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Augmented Survival of Bacteria Within Biofilms to Exposure to an Atmospheric Pressure Non-Thermal Plasma Source

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Abstract: Bacteria embedded within biofilms present a challenge to surface decontamination by conventional means. Atmospheric pressure non-thermal plasma processes have emerged as a promising approach to overcome this problem. We used a non-thermal atmospheric pressure plasma, operated in a humid atmosphere, to assess planctonic versus biofilm-resident bacterial (*Escherichia coli*) susceptibility to treatment. The concentrations of stable chemical species at the treatment reactor gas outlet were monitored by FTIR. The decontamination efficiency of the process was evaluated against bacteria embedded within a biofilm, as well as planctonic cells placed on a glass surface. Bacterial survival was assessed using a combination of Colony Forming Unit (CFU) ability and vital staining with a combination of DAPI plus Propidium Iodide. Both methods revealed an increased resistance of biofilm-resident bacteria, when compared to planctonic cells, after a 40 min exposure to the post discharge gas. Present results show that biofilm-resident bacteria demonstrate augmented survival when exposed to atmospheric pressure non-thermal plasma treatment and thus that decontamination procedures should take into account this survival when evaluating surface decontamination measures.

Key words: Biofilm, *Escherichia coli*, non-thermal plasma, fluorescence microscopy

INTRODUCTION

Biofilms consist of microorganisms embedded in biological polymers which can be composed of polysaccharides, proteins and DNA. They form complex structures notorious for their resistance to various treatments such as antibiotics or chemical/physical decontamination procedures. The exact mechanisms responsible for their resistance are, as yet, not fully understood, though the expolymeric substances (EPS) surrounding the cells can act as a physical/chemical protective barrier (Odic *et al*., 2002; O'Toole *et al*., 2000). Biofilm construction starts with bacterial cells adhering to a surface. Microcolonies then develop that become increasingly complex in structure and eventually shed cells that can act as founders for new biofilms (Cho *et al*., 2007; O'Toole *et al*., 2000; Patel, 2005). Bacteria within biofilms are noted to be difficult to completely remove or disinfect from a surface using known decontamination procedures (Kamgang *et al*., 2007; Nadakumar *et al*., 2004). Surface treatment methods, such as ultraviolet (UV) or X-ray irradiation, chemical cleaning, heat treatment (with or without chemical treatment), present various drawbacks, including high costs, inadequacy of complete bacterial inactivation and material damage to fragile and/or complex surfaces such as electronic components, catheters and other medical devices (Laroussi and Leipold, 2004). A serious need for research in this area is thus emerging.

Non-thermal plasma present a promising avenue for surface decontamination (Odic *et al*., 2002; Pointu *et al*., 2008). Non-thermal atmospheric pressure plasma processes are simple to implement and yield, at moderate temperatures (generally room temperature), reactive chemical species. The concentration and nature of these species (including free radicals, excited molecules and stable species such as ozone) can be controlled, depending upon the gas mixture and electrical parameters used. Light emission is also produced by non-thermal plasma and according to the surface exposure mode, the resultant UV emissions can contribute to surface treatment. Non-thermal plasma have been widely studied to treat surfaces for a variety of purposes such as polymer surface modification (Arefi-Khonsari *et al*., 2005) and

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wafer surface cleaning for electronic applications (Radio-frequency plasma) (Korner *et al*., 1995). Atmospheric pressure non-thermal plasma processes have been shown to be effective for surface decontamination of various agents, such as bacterial cells (Dodet *et al*, 2006; Kamgang *et al*., 2007; Kirkpatrick *et al*., 2007; Moreau *et al*., 2005) or prions (Baxter *et al*., 2005). It has also been shown that non-thermal plasma processes can irreversibly damage bacterial spores so that they are unable to grow and form colonies on Petri dishes (Odic *et al*., 2002). For the present study, an atmospheric pressure non-thermal plasma device was specifically developed for the treatment of biofilms present on surfaces. This device presents the following advantages: operation at atmospheric pressure, low investment (relatively simple device) and low energy costs (discharge input power <2 W), minor heating of the gas and treated surfaces, a post discharge exposure mode implying homogeneous treatment of the surface, adjustable geometry of the plasma generation electrodes and thus variable surface area treatment. For this study, the plasma device was operated in a humid atmosphere. The decontamination efficiency of the process was evaluated using differential staining of *Escherichia coli* bacteria directly within a biofilm, as well as planctonic cells placed on a glass surface. Present results show that biofilm-resident bacteria demonstrate greater survival to atmospheric pressure non-thermal plasma treatment than planctonic cells and thus that decontamination procedures should take into account this survival when evaluating surface decontamination measures.

