes of metabolically active bacteria lead to the formation of formazan crystals. If the formazans are water soluble, colorimetric
measurements can be made directly using the supernatant of the sample. If the formazan is not soluble in water, solubilization of the crystals must be performed before light absorbance measurements can be made. Tetrazolium salts most commonly used such as MTT, INT, NBT, and TNBT produce formazans which are not very soluble in water. [97] With an increase of popularity in industry, diagnostics, and the life sciences, efforts were made to produce tetrazolium salts that yielded formazan products which were more water soluble. These newer products include tetrazolium salts such as XTT and WST-1 [97]. Before use, XTT is pale yellow in color. Actively respiring cells convert the water-soluble XTT \((C_{22}H_{17}N_7NaO_{13}S_2)\) to a water-soluble, orange colored formazan product. Cleavage of XTT by dehydrogenase enzymes of metabolically active cells yields a richly colored formazan product [98]. XTT from Molecular Probes, Inc was used for all respiration results presented herein.

The stock solution of 10 mg/mL XTT was created by mixing 10 mL PBS with 100 mg of XTT (Invitrogen, X6493). Alliquots of 100μl samples were stored at -20°C. The working solution was prepared by mixing 1 ml dH2O, 50 μl stock solution (to yield a final concentration of 0.5 mg/mL), and 1μl of 50 mM menadione (to yield 50 μM final concentration).

A 65 μl sample of bacteria in a hanging-drop glass slide was exposed to plasma and 50 μl of the treated sample was collected and harvested by centrifugation at 10K for 6 minutes. The pellet was resuspended in 150 μl XTT working solution and incubated in the dark for 2 hours. Cells were pelleted by centrifugation at 10K for 6 minutes and 100 μl of the supernatant is transferred into a 96 well plate for analysis. The pellet was then
re-suspended in 100 µl distilled water and plated for colony count comparison. Absorbance measurements were then acquired at 492 nm using a BioTek Synergy 4 Hybrid Multi-Mide Microplate reader and Gen5 (1.06) software.

**Plate Counts**

The traditional method to quantify the “viable” population of bacteria is by inoculation on agar and observing the resulting growth. The time of incubation on agar and the type of agar used varies by bacteria. *B. stratosphericus* and *E. coli* were plated on brain heart infusion (BHI) agar and incubated at 37°C for 16 hours. *D. radiodurans* on the other hand was plated on tryptone/glucose/yeast (TGY) (0.8% Tryptone, 0.1% glucose, 0.4% Yeast Extract) agar and incubated at 32°C for 2 days. Colony counts were performed in quadruplicate and observed daily for up to a week after plating to assess any latent growth.

**Scanning Electron Microscopy**

Samples were imaged using a FEI/Philips XL30 Field Emission Environmental SEM. During the imaging process, the bacteria are in high-vacuum mode with a pressure of $1.3 \times 10^{-4}$ mbar (Vac OK) to $1 \times 10^{-5}$ mbar.

**4.5 Application of the Correlation Methodology to Plasma Treated Bacteria**

*Bacillus stratosphericus*

Previous reports of sterilization efficacy by DBD plasma rely only upon cfu assays [33, 64-66]. In our case, when *B. stratosphericus* is treated in a liquid environment, it
completely loses its culturability within 120 sec of plasma treatment (Figure 38). Colony counts were performed in quadruplicate and observed daily for up to a week after plating to assess any latent growth.

At this point, it is not uncommon to assume complete sterilization has taken place; however with further analysis, it can be seen that viable *B. stratosphericus* remain.

![Graph](image)

**Culturable *B. stratosphericus* 24 hrs post-plasma**

Although we show zero colony counts after 120 sec, it must be compared with other methods to truly determine viability. LIVE/DEAD fluorescent technique shows that there are still bacteria which remain after treatment whose membrane is still intact even 24 hours after plasma treatment (Figure 39). This can be indicative of dead bacteria whose membrane remains intact. To verify the presence of viable bacteria, XTT is used and shows there are bacteria which continue to respire far after plasma treatment, although at a low rate (Figure 40). The XTT data can be observed in Table 2. Standard Error
Measurement (SEM) is calculated by dividing the standard deviation by the square root of the number of samples, in this case 4 samples.

The oxidants produced in plasma (described in section 3.4) are generated in a similar way as outlined and used by Gourmelon et al. [99] to induce the VBNC state via oxidative stress. In response to hydrogen peroxide and superoxides produced by DBD Plasma, *B. stratosphericus* inhibits its ability to culture while maintaining its membrane integrity and a baseline amount of respiration. A reduction in respiration rate >98% is expected for bacteria which enter a dormant or quiescent lifestyle change[100]. Transfer to growth media is typical to increase respiration in such microorganisms[100], and is observed in our case as well.

