Inactivation of Bacteria in Flight by Direct Exposure to Nonthermal Plasma

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***Abstract*—Plasma treatment is a promising technology for fast and effective sterilization of surfaces, waterflow, and airflow. The treatment of airflow is an important area of healthcare and biodefense that has recently gained the interest of many scientists. In this paper, we describe a dielectric barrier grating discharge (DBGD) which is used to study the inactivation of airborne *Escherichia coli* inside a closed air circulation system. Earlier published results indicate approximately 5-log reduction (99.999%) in the concentration of the airborne bacteria after single DBGD exposure of 10-s duration. This paper investigates plasma species influencing the inactivation. The two major factors that are studied are the effect of charged and short-lived species (direct exposure to plasma) and the effect of ozone. It is shown that for a 25% reduction in direct exposure, the inactivation falls from 97% to 29% in a single pass through the grating. The influence of ozone was studied by producing ozone remotely with an ozone generator and injecting the same concentration into the system, as that produced by the DBGD plasma. The results show a 10% reduction in the bacterial load after 10-s exposure to ozone; thus, ozone alone may not be one of the major inactivating factors in the plasma.**

***Index Terms*—Air sterilization, bioaerosol, dielectric barrier discharge (DBD), plasma medicine, plasma sterilization.**

I. Introduction

THE RECENT outbreaks of foodborne pathogens as well as the growing threat of bioterrorism have brought into focus the need for an effective method to sterilize air, water, and surfaces from microorganisms. In this paper, we will focus on air sterilization. Nonthermal plasma has been proposed as an effective way of surface sterilization of different materials, including living tissue [1], [2]. In our earlier work on air sterilization, we showed an $\sim$1.5-log reduction in the concentration of airborne bacteria after one single exposure to plasma where the residence time of a single bacterium in the discharge zone was 730 μs based on the flow velocity through the discharge zone [3]. In this paper, we further investigate this rapid inactivation effect. A gratinglike arrangement of a dielectric barrier discharge (DBD) configuration was designed for use in our air sterilization experiments [4]. The discharge was created between a grating made up of alternating high-voltage wires (covered by quartz dielectric) and grounded wires. An alternating-current pulsed high-voltage signal was utilized for discharge ignition. The details of the plasma power supply and setup have been previously reported by authors [1]. Reactive oxygen species (ROS) are well-known sterilization agents [5] and are produced in abundance in air plasma [6]. These ROS, such as hydroxyl radical, molecular and atomic oxygen ions (e.g., superoxide anion and singlet oxygen), and ozone, have bactericidal properties [7]. The sterilization effect produced by the plasma has been discussed in detail, and theories have been put forward to explain the cause and the mechanism of sterilization [8]–[11]. Most studies have used a DBD discharge point in the direction of the ROS as the main sterilizing agent. Plasma discharge produces a mixture of species that are highly reactive with various biomolecules as well as able to produce other bioreactive species due to their interaction with each other [9].

Role of Ozone in Inactivation of Bacteria: Ozone has been widely used commercially as a sterilizing agent [12]. The only method used to generate ozone is plasma discharge (DBD, usually) [4]. Ozone is a long-living specie and is predominantly present in the atmosphere after the plasma discharge. The sterilization effect by ozone is a complex phenomenon. Ozone reacts with almost every major component of the bacterial cell, and its effects have been observed on the cell membrane, DNA, and RNA [13]. SEM images of *Escherichia coli* treated with an ozone concentration of 0.167/mg/min/L for 30–60 min have shown major damage to surface morphology and membrane lysis [14], [15]. Ozone was shown to react with nucleic acid *in vitro*, and thymine was the base that was the most reactive with ozone [16]. Ozone acts as a general protoplasmic oxidant [17], [18]. Kowalski *et al.* [19] investigated the effects of the high concentration of airborne ozone on *E. coli* and *Staphylococcus aureus*. In their study, 30–1500 ppm of ozone was pumped into a chamber containing petri dishes with bacteria on their surface. It was observed that for high concentrations of ozone, there was a 4-log reduction in viable bacteria counts after 480 s of exposure. This would suggest that the action of ozone on the bacteria is slow in comparison with direct plasma treatment [3], [21].

Role of Direct Exposure to Plasma: Major Effect on Inactivation: Microorganisms may be treated by plasma in two ways:

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1) where the organism comes in direct contact with the whole contents of the plasma such as charged species, UV and VUV, long- and short-lived species, etc., or 2) where the organism is separated from the plasma and only the long-lived plasma-generated species and/or UV and VUV light are used for the treatment. We term the first way as a *direct* treatment and the second as an *indirect* one. For the case of air sterilization by plasma, direct exposure would mean that the bacteria in air are flown through the plasma discharge; while in the case of the indirect air sterilization, the bacteria are not flown through the plasma. The reactive species that have a shorter lifetime recombine with each other or may be deactivated before they can affect the bacteria [20]. Fridman *et al.* [21] showed that direct exposure is more effective in bacteria inactivation during surface treatment. Investigating the effects of direct exposure will help us determine if the short-lived species, such as ions, electrons, and ROS, are responsible for the inactivation.