MATERIALS AND METHODS

Strains and growth medium: *Escherichia coli* strain DH10B (F-, *mcrA*, Δ(*mrr-hsdRMS-mcrBC*), ϕ80d*lacZ*ΔM15, Δ*lacX*74, *deoR*, *recA*1, *relA*1, *endA*1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *galK*16, *galE*15, *rpsL*, *nupG*, *spoT*) (Durfee *et al*., 2008) was stored at -20°C in glycerol (20% v/v). Bacteria were cultured at 30°C in M63 minimal medium supplemented with glucose (0.2%) without agitation for all experiments (Miller, 1972).

Planctonic cell preparation: The cells of a stationary phase bacterial culture were collected by centrifugation (4000 x g, 10 min, 5°C) and the cells re-suspended in water. After dilution, 10 μL of a 10^8 CFU mL^{-1} suspension were spotted on sterile glass microscope covers slips (15×15 mm, Menzel Glaser, Braunschweig, Germany) and subsequently dried at room temperature for 45 min prior to atmospheric pressure non-thermal plasma treatment.

Biofilm formation on glass coverslips: Sterile glass microscope covers slips were placed in a 60 mm diameter plastic Petri dish containing 15 mL of M 63 medium (Miller, 1972). Then, 200 μL of stationary phase bacterial culture were added and the Petri dish incubated for a further 48 h at 37°C without agitation. The glass cover slips were then removed, washed with distilled water and dried at room temperature for 45 min prior to atmospheric pressure non-thermal plasma treatment.

Plasma treatment: The non-thermal plasma apparatus (Fig. 1) consists of two parallel insulated electrodes separated by a 1 mm gas gap g as the electrical discharge cell. Each insulated electrode is a borosilicate tube (external diameter 2×r = 6 mm, 1.5 mm thickness, 70 mm length) with its inner surface partially covered with silver paste acting as an electrode (the active length of the electrode is limited to 50 mm). One electrode is connected to ground through a measurement resistor R_m and the other is energized by a high voltage power supply (AC 28 kHz; 0-10 kV_{peak}). Micro filamentary discharges develop in the gas gap g between the two parallel

