CHALMERS
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Cold Plasma in Medicine

Combatting Bacterial Biofilms

Master's thesis in Applied Physics

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CHALMERS UNIVERSITY OF TECHNOLOGY
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Abstract

Solid, liquid and gas are what is generally called the three states of matter. Plasma is an ionized gas, where ionization is obtained by applying energy in the form of heat or high electrical fields to the gas, and it is the fourth state of matter. Plasmas can be of equilibrium or non-equilibrium type. In a plasma in an equilibrium state, the ions and electrons are in thermodynamic equilibrium, resulting in high gas temperatures. Plasmas used in medicine and healthcare are in highly non-equilibrium state and have gas temperatures that are suitable for treatment of living tissue without, or with minimal, damage to surrounding healthy tissue.

The field of cold plasma in medicine is growing quickly and the possibilities seem endless. For example, cold plasma has been shown to have potential to be effectively combined with chemotherapy in cancer treatment and to promote wound sterilization, blood coagulation, and cell proliferation in wound healing.

The main active agents in cold plasma have been proposed to be reactive chemical species, ions and electrons, heat radiation, electromagnetic fields, UV radiation, and visible light. The combination of the produced agents in the cold plasma is dependent on a number of things, for example, the type of gas used, air or noble gases, the input energy, and the pulse frequency. However, the mechanisms of action of the active agents are not fully understood, and more work has to be done in order be able to establish cold plasma as a solid technique in the field of medicine.

After a description of the physics of cold plasma, its efficacy on bacterial biofilms was tested experimentally. 48-hour biofilms of two gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, and two gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus epidermidis*, were exposed to the cold plasma at fixed exposure distance of 15 mm for exposure times of 0, 5, 10, 15, 30, and 60 minutes. The inactivation of bacteria was evaluated quantitatively with colony forming units (CFU) counting and qualitatively with live/dead fluorescent staining, and scanning electron microscope (SEM) was used to examine the morphological changes of the bacteria.

Significant decrease in the number of viable bacterial cells was observed for all the bacteria. For the gram-negative bacterium *E. coli*, almost 90% reduction was observed after only a 10-minute exposure, however a 100% reduction was not accomplished after exposures of up to one hour. For the other gram-negative bacterium, *P. aeruginosa*, a reduction over 90% was not accomplished until after a 60-minute exposure, where the reduction reached almost 100%. For the gram-positive bacterium *S. epidermidis*, only about 80% reduction was accomplished after a 60-minute exposure. However, for the other gram-positive bacterium, *B. subtilis*, almost a 100% reduction was accomplished after only 5 minutes of exposure.

The results obtained were not surprising. Even though results of gram-negative bacteria being more sensitive to a cold plasma exposure than gram positive ones have been published, there have also been publications of *B. subtilis* being very sensitive to cold plasma exposure. However, the time to reach a 90% reduction varies greatly in published results, indicating that results from cold plasma experiments are highly dependent on the device used each time. It can be concluded that further studies need to be conducted for a clearer view of the efficacy of cold plasma in biofilm inactivation.

Key words: Atmospheric pressure, bacteria, cold plasma, discharge, medicine, plasma, reactive species.

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1 Introduction

Solid, liquid and gas are what is generally called the three states of matter. If a gas is energized, by applying heat or high electrical fields, electrons can escape from atoms and molecules forming a mixture of neutral atoms and molecules, ions and electrons. This mixture is called plasma, and it is the fourth state of matter. Plasmas can be classified as either of equilibrium type or non-equilibrium type (Figure 1.1). In an equilibrium plasma the ions and electrons are in thermodynamic equilibrium, where all particles have the same temperature. Non-equilibrium plasmas have electrons that have much higher temperature than the ions and neutrals. Since the temperature of the gas is taken to be the same as the ion temperature, equilibrium and non-equilibrium plasmas are also referred to as thermal and non-thermal plasmas, respectively. That is, when the ions and electrons are in thermal equilibrium, in equilibrium plasmas, the ion temperature is as high as the electron temperature, hence the alternative name thermal plasmas. The same logic holds for non-equilibrium plasmas, the ions have much lower temperature than the electrons, and the plasma is therefore called non-thermal. This definition well describes the characteristics of the plasma, however, these non-thermal plasmas still cover a broad range of temperatures, up to several thousand degrees. Describing a plasma as non-thermal, when the gas temperature can still be thousand degrees, can thus be confusing.

Non-thermal plasmas used in medicine and healthcare are in highly non-equilibrium state and can be described clearer by defining three temperature bands suitable for different applications. In the upper band the gas temperatures are 100°-150°C and is useful for sterilization and decontamination of metallic medical devices. The middle band has gas temperatures of 60°-100°C and can be used for blood coagulation and

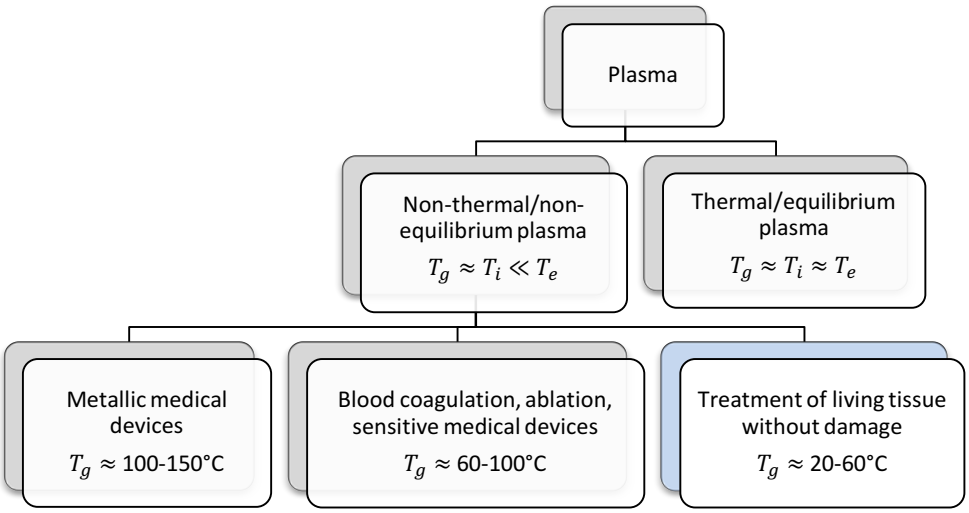

Figure 1.1: Classification of a plasma. Plasmas can be classified as either of equilibrium type or non-equilibrium type. In an equilibrium plasma the ions and electrons are in thermodynamic equilibrium, where all particles have the same temperature. Non-equilibrium plasmas have electrons that have much higher temperature than the ions and neutrals. Since the temperature of the gas is taken to be the same as the ion temperature, equilibrium and non-equilibrium plasmas are also referred to as thermal and non-thermal plasmas, respectively. Non-thermal plasmas used in medicine and healthcare are in highly non-equilibrium state and can be described clearer by defining three temperature bands suitable for different applications: A plasma with gas temperature of 100°-150°C is useful for sterilization and decontamination of metallic medical devices, a plasma with a gas temperature of 60°-100°C can be used for blood coagulation and ablation of living tissues as well as decontamination of more temperature-sensitive plastic and glass medical devices, and a plasma with gas temperature of 20°-60°C can be used for treatment of living tissue without, or with minimal, damage to surrounding healthy tissue.

ablation of living tissues, as well as decontamination of more temperature-sensitive plastic and glass medical devices. The lowest temperature band has gas temperatures of 20°-60°C, and can be used for treatment of living tissue without, or with minimal, damage to surrounding healthy tissue. [1, Ch. 1, 3.1] [2, Ch. 1] This plasma hierarchy can be seen in Figure 1.1.

Plasmas used in medicine are also often described as non-equilibrium/non-thermal atmospheric-pressure plasmas, where, in contrast to low-pressure plasmas, the gas temperature can be controlled at atmospheric pressure. In low-pressure plasmas the gas temperature is controlled by containing the gas in a vacuum chamber. Lower particle density lowers the total number of particles that can be ionized and able to carry charge, thus limiting the growth of the discharge current. Even though the electrons carry the majority of the current, the ions also gain some kinetic energy when the current increases, which subsequently increases the plasma gas temperature. Therefore, the ability to control the gas temperature of plasmas under ambient condition, that is to be able to control the discharge current, without the use of vacuum chambers, opened the door for applications of plasmas on living tissues. [1, Ch. 2.2] How this is accomplished will be explained in details in later chapters. For the purpose of this report, from here on, the term cold plasma will be used to describe a plasma formed at atmospheric pressure in the lowest temperature band, the type of plasma suitable for treatment of living tissue.

Plasma-based electrosurgical devices have been used for a long time in medical applications like tissue cutting, tissue ablation, blood coagulation, and desiccation. In these applications electrical impulses and electrical discharges are used to provide heating of the tissue, thus damaging it. This is not to be confused with cold atmospheric plasmas defined above, where thermal effects are negligible. [1, Ch. 12.1.1] [3]

Cold plasma has been shown to have potential in sterilization of thermo-sensitive materials and instruments, cancer treatment, and wound healing. In cancer therapy, when treated with cold plasma, cells are observed to undergo apoptosis and the normal cell cycle stops, leaving the cells vulnerable for cold plasma treatment to be effectively combined with chemotherapy. [3] In wound healing cold plasma has many benefits, such as, wound sterilization, blood coagulation, and cell proliferation. [4] Interestingly, depending on the dosing of the cold plasma, it can both stimulate proliferation of mammalian cells and lead to their apoptosis. Furthermore, cancer cells seem to be more sensitive to a cold plasma treatment than non-malignant cells, giving rise to the finding of a “sweet spot” in the dosing. The main medical application areas that have been established with clinical trials are the treatment of chronic wounds, infected skin diseases, and dermatitis. In addition, four cold plasma sources have received a CE certification as medical devices. [5]

The main active agents in cold plasma have been proposed to be reactive chemical species, ions and electrons, heat radiation, electromagnetic fields, UV radiation, and visible light. The combination of the produced agents in the cold plasma depends on, for example, the type of gas used, air or noble gases, the input energy, the pulse frequency. However, the mechanisms of action of the agents are not fully understood, and more work has to be done in order be able to establish cold plasma as a solid technique in the field of medicine, having the ability to produce specific combinations of active agents for variety of applications. [1, Ch. Foreword]

The field of cold plasma in medicine is growing quickly and the possibilities seem endless. This study aims to give a description of the physics of cold plasma, with the addition of experimentally observing what effects the active agents in the cold plasma have on bacterial biofilms. The purpose of the experimental work to be presented is to contribute to the basic research needed to get a deeper understanding of the cold plasma's effects on living organisms. [5]

Most experiments on the antibacterial effects of cold plasma have been carried out using planktonic cells, which have been shown to be easily inactivated. [6] However, in a bacterial biofilm, bacteria have arranged themselves in a highly organized three dimensional structure that can attach to surfaces, living and nonliving, leading to colonization and infection. Because of how closely cells in a biofilm interact with each other, they become very resistant to disinfectant chemicals and antibiotics, causing chronic infections. [7] There is thus a critical difference in the destruction of planktonic bacterial cells and bacterial biofilms.

An important example is biofilm formation on medical devices, a foreign-body infection. It is the largest contributor to healthcare-associated infections, three out of four most common infection sites on the body are sites common for medical devices, the urinary tract, respiratory tract, and bloodstream, leaving out only surgical wound sites. And, in fact, 95% of urinary tract infections are associated with urinary catheters. [7]

When a biofilm has formed on a foreign body it has to be taken out or treated for a long time with antibiotics but its overuse, and inappropriate use, has caused bacteria to develop antibiotic resistance, making bacterial infections an emerging threat again. The world is said to be going into a post-antibiotics era and the antibiotic resistance has become a global threat according to the WHO (World Health Organization). Thus, in addition to manage the usage of antibiotics, there is a great need for novel approaches for treatment of bacterial infections. One of the most promising approaches at the present time has been suggested to be cold plasma. [7]–[10]

This thesis comprises two chapters of theoretical discussion about non-thermal plasma and bacteria, with the relevant subsections, a computer simulation chapter, a chapter presenting the experimental work conducted, and chapters of discussion and conclusion.

2 Non-Thermal Plasma

This chapter discusses non-thermal plasmas in detail, from a theoretical characterization of the plasma, how it is ignited and generated at the medically relevant atmospheric pressure and room temperature, and to the main agents acting in the plasma.

2.1 Plasma characterization

To characterize a plasma, it is necessary to consider various parameters of the gas, such as particle number densities, the degree of ionization, and different length scales and forces between particles. In addition, it is necessary to consider how the particles are affected by external fields, and how the particles contribute to macroscopic properties of the gas, such as the gas temperature. [11, Ch. 1]

2.1.1 Particle densities and degree of ionization

Like stated above, a plasma comprises neutral gas atoms and molecules, positive ions, and electrons. Corresponding particle densities, n, are the number of particles in the volume:

$$n = \frac{number\ of\ particles}{volume} \tag{1}$$

The electron and ion densities of the gas depend on the degree of ionization of the plasma. The degree of ionization of a gas is the number of ionized atoms or molecules in the gas, compared to the total particle density, n_{tot}. Quasi-neutrality, which is described in more detail below, is the property of ionized gas making it a plasma, and it means that ion and electron densities are almost the same:

$$n_i \approx n_e \tag{2}$$

In general, ions can be multiply charged, and the quasi-neutrality relation becomes;

$$n_e \approx \sum_z z \cdot n_z \tag{3}$$

where n_z is the density of z-charged ions.

The definition of the degree of ionization, α, of the plasma is:

$$\alpha = \frac{n_e}{n_{tot}} \tag{4}$$

Only a small fraction of atoms in a gas discharge are ionized, typically around 10^{-6} to 10^{-5}. As an example, the density of a gas at an atmospheric pressure and room

temperature is around $n_{tot} = 10^{22}$ m^{-3} and for a gas ionization of $\alpha = 10^{-6}$, the ion and electron densities are about $n_e = 10^{16}$ m^{-3}.

2.1.2 Distance between particles

The average distance between particles, λ_n, in plasma is related to the particle density, n, as:

$$\lambda_n = \frac{1}{(n)^{1/3}} \tag{5}$$

In the gas described above, at an atmospheric pressure and room temperature, the distances between electrons/ions is around 10^{-6} m $= 1$ μm and the general distance between particles in the gas is around 10^{-8} m $= 10$ nm. An example of distance between particles in a ionized gas can be seen in Figure 2.1.

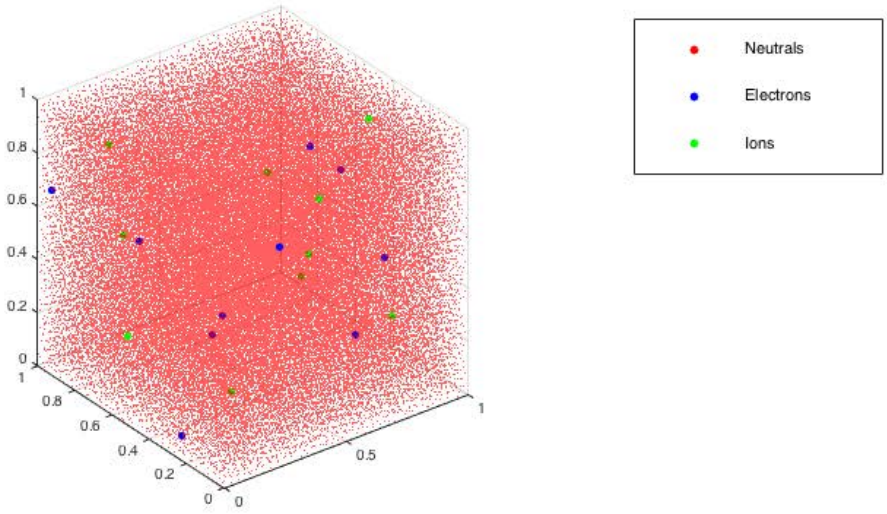

Figure 2.1: Visualization of a gas with a degree of ionization of $\alpha = 10^{-3}$, that is, for total of 10^4 particles there are 10 electrons (blue) and ions (green). This would represent a highly ionized gas, but is used here for better visual effects. For a gas at atmospheric pressure and room temperature, the total particle density is around $n_{tot} = 10^{22}$ m^{-3} and, in this visualization example, the ion and electron densities are about $n_e \approx n_i = 10^{19}$ m^{-3}. This gives the distances between electrons/ions of around $\lambda_e \approx \lambda_i = 10^{-7}$m $= 100$ nm and the distance between particles in the gas is around $\lambda_{tot} = 10^{-8}$ m $= 10$ nm. It should be noted that the sizes of neutrals, ions, and electrons are not to scale, also to provide better visual effects.