II. Materials and Methods

A. Experimental Setup

The previously described pathogen detection and remediation facility (PDRF) was used to perform the airflow sterilization experiments [3]. In general, the PDRF setup is a closed-loop air circulating system that consisted of a large 250-L drum connected by pipes to a square box that contains the electrode arrangement of the dielectric barrier grating discharge (DBGD). The drum with internal baffles provides a desirable volume of air for the experiments and arranges the airflow treatment in the plug flow reactor mode. Air sampling ports on both sides of the DBGD are used to sample the air from inside the system. The flow inside the system was not interrupted throughout the plasma treatment or the sampling procedure. The flow rate is maintained at 25 L/s, so the entire volume is circulated within 10 s; for this reason, the plasma treatment procedure consisted of turning the plasma discharge on for 10 s to treat all air in the chamber. The sampling time points were kept constant for all of the experiments. All experiments were performed with the same initial conditions, and the only parameter that was changed was the type of treatment:

1) direct plasma exposure (same as reported in earlier publication [3]);
2) 75% direct exposure (where 75% of the bacteria pass through the plasma and 25% do not);
3) indirect plasma exposure: treatment by ozone (injection of the same amount of ozone as is produced by the plasma).

Direct Exposure: The plasma discharge setup consists of 21 high-voltage wire electrodes insulated by quartz and 22 grounded wires. When the discharge is initiated, DBD plasma is produced in the air gaps between the electrodes and grounded wires. This creates a screen of the plasma that the bacteria have to pass through. This arrangement of the plasma discharge is shown in Fig. 1.

The direct exposure experiments performed with this setup are reported in our earlier publication [3]. The setup modification for the indirect treatment is described hereinafter.

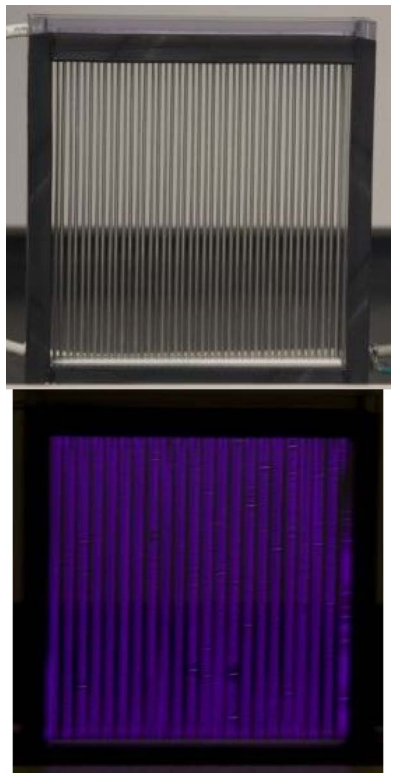


Fig. 1. (Top) Plasma unit with its multiple-electrode configuration. (Bottom) Same plasma unit, with the plasma discharge initiated. Notice the screen of plasma covering the entire cross section of air passage.

75% Direct Exposure: There are two ways of investigating the effect of direct exposure to plasma.

1) Introducing a barrier between the plasma and the sample to be treated, thus removing the influence of the ions and ROS produced by the plasma: This method is not possible for these experiments as the flow of air is perpendicular to the electrodes, any obstruction will lead to changes in airflow, and a pressure drop will be introduced inside the system;
2) Reducing the total area of direct exposure and letting a certain percentage of the sample (in this case, the bioaerosol) be treated indirectly by the long-living species such as ozone. In the DBGD setup, this can be achieved by reducing the number of active electrodes, creating gaps in the screen produced by the plasma. This way, a certain percentage of the bacteria escape coming in contact with the plasma and are treated by plasma indirectly.

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

This discharge is shown in Fig. 2. This discharge occurs across the air gaps between the wires. Since the area of the discharge is 75% of the total cross-sectional area, this can be termed as the 75% direct and 25% indirect exposure. In this case, as the plasma discharge is away from the path of 25% of the bacteria flow, it can be considered indirect treatment



Fig. 2. (Top) Plasma unit with every fourth high-voltage electrode wire removed. Quartz tubes are retained in order to maintain the same airflow. This produces (A) zones of indirect exposure. Image shown on the bottom is with the plasma.

