Air-Based Coaxial Dielectric Barrier Discharge Plasma Source for *Pseudomonas aeruginosa* Biofilm Eradication

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ABSTRACT: Biofilms cause biofouling, pipe plugging, prostheses colonization, disease, and nosocomial infections. Bacterial biofilms are more resilient to sterilization methods than planktonic bacteria; therefore, better control methods are required. The use of gas discharge plasmas is an appropriate alternative because plasmas contain a mixture of reactive agents that are well known for bacterial decontamination. This study assesses culture medium-abiotic surface combinations leading to robust biofilms and tests an air-based coaxial dielectric barrier discharge (DBD) plasma source on *Pseudomonas aeruginosa* biofilms grown in continuous culture under those selected conditions. Biofilms were eradicated after a 15-min plasma treatment, resulting in a CFU/mL decrease of 5.6 $\log_{10}$ units. CFU/mL decreases of 1.6 and 2.7 $\log_{10}$ units were achieved after a 3-min plasma exposure to ambient and moistened air plasma, respectively, although viability assays showed that some cells were alive. Moistened-air plasma resulted in a faster biofilm inactivation, with decimal reduction times of 1.14 and 4.36 min. The coaxial DBD air-based plasma source presented here is effective for *Pseudomonas* biofilm inactivation, affordable because it does not rely on expensive gases, and easy to handle for indirect surface treatment. To the best of our knowledge, the search for the best combination medium surface leading to robust biofilms before plasma treatment has not been previously assessed.

KEY WORDS: biofilms, *Pseudomonas aeruginosa*, sterilization, biofilm eradication, non-thermal plasmas, air-based plasma, *Pseudomonas* biofilms

I. INTRODUCTION

Biofilms are microbial communities that grow attached to a surface and embed in a matrix composed of exopolysaccharides together with proteins and extracellular nucleic acids. Biofilms are present almost everywhere and, therefore, wherever there is a surface and some moisture, it is likely that a biofilm will develop. Biofilm formation takes place

in several steps, starting with free-living (planktonic) bacteria that recognize a surface. Bacteria reversibly adsorb and then irreversibly attach to the surface. The attached cells grow, divide, and recruit additional planktonic cells that attach to the cells already present on the surface. This process results in the development of a mature biofilm with a particular architecture in which cell clusters and water channels among them form a primitive circulatory system.[1] The emerging biofilm results in a tridimensional structure, where cells are protected by the matrix and cooperative interactions become important.[2]

Bacterial biofilms are of concern in industrial settings because they result in biofouling, equipment damage, contamination of water sources, pipe and duct plugging, and product contamination, to mention a few. They also cause prostheses colonization, tooth decay, and dental plaque and produce or contribute to diseases such as otitis media, cystic fibrosis, and Legionnaire's disease.[3] As biofilms contaminate water sources, they could spread disease.[4] Biofilms are also responsible for ~90% of infections in humans, according to the National Institutes of Health (NIH), and 65% of nosocomial infections, according to the Centers for Disease Control (CDC). Biofilms are more resilient to typical decontamination/sterilization methods that are used for free-living bacteria due to their encased, protected tridimensional structure, in which the exopolysaccharide functions as a diffusion barrier for compounds such as antimicrobial agents, and also because bacteria within the biofilm are physiologically different from those in planktonic life. The mechanisms explaining the unusual resistance of biofilms to nearly all forms of sterilization are diverse and vary from nongenetic antibiotic resistance to phenotypic changes into persistent cells.[5–10] Therefore, the problem of biofilm destruction demands the development of alternative methods. The use of nonthermal atmospheric-pressure plasma is one of the techniques that has been researched for more than a decade and has produced promising results.[11–28]

Nonthermal atmospheric-pressure plasma can be generated by an electrical discharge using different gases such as argon, helium, and air at atmospheric pressure. There are several types of electrical discharge configurations for nonthermal plasma production, and the most common are the corona, plasma jet, and dielectric barrier discharge (DBD). In the case of the DBD, an alternating or pulsed voltage is applied between two electrodes separated by a narrow gap. At least one of the electrodes is covered by a solid dielectric material (glass, plastic), or a dielectric structure is placed inside the electrode gap. There are many electrode configurations, the most common being the planar or cylindrical. The discharge can be generated in ambient air or by a gas flowing through the electrodes' gap. When the electric field at the discharge gap is high enough, it induces gas breakdown. Normally, at atmospheric pressure, a large number of individual discharge channels within the discharge gap are generated (filamentary mode). The charge carriers deposited on the surface of the dielectric reduce the electric field strength and quench the discharge. When the alternating applied voltage changes its polarity, the electric field at the discharge gap builds up and the discharge reignites.[29] In the discharge channels, the gas is partially ionized and transitions to a plasma phase. In this type of discharge, the majority of the electrical energy results in the production of

energetic electrons; the electron temperature is considerably higher than the temperature of the ions and neutrals, which remains almost at room temperature. The plasma is not in thermodynamic equilibrium and is a nonthermal plasma. The energetic electrons consume the energy by electron impact dissociation and ionization of the background gas for the production of various active agents, such as ultraviolet photons, and particles, such as neutral or excited atoms and molecules, negative and positive ions, and radicals. This type of plasma has proven to be well suited for disinfection or sterilization processes and enables the treatment of thermosensitive materials and living tissue that cannot undergo autoclaving.[30–32] In a DBD operating in air, the most significant reactive species for sterilization are ozone, atomic oxygen, hydroxyl radical, and nitric oxide, among others, but the mechanism for the deactivation of microorganisms is still under discussion.[33–35] The typical configuration is planar, and the discharge is applied directly onto the sample to be treated or indirectly with the sample outside the discharge region.[27,28,33,36,37]

Most of the studies aimed at elucidating the effects of plasmas on bacterial cells were carried out with planktonic microorganisms or spores. By the middle of the last decade, our group and a few others reported for the first time on the use of plasma for biofilm eradication.[11,38–41] Since then, there has been an increasing amount of research aimed at understanding plasma-mediated biofilm inactivation, and some of the contributions have focused on *Pseudomonas aeruginosa* biofilms.[12,13,27,28,37,42–44]