2.1.3 Quasi-neutrality and the Debye-Hückel length

Plasma is quasi-neutral on a large macroscopic scale, meaning that the number of positive ions and electrons are almost the same, as mentioned above. However, on a microscopic scale, there are local deviations from neutrality, owing to the thermal energy of the electrons, $k_B T_e$, where k_B is the Boltzmann constant and T_e is the temperature of the electrons. Thermal energy of the ions can be neglected because of the difference of electron and ion temperatures, $T_e \gg T_i$, of non-thermal plasmas, as will be explained in detail later on. These local deviations correspond to a charge separation, that is deviations from charge neutrality, over the so-called Debye-Hückel length;

$$\lambda_D = \left(\frac{\varepsilon_0 k_B T_e}{e^2 n_e}\right)^{1/2} \quad (6)$$

the radius of a sphere around a charged particle, approximated as a point charge, outside of which the charges are screened, with ε_0 being the permittivity of free space and e the electron charge. To fulfill the condition of a gas being a plasma, that is being quasi-neutral, the characteristic plasma length, L, that is the distance over which the plasma extends, has to be large compared to the length scale of deviations from neutrality, the Debye-Hückel length:

$$L \gg \lambda_D \quad (7)$$

For an electron of a temperature $T_e \approx 30000$ K in the already mentioned example of a gas at an atmospheric pressure and room temperature with an electron density $n_e \approx 10^{16}$ m^{-3}, the the Debye-Hückel length for the electrons in the gas is of the order of $\lambda_D = 0.12$ mm, which is small compared to, for an example, a plasma of $L = 1$ cm. [12]

2.1.4 Forces acting on particles

Between two charged particles there is always an electrostatic force, the Coulomb force. There is attraction between opposite charges and repulsion between like charges. The Coulomb law gives the electrostatic force between two point charges, q_1 and q_2, separated by distance r:

$$F_{Coulomb} = \frac{q_1 q_2}{4\pi r^2 \varepsilon_0} \quad (8)$$

Electrons can be considered point charges, as well as ions if it is assumed that their charge is concentrated in the center. Continuing with the example of a gas at an atmospheric pressure and room temperature, the Coulomb force between two electrons at a distance of around $\lambda_e = 10^{-6}$ m $= 1$ µm is about $F_{Coulomb} = 2.3 \cdot 10^{-16}$ N.

When an external electric field, E, is applied, charges, q, are acted up on by a constant force;

$$F_{Electric\ field} = qE \quad (9)$$

positively charged ions will move in the direction of the electric field, while electrons move in the opposite direction.

If the two particles are close, the Coulomb force is strong and can be much greater than the force on charges from the applied electric field. On the other hand, if the particle density is low, the external field can have much greater affect than the Coulomb force.

Finally, the potential describing the interaction between two charges at a distance r is;

$$V_{Coulomb} = \frac{q_1 q_2}{4\pi r \varepsilon_0} \tag{10}$$

and in the ongoing example here with the distance between electrons being around $\lambda_e = 10^{-6}$ m $= 1$ μm, the potential felt by the electrons is about $V_{Coulomb} = 1$ mV.

For a system with more than two charges the interaction potential between two charges is modified, screened, by other charges and a typical Coulomb interaction is reduced by a factor exp $(\frac{-r}{\lambda_D})$ where λ_D is the Debye-Hückel length explained above. [11, Ch. 1] [12]

2.1.5 Particle movement

From the force from the electric field (eq. 9), a charged particle will attain a drift velocity. This velocity is dependent on the particle density. As the particle density becomes higher, friction due to more frequent collisions, known as Brownian motion, between particles affects the mobility, b, of the particles. The drift velocity thus becomes:

$$\bar{v}_D = bq\bar{E} \tag{11}$$

However, the drift velocity can also be affected by electrostatic forces between particles. Like mentioned above, the Coulomb force becomes large if two particles are close, and can then overcome the force on a charged particle from the external field. [12]

2.1.6 Classical plasma

A plasma gas is called classical plasma, for which the plasma kinetic theory is classical Boltzmann statistics, if the distance between gas particles is sufficiently large. The distance between electrons in a plasma (eq. 5) is sufficiently large if it is large compared to the average electron, of mass m_e and speed v_{th} from its thermal energy, de Broglie wavelength:

$$\lambda_B = \frac{h}{m_e v_{th}} \tag{12}$$

The de Broglie wavelength describes the wave-like properties of particles, a particle of mass m moving at speed v will have the properties of a wave of the de Broglie wavelength, where h is the Planck constant.

Furthermore, a plasma can be described as an ideal gas, where particles only interact elastically, if the mutual potential energy of electrons and ions is small compared to the average kinetic energy:

$$\frac{3}{2} k_B T_e \gg \frac{e^2}{4\pi\varepsilon_0 \lambda_e} \tag{13}$$

That is, there is large enough distance between particles in the plasma so that the electrostatic force between them is weak. Since, as will be discussed in the next section, ions have low temperature compared to electrons, their thermal effects can be neglected in the equation above. Using the relations of $k_B T_e$ and Debye-Hückel length, λ_D, (eq. 6) we get the equivalent conditions for plasma being an ideal gas as:

$$\lambda_D \gg \lambda_e = \frac{1}{n_e^{1/3}} \tag{14}$$

[12]

2.1.7 Gas temperature

As has been stated above, the gas temperature of non-thermal plasmas is taken to be approximately the same as the ion temperature, $T_g \approx T_i$. This is true even though the electrons can have temperatures of several tens of thousands degrees. Since the mass of the electrons is much lower than the mass of the heavier gas components of ions and neutrals, $m_e \ll M$, the electrons do not transfer much of their thermal energy as heat to the gas component or the environment. [12]

It is in elastic collisions that particles exchange momentum, but the total kinetic energy is conserved. For example, for electron colliding with a heavier gas molecule, A, the kinetic energy transfer is:

$$e^-_{fast} + A_{slow} \rightarrow e^-_{less\ fast} + A_{less\ slow} \tag{15}$$

From the conservation of energy and momentum, the fraction of the electron kinetic energy which is transferred, k, in an elastic collision from a particle of mass m to a stationary particle of mass M, for impact on the axis of the two particles, can be derived as:

$$k = \frac{\frac{4m}{M}}{(1+\frac{m}{M})^2} \tag{16}$$

For electrons colliding with with a particle of mass $M \gg m$, the energy transferred is approximately:

$$k \approx \frac{4m}{M} \tag{17}$$

The mass of an electron is $m_e = 9.11 \cdot 10^{-31}$ kg and the mass of a proton, M_p, is around 2000 (1837) times that of an electron. For an atom of atomic number, N, the transferred energy can be expressed as:

$$k \approx \frac{4m}{NM_p} \approx \frac{1}{500N} \tag{18}$$

As an example, the atomic number of nitrogen is 7, and therefore the transferred energy is only about 0.03% of the initial electron energy. [11, Ch. 1] [13]

2.2 Plasma as a gas discharge

Electric discharge in a gas means that electric current starts to flow between two points of different electrical potential in the gas. The discharge can be temporary or permanent, and is a way of equalizing the two potentials. An example of a temporary gas discharge in nature is lightning in a thunderstorm, where there is, for an example, a difference in potential between a cloud region of excess charge and the ground. If there is a continuous supply of electrical charge the discharge is permanent, which would be the case for fabricated plasma sources.

The plasma of interest here is classified as a capacitively coupled plasmas (CCPs). In CCPs, the distance between the two electrodes resembles a capacitor of an electric circuit, thus creating a potential difference between the two electrodes when an external field is applied over the gas between two electrodes, which is usually how non-thermal plasma-forming gases are supplied with energy. The higher potential is at the positively charged anode, and the lower potential is at the negatively charged cathode. There will then be a consequent current flow between two electrodes. The anode attracts electrons and the cathode positive ions, and, the conventional current is defined to flow in the direction of the positive charge, that is, from the anode to the cathode. So both electrons and ions carry charge, however, since the electrons are much lighter than the ions they move considerably faster and carry the majority of the current. [14] [1, Ch. 3.2.3] [15]

Another type of plasmas, inductively coupled plasmas (ICPs), differ in power input. In ICPs, a current carrying coil in the plasma induces an electric field. At atmospheric pressures, inductive plasmas have higher temperature compared to capacitive plasmas, making them less interesting for medical applications. [1, Ch. 3.2.3] [15]

2.3 Plasma ignition

In the absence of an electric field, gases are electrically neutral, that is, the total charge of electrons and protons in atoms or molecules is equal. When an external field is applied there will be a difference in potential between the anode and cathode, called voltage. The voltage over the distance between the anode and cathode, the average potential gradient, is called the electric field and has the unit V/m. Like stated above, this average field will drive the ions to the cathode and the electrons to the anode. Initially, a very weak current is produced from the flow of electrons and ions created by ionization from background cosmic radiation. When the electrons are accelerated by the applied electric field they can gain enough kinetic energy to start to ionize the neutral gas molecules, *A*:

$$e^-_{fast} + A \rightarrow A^+ + e^-_{slow} + e^-_{slow} \tag{19}$$

The strength of the electric field needed increases with higher gas pressures, that is if the mean free path of the electrons gets smaller and the probability of collisions is

increased. When the free electrons have gained enough kinetic energy, they can transfer kinetic energy to orbital electrons via collisions. The orbital electrons are excited to a higher-energy orbit, and eventually have high enough energy to escape the electrostatic confinement of the nucleus, leaving a positively charged ion. The gas is then said to be ionized. The energy required to excite a molecule from its ground state to its first excited state is called the resonance energy, and the energy required to ionize a molecule is called the ionization energy. The ionization energy is far too large for electron-molecule collisions to result in ionization of the molecule. The noble gases, often used in plasma devices instead of simply air, have very large resonance energies. However, their energy levels are also metastable, meaning that de-excitation back to the ground state by radiation is difficult. They may thus hold on to the excitation energy until they experience another collision. If this next collision is with another electron, the molecule can at last be ionized, freeing an additional electron. These newly unbound electrons now join the original free electrons in further ionizing the gas by colliding with other gas molecules, creating an electron avalanche. This ionization process is often referred to as the Townsend ionization, and is visualized in Figure 2.2. These ionization processes multiples the initial electrons and the current they carry. This is the start of an electric discharge of the gas, which can be sustained if the electron multiplication is more frequent than electron loss. The main electron loss mechanisms are electron-ion recombination and electron absorption or attachment to the anode. But, recombination of ion and electron is a very difficult and improbable process. Instead, the cloud of slowly moving positive ions will at some point collide with the cathode, recombine with the surface, and sometimes release electrons from it:

$$A^+ \rightarrow A + e^- \tag{20}$$

Now the discharge produces its own electrons and becomes self-sustained. This results in an electrical breakdown, at which the whole path between the anode and cathode becomes conducting. The current can now have unlimited growth. The ions

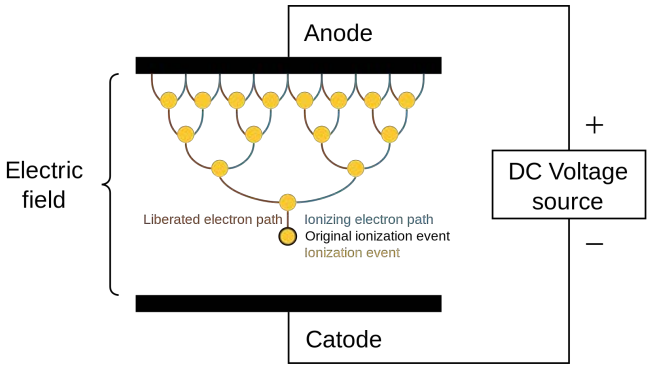

Figure 2.2: Townsend electron avalanche. Original free electron gains enough kinetic energy from the applied electric field to ionize a gas molecule. The newly unbound electron joins the original free electron in further ionization and so on. [16]

colliding with the cathode heat up its surface, which then starts to emit electrons thermionically. [12]–[14]

2.4 Plasma modes

When an electric field is applied over the gas between two electrodes, as described in above chapter, a gas discharge (basic DC glow discharge) can go through three different modes.

The first mode is called the dark discharge mode. When the applied voltage becomes high enough so that the free electrons have gained enough kinetic energy to ionize neutral atoms in the gas, the movement of the electrons and ions in the ionized gas make up another electromagnetic field, in addition to the external field, called the space-charge field. But, immediately after the discharge ignition, the number of ions and electrons is low and the discharge current and the space-charge field are weak. Because of how low the kinetic energy of the ions is and how low their concentration is, the secondary electron emission, the process of ions being accelerated to hit the cathode and release electrons from it, is low compared to electrons produced in electron-neutral collisions. Gas ionization is thus sustained mostly by the Townsend ionization in this mode. In addition, also because of the low kinetic energy of the electrons, photon emission, resulting from atoms being excited and subsequently de-excited, is infrequent and the plasma is barely visible to the naked eye, hence the name dark discharge. The discharge in this region can have a glow, if the discharge is at high pressure near points of high fields that are usually caused by small radius of curvature. The discharge is then called corona discharge.

When the external field is further increased more electrons and ions are generated, resulting in a stronger space-charge field. Since the ions and the electrons now have more energy, secondary electrons emission from the cathode gets higher, which, in addition to electrons produced in collisions, cause electrical breakdown and the voltage over the gas drops. The higher energy of the electrons also increases photon-emission, making the plasma visible. The plasma has now entered the glow discharge mode and is a self-sustaining glow discharge.

The visible appearance corresponds to the formation of a plasma structure between the two electrodes. Since the electrons are much lighter than the ions, they accelerate quicker from the cathode than the ions to the cathode, resulting in a strong electric field over a narrow region between the ions and the cathode. This is called the sheath region. At the edge of the sheath region the electrons have accelerated and have high kinetic energy, thus inducing localized photon emission. The sheath edge appears very bright and is called the negative glow. Since the electrons have lost a lot of their kinetic energy for photon emission, and of course ionization, there is much less photon emission in the region between the negative glow and the anode. This region is the plasma bulk, it is quasi-neutral and therefore has relatively low electric field.

At some point, when the voltage across the discharge increases, the cathode heating gets sufficient for it to thermionically emit electrons. The voltage across the discharge drops and there is unlimited discharge current. The gas discharge has made a transition to the third mode, the arc mode, where the discharge becomes unstable. [1, Ch. 2.3] [17]

2.5 Non-thermal plasma sources

For low temperature applications of gas plasma, the plasma reactivity is the desired function. The higher the concentration of ions and electrons, that is, the higher the discharge current, the higher the reactivity of the gas discharge. Therefore, it is desirable to operate the gas discharge in the glow mode. However, when the gas ionization is significant, its growth can get out of control and the gas discharge can go through the something called the glow-to-arc transition, where the discharge current rapidly increases, resulting in uncontrolled growth in the gas temperature. This would not be suitable for low temperature applications, and poses a dilemma regarding the reactivity and stability of the gas discharge.

2.5.1 Low-pressure plasma

Like stated earlier, one way to lower the temperature is containing the plasma gas in a vacuum chamber. With the lower gas pressure, the number of atoms available for ionization is lowered, thus reducing the number of charge carriers and the current they carry. However, the application of cold plasma in medicine requires a contact to the tissue, therefore requiring cold plasma generation at atmospheric pressure. The revolution of being able to control the plasma instabilities at atmospheric pressure was the ability to limit the growth of the discharge current.

2.5.2 Atmospheric-pressure plasma

There are mainly three ways to limit the growth of the discharge current at atmospheric pressure, using a pulsed applied voltage, introducing a current-limiting capacitor, and using very high excitation frequencies above 1 MHz.

Pulsing the applied voltage (DC) is based on the fact that plasma instabilities take time to develop. Therefore, the applied voltage needs to be reduced before the development of the plasma instabilities becomes irreversible. Since for plasmas at atmospheric pressure, the glow-to-arc transition can take place in a few to few hundreds of nanoseconds, the pulsing of the applied voltage has to take place on a shorter time scale. The pulsed voltage discharge simply resembles a pulsed glow discharge, described above for the time independent DC voltage.

In the second technique, the capacitor is in the form of one or two dielectric barriers that are attached to the electrodes, separating the plasma from the electrodes. The resulting gas plasma is therefore called dielectric barrier discharge (DBD). Displacement polarization of the dielectric creates an electric field over the plasma gas opposite to the applied field, reducing the electric field over the plasma and thus decreasing the growth of the discharge current. The mechanism is explained in detail below. The current limitation of the dielectric barrier only happens after the discharge current has reached the high levels for required plasma reactivity. When the voltage over the plasma drops, the discharge collapses. AC voltages are typically used to drive a DBD by displacement current, which is determined by the dielectric constant and the thickness of the dielectric barrier.

The dielectric barrier discharge can either be homogeneous or filamentary, depending on the discharge conditions. A filamentary discharge composes of random, individual,

narrow discharge strips called streamers. Streamer breakdown is when electron avalanche grows quickly in on direction, occurring in breakdown without the secondary electrons from the cathode. In contrast to this, in the homogeneous mode the discharge covers the entire electrode uniformly. The breakdown is driven by Townsend ionization and secondary electron emission at the cathode. Homogeneous mode is mainly observed in DBD plasmas using noble gases, where the ionization is slowed down, thus, inhibiting streamer breakdowns.