![Living B stratosphericus using Live/Dead fluorescence technique](image)

Figure 39. Viable *B. stratosphericus* using LIVE/DEAD fluorescence technique.
Table 9. *B. stratosphericus* respiration and Standard Error Measurement (SEM) post-plasma treatment using XTT technique.

<table>
<thead>
<tr>
<th>Observation time Post-plasma</th>
<th>2 hrs</th>
<th>SEM</th>
<th>8 hrs</th>
<th>SEM</th>
<th>20 hrs</th>
<th>SEM</th>
<th>26 hrs</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.00</td>
<td>3.19</td>
<td>100.00</td>
<td>2.65</td>
<td>100.00</td>
<td>11.04</td>
<td>100.00</td>
<td>1.05</td>
</tr>
<tr>
<td>60</td>
<td>2.31</td>
<td>0.50</td>
<td>0.45</td>
<td>0.06</td>
<td>0.31</td>
<td>0.04</td>
<td>0.65</td>
<td>0.12</td>
</tr>
<tr>
<td>120</td>
<td>0.08</td>
<td>0.13</td>
<td>0.69</td>
<td>0.08</td>
<td>0.67</td>
<td>0.19</td>
<td>0.65</td>
<td>0.13</td>
</tr>
<tr>
<td>70% Iso</td>
<td>1.17</td>
<td>0.26</td>
<td>1.92</td>
<td>0.14</td>
<td>2.14</td>
<td>0.24</td>
<td>1.77</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Further observation reveals that the bacteria producing the baseline signal after plasma treatment represent a small fraction of surviving bacteria (Figure 41, top); 24 hours following the treatment, 8-times the amount of bacteria are respiring (Figure 41, bottom) although they remain non-culturable. This increase of respiration gives rise to the question of whether this viability is reversible or irreversible. This change in the level of respiration may have resulted from incubation in LB medium for 24 hours post
plasma treatment, as it is known to enhance respiration of bacteria that has experienced an external stressor.

Correlation of the culturability with membrane and respiration activity is indicative of the VBNC state. Further observation of morphology shows that the *B. stratosphericus*
vegetative cells are also able to elongate (Figure 42), an additional indication of VBNC bacteria as was reported by Roszak et. al [90].

![Image](image_url)

**Figure 42.** 120 sec of plasma treatment of wet *B. stratosphericus* shows elongation (white arrow), a morphological state associated with VBNC bacteria.

It has been shown that exposure to DBD plasma at otherwise “lethal” doses induces a viable but non-culturable state in bacteria which are not completely disintegrated by treatment. This has been demonstrated through observations of a drastic reduction in respiration to a baseline amount, intact membrane, and ability to elongate. It is hypothesized that the oxygenated species produced in plasma induce this state, although it has not been determined whether this state is temporary or permanent. We observe, at lower doses, that bacteria are able to recover and remain both viable and culturable.
4.6 Mechanisms of inducing Viable But Non-Culturable state in Bacteria by Dielectric Barrier Discharge Plasma

The presented results exemplify the need for correlation methodologies to be used when assessing the viability of a microorganism. Scientists especially within the plasma community may feel they are achieving 100% kill without this method, and are falsely misled. The induction of the VBNC state may depend on the environment as well as other parameters which are associated with that state. Table 10 is a compilation of specific parameters associated with E. coli in the VBNC state. As hypothesized in the previous section, reactive oxygen species have the ability to trigger a VBNC response from bacteria (e.g. E. coli). Plasma is a known producer of O, OH, O₂⁻ and other reactive oxygenated species, which are believed to be the main cause of prompting this state in bacteria suspended in a liquid environment.

Plasma is also known to change the pH of a liquid to a more acidic state. Although it has been shown in previous experiments that the change in acidity does not contribute to bacterial inactivation[33], the VBNC state is known to be induced by the addition of acid [101]. The VBNC state in C. jejuni was induced by Gangaiah et al. [101] following the method of Chaveerach et al. [102] where 1 ml of overnight culture with concentration of 5×10⁸ bacterial cells/ml was added to 4 ml of MH broth with pH adjusted to 4.0 using formic acid and incubated under microaerophilic conditions at 42⁰ C for 3 h. This group confirmed their induction of VBNC by comparing culturable cell counts via plate method and viable cell counts via fluorescent microscopy using CTC-DAPI staining. The acidic
conditions plasma creates may also add to factors which induce a VBNC state in bacteria.