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

Indirect Exposure (Ozone Treatment): The major long-living specie created by the DBD plasma in volume is ozone. The dissociation of O_2 molecules in air by energetic electrons is the first reaction in this process. This reaction followed by a three-body reaction between O, O_2, and M leads to the formation of ozone [22], where M is another molecule or wall

$$O + O_2 + M \rightarrow O_3^* + M \rightarrow O_3 + M.$$

To isolate the effect of the plasma-generated ozone on the airborne bacteria, we produce ozone elsewhere and inject it into the chamber. For this, the ozone concentration generated by the DBGD inside the system was measured, the total ozone production was calculated, and then the ozone generator (MedOzone, Russia) was adjusted to produce the same amount of ozone. The bacteria were introduced into the system through the process of nebulization. A pretreatment air sample was taken, and the ozone generator was switched on for 10 s, the same time as the plasma treatment. The ozone was allowed to pass through the experimental system, and a postozone treatment sample was taken. The time of sampling was kept the same as that in the plasma experiment. Further samples were taken pre- and postozone exposure.

B. Growth and Preparation of Bacterial Strains for Nebulization

The microorganism used in this paper was *E. coli* K12 substr. MG1655. Strains were prepared as frozen stocks. The frozen stock was transferred to a 10-mL culture tube containing Luria Bertani media. The culture was grown overnight in an incubator shaker at 37 °C. The culture was then transferred to centrifuge tubes and spun at 3500 r/min for 1 min. The supernatant was removed, and the pellet was again washed with deionized water. The final solution was prepared by adding the bacterial pellet to 30 mL of deionized water.

C. Injection of Bacteria and Air Sampling

A 24-jet collision nebulizer (BGI Inc., Waltham, MA) was connected to the system. Deionized water was added to the nebulizer, and the nebulizer was operated at a 40 lbf/in^2 input pressure. This injection was performed to increase the humidity inside the system. The humidity was increased to 70% RH, and the nebulizer was disconnected. The bacterial solution was then added to the nebulizer, and it was connected back to the system. The nebulizer was run again at 40 lbf/in^2 for 45 s. The nebulizer was then disconnected and removed. The sampling of the air inside the system was performed using specially modified AGI impingers (Ace Glass Inc., Vineland, NJ) [23]. A negative air pressure system was used for acquiring the samples from the uninterrupted circular flow system.

D. Analysis of Surviving Cells

The samples were taken in a 1 × phosphate buffered saline solution. Each sample was then diluted using the serial dilution method. The dilutions were then plated on BHI agar plates. These plates were incubated overnight at 37 °C inside an aerobic incubator. The number of colonies growing on the plates was counted the next day to determine the number of bacteria present in the sample.

III. Results

A. Measurement of Ozone

The concentration of ozone produced by the plasma was measured. This was done using an ozone meter. The plasma discharge was initiated and kept on for 10 s. It was observed that the concentration of ozone generated was 28 ppm. A separate ozone generator was employed for producing ozone. It has an intake for air and outlet for the ozone generated. It was observed that at 0.5 SLPM, the amount of the ozone generated inside the system by the generator is the same as that generated by the DBD plasma for 10 s, i.e., 28 ppm (see Fig. 3).

B. Direct and Indirect Exposure

Fig. 4 shows the summary of the experimental results performed with the PDRF system. 100% direct exposure to plasma leads to the greatest degree of inactivation. A 25% drop in the direct exposure leads to the inactivation due to plasma exposure

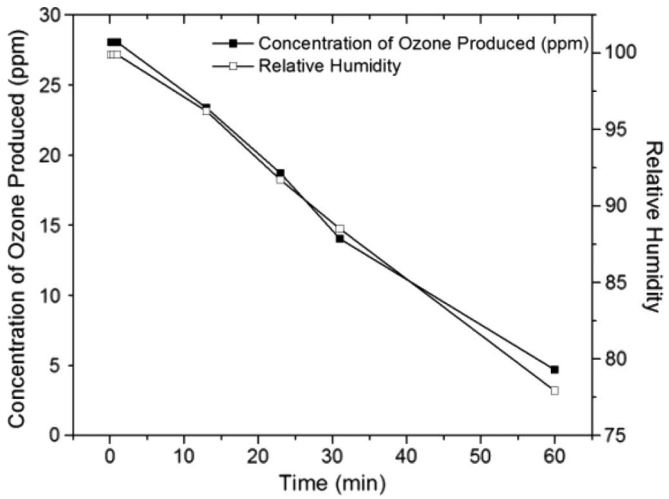


Fig. 3. Evolution of ozone generated by DBGD discharge and relative humidity inside the system.