A homogeneous DBD plasma shows an intensity distribution, similar to the basic DC glow discharge, that is, a region of bright intensity near the cathode. Since a DBD is a discontinuous plasma, driven by alternating voltages allowing one breakdown per half cycle, it can be described as a glow discharge igniting once every half cycle. When a high voltage is applied (E_a), an equivalent field is established over the plasma gas (E_{gap}). Displacement polarization of the dielectric creates an opposite electric field (E_{diel}), reducing the electric field over the plasma gas (see Figure 2.3a):

$$E_{gap} = E_a - 2E_{diel}$$

If E_{gap} reaches the breakdown voltage (E_b, Figure 2.3b), a discharge ignites, with free charges flowing to the surface of the dielectric barriers and accumulate there (E_c, Figure 2.3c), reducing the electric field over the plasma (neglecting different motilities of ions and electrons):

$$E_{gap} = E_a - 2E_{diel} - E_c$$

This reduction leads to a termination of the discharge. In the next half cycle, the polarity of the applied voltage is reversed (Figure 2.3d). The charges accumulating the dielectric barriers now flow in the other direction, inducing another breakdown:

$$E_{gap} = E_a - 2E_{diel} + E_c$$

The dielectric barriers thus have a twofold role; hindering the development of continuous discharge and decreasing the required applied voltage to reignite the plasma discharge.

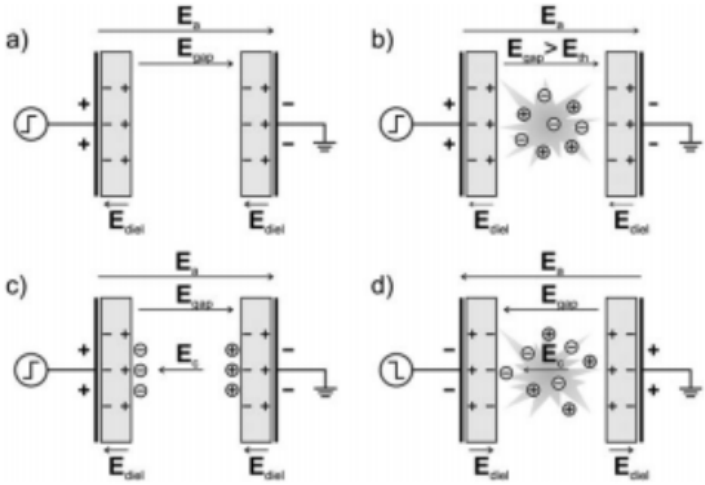

Figure 2.3: The role of the dielectric barrier in changing the polarity of the applied voltage; hindering the development of continuous discharge and decreasing the required applied voltage to reignite the plasma discharge. [15]

Both pulsed discharges and dielectric barrier discharges use ignition-collapse mechanism for limiting the current. By using high frequencies above 1 MHz, the growth of the discharge current can be limited without collapsing the discharge. Using high excitation frequency, usually radio frequency (RF), the excitation voltages are alternated with frequencies higher than the ion plasma frequency, but lower than the electron plasma frequency. Due to the high mobility of the electrons, they respond instantaneously to the electric field, while the ions remain fixed, forming a static positive space charge. And before the majority of the electrons can reach the anode, the polarity of the applied voltage changes, the anode becomes the cathode, and the direction of the electron acceleration is reversed.

These three techniques can also be combined to better control the current. For example, pulsed voltages can be used in the dielectric barrier discharge. Dielectric barriers can also be used with RF voltage, however, most RF discharges can be maintained in a stable mode without dielectric barriers by the means of the electron trapping. [1, Ch. 2.2, 2.4, 3.2.2] [15]

2.5.3 Plasma jets

In a medical application, the plasma needs to come into contact with the tissue to be treated. The plasma described in all the chapters above is confined between two electrodes, making it difficult for treatments. This problem is addressed with using a configuration called a remote plasma jet (RPJ). In the RPJ, the plasma is sustained in a tube through which the operating gas flows rapidly through, creating a plasma flux onto the tissue, and the plasma is said to be in indirect contact with the tissue. Various plasma jets have been developed, using the different current limiting techniques to sustain the plasma in a stable mode. An example of an RF-driven plasma jet is shown in Figure 2.4. The length of the plasma plume depends on the gas flow rate and the applied voltage. [1, Ch. 2.5, 3.3]

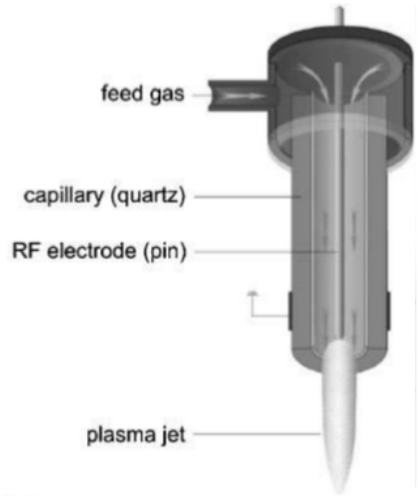

Figure 2.4: An example of RF-driven plasma jet. This plasma jet is a capacitively coupled plasma device where the operating gas flows between two co-axial electrodes. The inner electrode (pin) is excited by RF power while the outer electrode is grounded. The capillary (quartz) isolates the outer electrode from the gas. Electrons accelerated by the RF field ignite the discharge between the electrodes and the molecules of the plasma gas flow out of the nozzle. [18]

To enable treatment of larger areas, plasma jets can be arranged in arrays and ignited simultaneously. The distance between the jets is large enough for the jets not to combine but short enough for the effects of the jets to overlap. Arrays of plasma jets are often called plasma brushes. [1, Ch. 3.3.5] An example of a plasma array can be seen in Figure 2.5.

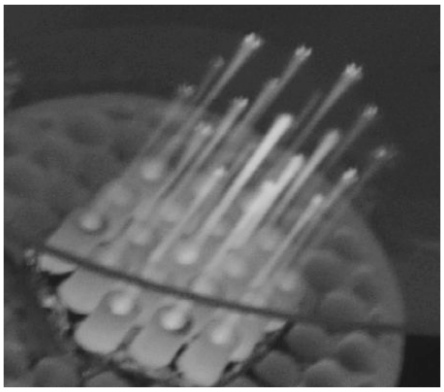

Figure 2.5: An example of plasma array. Plasma jets are arranged in arrays and ignited simultaneously to treatment of larger areas. The distance between the jets is large enough for the jets not to combine but short enough for the effects of the jets to overlap. [1, Ch. 3.3.5]

2.6 Active mechanisms in atmospheric pressure non-thermal plasma

This section lists the main active mechanisms in cold plasma in direct and indirect exposure of the sample to the plasma. How these mechanisms affect bacteria and bacterial biofilms will be discussed in later chapters.

Like stated in the above section, samples can be exposed to the plasma directly or indirectly (remotely). In direct exposure the sample comes in direct contact with the plasma and can be affected by all agents generated in the plasma, that is, heat radiation, UV radiation, visible light, electromagnetic fields, charged particles, and reactive species, see Figure 2.6. In indirect or remote exposure, the sample does not come into direct contact with the plasma, rather a gas flow produces a plasma plume from which the sample is placed at some distance. The charged particles are short-lived, lifetime up to only tens of nanoseconds, and recombine quickly before they can reach the sample in the indirect exposure, even though they have been shown effective in direct exposure by creating an electrostatic force on the cell membrane and rupture it. [1, Ch. 2.5] [1, Ch. 9.4] Heat flux and radiation flux are also reduced in the indirect exposure. Since plasma is not in contact to the sample in indirect exposure, there is no significant electric field exposure to the the sample. [19] In addition, cold plasmas do not produce high temperatures so it can be concluded that heat is not an important agent in medical treatments. Plasmas can produce UV radiation in wavelength up to 380 nm, depending on the operating gas. However, plasmas operating with air have been shown to produce very little UV radiation. [4] Also, wavelengths of UV light below 200 nm, also called vacuum UV, the most energetic wavelengths, are not able to propagate at atmospheric pressure due to absorption in air. UV radiation in plasmas operating with air can therefore be dismissed in medical treatment. However, plasmas operating with rare gases, such as argon, can produce UV radiation that can be effective in indirect exposure, the vacuum UV can reach a target at some distance. [20]

The collisions of high-energy electrons with gas atoms or molecules result in many microscopic physiochemical events generating different reactive plasma species, the electrons can dissociate, ionize, or excite the gas molecules. Following these electron-molecule reactions, ion-molecule reactions may also occur resulting in different types of reactive species, especially reactive oxygen and nitrogen species (RONS). The type and amount of reactive species in the plasma can be controlled with the choice of gas mixture. Most devices use air or noble gases (He, Ne, Ar, Kr, or Xe) with a few percent of reactive gases such as O_2, N_2, or H_2O. Since air has high percent of the electronegative O_2, it is difficult to sustain an atmospheric pressure non-thermal air plasma jet, and the hundreds of reactions are difficult to control. Thus, noble gases are preferred for the plasma generation, and O_2, N_2, or H_2O is added for controllable reactivity. However, the plasma plume will always mix with air during treatment so total control of reactions will not be possible. [1, Ch. 2.5] [3] [21, Ch. 7.1, 7.2, 7.6] Apart from the controllability factor, using air has its benefits, it is cheaper, and more convenient devices can be designed, with continuous generation of RONS.

RONS have been suggested to play the most important role of the plasma agents, especially in indirect exposure since they can be longer-lived, their lifetime can be up to several milliseconds. Reactive oxygen species (ROS) are generated from reactions involving O_2 and H_2O and reactive nitrogen species (RNS) are generated from reactions involving N_2. ROS include species like O atoms, $O_2(^1\Delta)$ (electronically excited oxygen), OH (hydroxyl radical), O_3 (ozone), and H_2O_2 (hydrogen peroxide), and RNS include species like N, $N_2(A)$ (electronically excited nitrogen), NO, and NO_2. [1, Ch. 2.5, 9.2], [3]

The main reactions from electron impact on molecules existing in operating gases are;

$$e + N_2 \rightarrow N_2^+ + e + e$$
$$e + N_2 \rightarrow N_2(v) + e$$
$$e + N_2 \rightarrow N_2^* + e$$
$$e + N_2 \rightarrow N + N + e$$

$$e + O_2 \rightarrow O_2^+ + e + e$$
$$e + O_2 \rightarrow O_2^* + e$$
$$e + O_2 \rightarrow O + O + e$$
$$e + O_2 \rightarrow O + O^- + e$$
$$e + O_2 \rightarrow O_2^-$$
$$e + O_2 \rightarrow O + O^-$$
$$e + O_2 \rightarrow O_2^+ + e + e$$
$$e + O_2 \rightarrow O_2(^1\Delta) + e$$

$$e + H_2O \rightarrow H_2O^+ + e + e$$
$$e + H_2O \rightarrow OH + H + e$$
$$e + H_2O \rightarrow OH^- + H$$

where $M(v)$ and M_x^* are the vibrationally and electronically excited states of the M_x molecule, respectively, and M_x^+ is the positive ion of a M_x molecule. The RONS can then participate in other neutral chemical reactions, producing more types of reactive species:

$$O + O_2 \rightarrow O_3$$

$$O^* + H_2O \rightarrow OH + OH$$

$$OH + O_3 \rightarrow HO_2 + O_2$$

$$H + O_2 \rightarrow HO_2$$

$$N^* + O_2 \rightarrow NO + O$$

$$N + OH \rightarrow NO + H$$

The above reactions are just an example of over hundred reactions happening in the plasma, and give a hint of the complexity of using air as an operating gas. [1, Ch. 2.5] [22]

RONS are created in the plasma gas and when they hit a sample the RONS react with it and create other species that alter the sample or its environment. [3] The effects on RONS on bacteria and bacterial biofilms will be discussed in more detail in a later relevant chapter.

Generation of RONS can also be controlled by the frequency and format of the applied voltage which determine the electron energy distribution, f(ε), the fraction of electrons with energy ε. When an electron collides with an atom or molecule the probability that it generates a specific specie is dependent on the energy of the electron, each reaction has its own energy cost. [1, Ch. 2.5] That is, for shorter voltage pulses and higher peak voltage, more energetic electrons are generated and can trigger reactions that have higher energy threshold. [23]

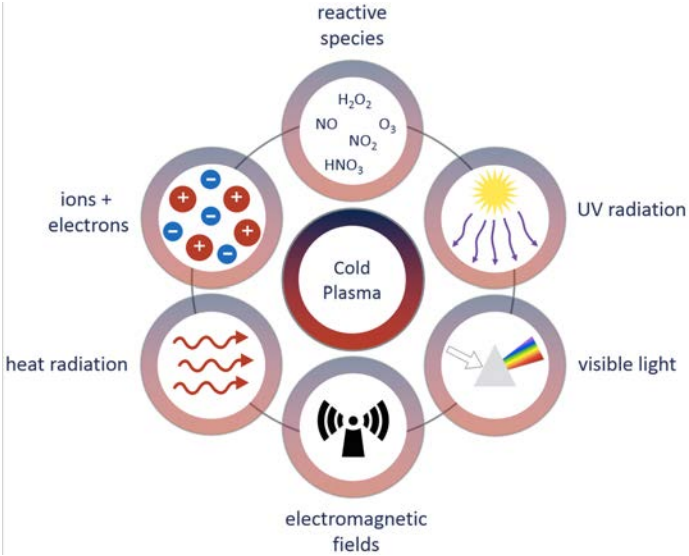

Figure 2.6: The active mechanisms in plasma. In direct exposure the sample comes in direct contact with the plasma and can be affected by all agents generated in the plasma, that is, heat radiation, UV radiation, visible light, electromagnetic fields, charged particles, and reactive species. In indirect or remote exposure, the sample does not come into direct contact with the plasma, rather a gas flow produces a plasma plume from which the sample is placed at some distance, eliminating all factors exept for the reactive species and UV radiation. [24]

3 Bacteria

This chapter presents the biological part relevant to later presented experiments. It comprises a description of the bacterial cell, their distinction into gram-negative and gram-positive bacteria, their metabolism, and their arrangement into biofilms, especially the biofilm formation on medical devices.

3.1 Bacterial cells

Bacteria are one type of prokaryotes, simple unicellular organisms with no cell nucleus or other membrane-bound organelles, as opposed to eukaryotes. Most bacteria have a size of the order of one micron, and the shape of rods, spheres, and spirals. The bacteria chromosomal DNA is a single loop that forms an irregular structure in the cytoplasm called the nucleoid. In addition, bacteria can also carry plasmids, that is small circular DNA molecules providing additional genes that are not essential to the bacteria, but can benefit their survival in harsher environments, it can for example make them resistant to antibiotics. All bacterial cells have a cell membrane in the form of a lipid bilayer. The membrane is a barrier between the cell interior, called cytoplasm, and the external world, and molecules can selectively be transported across it. In addition to the lipid membrane, bacterial cells have a cell wall that protects the cell from pressure caused by osmotic flow of water into the cell. [1, Ch. 4.3] [4]

3.2 Gram-positive and gram-negative bacteria

Based on the structure of their cell envelope, bacteria can be distinguished into two groups: Gram positive and gram negative. Gram-positive bacteria have a thick cell wall covering the inner lipid membrane, and gram-negative bacteria have a thinner cell wall, and, in addition to an inner lipid membrane, they have an outer membrane covering the cell wall. The cell envelope of all bacteria has a net negative charge. In the case of gram-positive bacteria, the thick cell wall is made of layers of peptidoglycan, and threading these layers are long negatively charged polymers, called teichoic acids. For gram-negative bacteria, the outer membrane, surrounding the thin peptidoglycan cell wall, contains negatively charged lipopolysaccharides. The structural difference of gram-positive and gram-negative bacteria can be seen in Figure 3.1.

