

Research Article

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Sterilization of bacteria suspensions and identification of radicals deposited during plasma treatment

Abstract: In this paper we will present results for plasma sterilization of planktonic samples of two reference strains of bacteria, *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 29212. We have used a plasma needle as a source of non-equilibrium atmospheric plasma in all treatments. This device is already well characterized by OES, derivative probes and mass spectrometry. It was shown that power delivered to the plasma is below 2 W and that it produces the main radical oxygen and nitrogen species believed to be responsible for the sterilization process. Here we will only present results obtained by electron paramagnetic resonance which was used to detect the OH, H and NO species. Treatment time and power delivered to the plasma were found to have the strongest influence on sterilization. In all cases we have observed a reduction of several orders of magnitude in the concentration of bacteria and for the longest treatment time complete eradication. A more efficient sterilization was achieved in the case of gram negative bacteria.

Keywords: plasma needle, sterilization, spin-trap, Electron Paramagnetic Resonance, radicals

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1 Introduction

Atmospheric non-equilibrium low temperature plasmas have proven their usefulness in many applications involving living cells, tissue and bacteria. In spite of the fact that ignition and sustaining of atmospheric non-equilibrium plasmas are much harder than for low pressure plasmas, the former have taken over the leading place in plasma applications in biology and medicine. This is because such plasmas fulfil the most important requirements such as low gas and substrate temperature (must be below 42°C to avoid thermal necrosis), they provide abundance of chemical species and do not pose any electrical and chemical hazards. In addition, the composition and plasma-chemical processes can be controlled, even tailored, depending on the desired effect.

Results of previous studies have shown that the low-temperature plasma can be used for modification of biomaterials and, at the same time, as an alternative to conventional methods of sterilization [1-3]. The majority of the latest research has focused on the possible use of low-temperature plasma *in vivo*. Given that low-temperature plasma exerts a strong lethal effect on microorganisms, in recent years a possibility of applying it *in vivo* for disinfection and antimicrobial therapy has been extensively examined [4-9].

One of the non-thermal atmospheric pressure plasma sources, operating in the mixture of ambient air and rare gas, convenient for biomedical applications is the plasma needle [10]. We have redesigned and improved the plasma needle and performed detailed investigation of the properties of the discharge. To achieve an effective and safe treatment it is necessary to optimize the conditions under which it would be possible to implement it. The derivative probe measurements of power delivered to the plasma and mass spectrometry of the plasma generated by the needle have already been presented in our previous work [11,12]. Plasma needle generates radical oxygen species (ROS) and radical nitrogen species (RNS) radicals

like N, O, O₃, OH and NO that may play a role in metabolism of cells, triggering different biological processes and in sterilization. When the samples are in direct contact with the discharge all these radicals easily reach the surface of the sample. However, when bacteria are suspended in liquid it is needed to additionally investigate whether and which radicals originated from the plasma can penetrate into the solution. Electron Paramagnetic Resonance (EPR) is one of the diagnostic methods that can be used to detect presence of radicals in the solutions.

In the present study, the antimicrobial potential of low-temperature atmospheric plasma, generated by a plasma needle, was tested on two bacterial species, notorious for their growing resistance to various antimicrobial agents. EPR was used in order to detect the OH, H and NO species in solutions that were used to prepare suspensions of bacteria.

2 Experimental procedure

2.1 Bacterial suspensions

In this study we used the reference strains *Pseudomonas aeruginosa* ATCC 27853 and *Enterococcus faecalis* ATCC 29212.

Bacterial suspensions were prepared at the Laboratory of Microbiology, Faculty of Dental Medicine, University of Belgrade. Standard strains of bacteria were kept in a deep freezer at -76°C. They were inoculated into dextrose broth (Torlak Institute of Immunology and Virology, Serbia), and incubated aerobically for 20 h at 37°C. The strains were then subcultured in solid nutritional media under the same conditions. *E. faecalis* on blood agar (Torlak) and *P. aeruginosa* on Müller-Hinton agar (Torlak). The next step was preparation of bacterial suspensions. The initial bacterial concentration was determined using a spectrophotometer, so that the optical density corresponded to 1.5×10^8 CFU mL⁻¹ of bacterial cells. From this initial suspension, 1:10, 1:100 and 1:1000 dilutions were made, and inoculated into 96-well microtiter plates, 0.2 mL per well.