In recent years, we reported on the use of a plasma jet to inactivate *P. aeruginosa* biofilms.[11,16,24–26] *P. aeruginosa* is a Gram-negative opportunistic pathogen that preys on victims with compromised immune systems, such as patients on respirators, and causes infections of burned tissue and colonization of catheters and medical devices. It also cocolonizes, together with *Burkholderia cenocepacia*, lung tissue in cystic fibrosis patients, contributing to mortality.[45] *P. aeruginosa* forms biofilms on implants and dead or living tissue, leading to a variety of persistent infections.[46] Most naturally occurring biofilm are mixed species. However, single species are of particular interest due to their clinical importance. Monospecies biofilms formed by *P. aeruginosa* have become a prominent model for studying this aspect of microbial biology.[46]

Several approaches have been used to inactivate *Pseudomonas* biofilms, including the use of diverse compounds, such as antimicrobial agents,[47,48] chelators,[49] furanone, and *N*-acyl homoserine lactones,[50,51] and surface modification.[52,53]

In previous contributions, we demonstrated that *P. aeruginosa* biofilms grown in batch culture on borosilicate, polycarbonate, and stainless-steel surfaces were almost completely inactivated after a 5-min exposure to plasma. Atomic force microscopy studies showed that the thicknesses of the biofilms grown on borosilicate and adhesiveness to the surface were reduced. In addition, sequential morphological changes and loss of viability were observed after plasma treatment.[25,26] We later presented data on plasma-mediated inactivation of *P. aeruginosa* PAO1 biofilms grown on borosilicate in continuous culture.[24] We showed a similar inactivation, kinetic to those reported for cells grown in batch cultures,[25,26] and demonstrated that cells underwent sequential changes, ranging from minimal modification without loss of viability at short plasma exposure times to

major structure and viability loss at longer exposure times. We reported that changes in biofilm structure leading to the loss of culturability and viability were related to a decrease in biofilm matrix adhesiveness.[24] Taken together, the results show the effectiveness and applicability of plasma to inactivate biofilms grown on different abiotic surfaces and conditions. All of the experiments were carried out using an inductively coupled commercially available plasma reactor (Atomflo 300 reactor; Surfx Technologies, Redondo Beach, CA) that delivers a plasma jet and operates in helium, an expensive inert gas.[54]

In this article, we present an inexpensive, easy-to-build, and easy-to-handle coaxial DBD plasma source operating in air and we demonstrate its usefulness for the inactivation of *Pseudomonas* biofilm grown on stainless-steel coupons in continuous culture and synthetic medium. We also show results on biofilm growth under different scenarios such as three abiotic surfaces and two culture media in order to determine the conditions that produce a more robust biofilm. The best combination abiotic surface/culture medium was selected to further carry out the plasma treatments. We also demonstrate the presence of extracellular DNA released by the biofilm and the presence of living cells at exposure times for which there were no culturable cells. These results support the statement that viability experiments should always be carried out before drawing the conclusion that plasma eradicates biofilms at short exposure times based solely on counting culturable cells.

II. MATERIALS AND METHODS

A. Plasma Generation and Conditions

An atmospheric-pressure gas discharge plasma was produced using a DBD configuration consisting of two axial electrodes and a double dielectric barrier. A schematic of the experimental device is shown in Fig. 1. The inner electrode consisted of a 1-mm-diameter iron wire inside a 6-mm-external-diameter glass capillary tube sealed at the tip. The outer electrode was a 25-mm-long aluminum tape attached to a 1-mm-thick acrylic 10-mm-diameter tube. The alternating current power supply was a commercially available transformer for neon light (8 kV, 70 mA, and 50 Hz) connected to a variable autotransformer (Variac) to control the operating voltage amplitude. The discharge operated with the power source at its maximum voltage of 8 kV (voltage amplitude at open circuit). Each electrode was connected to one of the two high-voltage transformer outputs and was out of phase with the other (16 kV between electrodes). The voltage was measured with a high-voltage probe (Tektronix 1000×/3.0 pf/100 mW) connected to the inner electrode. Current measurements are performed with a transformer (Bergoz CT-D5.0-B). These electrical waveforms were registered using a two-channel digitizing oscilloscope with a bandwidth of 60 MHz and a sampling rate of 1 GS/s.

The optical emission spectrum of the discharge was obtained with a spectrograph (Horiba IHR320) equipped with a charge-coupled device detector. The spectrum was analyzed and the rotational temperature determined using the transitions of the N_2 second positive system. SPECAIR software was used to model the optical emission spec-
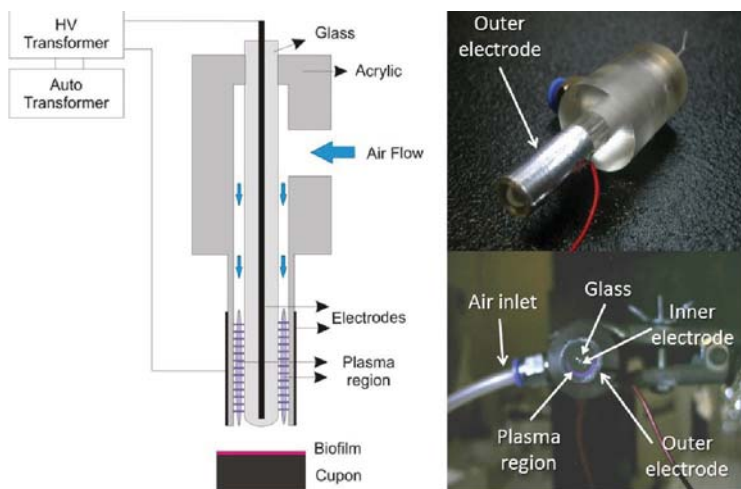
FIG. 1: Schematic and pictures of the DBD experimental device showing the axial electrodes and a double dielectric barrier. HV, High voltage.

trum and to determine the rotational temperature by comparison between the model and the experimental spectrum.[55]

For the biofilm experiments, the plasma applicator was mounted such that the discharge was 1 or 4 mm away from the biofilm. Three different setups were tested: Discharge generated in (1) airflow of 1 L/min, (2) ambient air, and (3) moistened airflow of 1 L/min. The relative humidity of the air under the first condition was ~32% ± 5% and 60% in the second condition, whereas it was 80% ± 5% in the moistened airflow. Air was moistened by passing it through a humidifier filled with water, and airflow was determined using a commercial flow meter. Humidity was determined with an analog hygrometer (Luft48HIG-DH) at the plasma applicator gaseous output. Temperature reaching the surface of the coupon was assessed using a thermocouple.