Gram-positive bacteria can be in either vegetative state or the spore state, while gram-negative bacteria can only be in vegetative state. When environmental conditions are favorable, cells are in the vegetative state and can uptake nutrient, divide, exhibiting logarithmic growth (very fast in the beginning when they have space and nutrients), and form colonies. Under harsh and unfavorable conditions, such as depletion of nutrients, acidic pH or unfavorable temperature, the first response of bacteria, gram-positive and gram-negative, is to transfer to another environment. Bacteria can move using gas vesicles, or, more commonly, using a long, thin, hair-like organelles called flagella. In the state of starvation, the next response can be synthesis of antibiotic substances in nutrient competition with other organisms, or synthesis of enzymes for access to more difficult nutrient sources. The ultimate response for gram-positive

bacteria is to form spores which can overcome extremely harsh conditions. When the conditions get favorable again, the spores can revert to vegetative cells. [1, Ch. 4.2, 4.3, 4.4, 4.5] [4] [25]

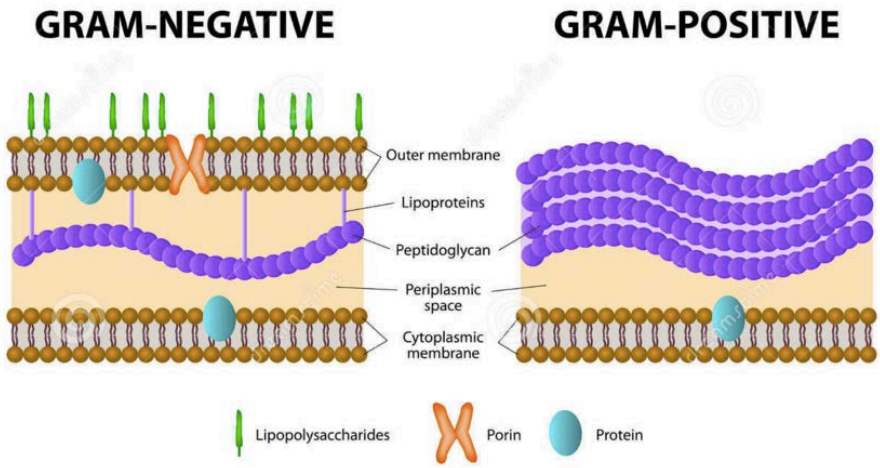

Figure 3.1: Based on the structure of their cell envelope, bacteria can be distinguished into two groups: Gram-positive and Gram-negative. Gram-positive bacteria have a thick cell wall made of layers of peptidoglycan covering the inner lipid membrane, and gram-negative bacteria have a thinner cell wall of peptidoglycan, and, in addition to an inner lipid membrane, they have an outer membrane containing lipopolysaccharides covering the cell wall. [26]

The effective dielectric constant of both gram-negative and gram-positive bacteria has recently been investigated, the effective dielectric constant, or relative permittivity, of a material being the ratio of its permittivity to the permittivity of free space. Under dry air conditions (room temperature and $< 1\%$ relative humidity) the effective dielectric constant was found $\varepsilon_{r,eff} \sim 3-5$ for both gram-negative and gram-positive bacteria. Under ambient condition (room temperature and $> 30\%$ relative humidity), it increased to $\varepsilon_{r,eff} \sim 6-7$ and $\varepsilon_{r,eff} \sim 15-20$ for gram-negative and gram-positive bacteria, respectively. The effective dielectric constants were also shown to depend on the gram-type of the bacteria rather than the bacterial type itself. The results under dry air conditions correlate with the expected dielectric response from the bacteria's biochemical constituents, such as lipids, proteins, and nucleic acids. Also, the results under ambient conditions suggest that the moisture has a larger impact in the gram-positive bacteria due to their thicker and more hydrophilic peptidoglycan cell wall, the much higher dielectric constant of water ($\varepsilon_r = 81$) thus contributes significantly to the dielectric response of the gram-positive bacteria. [27]

3.3 Bacterial metabolism

Bacterial metabolism is all the biochemical reactions occurring in the cell. Metabolism comprises two complementary processes, catabolism and anabolism. Catabolism creates chemical energy by oxidizing substrates, where the energy is stored in ADP and ATP as high-energy phosphate bonds, and anabolism uses that energy to synthesize building material needed by the cell. From a metabolic viewpoint, bacteria can be categorized into three physiologic types: heterotrophs (or chemoorganotrophs), autotrophs (or chemolithotrophs), and photosynthetic bacteria (or phototrophs).

All pathogens, that is bacteria that can cause infection, are heterotrophic bacteria, and therefore heterotrophic bacteria will be the main focus here. Heterotrophic bacteria obtain energy from oxidation of organic compounds, such as carbohydrates (particularly glucose), lipids, and protein. This process also results in the generation of simpler organic compounds needed for the biosynthesis of complex organic compounds. In contrast, photosynthetic bacteria use sunlight as an energy source to reduce carbon dioxide (CO_2) to glucose, and autotrophs oxidize inorganic compounds, without using sunlight, to create energy for CO_2 reduction. The glucose is then used to synthesize complex organic cellular matter.

The preformed organic compounds can be oxidized aerobically or anaerobically. In aerobic respiration, O_2 is used as the terminal acceptor of electrons. Bacteria exhibiting anaerobic respiration use specific chemical components other than O_2 as a terminal electron acceptor. The complete oxidation of organic compounds (usually carbohydrate), resulting primarily in CO_2 and H_2O, is called respiration. The glucose oxidation reaction can involve three biochemical pathways: The glycolysis, the Krebs cycle, and electron transport.

In glycolysis the glucose is split, which is exactly what the word glycolysis means. One molecule of glucose is split into two molecules of pyruvate (pyruvic acid). Two molecules of ATP are needed to initiate the glucose splitting. However, the glycolysis results in four molecules of ATP, so the net gain is two ATP molecules. Also, high-energy electrons are also transferred to NAD^+ to produce NADH, which also carries energy. The Krebs cycle starts with the two pyruvates from the glycolysis. In the Krebs cycle, all six carbon atoms of the glucose molecule are combined with O_2 and form CO_2, and the total energy is now stored in four ATP molecules, as well as high-energy electrons have been transferred to form 10 NADH molecules and two $FADH_2$ molecules. The glycolysis and Krebs cycle is performed in the cytosol, and the electron transport occurs over the lipid membrane of the cell. The high-energy electrons in NADH and $FADH_2$ are released and move along a series of membrane bound carrier molecules, called the electron transport chain, ultimately to a final electron acceptor. The energy of the electrons is used to pump protons (H^+), from NADH and $FADH_2$) across the membrane, creating a gradient of ion concentration across the membrane. The higher concentration of protons outside the cell causes them to flow back into the cytoplasm, to their lower concentration. They flow back through a channel protein (ATP synthase) which utilizes their flow to act as an enzyme in the synthesizing of ATP from ADP and inorganic phosphate. In total can up to 38 molecules of ATP be produces from one molecule of glucose, making a net gain of 36 ATP molecules. [28, Ch. 4]

3.4 Bacterial biofilm

Bacteria can attach to surfaces using hair-like surface structures called pili, allowing them to attach to surfaces. Indeed, in natural settings, bacteria are most often found in a surface-attached biofilm structure, rather than floating isolated planktonic cells. Despite the fact that the way bacteria form biofilms varies enormously depending on species and environmental conditions, there are features that are common to all biofilms. The floating planktonic cells adhere to the surface and form an initial monolayer. They then replicate and differentiate, that is change their morphology, to

form multilayered micro-colonies. An extracellular matrix comprising exopolysaccharides (EPS), proteins, and even nucleic acids, is produced to hold the cells in the biofilm together, resulting in the maturation of the biofilm. The biofilm formation protects bacteria from hostile environmental agents, even making the bacteria resistant to antibiotics. The biofilm development is ignited by environmental and self-produced extracellular signals. [29, Ch. 1.2]

Among the environmental signals affecting the biofilm development are temperature, pH, O_2 levels, hydrodynamics, osmolarity, the presence of specific ions, nutrients, and factors derived from the biotic environment. [28, Ch. 1.2] The intercellular signaling in the biofilm development and maintenance, such as recruitment of bacteria to the biofilm, the formation of the extracellular matrix, and the detachment of bacteria from the biofilm, is called quorum sensing (QS - Figure 3.2), and it works as a secretion of small molecules that can be self-recognized to coordinate cell behavior. That is, when the molecule concentration reaches a threshold value in the environment, the molecules can diffuse into the cells to participate in gene regulatory processes, controlling the expression of specific genes. [1, Ch. 6.2.1] [29, Ch. 4]

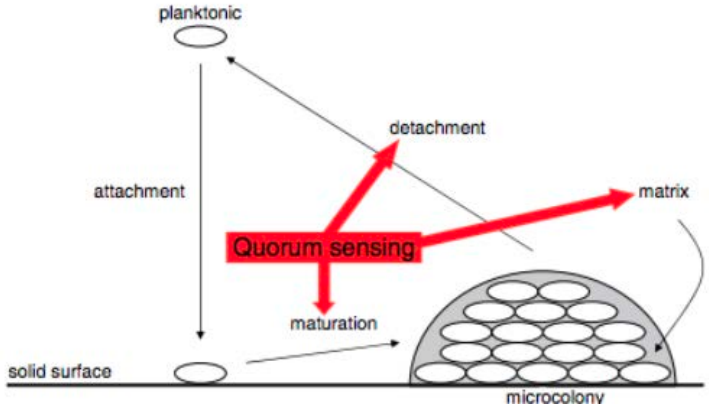

Figure 3.2: Quorum sensing is the intercellular signaling in the biofilm development and maintenance, such as recruitment of bacteria to the biofilm, the formation of the extracellular matrix, and the detachment of bacteria from the biofilm. It works as a secretion of small molecules that can be self-recognized to coordinate cell behavior. That is, when the molecule concentration reaches a threshold value in the environment, the molecules can diffuse into the cells to participate in gene regulatory processes, controlling the expression of specific genes. [29, Ch. 4]

As has been mentioned, bacterial biofilms are highly resistant to antibiotics, from 10 to 1000 times more resistant than planktonic bacteria. This resistance has been shown to be a mixture of two genetic mechanisms: innate and induced resistance. Innate resistance results from the development of the biofilm, that is the biofilm's structure and physiology hinder diffusion and penetration of antibiotics into the biofilm matrix, decrease oxygen and nutrient diffusion and availability in the biofilm matrix resulting in altered metabolism, and increase the formation of persisters, that is cells that tolerate antibiotic treatment without being antibiotic resistant mutants. On the other hand, induced resistance is a resistance that is induced in the biofilm by the antibiotics themselves, that is, the bacteria can adapt genetically to the antibiotics. The two resistance mechanisms have been suggested to work together in the way that even though it is possible for antibiotics to diffuse through the biofilm matrix, their diffusion is sufficiently slowed down by the matrix so that the antibiotic concentration is smaller inside the biofilm, which gives the interior bacteria time to adapt genetically and become resistant. However, there are also cases where antibiotics can easily diffuse through the biofilm matrix without being slowed down. In that case, the

innate mechanism of altered microenvironments, for example oxygen and nutrient limitation, decreases the bacterial metabolic activity resulting in slower growth rates of the interior bacteria, which protects the bacteria since antibiotics usually act upon rapidly growing bacteria. Figure 3.3 demonstrates the gradient of the diffusion of antibiotics, and consequently the different gene expressions of cells, and the micro-environmental gradient, resulting in different growth rates of cells. [29, Ch. 5]

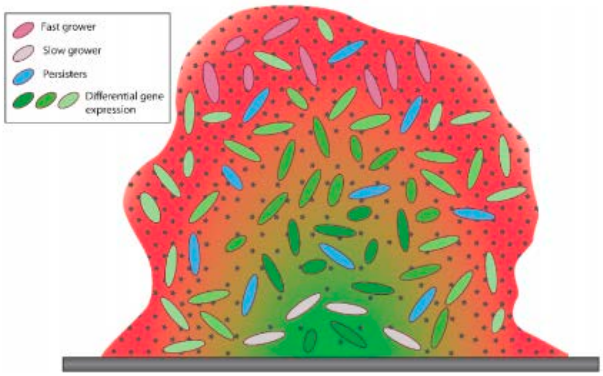

Figure 3.3: The mixing of innate and induced antibiotic resistance mechanisms in a bacterial biofilm made up of bacteria surrounded by an extracellular matrix. The small dots represent antibiotics present in the biofilm and the dot's concentration represents the diffusion gradient of the antibiotics through the biofilm matrix. The different shades of green bacteria represent the activation of resistant genes, the darker green bacteria having had more time to adapt to the antibiotics because of their slowed down diffusion. The red to green gradient represents the gradient of different microenvironments, red being high oxygen and nutrient concentrations and green the opposite, resulting in a gradient of the slower growth rates of the innermost bacterial cells. Blue bacterial cells represent persisters that survive the present of antibiotics. [29, Ch. 5]

Even if no bacteria in the biofilm have time to adapt genetically to the antibiotics, persisters tolerant to antibiotics will form, also shown in Figure 3.4. After the antibiotics have killed most of the biofilm cells, the immune system cleans up the planktonic persisters, but is unable to kill persisters that are protected by the exopolysaccharide matrix. After the antibiotic concentration drops, the persisters can regrow the biofilm, and the infection relapses. Killing the persisters is thus the last step in curing infections. [29, Ch. 6]

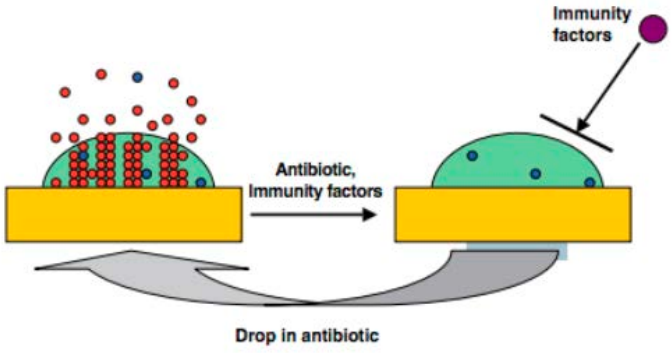

Figure 3.4: Biofilm resistance based on persister survival. Treatment with antibiotics kills the planktonic cells outside the biofilm and most of the cells inside the biofilm matrix, leaving only persisters. The immune system then kills the planktonic persisters, but is unable to kill the persisters that are protected by the exopolysaccharide matrix. After the antibiotic concentration drops, the persisters can regrow the biofilm and the infection relapses. [29, Ch. 6]

3.5 Catheter biofilm formation

Healthcare-associated infections (HAI) are infections acquired while other medical conditions are being treated, and are caused by various infectious agents from either endogenous or exogenous sources, 90% of the infectious agents being bacteria.[30, Ch. 14]

The largest contributor to healthcare-associated infections is biofilm formation on medical devices, three out of four most common infection sites on the body are sites common for medical devices, the urinary tract, respiratory tract, and bloodstream, leaving out only surgical wound sites. In fact, 95% of urinary tract infections are associated with urinary catheters. [7] And, 70-80% of these are attributed to the use of an indwelling urethral catheter. [31]

Indwelling urinary catheters are urinary catheters that are left in the bladder, and are standard medical devices used to relive urinary retention. The most common indwelling urinary catheter is the so called Foley catheter, were the catheter is inserted into the bladder through the urethra. The catheter can also be inserted through a small hole in the belly. A Foley catheter is a soft, flexible tube made of plastic or rubber. It has two separated channels, one that is open in both ends and drains the urine to a drainage bag, and one that has a small balloon on one end that is inflated with sterile water to prevent the catheter from sliding out of the bladder. [32] Figure 3.5 is a schematic picture of a Foley catheter.

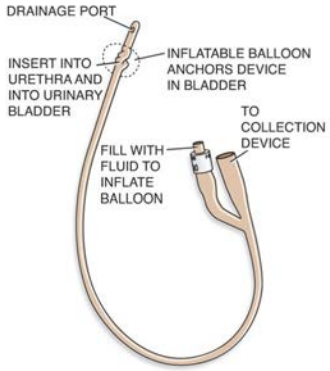

Figure 3.5: Foley catheter. Indwelling urinary catheters are urinary catheters that are left in the bladder, and are standard medical devices used to relive urinary retention. The most common indwelling urinary catheter is the so called Foley catheter, were the catheter is inserted into the bladder through the urethra. A Foley catheter is a soft, flexible tube made of plastic or rubber. It has two separated channels, one that is open in both ends and drains the urine to a drainage bag, and one that has a small balloon on one end that is inflated with sterile water to prevent the catheter from sliding out of the bladder. [32] [33]

Duration of the catheterization is the most determinant factor of the infection, as a biofilm starts to form immediately after the catheter is inserted, both on the interior and exterior catheter surfaces, and the chance of resistance increases with the age of the biofilm. [31]

The most common origination of bacteria is the periurethral area or the drainage bag, although microorganisms, from the patient's skin or the hospital environment, can be introduced to the bladder following catheter insertion. The most common infecting bacterium in urinary catheters is *Escherichia coli*. Figure 3.6 shows the process of a biofilm formation on urinary catheters. Immediately after the catheter is inserted a film of host cell surface and extracellular matrix components, such as proteins, glycoproteins, glycolipids, and carbohydrates, forms along the catheter surface.