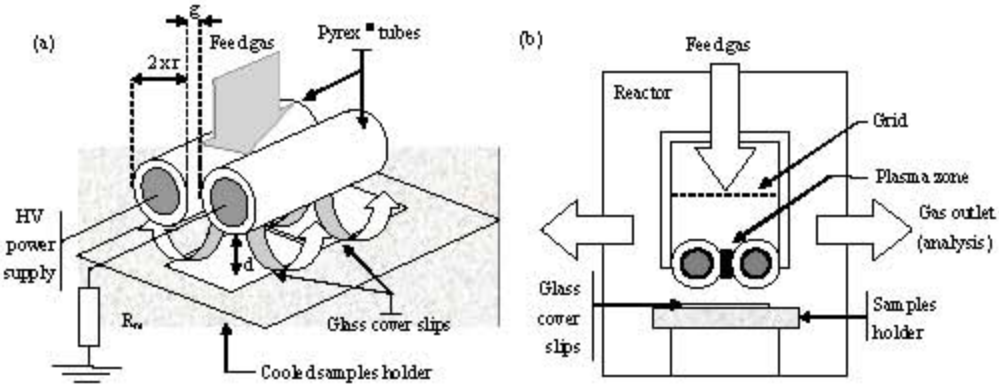


Fig. 1: Diagram of the (a) discharge cell and (b) plasma treatment apparatus

electrodes along the 50 mm active length. Arcing is prevented by the borosilicate insulators (relative permitivity ε_r = 4.5) through a dielectric surface charge build-up mechanism leading to a decrease of the local electrical field. Voltage and discharge current measurements were made using a LeCroy PPE20kV 1:1000 voltage probe (Lecroy France, Courtaboeuf, France) and the R_m 25 Ω resistor, respectively. Electrical signals were recorded using a Tektronix TDS 544A 500 MHz oscilloscope (Tektronix S.A., Les Ulis, France). The mean discharge input power P was calculated by the instantaneous voltage current product method integrated over two periods of the applied voltage signal. The distance d between the dielectric tube arrangement and the surface to be treated was fixed at 3 mm (Fig. 1a) and the plasma zone was then located approximately 6 mm above the sample surface. The feed gas was an air-like mixture (N_2/O_2-80/20) saturated, by bubbling distilled water through a gas sparging bottle, with water vapor at room temperature. The feed gas is homogeneously distributed in the electrode gap along the active zone (50 mm) using a grid device (Fig. 1b). Two glass cover slips, containing either planctonic or biofilm-resident bacteria, were simultaneously exposed to the discharge effluents. This arrangement allowed the entire surface of the glass cover slips to be exposed to the plasma effluent. The sample holder (microscope slide covering a brass plate) was cooled to enhance water condensation. The entire device, including sample holder discharge cell and feed gas injector, was embedded in a treatment reactor. The gas outlet composition was monitored using a Bruker FTIR absorption spectrophotometer (Bruker Optics, Champs sur Marne, France). The experimental spectra were acquired using a feed gas consisting of air at atmospheric pressure and ambient temperature. In order to subtract overlapping peaks of water vapor absorption, the reference transmittance spectrum was acquired in atmospheric air saturated (RH≥95%) at 50°C with water vapor.

For each treatment, four glass cover slips with the biofilm or planctonic cells were prepared. One was kept at room temperature as a non-exposed control, while the other two were placed on a microscope slide for plasma treatment. The slide was then placed into the treatment zone. Typical operating conditions were as follows:

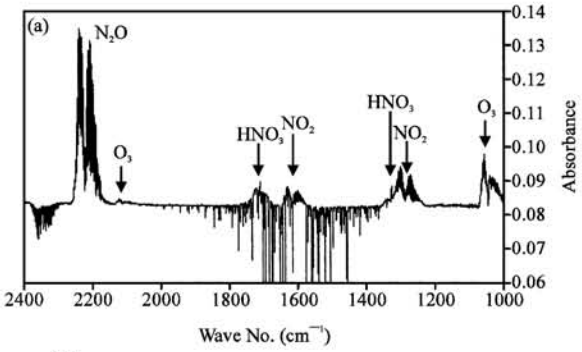
- Exposure duration (treatment time): 40 min
- Mean discharge input power P: 1.7-1.8 W
- Gas flow rate: 2 NL min^{-1} (N_2/O_2-80/20, RH≥95%)

Viability assays: After treatment, one of the cover slips was used for determining cell viability by Colony Forming Unit (CFU). Using a sterile glass microscope slide, the bacteria in the biofilm, or deposited planctonic cells, were scraped from the cover slips into a 60 mm Petri dish containing 5 mL of LB media (Miller, 1972) and then dilutions were spread on LB agar. The colonies were then counted after a 30 h incubation at 30°C. The other cover slip was stained and examined using DAPI (10 μg mL^{-1}) plus propidium iodide (5 μg mL^{-1}), as previously described by Doolittle *et al*. (1996), Jenkins *et al*. (1997) and Lecoeur (2002). The cover slips were then placed in the dark for 15 min prior to observation by fluorescence microscopy.