B. Strain and Culture Media

All of the experiments were carried out with *P. aeruginosa* PAO1 strain cultured in either rich or minimal (also called synthetic) medium. Luria broth (LB) was used as a rich medium[56] and agrobacterium (AB) supplemented with 0.5% w/v glucose as a minimal medium.[57] Inocula were prepared from a fresh plate in either LB or AB medium and grown at 37ºC with agitation in the corresponding medium.

C. Biofilm Growth

P. aeruginosa biofilms were grown on stainless-steel 316L, polycarbonate, and borosilicate (glass) 12.7-mm-diameter coupons in the CDC biofilm reactor (BioSurface Tech-

nologies; Bozeman, MT). The biofilm reactor was inoculated with a fresh bacterial suspension in LB or AB liquid broth to reach an optical density (OD) of 0.1 (550 nm) within the reactor vessel and operated under batch culture regime for 24 hr at 37ºC with agitation (130 revolutions per min) to obtain a dense culture. Thereafter, fresh medium was pumped through the biofilm reactor, and spent medium was removed at a constant rate. The reactor operated as a chemostat with a fixed volume of 350 mL until constant OD for 24 hr. The flow that ensured constant OD was 2.6 mL/min for LB and 0.6 mL/min for AB medium. Flow was controlled with a LKB Bromma 2120 Varioperpex pump. After running the chemostat at constant OD for 24 hr, coupons were aseptically removed from the reactor and unbound bacteria removed by rinsing the coupons twice with sterile saline solution. Coupons were air-dried for 10 min and then placed biofilm-side up into empty Petri dishes before the attachment assay (see Attachment Assay section below) or the plasma treatment (see Plasma Treatment section below).

D. Attachment Assay

Biofilm formation on the three abiotic surfaces using two culture media was indirectly assessed by determining bacterial attachment to each surface with a modification of the crystal violet method.[58] Briefly, the upper surface of air-dried coupons was covered with 28 µL of 1% v/v crystal violet for 15 min in the dark. The unbound crystal violet was removed, and coupons were rinsed in sterile saline solution and air-dried. Each coupon was placed in a tube with 3 mL of 95% alcohol to solubilize the dye, and the OD (540 nm) of the solution (or appropriate dilutions) was determined. Coupons with no bacteria attached were used as controls, and 95% alcohol was used as a blank. The absorbance of the controls was subtracted from the absorbance of the samples.

E. Plasma Treatment

P. aeruginosa biofilms were grown on stainless-steel coupons using the CDC biofilm reactor and AB liquid broth as described above. Air-dried coupons were subjected to gas discharge plasma for various exposure times (1, 3, 5, 15, and 30 min) under sterile conditions. A control without plasma treatment (0-min exposure time) was included. Coupons were placed in a wet chamber after treatment and incubated with 35 μL of sterile saline for 10 min. Biofilms were then scraped off and suspended in 1 mL of sterile saline, serially diluted, and 100 μL of each suspension was plated in duplicates on AB medium. Plates were incubated at 37ºC and evaluated for colony-forming-unit (CFU) formation by counting the colonies.

F. Bacterial Viability Determination

Biofilms were grown on stainless-steel coupons in the CDC biofilm reactor using AB medium under the conditions described in the Biofilm Growth section above. Coupons were removed from the reactor, rinsed twice with sterile saline, air dried for 10 min, and

treated with humidified air plasma for 0 (control), 3, 15, and 30 min as indicated in the section immediately above.

To each coupon we added 20 µl of 0.1-mg/mL DNAse I from bovine pancreas (Sigma-Aldrich). A control with no enzyme and no plasma treatment was included. Coupons were incubated for 60 min at 30°C. After incubation, enzyme suspension was removed with sterile paper towels and coupons rinsed three times with 150 µL of sterile saline. Coupons were further treated with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Molecular Probes), consisting of two dyes: SYTO® 9 and propidium iodide. We added 20 µl of a dye mixture containing a 1:2 ratio of SYTO® 9 and propidium iodide to each coupon, and the coupons were incubated for 20 min at room temperature in the dark. Coupons were visualized with a Leica epifluorescence microscope using a 480/500-nm excitation/emission filter for SYTO® 9 and a 490/635-nm excitation/emission filter for propidium iodide. Images were acquired with the Leica Application Suite (2.5.0 R1), and image analyses were performed using ImageJ software.

G. Statistical Analyses

The assumptions of normality and homoscedasticity for the analysis of variance (ANOVA) were contrasted with the Kolmogorov–Smirnov test for normality and the Levene test for homogeneity of variances. To compare means, the ANOVA and Tukey test (α = 0.05) were carried out using STATISTICA (StatSoft 2011) software. All of the possible combinations of medium/surface/growth conditions were statistically contrasted.

III. RESULTS AND DISCUSSION

A. Discharge Characterization

Electric discharge, produced in the space between the two dielectric tubes, consists of a series of filament discharges. Typical waveforms of current (I) and voltage (V) applied to the inner electrode and taken during the discharge are shown in Fig. 2. The voltage waveform has amplitude of 8 kV and frequency of 50 Hz, corresponding to the line frequency. The discharge current waveform has a number of current pulses of ~10 mA and durations of ~20 ns that are superimposed on the almost negligible displacement current of ~0.01 mA amplitude. These I-V waveforms are characteristics of a DBD discharge with filamentary structure. The same features are shown under the three discharge conditions tested.