Table 10. Environmental and Local Parameters associated with E. coli entering a VBNC State [103].

<table>
<thead>
<tr>
<th>Environment</th>
<th>Parameters related to the VBNC state</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Municipal, reservoir, lake, and saltwater</td>
<td>Temperature</td>
<td>When kept at 25°C E. coli is more likely to go into a VBNC state when compared to 10°C [104]. This effect is regardless the water source based on Municipal, reservoir, and lake, water [105].</td>
</tr>
<tr>
<td>River water</td>
<td>Visible light irradiation</td>
<td>Uptake and respiration of $^{14}$C glucose were drastically inhibited in illuminated systems, and cells rapidly fell into the VBNC state [106, 107]. The effect of visible light on culturability loss of E. coli could result from photochemical generation of hydrogen peroxide in cells [108]. E. coli is able to maintain its enteropathogenicity in seawater [109].</td>
</tr>
<tr>
<td>Saltwater</td>
<td>Osmolarity, salts</td>
<td>There is a 40-fold higher number of VBNC forms of E. coli after 96 h of incubation of 25% salinity in comparison to 5% salinity[104]. 80-90% of E. coli lost culturability within 2 to 3 hours but retained viability when exposed to a high salt concentration (0.8 M NaCl) [110]. Osmoregulatory processes involving organic compatible osmolytes can help survival of enterobacteriaceae in saline aquatic environments [111]. The resuscitation of E. coli from osmotic upshift by betaine suggests that osmotic stress may contribute to the physiology of the VBNC state [110]. Osmotic upshift drastically inhibits transport of carbohydrates and amino acids [112] and thus the VBNC response of enteric bacteria may be triggered through nutrient starvation.</td>
</tr>
<tr>
<td>fresh water, sea water</td>
<td>nutrient scarcity and humic acids</td>
<td>E coli survives in autoclaved filtered riverwater for up to 260 days [113]. The presence of humic acids significantly decreased the entry of E. coli into the VBNC state in freshwater but not in seawater [114].</td>
</tr>
<tr>
<td>buffered fresh water</td>
<td>biocidal agent</td>
<td>Enterotoxigenic E. coli developed VBNC cells when exposed to natural freshwater containing toxic mineral and organic chemicals [115]. Exposure of E coli strains to copper and chlorine in buffered water at 4°C produced VBNC cells that retained enterotoxigenic activity when resuscitated [116]. Copper induces nutrient-starved cells to become VBNC [117]. Evolution of 10% of population to VBNC state by suspending in buffer and treatment with 10 μM HClO is shown to regain its culturability [118]. They also show that stressed bacteria grow at a higher rate than unstressed bacteria.</td>
</tr>
<tr>
<td>Culture medium</td>
<td>toxic forms of oxygen</td>
<td>Compounds which eliminate reactive oxygen species (catalase, pyruvate, etc) also exert a protective effect on E. coli [99, 108].</td>
</tr>
</tbody>
</table>
CHAPTER 5: COMPLETE DESTRUCTION OF BACTERIA THROUGH ION ETCHING
BY DIELECTRIC BARRIER DISCHARGE PLASMA

Surface treatment of polytetrafluoroethylene (PTFE) by chemical reduction with sodium naphthalene, ion beam bombardment, flame treatment and plasma modification have been reported previously for sterilization and functionilization of surfaces [119]. Plasma treatment of polymers is also a common procedure used to modify adhesion, friction, wettability, biocompatibility, and other surface properties of polymer materials [119-121]. These changes in properties are due to functionalization of the surface by active molecules, atoms, radicals, ions and UV radiation generated in plasma. The breaking of polymer surface bonds by plasma leads to the creation of polymeric surface free radicals. In case of oxygen containing gases, plasma treatment leads to oxidizing and increasing of the surface energy [119-121]. Plasma etching or ablation produces a continuous loss of material from the polymer surface. The rate of this process is a function of polymer composition, chemical structure (degree of branching, crosslinking) and plasma conditions. Fluorocarbon polymers are known as the most plasma resistant materials with low etching rate [120]. Hydrocarbon polymers generally exhibit a larger etching rate and oxygen containing polymers are characterized by the largest ablation at the same plasma conditions. Our unpublished results show that long-term plasma treatment of PTFE generates a new topography (Figure 43) and reduces the total mass of the treated area by 57% [122].
Figure 43. Long-term exposure of PTFE to DBD plasma (90 min) results in topographical changes to the polymer surface on both the large scale (top) and small scale (bottom).