Bacteria enter the bladder and recognize and attach to the host cell components, forming a monolayer. Bacteria replication and recruitment build up micro-colonies that eventually mature into biofilms. During the development of the biofilm, the bacteria cells communicate by quorum sensing, regulating detachment of cells when the concentration of bacteria cells in a biofilm reaches a threshold. These daughter cells can then seed other sections of the catheter. [31]

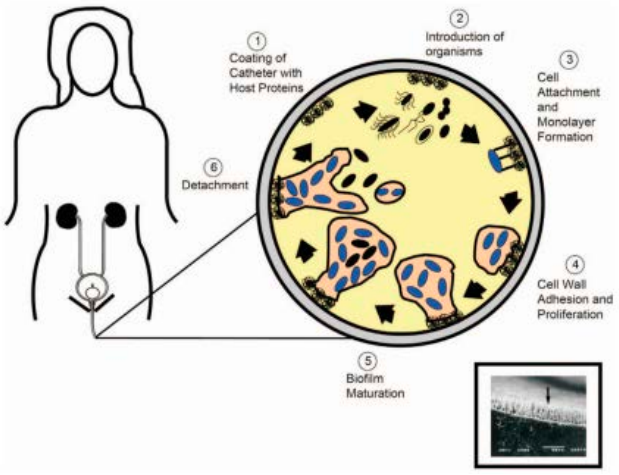

Figure 3.6: The process of biofilm formation on urinary catheters during catheter-associated urinary tract infection (CAUTI), and a SEM of a biofilm on a urinary catheter. 1) Host cell surface and extracellular matrix components such as proteins, glycoproteins, glycolipids, and carbohydrates attach to the catheter surface. 2) Micro-organisms enter the bladder extraluminally or intraluminally. 3) Bacterial adhesins recognize specific host cell components on the catheter surface, and attach to them, forming a monolayer. 4) The bacteria replicate and form micro colonies. 5) Bacterial micro-colonies eventually mature into biofilms. 6) During the development of the biofilm, the bacteria cells communicate by quorum sensing, regulating detachment of cells when the concentration of bacteria cells in a biofilm reaches a threshold. These daughter cells can then seed other sections of the catheter. [34]

3.6 Effects of cold plasma on bacterial cells

Like stated earlier (Section 2.6), reactive oxygen and nitrogen species are probably the main active agents of cold plasma, even though many of the different mechanism may act simultaneously to achieve maximum results. However, in indirect exposure of the plasma, all mechanisms other than UV radiation and RONS have been eliminated, and, in the case of operating with compressed air, even UV radiation can be eliminated.

The fact that RONS are created by cells and released extracellularly in inflammatory response to infection, tumors, and wounds supports the claim that RONS must be the main active mechanism in cold plasma. That is, RONS are believed to be a part of an intracellular signalling pathway. This class of RONS are called primary species and they are well controlled in the cell and their reactions with biomolecules are reversible. The signalling mechanisms include modifications of the intracellular redox state and proteins in signalling pathways by oxidation. Another class of RONS are secondary species and they are toxic and their reactions with biomolecules cause irreversible damage. The primary RONS include superoxide, hydrogen peroxide, and nitric oxide, and the secondary RONS include hydroxyl and peroxynitrite ($ONOO^-$). The secondary species only form in the cell if primary species react with one another

or a transition metal. The formation of hydroxyl from hydrogen peroxide is catalysed by iron ions and is called the Fenton reaction. Interestingly, in a reaction causing oxidative damage to pathogen membranes upon immune response, called lipid peroxidation, the cleavage of lipid peroxides is catalysed by ferrous ions, which is similar to the Fenton reaction. Another way of forming hydroxyl is through the Haber-Weiss reaction, where O_2^- is converted into H_2O_2 which is then converted to OH. Peroxynitrite is formed in a reaction between superoxide and nitrogen oxide. The reaction between peroxynitrate and amino acids result in the formation of nitrated amino acids which causes oxidative damage to proteins. [3], [35], [36] So, RONS generated in cold plasma can both act as a immune response (primary RONS) and a highly reactive damaging species (secondary RONS).

RONS in cold plasma have been reported to have oxidative effects on the outer structures of the cells, that is on the membrane and the cell wall, affecting their structural integrity. Ozone interferes with cellular respiration, OH (hydroxyl radical) compromises the function of unsaturated fatty acids in the lipid membrane, H_2O_2 (hydrogen peroxide) has oxidative effect on proteins, lipids, and DNA, and atomic oxygen, metastable oxygen molecules, and superoxide (O_2^-) can oxidize proteins. [1, Ch. 9.2] However, the way RONS affect cells depends on their concentration. For an example, H_2O_2 (hydrogen peroxide) can stimulate cell proliferation or inhibit it and induce cell apoptosis and Nitric oxide (NO) acts as an anti-inflammatory agent at low concentration, but at higher concentration it can damage cells.[36] When the concentration of RONS are overwhelming to the cell, it is said to be in a state of oxidative stress, damaging lipids, protein, and DNA. [37]

As for the metabolic part, ATP (Adenosine triphosphate), the molecule used as the energy currency of life, is the indicator of the metabolic activity in cells and can be used to monitor how bacterial cells in a biofilm respond to the stress that follows a cold plasma exposure. For short exposure times, bacteria have been shown to try to cope with the stress by either increasing respiration and therefore the ATP production or to uncouple the ATP production from respiration. [6]

In a liquid environment, RONS have been shown to cause acidification and thus inactivation of suspended bacteria. [38]

This effects of RONS on bacterial cells, and cells in general, are not fully understood, however, it is clear that the cellular regulation by RONS is a complex and diverse process.

4 Computer simulation

An application to simulate a capacitively coupled plasma (CCP) [39][22], provided in the plasma module in the Comsol Multiphysics® software, was used to gain a better understanding of the physics in such a plasma. Physical properties of the plasma are visualized in 1D at different times over one RF cycle. The 1D geometry is over a 2.54 cm plasma region and, in the figures below, and is termed arc length.

This application uses argon gas because of its simple plasma chemistry, for which a plasma gas at low pressure (1 torr) well demonstrates the behavior of certain plasma characteristics, such as, electron and ion densities, electron temperature, and electric field, where the gas temperature is around room temperature (300K). The clear visualization effect was lost as the pressure of the gas was increased. However, the electron and ion densities in atmospheric pressure plasmas are not very high and comparable to low pressure plasmas (less than 10^{16} m^{-3}) [40]. The application uses a potential of 300 V at a frequency of 13.56 MHz to drive the plasma.

Figure 4.1 shows how the electron density in the plasma builds up, in the process of the gas ionization described in above chapters, and oscillates with the applied electric field. It can be seen that most of the electrons are gained around the middle of the gas gap, called the plasma bulk, where other unbound electrons have gained enough kinetic energy from the applied electric field to free new electrons from the gas molecules. It can also be seen how the electrons drift towards one electrode, in the direction opposite to the direction of the electric field, and how their direction changes after one half of the RF cycle.

The electron temperature (kinetic energy) at different times of the RF cycle is visualized in Figure 4.2. It both shows how the energy of the free electrons oscillates with the applied electric field and how the free electrons gain and lose kinetic energy over the gap. Their kinetic energy increases when they start to accelerate from one of the electrons and when approaching the middle of the gap their kinetic energy drastically decreases due to the events of freeing new electrons from the gas molecules.

The fact that the ions are much heavier than the electrons, they do not respond to the applied electric field as quickly as the electrons so the ion density remains stationary over one RF cycle, as can be seen in Figure 4.3. This creates a separation of charges in the sheath region near the electrodes, which in turn produces an electric field. This charge separation only occurs in the sheath regions so the plasma bulk is quasi-neutral and therefore has a relatively low electric field.

The changes in the electric field over one RF cycle can be seen in Figure 4.4: The electric field over the plasma gap at different times of the RF cycle, the x component represents the electric field in the direction perpendicular to the electrodes, that is on the 1D geometry. Because of the charge separation occurring in the sheath regions but not in the plasma bulk there is only an electric field in the sheath regions, which oscillates with the applied electric field over the RF cycle.

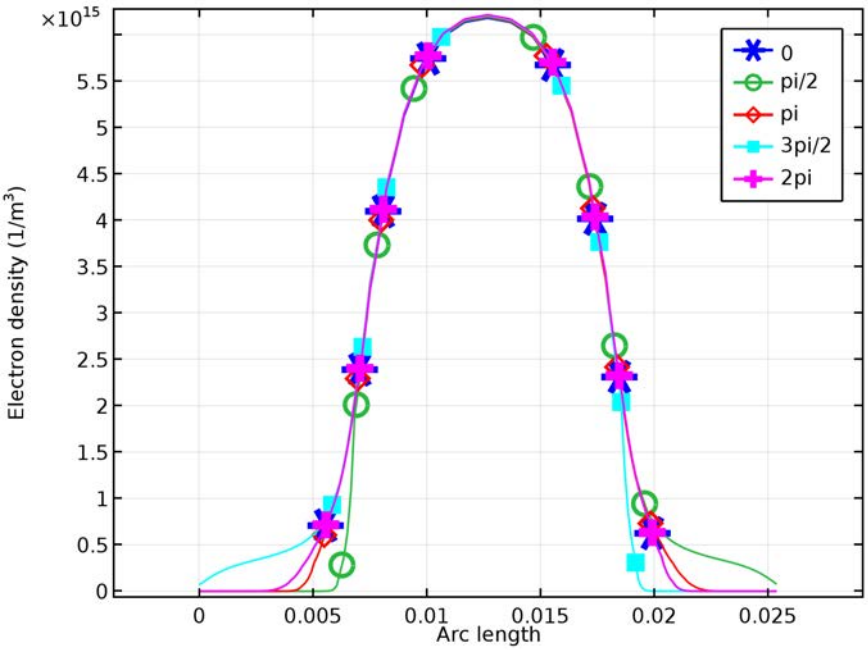

Figure 4.1: The electron density over the plasma gap (arc length) at different times of the RF cycle. The electron density is highest in the plasma bulk since there the free electrons in the plasma have been accelerated to a sufficient high kinetic energy to free more electrons from the gas molecules. In addition, the electron density in the sheath region oscillates with the applied electric field.

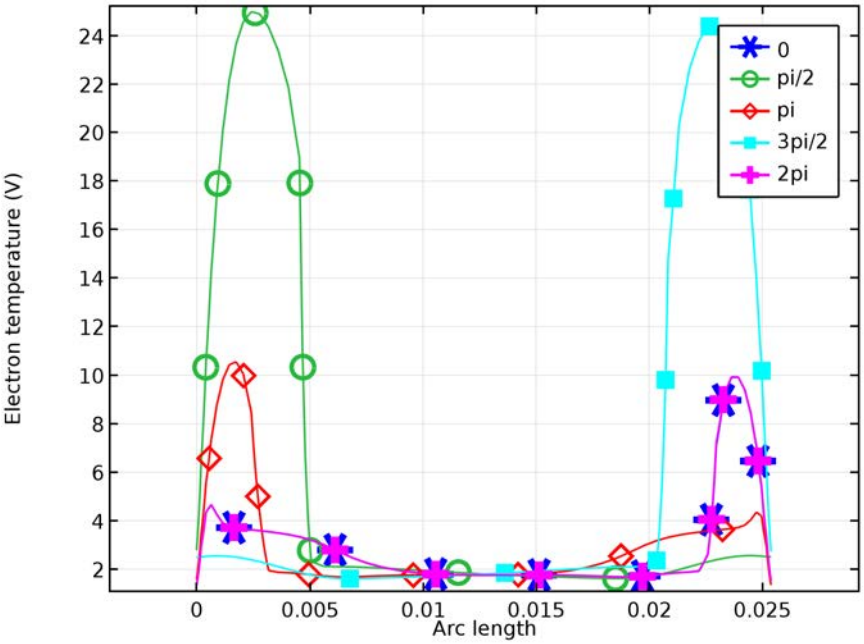

Figure 4.2: The electron temperature over the plasma gap at different times of the RF cycle. The temperature is highest in the sheath regions since there the electrons gain kinetic energy which they again lose in the bulk region due to collisions leading to ionized gas molecules or molecules in an excited state. Also, the temperature is the highest at times pi/2 and 3pi/2 in the RF cycle which correspond to the times right before the applied electric field changes direction.

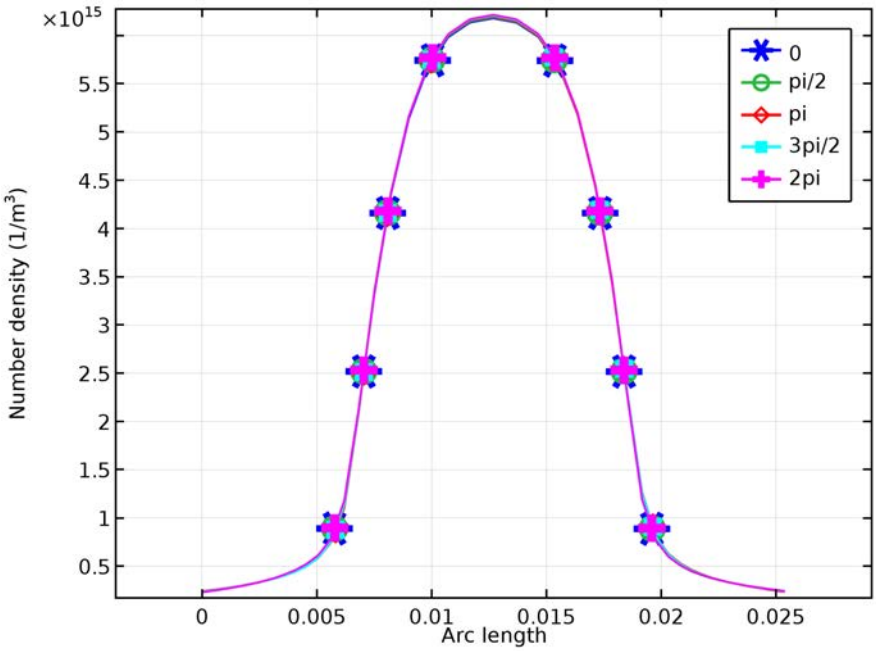

Figure 4.3: The ion density over the plasma gap at different times of the RF cycle. The ions are much heavier than the electrons so they do not respond to the applied electric field as quickly and the ion density remains almost stationary over one RF cycle.

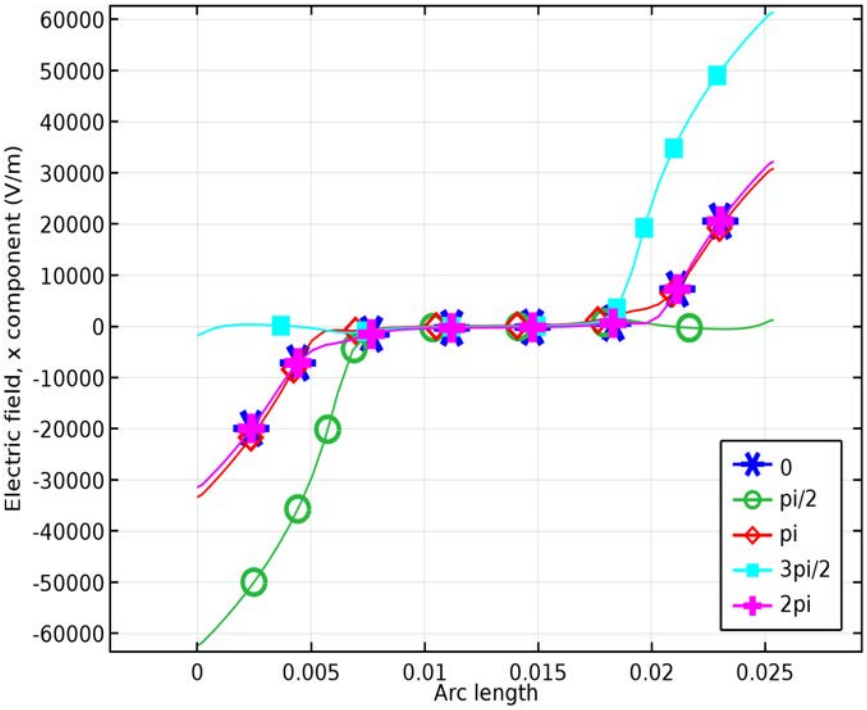

Figure 4.4: The electric field over the plasma gap at different times of the RF cycle, the x component represents the electric field in the direction perpendicular to the electrodes, that is on the 1D geometry. Because of the charge separation occurring in the sheath regions but not in the plasma bulk there is only an electric field in the sheath regions, which oscillates with the applied electric field over the RF cycle.

5 Experimental work

The antibacterial activity of the cold plasma against 48-hour biofilms of two gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*, and two gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus epidermidis*, is evaluated with colony forming units (CFU) counting, live/dead fluorescent staining, and scanning electron microscope (SEM). It was decided to use a fixed operating distance with various exposure times. The number of CFU after cold plasma exposure is used as a quantitative assessment of the activity of the cold plasma while the live/dead fluorescent staining is used as a qualitative conformation of the activity. The morphological changes of the bacteria are examined using SEM. In addition, the size of the cold plasma exposure area is determined by exposing planktonic *S. epidermidis* bacteria on agar plates.