Figure 3 shows the simulated and experimental emission profile from the N_2 second positive system (dots + lines) band between 305 and 345 nm. The determination of best fit between the experimental spectrum and the spectrum calculated with the SPECAIR software provides the rotational temperature, an estimation of the gas temperature in the interelectrode region.[59] The best fit was found at the rotational temperature of 27°C. Such temperatures are consistent with nonthermal equilibrium plasma, where the gas is relatively cold compared to the electrons that are found at ~1 eV (11,327°C).[60,61] Nev-

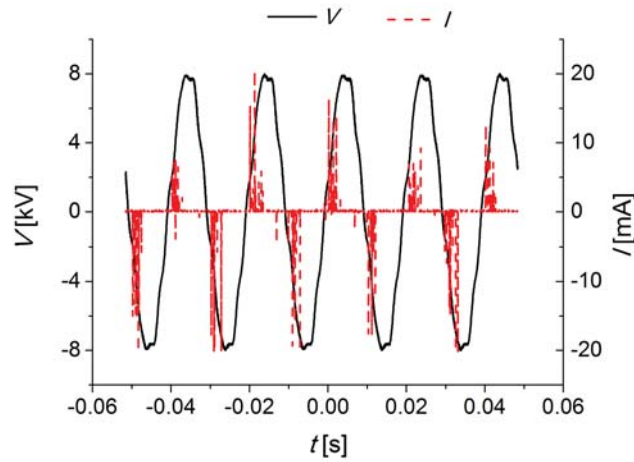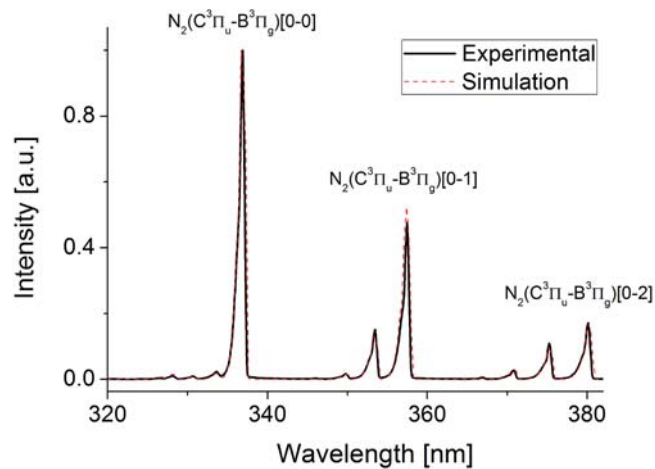**FIG. 2:** Waveforms of voltage (kV) and current (mA) of the DBD discharge

FIG. 3: Experimental spectra (solid line) and SPECAIR software spectral simulations (dashed line) of the N_2 second positive system emission. The SPECAIR best fit provides a rotational temperature of 300ºK ± 20ºK.

ertheless, the temperature of the gas reaching the coupon under the three conditions tested was 30ºC ± 2ºC, as determined by the thermocouple. Therefore, temperature is not responsible for biofilm inactivation. Both the I-V waveform and emission spectrum have the same characteristics for all of the discharge conditions tested.

B. Biofilm Formation on Different Surfaces and Culture Media

Biofilm formation was indirectly assessed under different scenarios: two culture media (rich and minimal) and three abiotic surfaces (polycarbonate, stainless steel, and glass).

The aim of this part of the work was to find the best combination of culture medium and surface leading to the formation of a robust biofilm to be further treated with gas discharge plasma.

The following situations were statistically analyzed and compared:
1. Biofilm formation in rich medium versus minimal medium
2. Biofilm formation on diverse surfaces for each medium.

Figure 4 shows the indirect assessment of *P. aeruginosa* biofilm formation in continuous culture, on three abiotic surfaces and two culture media. It is clear from the graph that bacterial attachment depends on the abiotic surface on which the biofilm grows. Biofilm formation, measured as bacterial attachment, occurred to a significantly degree on stainless-steel coupons for both culture media.

Bacterial attachment to surfaces and subsequent biofilm formation are undoubtedly multifactorial. Bacteria may contribute surface appendages in addition to the bacterial outer envelope and metabolites produced at high cell density. Substrate surface characteristics such as roughness, hydrophobicity, or hydrophilicity are also important for attachment. In addition, components of the culture medium could also affect the substratum surface properties and consequently the adhesion process. Any substrate immersed in an aqueous fluid such as a culture medium will be rapidly covered by a film, called conditioning film, produced by the adsorption of molecules to the substrate.[62,63] Conditioning films change physicochemical properties of the surface such as roughness, hydrophobicity, surface tension, charge density, and the chemical composition affecting
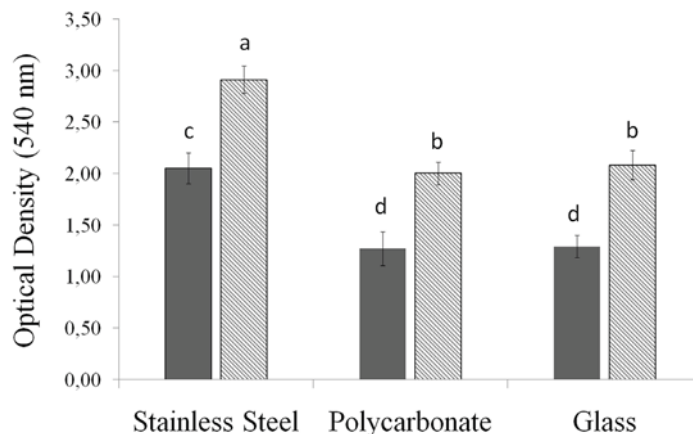

FIG. 4: Attachment assay: OD (540 nm) of a 95% ethanol solution of crystal violet solubilized from *P. aeruginosa* biofilms. Biofilms were produced in continuous culture, on three abiotic surfaces, and two culture media, as indicated in Materials and Methods section. (Gray columns) LB medium; (hatched columns) AB medium. Results are the average of at least four independent experiments. Error bars represent the standard deviation of the mean. (a–d) Significant differences at $\alpha = 0.05$.

bacterial adhesion and further attachment.[62–64] Lorite et al. demonstrated that conditioning film formation due to Periwinkle wilt culture medium decreased the degree of surface hydrophilicity and roughness of glass surfaces.[64] These authors also demonstrated that the contribution of phosphate groups due to the conditioning film was more important than the changes in roughness and hydrophilicity. Chemical surface change resulted in a facilitation of biofilm growth in *Xylella fastidiosa*.