Similarly to PTFE, the exposure of dry bacteria on a surface shows selective etching of bacteria with a mechanical degradation to the bacterial membrane over longer-term
treatments relative to the time needed for inactivation. Bacteria themselves can be considered as a polymer. Bacterial polysaccharides, \( C_x(H_2O)_y \), comprise its peptidoglycan, lipopolysaccharides, capsules and exopolysaccharides. Furthermore, DNA and proteins are also oxygen-containing polymer chains. Translating the same chemistry described above to bacteria, hydrocarbon and oxygen containing polymers (bacterial polysaccharides) exhibit two of the largest etching rates compared to any other polymer surface. Evidence of this selective etching of bacteria and DNA at a higher rate than the surface and measurements of surface morphology of bacteria has been gathered and will be presented in this chapter.

5.1 Scanning Electron Microscopy and Atomic Force Microscopy analysis of morphological changes in bacteria.

Imaging techniques were applied to visualize the effect of treatment with cold plasma on the morphology of microorganisms. Viability measurements were supported by Scanning Electron Microscopy (SEM) images taken before and after 30 minutes of DBD treatment of D. radiodurans on blue steel at 1 Watt/cm\(^2\). D. radiodurans was dried on stainless steel substrates and

1. Exposed to DBD plasma for 10 minutes and Atomic Force Microscope (AFM) images were collected before and after plasma treatment.

2. Exposed to plasma for 20 and 30 minutes and Scanning Electron Microscope (SEM) images of the same bacteria were collected before and after plasma treatment (Figure 44).
Figure 44. Flowchart of the SEM visualization procedure of plasma treated bacteria: bacteria are deposited on an aluminum SEM stub and allowed to air dry; the sample is then imaged by the SEM in high vacuum mode; next, it is treated by plasma for the prescribed period of time; lastly, the sample is imaged to determine the level of damage.

Atomic force microscopy in tapping mode is used to discern topographical characteristics of surfaces and has the ability to quantify these characteristics. In this mode, the cantilever oscillates near its resonance frequency as it scans the surface without touching the sample. When the tip is close to the sample surface, the Van der Waals force causes the amplitude of the oscillation to decrease. The tapping AFM image is an improvement on contact mode, where the cantilever drags across the surface at a constant force. This results in damage of the membrane and polysaccharide surface which results in deformities and inaccurate measurements of the surface when scanned, and especially when re-scanned. AFM images clearly show a decrease in height of bacteria from approximately 500 to 100 μm (Figure 45).
Figure 45. AFM images showing morphological changes before (left column) and after (right column) 10 minutes of DBD treatment.

Comparison of Figure 46a (before DBD plasma treatment) and Figure 46b (after 30 min DBD plasma treatment) clearly shows that DBD plasma causes significant morphological changes and “physical” destruction of D. radiodurans on the sterilization surface at 30°C. Although the cell was considered to be dead, its remnants continue to inhabit the surface.
Figure 46. SEM images of Deinococcus radiodurans on blue steel before (a) and after (b) 30 minutes of DBD plasma treatment.
SEM images were also taken of D. radiodurans on surgical grade stainless steel, where the bacteria were treated for 20 minutes (Figure 47). It is believed that chemical etching resulting from plasma treatment is the reason of morphological changes observed in the SEM images. To demonstrate that the damage observed in Figure 46 and Figure 47 was not a result of vacuum exposure and/or SEM ion beam exposure, a control experiment was also performed in the following manner: D. radiodurans was imaged and maintained under high vacuum for 30 minutes, removed from the chamber upon venting, successively replaced in the chamber, and re-imaged in high vacuum for a second time. A miniscule amount of drying of the extracellular polysaccharide compounds is observed as a result of vacuum and desiccation and Figure 48 is a representative sample of one set of control experiments.