RESULTS

In order to determine the relative sensitivity of biofilm-located versus planctonic *E. coli* cells to an atmospheric pressure non-thermal plasma source (post-discharge exposure mode), treatments were carried out using remote exposure of cover slip surfaces, containing the bacteria, to the effluent gas. A sketch of the experimental set-up and the plasma source are presented in Fig. 1. The plasma produced in the transient micro-discharges does not reach thermodynamic equilibrium (non-thermal plasma) and thus the bulk gas temperature remains close to ambient (data not shown). The gaseous feed mixture, streaming through the gas gap at atmospheric pressure, was activated by the discharge. The resultant non-thermal plasma-activated gas mixture was then allowed to flow over the sample surface. Thus, there was no direct interaction between the plasma and the samples. The non-thermal plasma-activated gas mixture which flowed over the sample surface was characterized using absorption spectroscopy (Fig. 2a). Corresponding stable species are shown along with the detected concentrations expressed in ppm (Fig. 2b). The negative absorption bands observed in the spectrum are due to (1) the CO_2 (2300-2380 cm^{-1}) absence in the non-thermal plasma-activated gas mixture and (2) its lower water vapor content (1350-1900 cm^{-1}) when compared with the reference spectrum.

In order to test the efficacy of the non-thermal plasma-activated gas mixture to inactivate bacteria, *E. coli* samples were prepared as either planctonic cells or as cells embedded within a biofilm. Planctonic *E. coli* cells were grown and subsequently deposited on sterile glass cover slips. Bacteria in the stationary phase of growth were used because they are known to be more resistant to chemical treatment (Dodd *et al*., 2007). The viability of the bacteria before and after non-thermal plasma-activated gas mixture exposure was determined by scraping bacteria from the cover slip, followed by re-suspension, spreading and colony growth on LB agar. In addition, cells were



(b)

Species	O_3	N_2O	NO_2	HNO_3
Concentration (ppm)	157	76	6	58

Fig. 2: FTIR absorption spectrum of the treatment reactor gas outlet performed at ambient temperature and atmospheric pressure. (a) The spectra and (b) Stable gaseous oxidative species at the plasma reactor outlet were measured