Several characteristics of the aqueous medium, such as pH, nutrient levels, ionic strength, and temperature, may have a role in microbial attachment to a substratum.[65] Throughout all of the abiotic surfaces and culture systems tested, the minimal medium resulted in a larger amount of cells attached and, therefore, superior biofilm formation. The rich medium is composed of glucose, tryptone, and yeast extract, whereas the synthetic culture medium consists of a mixture of ionic salts and glucose, providing a higher concentration of ions to the environment. Fletcher found that an increase in the concentration of several cations (sodium, calcium, lanthanum, ferric iron) affects the attachment of *Pseudomonas fluorescens* to glass surfaces, presumably by reducing the repulsive forces between the negatively charged bacterial cells and the glass surfaces.[66,67] The presence of sodium, calcium, ferric ion, and magnesium in the AB medium may explain the better attachment of cells to the three surfaces obtained with this medium.

It has been reported that although there is no generic pattern of attachment to test surfaces, the majority of microorganisms isolated show a preference for a hydrophobic surface.[68] Hydrophobic interactions that may occur between the bacterial cell and the surface would enable the cell to overcome repulsive forces and irreversibly attach.[65] Laboratory studies of the initial attachment of a marine Pseudomonad have shown that these bacteria have a preference for low-energy (hydrophobic) surfaces.[69,70] Fletcher and Loeb reported low attachment of *Pseudomonas* spp to glass and a higher attachment to hydrophobic surfaces.[70]

From the three surfaces we tested, polycarbonate is hydrophobic and glass is clearly hydrophilic. As for stainless steel, based on literature contact angles, the starting surface is hydrophilic because the stainless-steel discs are milled from 316L stainless-steel rods, and no surface coating or treatments are added after milling. We determined contact angles for the three materials: 70º ± 5º for stainless steel, 55º ± 5º for glass, and 70º ± 5º for polycarbonate. Therefore, stainless steel and polycarbonate do not differ in hydrophobicity. We cannot conclude whether the original stainless-steel coupons were hydrophilic and then became more hydrophobic, perhaps due to deposition of material in the streaks left by the machinery of the coupon.

Electrostatic forces between a cell and a potential substrate for biofilm formation play an initial part in the attachment process. Extracellular polymers may bridge the gap between bacterium and substratum, resulting in more permanent attachment.[71] The bacterial cell surface interacts with the substrate surface, and both contribute to the hydrophobicity or hydrophilicity of the interaction. Most bacteria, especially those that are Gram-negative, are negatively charged but still contain hydrophobic surface com-

ponents such as fimbriae and flagella that may help the cell to overcome the initial electrostatic repulsion between the cell and the substratum surface.[72] In the case of biofilms, bacteria are assumed to have their motility genes turned off, so we do not expect a major contribution of flagella besides the initial steps.

A bacterial component that may have a role in adhesion and attachment is the exopolysaccharide excreted by biofilm-forming bacteria and part of the extracellular polymeric substances (EPS) composing the matrix. Exopolysaccharide production is affected by the nutrient status of the bacterium. It has been demonstrated that a growth medium with excess available carbon together with nitrogen, potassium, or phosphate limitation promotes exopolysaccharide synthesis.[73,74] Because of its acidic structure, the exopolysaccharide binds to hydrophilic surfaces but may also associate with metal ions, divalent cations, and other components of the culture medium.[75] We mentioned before that the AB synthetic medium used is rich in divalent cations that may interact with the EPS excreted by the biofilm.

Besides the physicochemical properties of a surface, the solid-surface characteristics are also an important factor to consider in the bigger picture of bacterial attachment to surfaces. Rougher surfaces offer an increased surface area and decreased shear forces. The extent of microbial colonization appears to increase as surface roughness increases.[76] In our case, the roughness of each surface was assessed by atomic-force microscopy, and stainless steel proved to be the roughest surface because the streaks left by the machinery of the coupons were easily visible (Vandervoort and Brelles-Mariño, unpubl.). A better attachment to stainless steel compared to polycarbonate might be related to the roughness of the stainless-steel coupons that "trap" microorganisms between the streaks left by the machinery of the surface. A recent contribution by Gabriel et al. showed that even the same abiotic surface, but with distinct finishes, results in a different attachment to the material.[13] The authors tested two types of stainless steel with different finishes, namely, mirror, hairline, and 2B surfaces, and found that attachment rates were significantly affected by the surface finish rather than by the stainless-steel type.

From the conditions tested, the best biofilm was obtained on a stainless-steel surface and in synthetic AB medium. Electrostatic forces may have a role in the initial attachment of cells to the surface, and ions present in the AB synthetic medium may also contribute to the process. In continuous culture, the input of fresh medium into the reactor ensures a continuous supply of nutrients, especially the carbon source, and therefore other compounds formed through bacterial metabolism may contribute to later steps of biofilm formation besides the initial attachment. The amount of matrix EPS produced will be favored by the continuous supply of the carbon source in the continuous culture. Later, hydrophobic interactions among newly synthesized compounds such as rhamnolipids and hydrophobic surfaces such as polycarbonate and stainless steel may become prevalent. The roughness of the stainless-steel surface may promote a better bacterial attachment to the surface. Based on the results obtained, the conditions resulting in better biofilms were AB medium and stainless steel coupons. Therefore, plasma treatment was performed on coupons with biofilms grown in continuous culture under those conditions.