During the imaging process, the bacteria are in high vacuum mode with a pressure of $1.3 \times 10^{-4}$ mBar (Vac OK) to $1 \times 10^{-5}$ mBar. Previous research by Saffary et. al.[45] has shown that exposure of D. radiodurans to $1 \times 10^{-8}$ mBar vacuum results in a 1-log reduction and desiccation of D. radiodurans results in approximately a 0.5-log reduction. This resistance to desiccation was further proved by Mattimore et. al. [123].
Figure 47. SEM images of D. radiodurans on surgical-grade stainless steel before (a) and after (b) 20 minutes of DBD plasma treatment.
Figure 48. Control experiments on stainless steel reveal only a miniscule amount of drying of the extracellular polysaccharide compounds due to SEM imaging (top) and re-imaging (bottom) in high-vacuum mode.
5.2 DNA Amplification Protocol

A polymerase chain reaction (PCR) technique was used to amplify a piece of DNA generating millions of copies of a particular DNA sequence. PCR was carried out based on primers for the 16S rRNA gene. Primers used were B27F forward primer (5'AGA GTT TGA TCC TGG CTC AG-3') and 1492 (reverse: 5'-TACGGYTACCTTGTTACGACTT-3'). The PCR master mix contains per sample:

1. 1 μl forward primer 27F
2. 1 μl reverse primer 1492R
3. 5 μl σ-buffer
4. 12.9 μl water
5. 0.1 μl Taq (Fermentas 5x Green GoTaq M791B), and
6. 1 μl of the bacterial sample

Deposition, treatment, and collection of bacteria were done in the following manner:

1. A 5 μl of bacteria suspension from the stock solution was placed on a scanning electron microscope (SEM) stub and allowed to dry for 30 min.
2. Samples were exposed to plasma for 0 sec (control), 60 sec, and additional time points which vary by experiment.
3. Bacteria were collected by adding 5 μl of distilled water to the sample and removed by pipette.
4. Of the 5 μl sample, 1 μl was used for PCR analysis. The amplification reactions were performed with a thermal cycler.
a. Initial denaturation at 94 °C for 5 minutes followed by 35 cycles 94 °C for 30 sec, 59 °C for 60 sec, 72 °C for 90 sec; and lastly a final extension at 72 °C for 7 minutes.

The 1% gels were prepared with 1 μl of SYBR Safe DNA Gel Stain 10,000 x concentration in DMSO (Invitrogen Molecular Probes, 489230) for visualization of the gel. A 1 kb DNA Ladder (Promega, G571A) and 100 bp ladder (Fermentas, SM1153) was used as a reference and 1 μl of 6X Orange Loading Dye Solution (Fermentas, R0631) was mixed with each sample for visualization in the gel. The gels were run at 100 Volts on RunOne™ Power Supply for 15 minutes. It was then removed from the power supply and imaged with the UVP EPI Chem II Darkroom at 254 nm.

5.3 NanoDrop Spectrophotometer Instrument and Protocol

The patented sample retention system (Figure 49) which holds 1 μl of sample makes the NanoDrop ND-1000 spectrophotometer an optimum choice for detection of low quantities of DNA. Each sample is held in place using fiber optic technology and surface tension between two optical surfaces in a vertical orientation that define the pathlength. As a result of this design, the pathlength is allowed to adjust in real time for a given sample. For each cycle of measurement, the sample is evaluated at both a 1 mm and 0.2 mm path, offering a broad dynamic range of nucleic acid detection (2 ng/μl to 3700 ng/μl for double strand DNA) [124]. The NanoDrop ND-1000 spectrophotometer is used in the experiments presented in the following sections to accurately quantify the change in nucleic acid over a course of plasma treatment durations. The amount of DNA is quantified using the following protocol (Figure 49):
1. The sample volume of 1 μl is dispensed onto the lower optical surface.

2. The instrument lever arm is lowered, engaging the upper optical surface with the sample and forming a liquid column.

3. The sample is assessed at both a 1-mm and 0.2-mm path.

The sensitivity of the instrument has been demonstrated to be at the level of measuring only a few ng/μl of nucleic acid.

Figure 49. The NanoDrop ND-1000 micro-volume sample retention system. (A) A sample volume of 1 μl is dispensed onto the lower optical surface. (B) Once the instrument lever arm is lowered, the upper optical surface engages with the sample, forming a liquid column. The sample is assessed at both a 1-mm and 0.2-mm path. [124]

5.4 Plasma treatment of dried plasmids to quantify level of destruction

Even when in a VBNC state, bacteria such as *E. coli* are able to retain the integrity of their plasmids [125]. For this reason, it is important to be able to completely disintegrate plasmids on a surface to eliminate the presence of this microorganism.
From a stock of 361.7 ng/μl plasmids, 5 μl (1808.5 ng) was dried on an Al stub. The sample was reconstituted in 20 μl water after plasma treatment, of which 5 μl was used for gel electrophoresis analysis.

Results show that plasmids are degraded by DBD plasma to an undetectable limit at >20 sec DBD exposure (Figure 50). The concentration of products is also verified with spectrophotometer measurements for each repetition of the experiment. Figure 51 represents a collection of n=3 plasmid experiments.