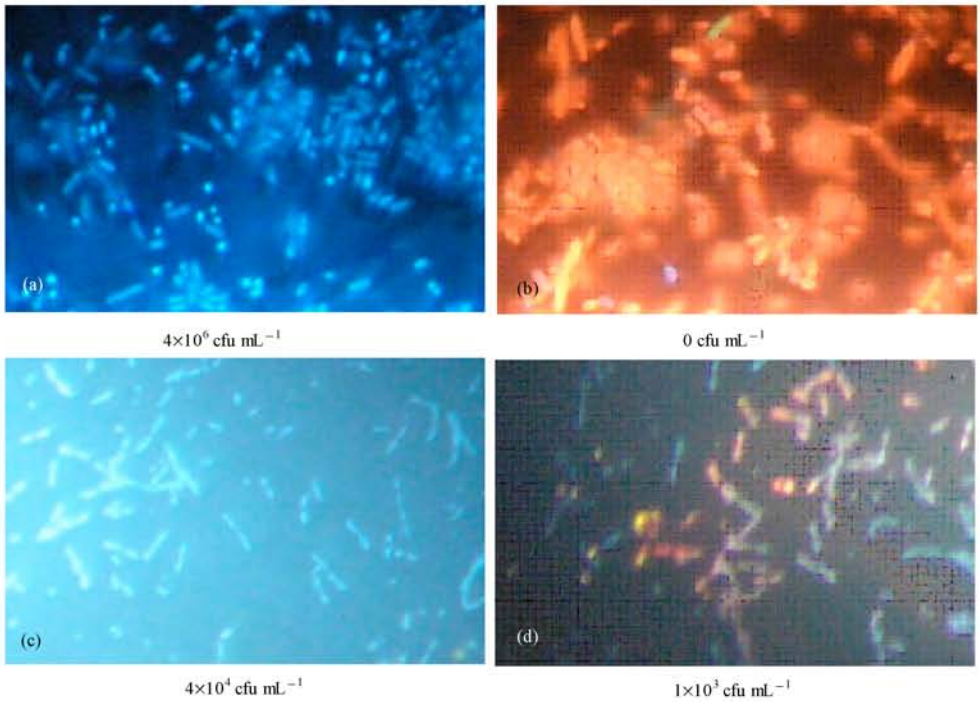


Fig. 3: Fluorescence microscopic examination of cells prior to and after exposure to an atmospheric pressure non-thermal plasma. (a) Stained planctonic cells after 40 min exposure to ambient air (control); (b) Stained planctonic cells after a 40 min non-thermal plasma treatment; (c) Stained biofilm cells after 40 min exposure to ambient air (control) and (d) Stained biofilm cells after a 40 min non-thermal plasma treatment. The corresponding CFU mL^{-1} results for the recovered *E. coli* planctonic cell and biofilm samples are displayed under each photograph

stained with DAPI and Propidium Iodide (PI) followed by direct fluorescence microscopic observation. Propidium Iodide (PI) will only enter and stain DNA in membrane-damaged bacteria and thus cells which are heavily damaged will appear red, while intact cells will appear blue (Jenkins *et al.*, 1997). Prior to exposure to the non-thermal plasma-activated gas mixture, the vast majority of cells stained blue (Fig. 3a), while after exposure all cells stained red (Fig. 3b). These results were supported by those using colony formation to asses cell viability, as no colonies were observable after non-thermal plasma-activated gas mixture exposure of planctonic cells, while the initial cell population was 4×10^6 CFU mL^{-1} prior to exposure (Fig. 3a, b).

Similar experiments were performed to determine the sensitivity of *E. coli* cells embedded within biofilms on glass cover slips to non-thermal plasma-activated gas mixture exposure. Scraping of cells from the cover slips, followed by re-suspension and growth on LB agar, revealed an approximate 40 fold reduction of apparent cell viability for the non-thermal plasma-activated gas mixture treated sample compared to unexposed controls. Fluorescence microscopic examination, after staining the biofilms with PI plus DAPI (Fig. 3c, d), showed that approximately 50% of cells stained red following non-thermal plasma-activated gas mixture exposure (Fig. 3d), whereas after a 40 min exposure to ambient air only a relatively minor fraction of the cells were found to stain red (Fig. 3c).

DISCUSSION

The decontamination of fragile material, such as plastics, has led to the search for alternative treatments to those (e.g., wet or dry heat) currently in use. As an example, atmospheric pressure ozonizers have been used to prevent microorganisms from proliferating in drinking water (Kogelschatz, 2000). Large quantities of ozone are required (in the range of 10,000 ppm) which, if utilized for surface treatment, could affect the integrity of fragile polymeric materials. Moreover, the recognition of the ubiquity of bacterial biofilms has also added to the necessity to discover efficient bacterial inactivation processes. Bacteria within biofilms are known to be more resistant to chemical and physical treatments (Nadakumar *et al*., 2004; Patel, 2005), with the comcomitant necessity for the use of alternative procedures for their removal and/or inactivation. One of these alternatives is the use of electrically-generated plasma processes as a surface decontamination technique (Laroussi *et al*., 2002; Moreau *et al*., 2005). These processes can include direct exposure to thermal or non-thermally generated plasma, but may also comprise exposure of the test surface to reactive chemical species generated in a gas presented to the plasma discharge.

The use of an atmospheric pressure non-thermal plasma process presents several advantages for surface contamination over thermally-generated plasma, including the lack of heating the substrate. In addition, there is no direct interaction between the electrically-generated plasma and the treated sample. Thus, surface decontamination is caused by exposure of the surface to be treated to short-lived chemical species generated in the surrounding gas by the electrically-generated plasma processes. Only the active chemical species produced by the plasma are transferred to the surface, thus ensuring a generally homogeneous treatment of the sample. Additionally, the active treatment chemical species can be controlled by the initial composition of the input gas and the electrical parameters of the plasma discharge.