C. Survival Curves and Inactivation Kinetics

Preliminary experiments were carried out using biofilms treated with plasma generated in air at a flow of 1 L/min. Almost no colonies were recovered after the treatment. To determine whether cells were killed by plasma or dried out by the simple flow of air, the same experiments were repeated with no discharge ignition and using solely air at the same flow. Only very few cells survived after the air treatment, demonstrating that cells were dehydrated by the simple flow of air and not killed by plasma (data not shown). As dehydrated cells remain alive and recover once hydrated, we no longer continued experiments with plasma ignited in air at 1-L/min flow and moved to the other experimental setups: plasma operating in ambient air with no flow or in moistened air at 1-L/min flow. The rationale for using moistened air was based on the fact that preliminary experiments with no plasma ignition and performed with dry or moistened air at various airflows showed a higher cell survival for the latter, regardless of the flow. Biofilm inactivation was not due to temperature either, because temperatures measured on the surface of the coupon were 30ºC ± 2ºC.

Figures 5 and 6 depict the survival curves for *P. aeruginosa* biofilms grown in synthetic AB medium on stainless-steel coupons and treated with plasma operating in ambient air (Fig. 5) or with a moistened airflow of 1 L/min (Fig. 6). The experiments with plasma operating in ambient air were carried out at two distances from the plasma applicator to the sample: 1 and 4 mm, whereas only the distance of 4 mm was used for
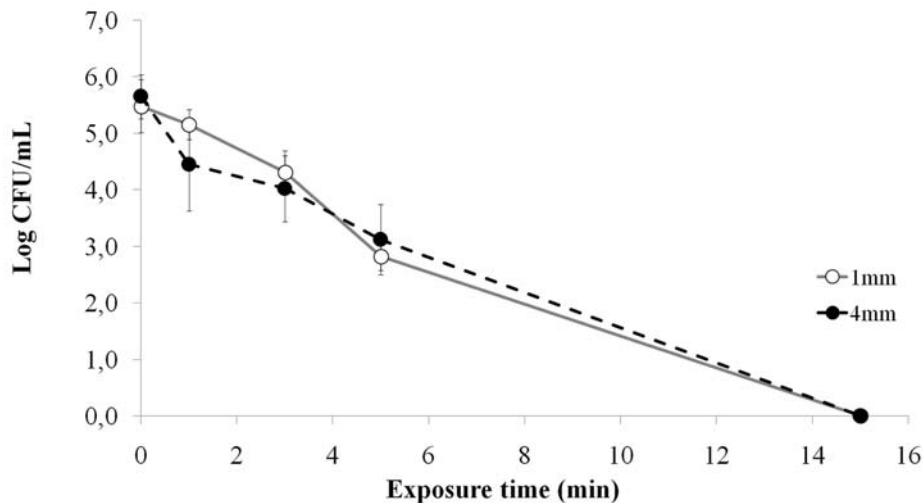

FIG. 5: Survival curve: log of the number of CFU/mL vs. plasma exposure time. *P. aeruginosa* biofilms grown in continuous culture on stainless-steel coupons in AB synthetic medium were treated with DBD plasma generated in ambient air. The plasma source was located 1 or 4 mm from the sample surface. Results are the average of four independent experiments, and each experiment was performed in duplicate. Error bars represent the standard error of the mean.
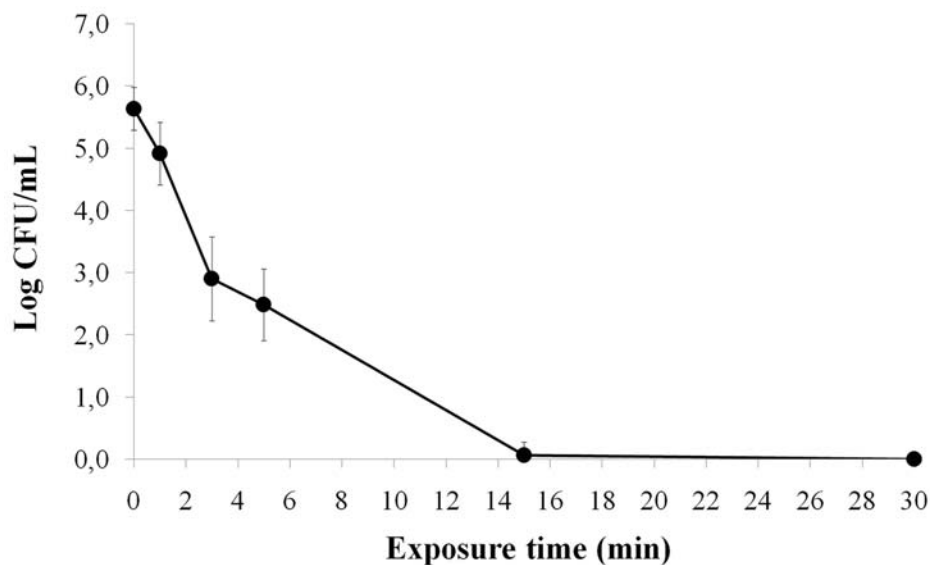
FIG. 6: Survival curve: log of the number of CFU/mL vs. exposure time. *P. aeruginosa* biofilms grown in continuous culture on stainless-steel coupons in AB synthetic medium were treated with DBD plasma generated in moistened air at a flow of 1 L/min. The plasma source was located 4 mm from the sample surface. Results are the average of four independent experiments, and each experiment was performed in duplicate. Error bars represent the standard error of the mean.

experiments with moistened air. Because there were no significant differences in the concentration of culturable cells treated with plasma operating in ambient air for the two distances used, and both D values (see below) were similar, a 4 mm-distance was chosen for the experiments with moistened air because of practical operative reasons (easier coupon manipulation). Cell concentration at a 0-min exposure time corresponds to the control without plasma treatment.

The graphs show that *P. aeurginosa* biofilms grown on stainless-steel coupons in continuous culture in AB medium were completely eradicated after a 15-min treatment with plasma for both treatment conditions, namely, ambient air and a moistened airflow of 1 L/min, resulting in a CFU/mL decrease of 5.6 $\log_{10}$ units (>99.999% killing efficacy). For plasma generated in ambient air, a CFU/mL decrease of 1.6 $\log_{10}$ units was determined for a 3-min exposure to plasma, whereas a decrease of 2.7 $\log_{10}$ units was determined for same plasma exposure time using moistened air. These results demonstrate that moistened air inactivates biofilms more rapidly than ambient air. CFU/coupon decreases of 5.50, 6.88, and 4.20 $\log_{10}$ were obtained, respectively, for *Escherichia coli* O157:H7 (ATCC 438), *Cronobacter sakazakii* (ATCC 29004), and *Staphylococcus aureus* (KCCM 40050) biofilms using an underwater DBD plasma source operating in dry air.[77] In this case, the biofilms were treated for 90 min. Reductions of 5.8 $\log_{10}$ were obtained for *P. aeruginosa* biofilms treated with O_2–N_2 plasma for 60 min.[12] Therefore, our methodology provides an excellent killing efficacy with shorter exposures to plasma.