2.2 Plasma system

The plasma needle that was used in these experiments consisted of long central electrode made of thin tungsten wire (0.5 mm in diameter) covered by a ceramic tube. Both were placed inside a glass tube with outer diameter of

6 mm. The purpose of the ceramic tube was to isolate central electrode from helium flow in order to avoid creation of plasma inside the glass tube and the Teflon body of the plasma needle. In the experiments helium was used as the feeding gas. The flow rates were adjusted to 0.5 and 1 sLm (standard litres per minute) by mass flow controller (OmegaFMA5400/5500). Sinusoidal signal at the frequency of 13.56 MHz was used for powering the plasma needle. In all experiments home-made calibrated derivative probes were used for measuring and controlling the power. Both probes were placed inside a metal case, as close as possible to the tip of the plasma needle. These probes were connected to the oscilloscope (Agilent DSO3202A) by the cables of equal length in order to acquire current and voltage signals. Obtained signals were converted from time to frequency domain by Fast Fourier Transform (FFT) and then multiplied by calibration curves. Finally, signals were converted back to the time domain using an Inverse Fast Fourier Transform (IFFT). We chose mean power as the main plasma parameter that was used to monitor and calculate from the calibrated signal. Powers transmitted to the plasma, obtained from the difference of calculated powers with and without plasma (no helium flow), were used to monitor plasma treatment conditions. In all treatments power delivered to the plasma did not go above few watts. Plasma was created in the mixture of helium and gas components from the surrounding atmosphere that can diffuse into helium plasma and produce more radicals such as atomic oxygen, nitrogen, nitrogen oxides and ozone. Plasma was generated only on the plane tip of the powered central electrode.

The plasma needle was placed vertically above the microtiter plate at the edge of each well containing bacterial cells. To examine the antibacterial effect of non-thermal plasma, the treatments were performed for three different powers of plasma (0.15, 0.9, 1.6 W), two flows of helium (0.5 and 1 sLm) and three exposure times (60, 120 and 180 s). To be sure that the helium flow has no influence on the treated cell samples we also treated samples in helium flow without ignition of plasma for the same exposure times. Untreated wells containing bacteria were used as control samples.

After exposing the bacterial suspensions to low-temperature atmospheric plasma, 0.05 mL of each suspension were taken and inoculated onto appropriate solid media, *E. faecalis* on blood agar (Torlak) and *P. aeruginosa* on Müller-Hinton agar (Torlak). After an incubation period of 20 h at 37°C in aerobic conditions, the effect of the plasma treatment was evaluated based on the number of bacterial colonies. Arbitrary units (0-6) were used to assess the bacterial growth: 0 - absence of

colony growth, 1 – number of colonies ≤ 50 , 2 - number of colonies 51-100, 3 - number of colonies 101-300, 4 - number of colonies 301-500, 5- number of colonies > 500 , fields with confluent growth; 6 - confluent growth.

2.3 Electron paramagnetic resonance

The spin-trap DEPMPO (5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide) was purified and tested for hydroxylamine impurities, as previously described [13]. The final concentration of DEPMPO in samples was 50 mM. The Fe(II)(DTCS)₂ complex was prepared by dissolving DTCS (N-(dithiocarboxy) sarcosine, diammonium salt) and FeSO₄•7H₂O in 18 MΩ deionized water. The final concentration of the complex in samples was 20 mM.

The samples for EPR measurements (60 μL) were drawn into 10 cm long gas-permeable Teflon tubes (wall thickness 0.025 mm and internal diameter 0.6 mm; Zeus industries, Raritan, USA). EPR spectra were recorded at room temperature using a Varian E104-A EPR spectrometer operating at X-band (9.51 GHz) with the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW; time constant, 0.032 s; field center, 3410 G; scan range, 200 G. The spectra were processed using EW software (Scientific Software, Bloomington, IL, USA). Spectral simulation was performed using EasySpin program for MATLAB environment [14].