Figure 50. Degradation of plasmids with increased DBD plasma treatment using gel electrophoresis. Here M is the 100 bp DNA ladder, and 0 sec to 60 sec is the plasma treatment times.
Treatment of dried naked plasmids for 20 sec shows in most cases complete disintegration; however, as shown in Figure 51, there are cases where a signal remains. According to the signal from the spectrophotometer, the detectible limit is reached after only 5 seconds of plasma treatment (Figure 52) and the numerical values obtained by the spectrophotometer beyond that point is in error.
Figure 52. Spectrophotometer signal of plasmid concentration nearly zero after only 5 sec plasma treatment.
It is an important factor that naked plasmids dried on an aluminum surface can be completely eliminated. It is now important to further this analysis to bacteria previously analyzed with plate count and fluorescence techniques presented in previous chapters. This will be done in Sections 5.5 through 5.7.

5.5 Plasma treatment of Chromosomal DNA to quantify level of destruction

Bacteria are hosts to both plasmid and chromosomal DNA. Thus it is also pertinent to prove that plasma has the ability to degrade chromosomes which are on an exposed dry surface. A low initial concentration of chromosomal DNA was used, 5x10^4 Chromosomes of DNA. Evidence was gathered to prove that only 2 seconds DBD treatment is needed for complete destruction (Figure 53). This is also verified by observing the lane spectra of the gel (Figure 54).

![Figure 53. Complete destruction of chromosomal DNA by DBD plasma after 2 sec plasma treatment](image)
5.6 Plasma treatment of B. stratosphericus and B. subtilis to enumerate efficacy and degree of sterilization

It is known that inactivation mechanisms differ based on the level of liquid present in the sample [42]. When B. stratosphericus and B. subtilis is dried on a stainless steel substrate in room air prior to plasma treatment and exposed to DBD plasma, etching by charged particles takes place to a point where DNA is completely destroyed. In 60 sec of plasma treatment, nearly all of the DNA has been eradicated, as seen in Figure 55. An interesting observation can be made from Figure 55: plasma disintegration occurs in such a way that exposed bacteria experience a total destruction of their chromosome, as indicated by the lack of DNA fragmentation. This lack of fragmentation may be explained as a result of DNA destruction to the point where remaining pieces are too
small to be amplified by the PCR technique. The lack of fragmentation may also be explained by a linearization of the chromosomal DNA by plasma treatment and the cleavage of nucleotides by exonuclease enzymes, thus rapidly degrading the remaining DNA [126]. Nonetheless, DNA digestion by either plasma and/or enzymes concludes that DNA is destroyed to the point where it drastically reduces the probability of transferring information to neighboring bacteria.

A direct flux of charged particles within the plasma filaments interacts directly with the membrane and penetrates the bacteria in numerous locations thus exposing internal
components directly to plasma. In 120 sec of plasma treatment, the physical disintegration of *B. stratosphericus* can be clearly observed (Figure 56). Remarkably, the polysaccharide exoskeleton remains structurally intact in the areas which are not directly exposed to the DBD filaments.

![Figure 56. SEM images of 120 sec of plasma treatment of dry *B. stratosphericus* shows etching of bacteria. Etching of the bacterial membrane is clearly visible on the image. Scale bar represents 2 μm.](image)

### 5.7 Plasma treatment of SAFR-032 to enumerate efficacy and degree of sterilization

As introduced in Section 1.5, SAFR-032 spores are endowed with UV radiation and H₂O₂ resistance capabilities which significantly exceed other Bacillus species. This resistance
allows for its survival against standard sterilization practices. In this section, evidence of physical damage on the DNA level of dry SAFR-032 spores is presented. Plasma exposure was conducted incrementally at 0, 2, 5, 10, 20, and 60 seconds to understand the type of damage plasma induces to DNA. For example, plasma may be indiscriminately disintegrating bacteria, or in the case of B. stratosphericus, it may destroy the DNA to a point where natural mechanisms digest the remaining DNA fragments which were exposed. After plasma treatment, bacteria were collected from the surface and processed as described in Section 5.2. The initial ramp in temperature of the amplification reaction cycles denatures the membrane of bacteria allowing for DNA from both the environment (destroyed bacteria) and DNA in intact bacteria to be amplified.

Results indicate that damage of nucleic acid in dry SAFR-032 Spores post-DBD plasma treatment occurs proportionately with treatment time without DNA fragmentation (Figure 57).