A good measure of the removal efficiency is the determination of decimal reduction time (D value), the time required to reduce an original concentration of microorganisms by 90%. This parameter has been originally defined for thermal killing of microorganisms by autoclaving. For plasma generated in ambient air, results show a constant decline in CFU/mL, with a D value of 2.78 ± 0.14 min for the 1-mm distance and a D value of 2.49 ± 0.38 min for the 4-mm distance. Single-slope kinetics was reported by Ziuzina et al. for *Listeria monocytogenes* biofilms treated with atmospheric cold plasmas produced in a DBD configuration.[28] In a recent contribution by Gabriel et al., single-slope kinetics and D values on the order of 2–3 s were reported for *P. aeruginosa* biofilms grown on stainless-steel surfaces and treated with an air-based microwave plasma jet.[13] However, these authors incubated the bacterial inoculum for just 4 hr and, even if they had a biofilm and not a simple layer of attached cells, it is likely easier to remove such a young structure compared to a mature biofilm. In addition, the reported D values were obtained at temperatures of 143.42ºC to 174.05ºC, much higher than those achieved in our work.

For the plasma generated in moistened air, the survival curve shows a typical double-slope kinetics. Therefore, two D values were determined: one for short exposure times (D_1) and another for the portion of the curve showing a slower decline (D_2). D_1 measured 1.14 ± 0.28 min, and D_2 4.36 ± 0.63 min. This inactivation kinetics is similar to what has been previously described.[25,26] Vandervoort and Brelles-Mariño reported double-slope kinetics for *P. aeruginosa* biofilms grown on borosilicate coupons under continuous culture: D_1 measured 27 s and D_2 6.13 min.[24] Zelaya et al. reported double-slope kinetics for *P. aeurginosa* biofilm grown in batch cultures, although D values were not calculated.[25] In that work, results showed a clear decrease in the percentage of surviving cells versus time, regardless of the biofilm age. The same group reported similar results for *Chromobacterium violaceum* biofilms grown for 4 or 7 d on polystyrene microtiter plates.[11,16]

The removal efficiency of the treatments can be assessed by comparing D values. The D value corresponding to biofilms treated with plasma generated in ambient air is twice as high as D_1 obtained for biofilms treated with plasma generated in moistened air. The difference might be due to the presence of more reactive oxygen and nitrogen species and ozone in the moistened air plasma. Purevdorj et al. studied plasma-mediated killing of spore-forming *Bacillus pumilus*.[22] In this case, spore mortality varied depending on the composition of the gas feed and was higher with moistened air plasma, suggesting that the inactivation may occur through hydroxyl radicals generated from water molecules.

In Vandervoort and Brelles-Mariño's contribution with *P. aeruginosa* biofilms grown in continuous culture, the decrease in the percentage of viable cells was more dramatic than the results we report here, because there were almost no culturable cells after a 5-min treatment with plasma, and most of the inactivation occurred during a biofilm exposure to plasma of <1 min. However, it was also reported that cells retained viability and virulence at short periods of exposure to plasma, even though cells were not culturable.[24] Moreover, it is important to point out that previous work was carried out using a commercially available radiofrequency plasma jet operating in helium, which is an expensive inert gas.

Here, we propose a plasma source prototype operating in simple moistened air, which makes the design affordable, easy to build, and easy to use but still effective.

D. Viability Experiments

Epifluorescence microscopy and the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit allow one to assess plasma-treated biofilm cell viability by monitoring viability as a function of the membrane integrity of the cell. The LIVE/DEAD assay consists of a mixture of two dyes: the green fluorescent nucleic acid stain SYTO® 9 and the red fluorescent nucleic acid stain propidium iodide. Both stains differ in their ability to penetrate bacterial membranes. When used alone, SYTO 9 stains cells with both intact and damaged membranes, whereas propidium iodide penetrates only bacteria with damaged membranes. Propidium iodide competes with SYTO 9 stain for nucleic acid binding sites when both dyes are present. Therefore, when mixed in recommended proportions, bacteria with intact cell membranes fluoresce green, and bacteria with damaged membranes fluoresce red. Thus, the ratio of green to red fluorescence provides an estimation of bacterial viability.

Figure 7 shows the fluorescent images of biofilms stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit. These images correspond to biofilms not exposed to
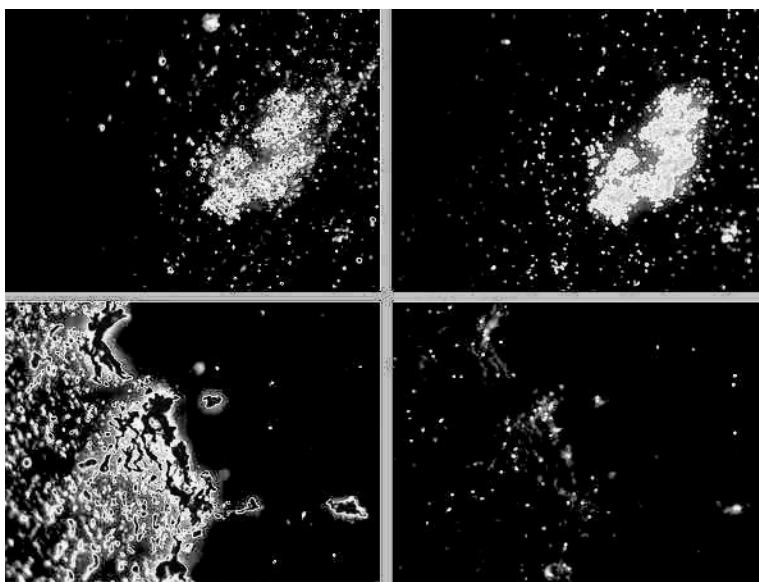