The approach of CFU counting only takes into account viable cells but not cells that might be alive after the cold plasma exposure, but non-cultivable. This motivates the incorporation of the live/dead fluorescent staining. [6]

This study used the commercially available CE certificated RF atmospheric pressure plasma jet kINPen® 11 (neoplas tools GmbH, Greifswald, Germany), seen in Figure 5.1. The discharge is generated with a radio frequency of 5 kHz and electric voltage of 2-3 kV. [41] The plasma pen was operated with compressed air of 2.5 bar pressure and a 4.5 SLM (Standard Liters per Minute) gas flow.

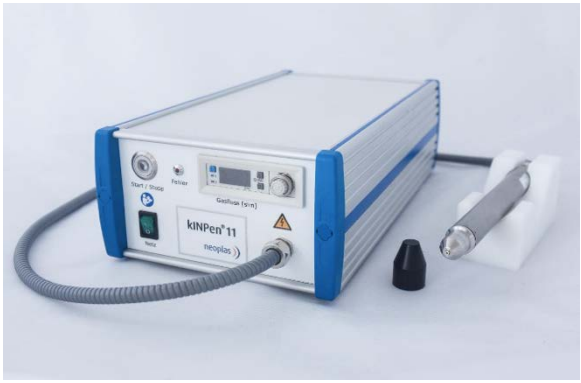

Figure 5.1: The commercially available CE certificated RF atmospheric pressure plasma jet kINPen® 11 (neoplas tools GmbH, Greifswald, Germany) used in the study. [41]

5.1 Bacteria characterization and biofilm cultivation

Escherichia coli is a versatile bacterium. As well as being important in the human intestinal microflora, it can also be a deadly pathogen, where urinary tract infection is the most common infection. [42]

Pseudomonas aeruginosa is a gram-negative human pathogen which has been described as opportunistic, it seldom infects healthy individuals, however, it can be held accountable for many hospital-acquired infections, especially of chronic wounds, and is highly problematic for patients with cystic fibrosis. [43] It can survive in many different environments and rather easily develops high resistant to antibiotics, making it clinically significant. [44]

Bacillus subtilis is a gram-positive non-pathogenic bacterium. It is found in soil as well as in the gastrointestinal tracts of humans, where it is beneficial. It is the best studied gram-positive bacterium and considered a model organism. [45]

Staphylococcus epidermidis is a gram-positive bacterium and a part of the normal skin flora in humans. This bacterium is also seen as an important opportunistic pathogen and, due to its permanent colonization on the human skin it is highly probable to contaminate indwelling medical devices during insertion. Even though these infections are rarely life-threatening, they are extremely difficult to treat, due to biofilm formation and antibiotic resistance. [46]

Bacterial biofilms were grown on 15 mm glass coverslips. Briefly, bacterial colonies were inoculated on the respective media and incubated overnight to prepare the bacterial inoculum. Herein, *E. coli* and *B. subtilis* were grown in LB broth and *P. aeruginosa* and *S. epidermidis* were grown in tryptic soy broth. Overnight grown bacterial suspensions were diluted to make 2-5$\times 10^6$ CFU/ml and 150 µl of diluted bacterial suspension were loaded on the glass coverslip and incubated for 24 hours at 37°C without agitation. After the 24 hour of incubation, the old medium was replaced by fresh medium and again incubated for 24 hours, resulting in 48-hour mature biofilms ready for treatment.

5.2 Evaluation of biofilms

5.2.1 Colony forming units

The viability of bacteria in the biofilms after the cold plasma exposure was analyzed by a normal plating method. The bacteria from the biofilm samples were collected from the glass coverslip and plated on agar plates for colony counting. The glass coverslip was put in a 5 ml NaCl (0.89) solution and this solution sonicated for 10 seconds to release all the bacteria from the glass coverslip, resulting in a homogenous bacterial NaCl solution. 100 µl from the homogenized suspension were diluted serially and plated on respective agar plates and incubated in a 37°C incubator. The number of colonies was then counted and the total number of CFU in 5 ml NaCl calculated, giving an estimate of the number of bacteria surviving the cold plasma treatment.

5.2.2 Live/dead fluorescent staining

Live/Dead BacLight Bacterial Viability test visualized with a confocal laser scanning microscope measures the membrane integrity of the bacterial cells by utilizing a mixture of two competing fluorescent dyes, one that is able to penetrate intact and damaged bacterial cells and the other is only able to penetrate damaged bacterial cells. When properly mixed, bacteria with intact cell membranes get green fluorescent staining while bacteria with damaged cell membranes get red fluorescent staining.

Cold plasma treated 48-hour biofilms were stained at room temperature in the dark for 20 min using 6.0 µM SYTO 9 and 30 µM potassium iodide (Live/Dead BacLight bacterial viability kit L13152, Invitrogen, Molecular Probes, Inc. Eugene, OR, USA). Fluorescence microscopic imaging of the biofilms was performed using Leica

fluorescence microscope. The excitation wavelengths for SYTO 9 and potassium iodide are 488 and 543 nm, respectively.

5.2.3 Scanning electron microscopy

In order to use scanning electron microscopy on biological samples they have to be preserved to handle the high vacuum environment that is necessary for the electron beams, both the primary beam and the second or backscattered electrons that are emitted from the sample generating the image. The steps required are stabilization of the biological structures by crosslinking with an optimal fixative, followed by a dehydration of the specimen by a method called critical point drying or by using a chemical dehydrant, and, finally, coating the specimen with a thin metal layer to ensure conductivity of the specimen for minimal damages and improved topological contrast. [47]

Here, cold plasma treated biofilms were fixed for 2 h in 3% glutaraldehyde solution and dehydrated with 30, 40, 50, 60, 70, 80, 90% of ethanol for 15 minutes each and with 100% ethanol for 20 min. All the dehydrated biofilms were dried in room temperature and coated with gold before SEM imaging.

5.3 Treatment

The experimental setup can be seen in Figure 5.2.

To begin with, the operating distance between the nozzle of the cold plasma device and the sample had to be determined for the rest of the experiments. 48-hour *E. coli* biofilms were exposed for 5 minutes at distances 5, 7, 9, 11, and 15 mm, two samples for each of the distances.

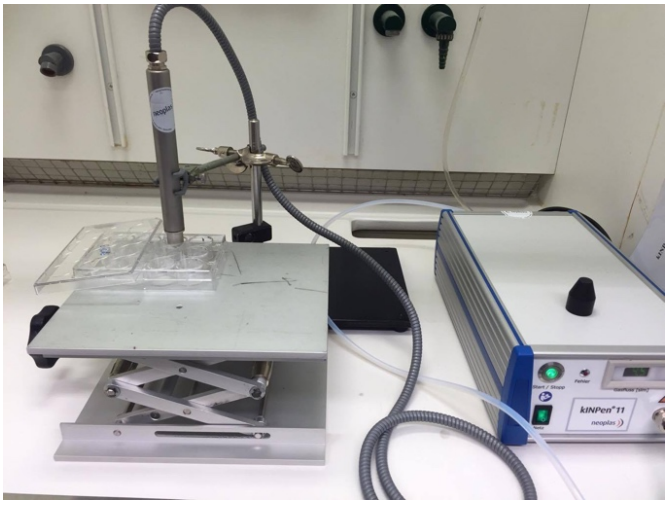

Figure 5.2: The experimental setup. The kINPen® 11 handheld device is held at a fixed position and the sample is moved to a desired location by moving the z-table.

To get an idea of the exposure area of the cold plasma gas flow, *S. epidermidis* planktonic bacteria was spread out on agar plates and exposed for 1, 2, 5, and 10 minutes.

For the main part of the experiments, 48-hour biofilms of *E. coli*, *P. aeruginosa*, and *S. epidermidis* were exposed with cold plasma for 5, 10, 15, 30 and 60 minutes, and 48-hour biofilms of *B. subtilis* were exposed for 5, 10, 15, and 30 minutes. The final number of exposures at each time for each of the bacterium varied depending on the standard deviation of the results of previous exposure.

For live/dead fluorescent staining and SEM, 48-hour bacterial biofilms of each of the bacterium were exposed with the cold plasma for 30 minutes.

5.4 Results

5.4.1 Exposure distance

The first experiment to decide on an operating exposure distance showed that the number of dead bacteria increases with an increasing exposure distance (at least up to 15 mm - results not shown). It was thus decided to fix the operating exposure distance to 15 mm.

5.4.2 Exposure area

The exposure diameter of the *S. epidermidis* bacterial agar plates (Figure 5.3) was qualitatively determined as below. The density of the CFU is very high in the control agar plate. Already after a 1-minute exposure there seems to be a decrease in the density of the CFU. After a 2-minute exposure, the area of the plasma gas flow, with even lower density, can be seen in the middle of the plate. After a 5-minute exposure the density of CFU has decreased to the point where the CFU could be counted, and an even larger and clearer area in the middle of the plate can has appeared. After a 10-minute exposure the density of CFU is even lower, however there is not as clear zone in the middle. The agar plates have a diameter of 86 mm. The diameter of the gas flow (visualized after 2-minute exposure) can thus be calculated to be about 20 mm.

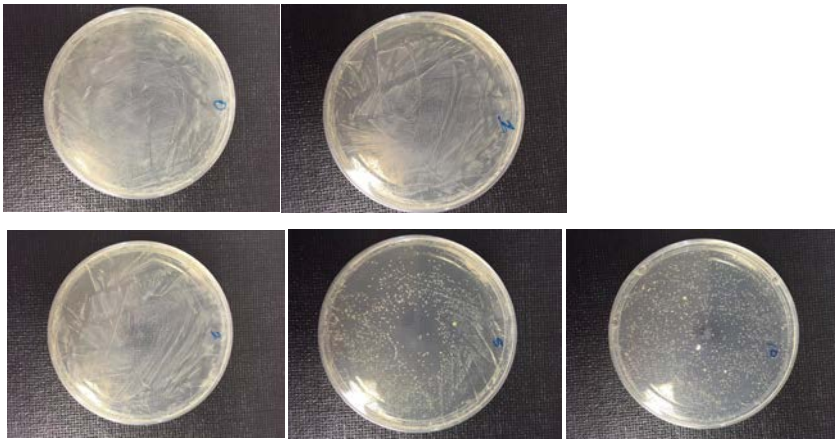

Figure 5.3: The *S. epidermidis* agar plates after 0, 1, 2, 5, and 10-minute exposures.

5.4.3 CFU counting

The results are presented as the percentage of average viable bacteria after a respective exposure time compared to the average number of viable bacteria in control samples, with errors as standard deviation of the average values. The results for *B. subtilis, S. epidermidis, E. coli, and P. aeruginosa* are shown in Figures 5.4, 5.5, 5.6, and 5.7, respectively. For all the species, a large initial drop in viable bacterial cells can be seen after only 5 minutes of exposure. For each of the gram-positive bacteria the results differ. *B. subtilis* shows a near-total loss of viable bacteria after only 5 minutes, while *S. epidermidis* shows over 70% loss of viability after a 5-minute exposure, and the loss of viability slowly increases up to over 80%. There is also a difference in behavior between the gram-negative bacteria. *E. coli* shows a behavior similar to *S. epidermidis* with a slightly higher loss of viability, going from over 80% after a 5-minute exposure and increasing to about and over 90% loss of viability. *P. aeruginosa* has a smaller initial drop in viability, of only about 58%, however, it reaches an almost 100% loss of viability after a 60-minute exposure. The loss of viability for all the species are compared in Figure 5.8.

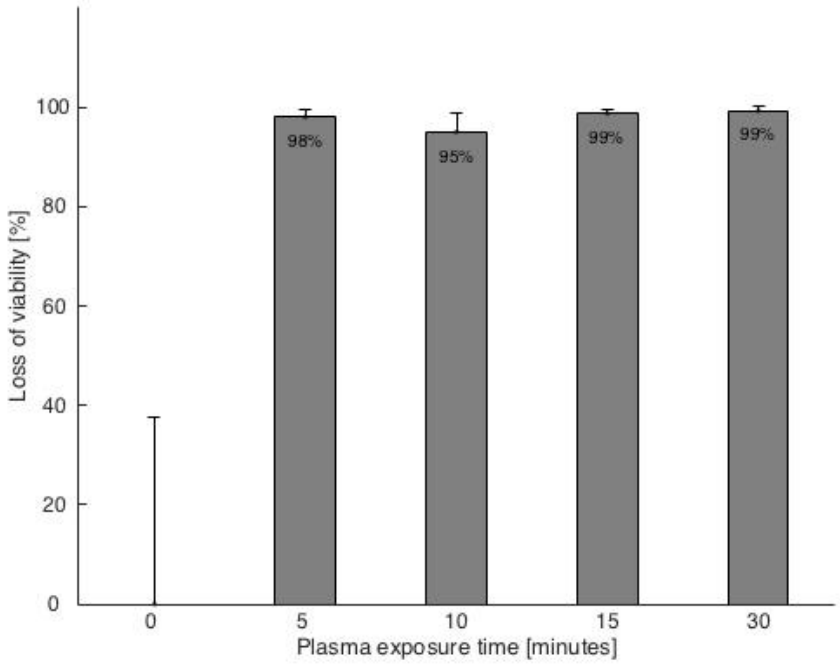

Figure 5.4: The loss of viability for *B. subtilis* after exposure times of 5, 10, 15, and 30 minutes compared to control samples.

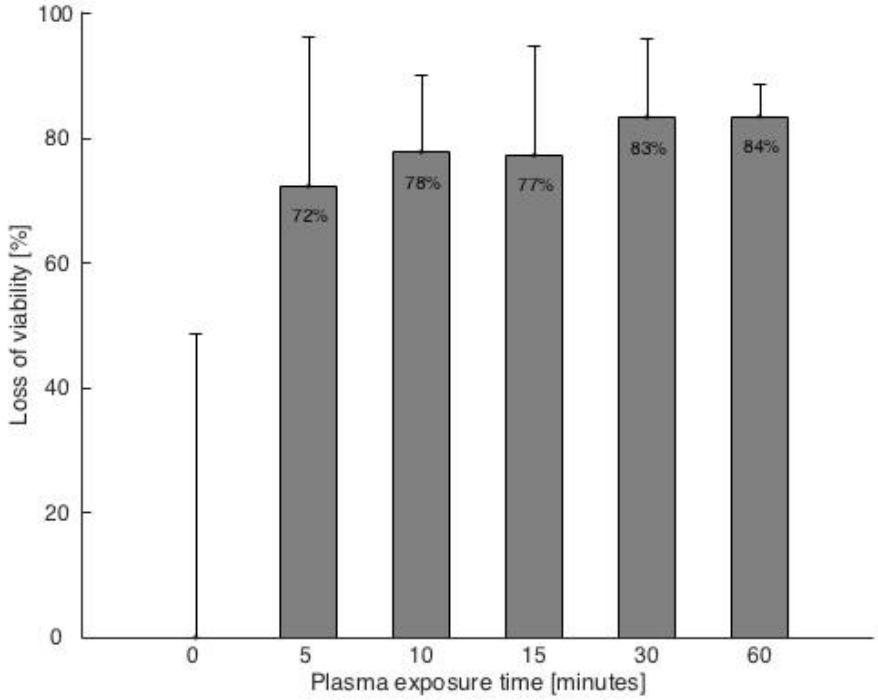

Figure 5.5: The loss of viability for *S. epidermidis* after exposure times of 5, 10, 15, 30, and 60 minutes compared to control samples.

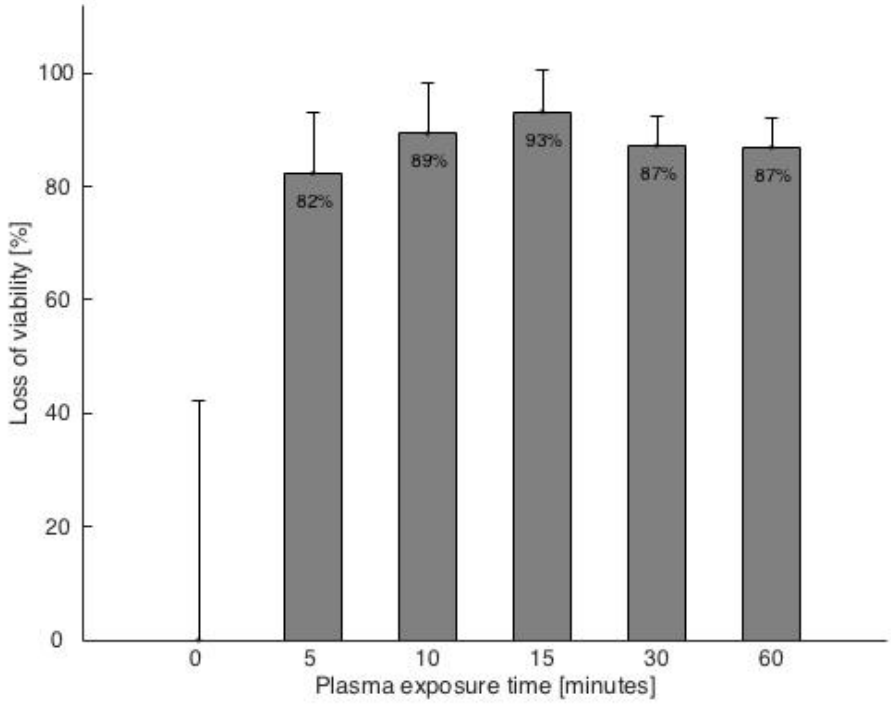

Figure 5.6: The loss of viability for *E. coli* after exposure times of 5, 10, 15, 30, and 60 minutes compared to control samples.