Figure 57. Degradation of nucleic acid of dry SAFR-032 spores with increased plasma treatment.
Spectral analysis of each lane is useful to verify contents without relying upon the human eye to discern the presence and intensity of bands. For this reason, our results were also verified by spectral analysis of each lane (Figure 58).

Figure 58. Lane spectra of Plasma treated dry SAFR-032 spores showing degradation of nucleic acid.

NanoDrop Spectrophotometer measurements also verify the degradation degree of DNA with increased doses of plasma (Figure 59).
5.7.1 Plasma treatment of SAFR-032 spores protected in Martian soil

Palagonite is a substantial component of Martian regolith. Its composition is: SiO$_2$ (42.1%), TiO$_2$ (2.31%), Al$_2$O$_3$ (12.6%), FeO (11.91%), MnO (0.19%), MgO (7.40%), CaO (8.40%), Na$_2$O (2.57%), K$_2$O (0.68%), P$_2$O$_5$ (0.18%), CO$_2$ (5.2%), H$_2$O(+) (6.2%) [127]. This solution was pre-prepared according to [128]. Palagonite which was filtered through a membrane and palagonite which remained unfiltered were used. A reduction in UV flux is predicted to occur when mixed with bacteria [129]; however because the mechanisms of sterilization through plasma is not primarily through UV, it is seen that the ability to sterilize is the same with or without palagonite (Figure 60).
Spectra for each lane in Figure 60 is taken and plotted in Figure 61. It shows a distinct difference in plasma sterilization efficiency when palagonite is added. Instead of a shielding effect, it actually enhances sterilization. This is hypothesized to occur because palagonite, composed of mostly Silicon, titanium, Aluminum and Iron, can act as mini-electrodes within the solution and thus enhance uniform and complete plasma treatment throughout the entire volume.
In summary, the exposure of dry bacteria on a surface shows selective etching of bacteria with a mechanical degradation to the bacterial membrane over longer-term treatments relative to the time needed for inactivation. Bacterial polysaccharides, as other hydrocarbon and oxygen-containing polymer chains exhibit the largest etching rate compared to the metal surface on which they are dried. This statement is supported by SEM analysis of surface morphology and PCR analysis of DNA from plasma treated samples of D. radiodurans, SAFR-032, and B. stratosphericus.
CHAPTER 6: CONCLUDING REMARKS

Plasma treatment was shown to inactivate bacteria on various surfaces of interest to NASA not only to stop bacteria from rapidly dividing (reduce culturability) but down to the DNA level where we showed complete inactivation, destruction, and removal of all genetic material. Traditionally, efficacy and efficiency of antimicrobial techniques or a sterilization method is characterized mainly through culturability of bacteria. In the presented work, a 4-log reduction in culturability of D. radiodurans was achieved in 15 sec and a 7-log reduction of E. coli in 30 sec, etc. A distinction between viable vs. VBNC vs. disintegrated/dead bacteria was also found. It was observed that the exposure of B. stratosphericus to plasma results in three viability states: 1) viable and culturable at low plasma doses, 2) viable but non-culturable injured bacteria, and 3) disintegrated bacteria at higher plasma doses. While remaining non-culturable, respiration levels are shown to be at relatively low levels immediately after plasma treatment while increasing up to 8 times over the course of 24 hours (while remaining non-culturable). To a large extent, the viability state induced by plasma is dose-dependent (~60-120 J/cm²). The loss of culturability is hypothesized to be induced as a response to oxidative stress, and it remains to be unclear if the response is temporary or indefinite. E. coli, which has less defenses against oxidative stress exhibits the peak amount of oxidative damage a 37.2 J/cm², whereas D. radiodurans is more robust against this type of damage due to its Mn(II) redox cycle.
The correlation studies suggest bacteria entered a dormant/VBNC state after plasma treatment; however complete destruction to DNA level can be achieved at higher doses. Extremophile bacteria are disintegrated to an undetectable DNA limit with plasma treatment in less than 1 minute; Plasmids become undetectable after 20 sec DBD exposure and Chromosomal DNA undetectable with 2 sec DBD and Elongated DBD treatment. The dose required to induce a particular state in bacteria is summarized in Table 11.