FIG. 7: Fluorescence microscopy images of biofilms: Biofilms grown as indicated in Materials and Methods section were stained with LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit and visualized using the 480/500-nm excitation/emission filter for SYTO® 9 (left) and the 490/635-nm excitation/emission filter for propidium iodide (right). (Upper panels) Biofilms with no DNAse treatment; (lower panels) biofilms treated with DNAse for 60 min before staining.

plasma (control). Therefore, most of the cells were thought to be alive and, thus, green. The upper panel depicts control biofilms with no DNAse treatment visualized using the 480/500-nm excitation/emission filter for SYTO® 9 (Fig. 7, left) and the 490/635-nm excitation/emission filter for propidium iodide (Fig. 7, right). As can be seen in the upper right image, the cells stain red, although we were not expecting to have a dead biofilm before the plasma treatment. We hypothesized that cells were not actually dead and that the red staining was due to extracellular DNA (eDNA) released from the biofilm and masking the cells that were alive.

Biofilm-forming strains of *P. aeruginosa*, including PAO1, have been shown to produce eDNA that may function as part of the biofilm matrix.[78] Evidence has been presented that suggests that eDNA functions as a cell-to-cell interconnecting compound in *P. aeruginosa* biofilms.[43,78] Allesen-Holm et al. have studied the distribution of eDNA on *P. aeruginosa* PAO1 biofilms grown in AB medium and have obtained similar results to those shown in Fig. 7, upper panel.[43] The researchers reported that DNAse treatment dissolved young *P. aeruginosa* biofilms, whereas established biofilms were only marginally affected by the treatment. This suggests that the cells in young biofilms are held together by eDNA, whereas the cells in older biofilms are held together primarily by other compounds. Based on these reports, we decided to treat the biofilms with DNAse before staining, and results are shown in Fig. 7, lower panel. It is clear by comparing images on the left (green-stained biofilm) and right (few red cells) that the biofilm had most of its cells alive and the red color shown on the upper panel on the right side was due to eDNA and not dead cells.

Figure 8 shows the fluorescent images of biofilms subjected to plasma treatment for 0, 3, 15, and 30 min, treated with DNAse for 60 min, and stained with the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit. As can be seen in the images, biofilms are predominantly green for the control and the 3-min treatment; therefore, cells are alive. The coupon subjected to a 30-min plasma treatment shows a completely red-stained biofilm, and, therefore, cells are dead. As for the biofilm treated with plasma for 15 min, although most of the cells are red and dead, live green cells are still visible. It is important to note that the survival curves (Figs. 5 and 6) showed no culturable cells after 15 min of exposure to plasma. However, the LIVE/DEAD® *Bac*Light™ demonstrates the presence of living cells that may eventually multiply. These results support our previous statement that viability experiments should always be carried out before drawing the conclusion that plasma eradicates biofilms at short exposure times, based solely on counting culturable cells.[16,24]

IV. CONCLUSION

In summary, results show that the coaxial DBD plasma source operating in air is a useful tool for the inactivation of *Pseudomonas* biofilms grown on stainless-steel coupons in continuous culture and synthetic medium. The coaxial DBD plasma source was used to generate the discharge, either in ambient air or in moistened air. For the latter, we report an inactivation kinetics similar to that in previously described results.[24,26] We also
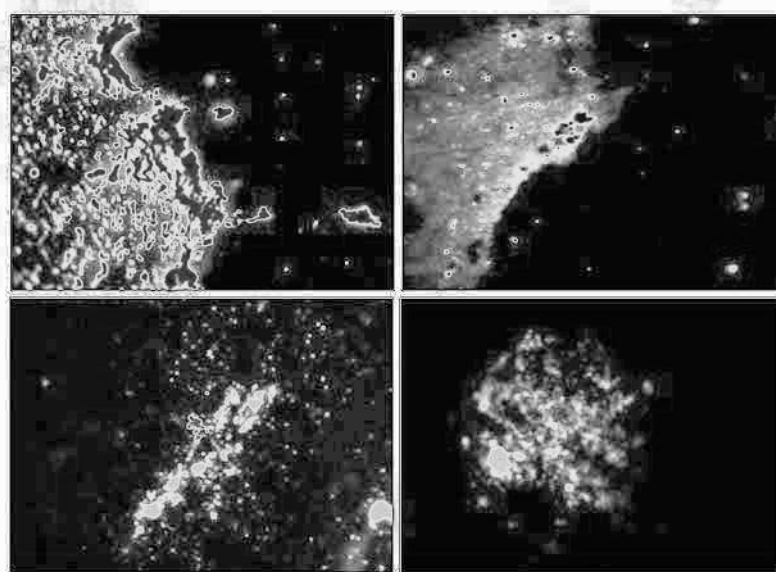
FIG. 8: Fluoresence microscopy of plasma-treated biofilms. Biofilms grown as indicated in the Materials and Methods sections were subjected to plasma treatment for 0, 3, 15, and 30 min, treated with DNAse for 60 min, and stained with a LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit. (Upper panels) 0 and 3 min of plasma treatment, left and right image, respectively; (lower panels) 15 and 30 min of plasma treatment, left and right image, respectively. Images were superimposed from images obtained using the 480/500-nm excitation/emission filter for SYTO® 9 and the 490/635-nm excitation/emission filter for propidium iodide.

demonstrated the presence of extracellular DNA on *Pseudomonas* biofilms grown in continuous culture and that biofilms treated with plasma for relatively short exposure times retain viability, even in the case of lack of cell culturability. Our results support the statement that viability experiments are indispensable before drawing the conclusion that plasma eradicates biofilms based solely on cell culturability.

The coaxial DBD presented in this work represents a more affordable, easy-to-build, and easy-to-handle but still effective plasma source, because it operates in air and does not rely on the use of helium or other expensive inert gases. In addition, the search for the best combination of culture medium and surface material leading to a robust biofilm before plasma treatment has not been previously assessed.

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