In this study, wet air was used as the feed gas, as it is readily available. Under our conditions, a water film was formed on the surface during the treatment through condensation of water from the humid air used as a feed gas. The water film pH was found to decrease from pH 6 to 1.8 at the end of the treatment (data not shown). In earlier studies using atmospheric pressure non-thermal plasma processes, the pH of aqueous samples was found to decrease to values ranging from pH 2 to 4, depending on experimental conditions (Kirkpatrick *et al*., 2007; Moreau *et al*., 2005; Odic *et al*., 2002; Pointu *et al*., 2008). It is thus possible that a decrease in pH played a role in cell inactivation, as *E. coli* is known to be sensitive to acidic pH conditions (Goodson and Rowbury, 1989).

The stable oxidative species, such as ozone (O_3), nitrogen oxides (mainly N_2O and NO_2) and the associated nitric acid, HNO_3, were identified and quantified. During the entire treatment, an average ozone concentration of 175 ppm was measured at the reactor outlet. Singlet oxygen O_2 ($^1\Delta_g$) biocidal activity has been shown to act as a membrane-disorganizing agent in *Staphyloccocus aureus* and as a DNA-damaging agent (Maisch *et al*., 2007; Ravanat *et al*., 2000). The OH and HO_2 radicals produced by means of an atmospheric pressure wet argon non-thermal plasma were found to be responsible for the inactivation of *E. coli* cells by 5 orders of magnitude within 20 min (Dodet *et al*., 2006). More generally, reactive oxygen species are known to be efficient at killing microorganisms (Waris and Ahsan, 2006). Thus, a combination of a decrease in pH and the presence of Reactive Oxygen Species (ROS) coming from the activated gas phase are likely to play important, though not necessarily exclusive, roles in cell morbidity and mortality casued by non-thermal atmospheric pressure plasma processes (Goodson and Rowbury, 1989).

In the present study, fluorescence microscopy examination of *in situ* stained cells, in addition to measurement of viable cells through CFU formation, were used to assess cell viability prior to and after non-thermal atmospheric pressure plasma treatment. We found that planctonic *E. coli* cells were very sensitive to treatment, with a significant loss of viability measured by both assays, as we observed a marked diminution of CFU ability and a marked uptake of propidium iodide by cells after treatment. This latter observation is in agreement with the results of Laroussi *et al*. (2002), who observed, using Scanning Electron Microscopy (SEM), that non-thermal atmospheric pressure plasma processes could

disorganize *E. coli* cell membranes. In addition, planctonic *Erwinia* sp. were also found to be sensitive to a similar plasma activated gas mixture (Moreau *et al*., 2005). In contrast, biofilm-resident cells appeared to be more resistant than planctonic cells to exposure to the plasma activated gas mixture, a result in concordance with those obtained using a gliding discharge plasma treatment in humid air with *Staphylococcus epidermidis*, a Gram positive eubacterial species, as the test microorganism (Kamgang *et al*., 2007). In both studies, CFU ability was measured prior to and after treatment and a macroscopic disorganization of the biofilm was observed using a scanning electron microscopy analysis (Moisan *et al*., 2001). As treatment of the biofilm by the non-thermal atmospheric pressure plasma process can damage the biofilm, it is possible that cell recovery by scraping and mechanical dispersal of the biofilm-resident cells to measure CFU ability was affected (Kamgang *et al*., 2007; Tryland *et al*., 1998). Thus, we examined the potential cell viability using a two-dye system and found that the CFU and staining/fluorescence microscopy results were concordant.

We have shown that biofilm-resident *E. coli*, a Gram negative bacterium, are more resistant to non-thermal atmospheric pressure plasma treatment than are planctonic cells. Clearly, further studies are required to determine bacterial resistance mechanisms within biofilms in order to optimize non-thermal atmospheric pressure plasma treatment for surface decontamination.

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