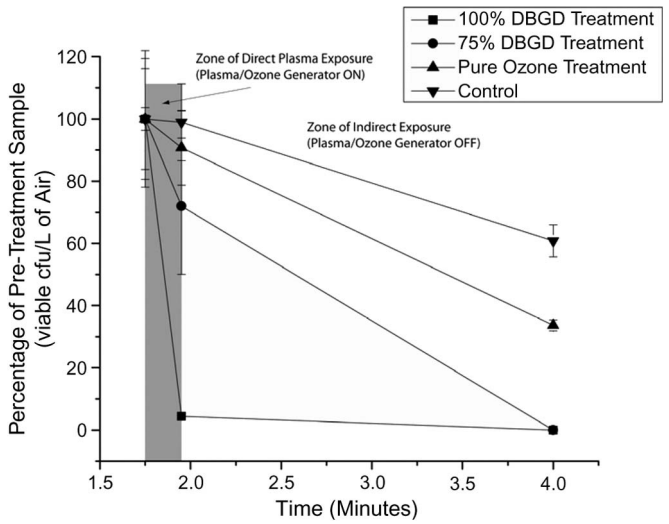


Fig. 4. Results of the experiments. Dark shaded region denotes the first 10-s treatment with (■) 100% plasma, (●) 75% plasma/(▲) ozone as compared to the control runs with (▼) no plasma/ozone. Error bars represent the standard deviation of mean of three trials. Time scale is from the instance of injection of bioaerosol. Third sample is pretreatment sample for second treatment.

to drop to 29% from the 97% observed in the 100% direct exposure experiments. The inactivation experiments with pure ozone produced the least degree of inactivation.

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

IV. Discussion

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

The direct exposure to the plasma disturbs the bacteria membrane, and the charges stick to the membrane. While a complete membrane breakdown requires a field of several kilovolts per centimeter and longer time periods [24], we know that charge absorption leads to pores opening much faster—in millisecond and tens of microseconds time range [25]. A follow-up 2-min ozone action on the bacteria with disturbed membranes provides complete sterilization.

The investigation of the influence of direct exposure shows that there is a 3.5-log reduction during the much longer post-plasma exposure. This means that, after the initial 97% reduction, the remaining bacteria keep flowing through the system when ozone enters the bacteria and further reacts with the membrane to inactivate them. Fan *et al.* [28] observed that there is a synergism between negative air ions (NAIs) produced by plasma and ozone on a bacterial cell death. The bactericidal effect of the NAIs in addition to the ozone was found to be far greater than the ozone itself. In their experiments, the viability of *E. coli* was reduced to 40% of the first sample after 11 h of NAI treatment, as compared with 70% in the ozone alone treatment.

The humidity inside the system plays a role in the inactivation. The bacteria in our experiments are in the form of a bioaerosol. This bioaerosol consists of the bacteria enclosed in a fine droplet of water. As the bioaerosol travels inside the system, the droplet shrinks. Dunklin and Puck [26] show that the shrinkage of the water droplet depends on the relative humidity and that, at 50% RH, the droplets shrink to one tenth of their size in 4 ms. Our experiments were made at a higher RH. As the liquid can act as a protective shield around the bacteria, the shrinking of the droplet causes the bacteria to be more vulnerable to the charges and ROS produced by the DBGD plasma. Although Muranyi *et al.* [27] have recently demonstrated that the fastest inactivation of plasma-treated *Aspergilus niger* spores occurs at a high relative humidity

(70%), their experiments consisted of treating bacteria placed on surfaces, whereas, in this paper, we discuss the inactivation of airborne bacteria.

Due to the humid air inside our system, an OH radical is expected to be formed. It is known that one main path for the generation of the hydroxyl radical in a DBD system is the photodissociation of ozone into atomic singlet oxygen and the reaction of this radical with water molecules [29]. This can be another synergetic mechanism that explains the nonlinearity in the direct plasma treatment. With this knowledge and our experimental results, we can conclude that the main cause of inactivation is the synergetic action of short-living plasma agents (charges, radiation, and radicals such as OH) that disturb the membrane and ozone. This synergy creates a toxic environment for the bacteria, ultimately resulting in inactivation.

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

V. Conclusion

The results of the air sterilization experiments led us to believe that the main reason of the fast in-flight bacteria inactivation is the synergetic action of short-living plasma agents (direct plasma treatment) and ozone. The direct exposure to plasma inactivates part of the bacteria and disturbs the membranes of others. The follow-up ozone treatment of the direct plasma-treated bacteria provides complete sterilization.

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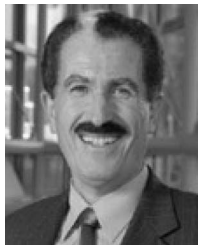
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