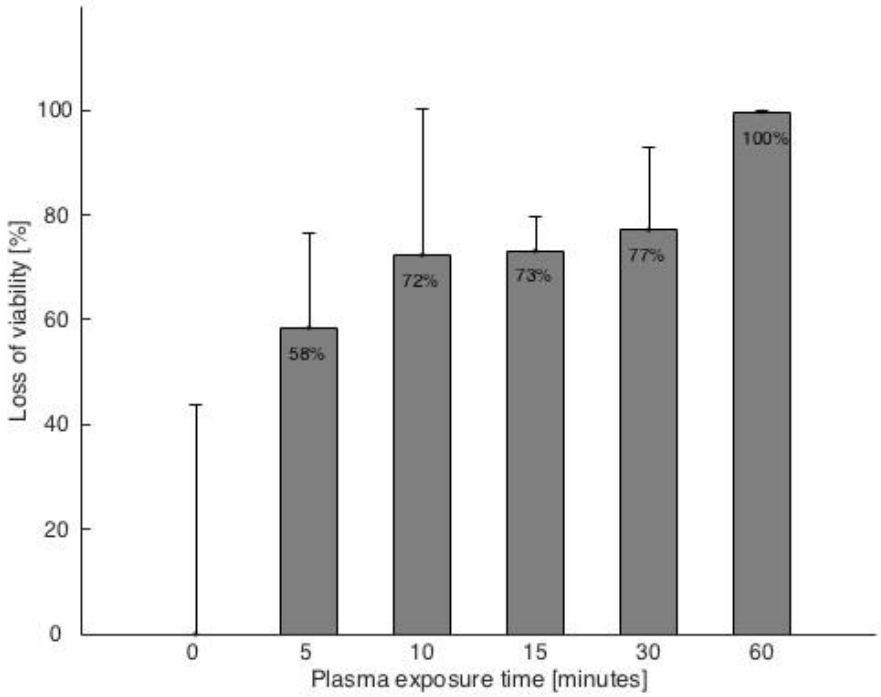

Figure 5.7: The loss of viability for *P. aeruginosa* after exposure times of 5, 10, 15, 30, and 60 minutes compared to control samples.

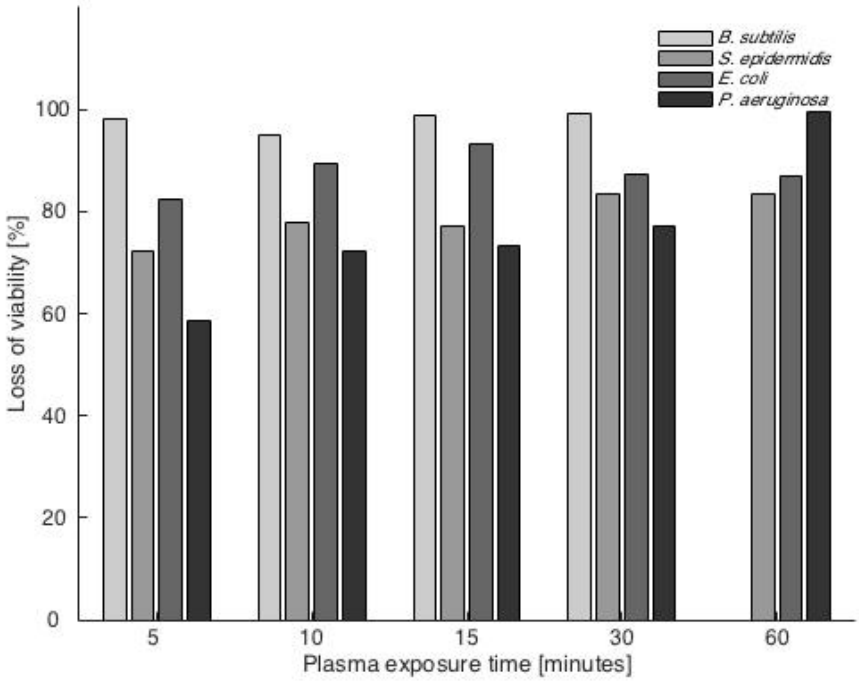

Figure 5.8: Comparison of loss of viability after exposures of times of 5, 10, 15, 30, and 60 minutes for all four bacterial species.

5.4.4 Live/dead fluorescent staining

Figures 9-12 show the live/dead fluorescent staining images for all the species. It can clearly be seen that there is a considerable difference in the amount of red coloring between the gram-positive *B. subtilis* (Figure 5.9) and *S. epidermidis* (Figure 5.10), with more and less amount of red respectively. For the gram-negative bacteria, *E. coli* (Figure 5.11) and *P. aeruginosa* (Figure 5.12), the brightness of the red color is similar. However, compared to the gram-positive bacteria, the amount of dead bacteria seems lower than for *B. subtilis* and higher than for *S. epidermidis*.

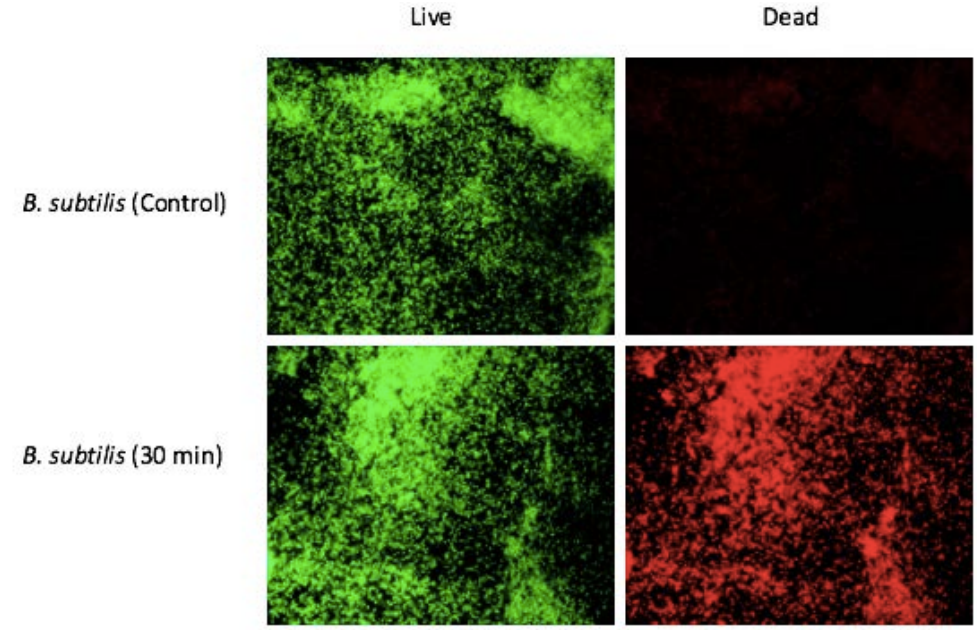

Figure 5.9: Live/dead fluorescent staining of *B. subtilis* after 30-minute cold plasma exposure.

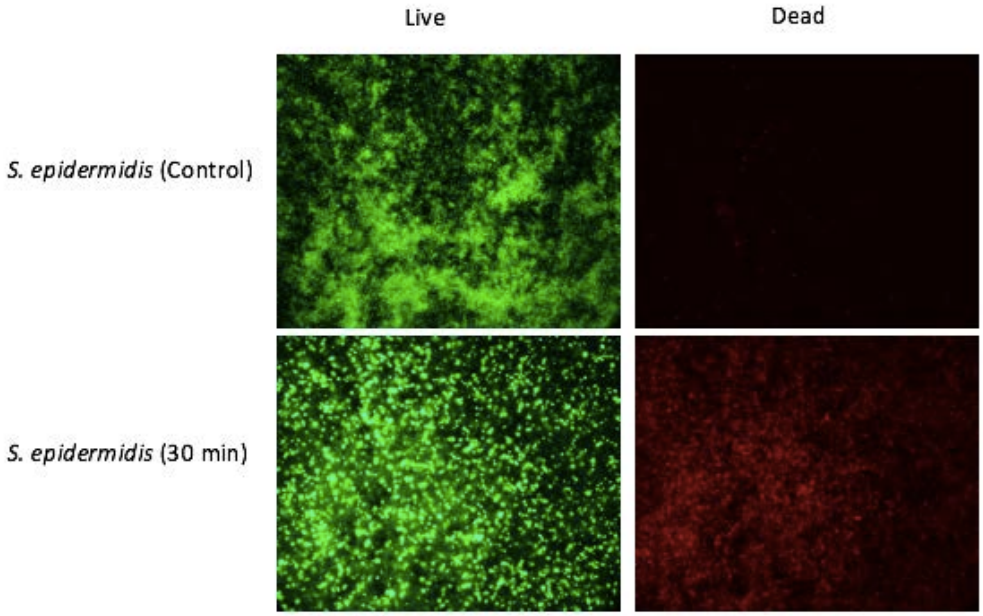

Figure 5.10: Live/dead fluorescent staining of *S. epidermidis* after 30-minute cold plasma exposure.

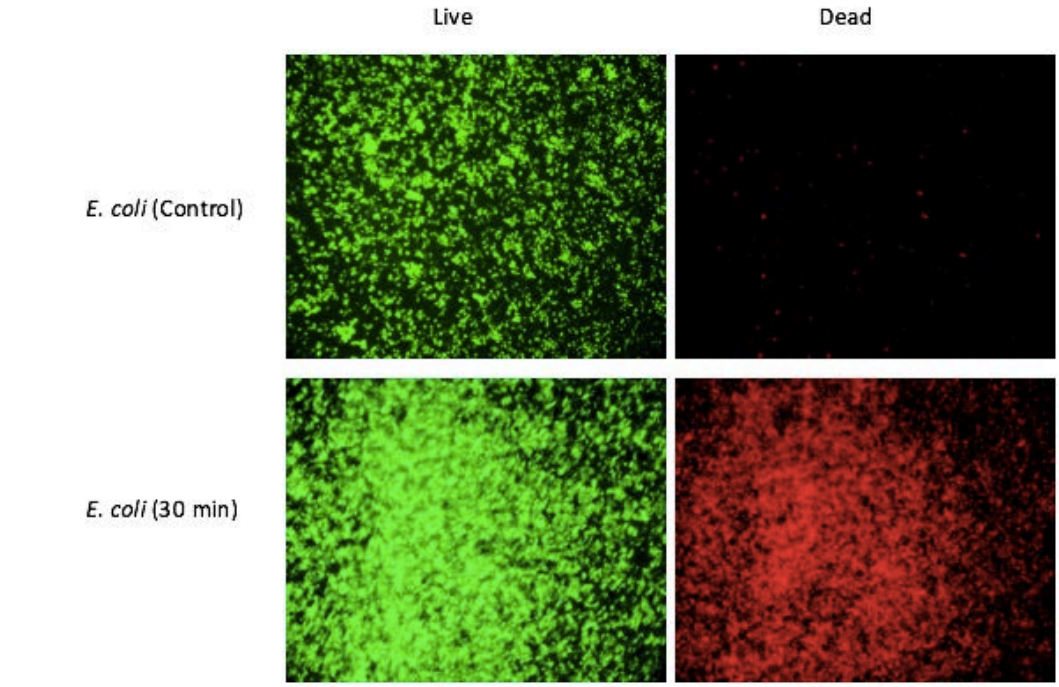

Figure 5.11: Live/dead fluorescent staining of *E. coli* after 30-minute cold plasma exposure.

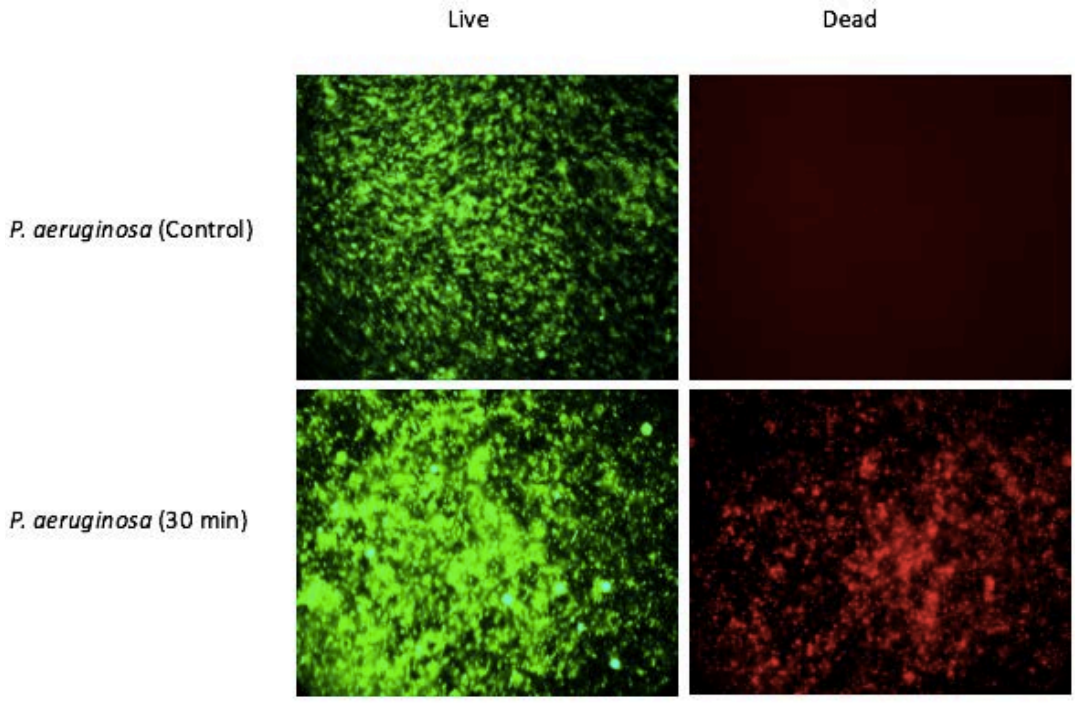

Figure 5.12: Live/dead fluorescent staining of *P. aeruginosa* after 30-minute cold plasma exposure.

5.4.5 Scanning electron microscopy

Figures 5.13-5.16 are images of all the species after a 30-minute exposure taken with scanning electron microscopy (SEM). Rupture of the bacterial cell membrane can be seen in all the figures. In addition, morphological changes can be seen in the gram-negative bacteria after a 30-minute exposure (**Figure** 5.15e-h and Figure 5.16e-h), compered to how the gram-positive bacteria seem to better hold their shapes and sizes.

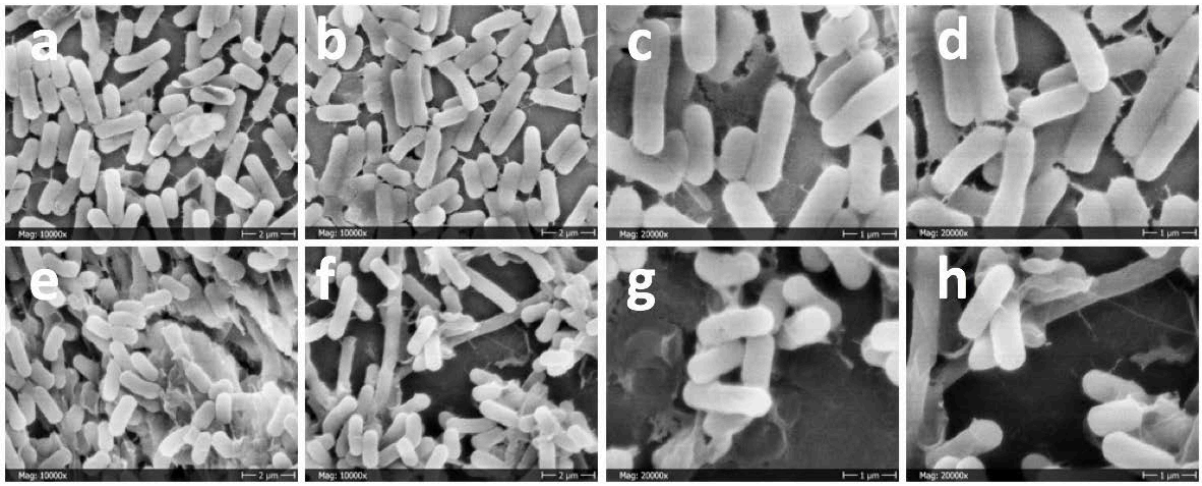

Figure 5.13: a-d) SEM of *B. subtilis* control samples. e-h) SEM of *B. subtilis* after a 30-minute exposure.