<table>
<thead>
<tr>
<th>Viability State</th>
<th>Plasma Dose Required [J/cm²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alive</td>
<td>0 - 60</td>
</tr>
<tr>
<td>VBNC</td>
<td>60-120</td>
</tr>
<tr>
<td>Dormancy</td>
<td>-</td>
</tr>
<tr>
<td>DNA Disintegration (on dry surface)</td>
<td>≥60</td>
</tr>
</tbody>
</table>

It is suggested that future work addresses the following issues: 1) sterilization of curved and non-planar surfaces; 2) Sterilization of non-conductive surfaces with ground far away; and 3) genetic analysis of bacteria to understand any adaptations/changes that the bacteria have developed resulting from plasma exposure. This will also further distinguish between dormant and VBNC bacteria.
REFERENCES


APPENDIX A - Capillary DBD Plasma Exposure

Dielectric barrier discharge (DBD) plasma is formed when at least one electrode is covered by a dielectric to prevent an arc from forming. By reducing the size of one electrode and allowing a capillary to serve as the dielectric barrier, it is possible to form plasma in millimeter-diameter tubes (Figure 62). Helium is used as a carrier gas to elongate the plasma outside of the capillary tube. This device is an atmospheric-pressure plasma which closely resembles a jet. Its characteristics are similar to a low-temperature glow discharge, where gas temperature is approximately room temperature.

![Capillary DBD Schematic.](image)

Plasma treatments were conducted on *D. radiodurans* suspended in 10 μl water and deposited on a stainless steel substrate. Exposure times were 15 sec, 30 sec, 1 min, and
3 min, and control samples were obtained by exposing the sample to helium for 3 min. This was done by placing the capillary at the same distance above the sample (2 mm) without initiating plasma (Figure 63). A microsecond pulsed voltage waveform power supply was used to generate plasma in these experiments.

Figure 63. Capillary DBD exposure of a "wet" sample of *D. radiodurans*.

Over the duration of the treatment the "wet" environment evolved to a "dry" at 35 seconds of plasma exposure. This results in two major slopes for sterilization in Figure 64. This is similar to results seen in Section 3.2 where there is a variance in sterilization efficiencies when in a wet versus dry environment due to the mechanisms of sterilization. In a wet environment, the main mechanism is through the production of superoxides and hydrogen peroxide in water. In a dry environment, the main mechanism is through ion etching of the membrane and cell contents.
Figure 64. Sterilization efficiencies of D. radiodurans by capillary DBD in wet and dry environments show two slopes over the evolution from wet to dry. Helium-only exposures show no significant drop in CFU by pure exposure to helium.

The initial 3 log reduction which occurs in 30 sec demonstrates the high-efficiency “wet” treatment of bacteria, whereas the 2-log reduction in 150 seconds is the result of the direct flux of charged species on the surface. The sterilization efficiency in general is lower than that of plasma generated by a 1 W/cm² continuous waveform, as the overall power density is much less. The propose of the experiment was to demonstrate that capillary DBD is an effective, yet lower-power method to inactivate DBD.
INDEX

A

Argon
  DBD in Argon, 34

B

Bacillus stratosphericus
  Complete DNA destruction of, 100
  Efficacy of Sterilization, 76
  Induction of VBNC state, 76

Bacillus subtilis
  Background, 27
  Complete DNA destruction of, 100

C

Chromosomal DNA
  Complete destruction of, 99
  Correlation Methodology, 72

D

Deinococcus radiodurans
  Background, 21
  Inactivation by DBD Plasma, 47
  Dielectric Barrier Discharge (DBD)
    Capillary DBD Plasma, 123
    Dielectric Barrier Discharge (DBD), 10
      Characterization, 29
      Dormancy
        Definition, 71

E

Escherichia coli
  background, 27
  Efficacy of Sterilization, 50

H

Helium
  DBD in Helium, 35

L

LIVE/DEAD
  Background, 74

M

Martian soil. See Palagonite

Medicine
  Plasma. See Plasma, Applications in Medicine
  Modeling
    Bacterial Inactivation by DBD Plasma, 65

N

Nitrogen
  DBD in Nitrogen, 38
  Shockwaves in Nitrogen DBD Plasma. See Shocks

O

Oxidative Damage
  Quantitation post-plasma treatment, 54

Oxygen
  DBD in Oxygen, 36

P

Palagonite, 105
  Planetary Protection Requirements, 1

  Plasma
    Applications in Industry, 15
    Applications in Medicine, 15
History, 4
Physics of Formation, 7
Plasmids
Complete DNA destruction of, 95
Plate Counts, Viability assessment using, 76
Polytetrafluoroethylene (PTFE)
Plasma treatment of, 84

SAFR-032
Complete DNA destruction of, 102
in Martian Soil, 105
Scanning Electron Microscopy (SEM)

Background, 76
Shocks
In a weakly ionized non-equilibrium plasma, 39
In Nitrogen DBD plasma, 41

VBNC
Causes, 83
Definition, 72
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