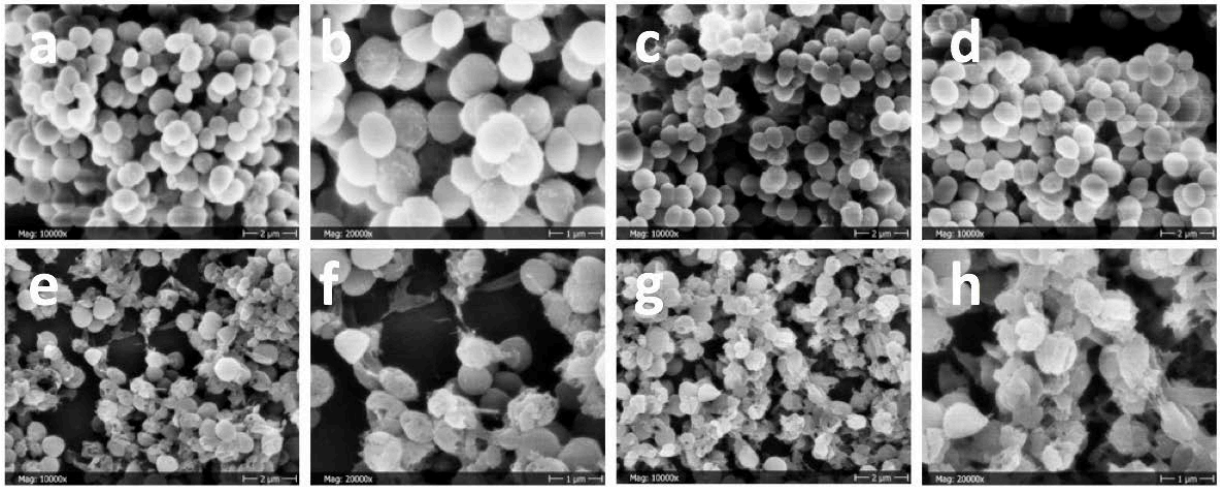

Figure 5.14: a-d) SEM of *S. epidermidis* control samples. e-h) SEM of *S. epidermidis* after a 30-minute exposure.

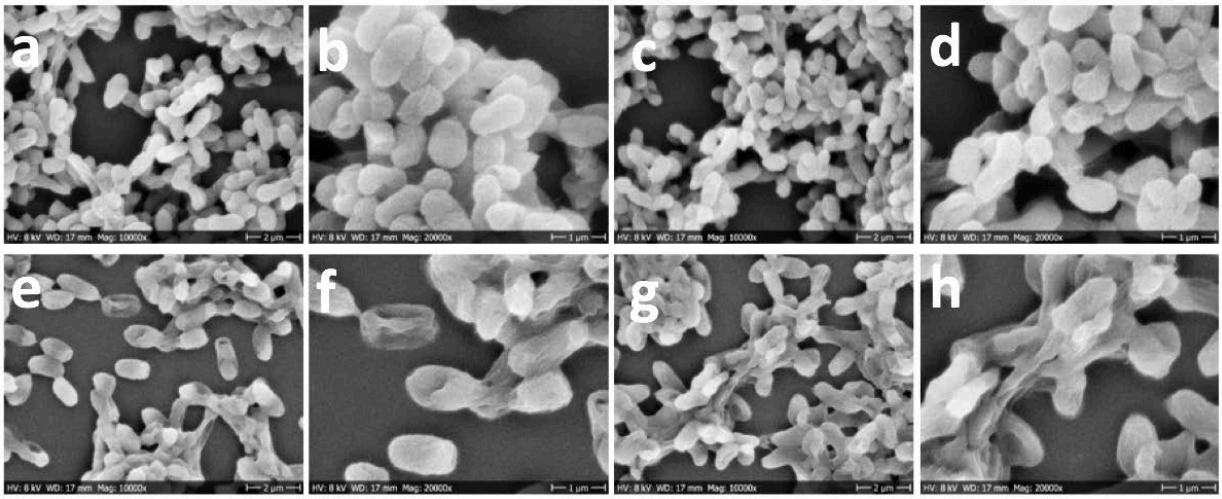

Figure 5.15: a-d) SEM of *E. coli* control samples. e-h) SEM of *E. coli* after a 30-minute exposure.

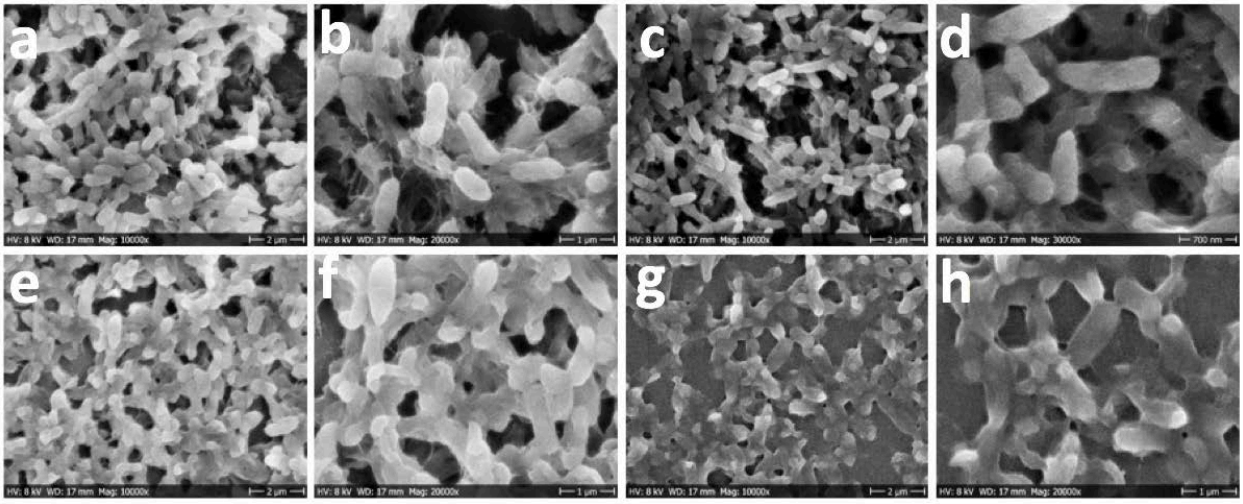

Figure 5.16: a-d) SEM of *P. aeruginosa* control samples. e-h) SEM of *P. aeruginosa* after a 30-minute exposure.

5.5 Discussion

5.5.1 Exposure distance

The increasing number of non-viable bacteria with an increasing exposure distance, at the fixed time of 5 minutes here, suggests that the area of exposure is increasing with the increasing distance. It can thus be concluded that the diameter of the plasma gas flow increases with increasing distance from the sample and clearly the reactive species are still very effective at least up to 15 mm. It was suspected that heat could have an effect on the samples exposed at the smaller distances, since the gas temperature is relatively high (maybe 70 degrees) at the nozzle. However, in this setup, the exposure area seems to outrank the heat. In addition, at 15 mm exposure distance heat can be discarded as an effective agent in the plasma.

5.5.2 Exposure area

From the evolution of the results when the planktonic *S. epidermidis* bacteria smeared on agar plates were exposed to the cold plasma, it can be concluded that for longer exposure times the reactive species of the plasma gas has time to evenly treat the whole plate, not just, or mostly, the area closest to the center of the plasma gas stream.

Similar results have been obtained [44]. bacterial inhibition zones were determined by exposing bacteria seeded agar plates with a cold plasma at a distance of 25 mm for 2 and 4 minutes. It was observed that the inhibition zone diameter increased with increasing exposure time and concluded that the density of reactive species decreases with increasing distance from the center of the plasma gas flow, thus requiring longer exposure times to inactivate bacteria at longer distances from the center.

This might lead to speculations about the exposure distance used in the study presented here, if a shorter exposure distance could have given better results.

5.5.3 CFU counting

The response to a cold plasma exposure for both the gram-negative and gram-positive bacteria seem to be species dependent. *B. subtilis* is highly sensitive to a cold plasma exposure, only needing 5 minutes of exposure for a near 100% inactivation of bacterial cells. *E. coli* also seems to be rather sensitive to the cold plasma exposure, showing a loss of viability between 80 and 90% already after a few minutes. For *S. epidermidis,* further studies with longer exposure time are needed to determine the time it takes to get over 90% inactivation. Even though an almost 100% inactivation of bacterial cells was reached for *P. aeruginosa,* this bacterium seems to be popular for cold plasma experiments and better results have been obtained.

As an example,48-hour *P. aeruginosa* biofilms have been exposed to cold plasma using a kINPen med source operating with argon as feeding gas at a flow rate of 4.2 SLM, with plasma pulses generated at a frequency of 1.82 MHz [43]. The sample was placed at a distance of 10 mm from the pen’s nozzle and after 5 minutes of exposure no viable cells were detected. This study also tested the effect of only an argon gas

flow which showed a slight decrease in number of CFU, most likely due to drying effect from the gas. The temperature was also monitored at the location of the sample and was recorded 32.6°C after 10 minutes, making it possible to conclude that the temperature is not affecting the killing. Another study [6] also ruled out the effects of the gas flow alone in the inactivation of biofilms. It showed that reduction in CFU of about 5 to 10% due to the gas flow drying out the cells was insignificant compared to the reduction produced by the cold plasma. Even though not tested, these conclusions about temperature and gas flow can also be assumed to apply here.

48-hour *P. aeruginosa* biofilms exposed to cold plasma at a distance of 15 mm have also previously been reported. [44] The biofilms were exposed for 15, 30, 35, 60, 120, and 240 seconds with a cold plasma device operated at a frequency of 20 and 40 kHz at a voltage of 6 kV, with a gas mixture of 99.5% helium and 0.5% oxygen at a flow rate of 2 SLM. When the plasma jet was operated at 20 kHz and 6kV, more than 4-log (99.99%) reduction in the number of viable cells was observed after only a 4-minute exposure. And a complete eradication was achieved after a 10-minute exposure (and for *E. coli* after 8-minutes-exposure). [48] When the plasma jet was operated at 40 kHz, the biofilm inactivation rate increased significantly, with a complete eradication of the 48-hour biofilm after only a 4-minute exposure. It should be noted that the gas temperature of the plasma jet increased for about 39°C to 57°C when the plasma operating frequency was doubled, limiting the potential application on living tissue.

In another study [49], using a different device at a distance of 3 mm from the sample, only a 40-fold reduction in the number of viable cells in a 48-hour *E. coli* biofilm was observed after 40-minutes exposure.

It can thus be concluded that biofilm inactivation is very dependent on the device used, and how they are operated. For example, the first two examples above used a much higher operating frequency and voltages than the device used in this study, frequency of 5 kHz and electric voltage of 2-3 kV. Plasmas operating with air have shown a larger increase in temperature with increasing applied power compared to argon plasmas, at least in the discharge region, which might restrain their usage at higher, more effective power. Also, the impact of argon gas, even when not in the plasma state, has been proposed to be higher than previously thought, causing anaerobic condition during experiments. And, the different reactive species created in plasmas operating with different gases make it difficult to compare the generated results. [50]

Biphasic behavior was observed for all four species tested here, with the initial drop in the number of viable cells followed by slower, further inactivation as a second phase. Two-slope inactivation curves have been reported before for biofilm inactivation and have been explained as being due to the initial exposure and fast destruction of the top layers of the biofilm, followed by slower destruction of the bottom layers since they are covered by dead cells and exopolysaccharide biofilm matrix that the cold plasma needs to penetrate. However, it has also been proposed that the first step is the bacterial cells entering a viable-but-non-cultivable (VBNC) survival state, corresponding to the initial drop in the inactivation curve, followed by the second step in which the cells are actually killed, corresponding to the rest of the curve. Since the CFU method does not discriminate between actually dead cells and cells in the VBNC state, it is important to combine it with other methods for confirmation. The VBNC state has been reported for many gram-negative bacteria, where they undergo morphological changes and decrease in size. [6]

5.5.4 Live/dead fluorescent staining

It can be stated that the live/dead fluorescent staining images (Figures 5.9-5.12) confirm the 30-minute results of the CFU counting, in the way that *B. subtilis* and *S. epidermidis* are the most sensitive and resistant, respectively, species tested here and that *E. coli* and *P. aeruginosa* lie there in between. It might seem that the amount of dead cells in the *E. coli* biofilm is closer to the *B. subtilis* biofilm than the two others, however, it can also be noted that the amount of live cells also seems higher than for all the other species, indicating that the *E. coli* image was taken at a dense spot in the biofilm. It can also be speculated that a lot of the cells in the *P. aeruginosa* and *S. epidermidis* biofilms are not actually dead rather than only non-cultivable after the 30-minute exposure, since their CFU count showed a larger increase in non-viable cells from the 30-minute exposure to the 60-minute exposure, after which more of the cells might be dead, compared to the CFU count for *B. subtilis* and *E. coli*.

5.5.5 Scanning electron microscopy

From the SEM images shown here (Figures 5.13-5.16), it is not clear if the morphological changes observed are because the cells have entered the VBNC state or simply because their cell envelope has ruptured. Since the images were only taken after a 30-minute exposure, where a significant decrease in the number of viable cells had been observed, that what is seen are mostly actual dead cells rather than cells in the VBNC state. Perhaps different images would have been obtained after only a 5-minute exposure, or, even in different areas of the 30-minute exposed biofilms.

6 Discussion

The results presented above showed, in agreement with other previously conducted experiments, that a cold plasma is effective in combatting bacterial biofilms. However, exactly how effective seems to be dependent on the device used. Like state in section 2.6 herein, shorter voltage pulses and higher peak voltage are able to generate more energetic electrons that can trigger reactions that have higher energy threshold. The RONS, and their amount, generated in the cold plasma are thus highly dependent on the device's operating voltage and frequency.

The perspective taken in this thesis was the problem of healthcare-associated infections, especially indwelling urinary catheters, in section 3.5. The results of the experiment showed that the most common infecting bacterium in urinary catheters, *E. coli*, is relatively sensitive to a cold plasma exposure. The remaining problem would be the application of cold plasma on the catheters, preferably escaping the need to remove the catheter. However, practical realizations of cold plasma sources capable of decontaminating the inside of tubes have already been presented [51], both sources that would be inserted in the catheter or implemented in the tubes.

7 Conclusion

This thesis has attempted to give a description of cold plasma and its medical applications. Cold plasma is the class of plasma which is in a highly non-equilibrium state, that is, in which the temperature of the gas is much lower than the electron temperature, a maximum 60°C gas temperature compared to the thousands of degrees of the electrons. This enables living tissue to be treated with cold plasma, giving it the status it has at this time as an emerging technology in the field of medicine, having shown to have potential in cancer therapy and wound healing. The main active agents generated in cold plasma had been proposed to be reactive chemical species, ions and electrons, heat radiation, electromagnetic fields, UV radiation and visible light. However, in an indirect plasma exposure, where the plasma itself does not come into contact with the tissue rather than a flow of ionized gas, suitable for medical treatments, all proposed agents other than reactive oxygen and nitrogen species (RONS) can be eliminated. RONS are believed to be a part of cells intracellular signaling pathway, however, the way RONS affect cells depends on their concentration, making it highly probable that RONS generated by cold plasma are affective by interference with cells' signaling pathways.

Here, the focus was on the beneficial effects of cold plasma on bacterial biofilms. In bacterial infections, bacteria have arranged themselves in a three dimensional structures, where the bacteria interact with each other and an extracellular matrix provides protection from hostile environmental agents, such as antibiotics. This so-called innate resistance of the biofilm has been suggested to induce an antibiotic resistance where bacteria have genetically adapted to the antibiotics, causing bacterial infections to become a global threat again.

The results of the experiment conducted here resembled the results of previously published studies, cold plasma is very effective in reducing the amount of viable cells in biofilms. The time it takes to get a considerable decrease of viable cells depends on the type of bacteria constructing the biofilm and the age of the biofilm. Even though a treatment with cold plasma generally does not result in a complete eradication of the biofilm, it could be sufficient when combined with other treatments, such as antibiotics. It is highly unlikely that bacteria will develop resistance to cold plasma, giving rise to a pre-treatment with cold plasma followed by a treatment with antibiotics. This would allow for a reduced amount of antibiotics required and, with a controlled use, could reverse the evolution of the antibiotic crisis. Cold plasma should be used with caution though, until more detailed results of its effect on bacteria are obtained, not to make the same mistake again.

Comparison of the time it takes to inactivate biofilms in different experiments, the one conducted here and previously published ones, indicates that the rate of inactivation depends strongly on the device used, and most likely also the experimental conditions. It is thus difficult to make a comparison of different results and to generate a credible knowledge gain in the field of cold plasma in medicine.

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