3 Results and discussion

3.1 EPR measurements of plasma treated samples

In our previous work we have shown that plasma needle produces significant amounts of ROS, RNS and ions [12]. Similar results were obtained by Stofells and coworkers [15]. The radical which is of most interest in biological processes is NO followed by the singlet delta (Δ) metastable O₂ molecule and hydrogen peroxide. All of these ROS can be detected in the plasma needle discharge. When the discharge is in direct contact with the sample we can be sure that ROS reaches the membranes of the treated cells or bacteria, but when the samples are in suspensions it is unclear which radicals enter the suspension and are responsible for the sterilization of the sample containing bacteria. In order to get a clearer picture about the role of ROS, we have evaluated the existence of free radicals in plasma-irradiated water samples by using DEPMPO

spin trap and Fe(II)(DTCS)₂ complex. DEPMPO is the most efficient spin trap for the detection of various short lived free radicals, which could be generated in the same system [16]. The DEPMPO free radical adducts give rise to characteristic EPR spectra as shown in [17]. The Fe(II)(DTCS)₂ complex traps nitric oxide radicals (NO•) and forms the ferrous nitrosyl complex Fe(NO)(DTCS)₂ which gives a characteristic three line EPR spectrum [18].

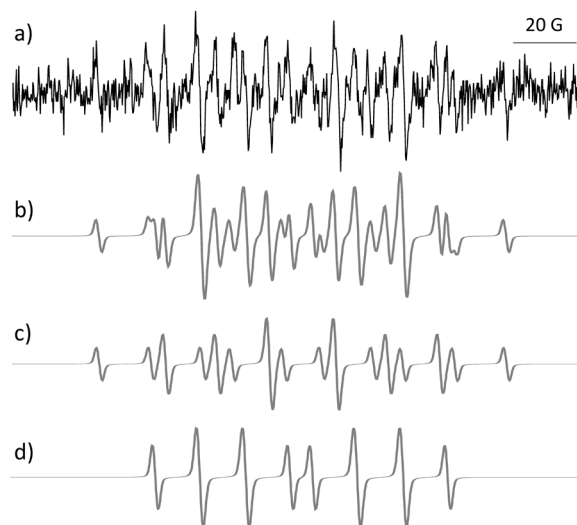


Figure 1: a) EPR spectrum of a water sample containing 50 mM DEPMPO irradiated with plasma for 9 min, power 1.6 W. b) Simulation of spectrum in a) obtained by spectral addition of simulations of 50% DEPMPO/OH and 50% DEPMPO/H adduct signals. c) Simulation of the DEPMPO/H adduct signal, hyperfine splitting constants (in Gauss); $a^P=50.7$; $a^N=15.4$; $a^H_{\beta}(1H)=19.5$, $a^H_{\beta}(1H)=20$, d) Simulation of the DEPMPO/OH adduct signal, hyperfine splitting constants (in Gauss); $a^P=46.7$; $a^N=13.64$, $a^H_{\beta}(1H)=12.78$.

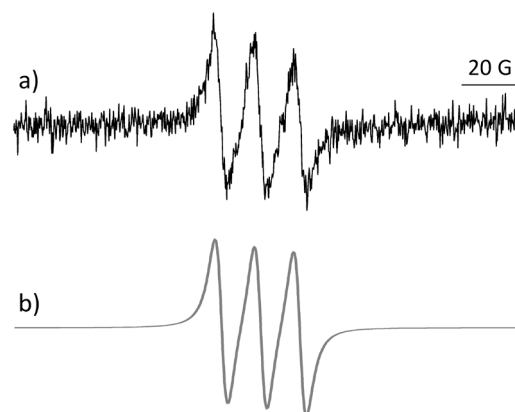


Figure 2: a) Experimental EPR spectrum of the Fe(NO)(DTCS)₂ complex obtained after plasma irradiation of a water sample containing Fe(DTCS)₂ for 9 min, power 1.6 W. b) Simulated EPR spectrum of Fe(NO)(DTCS)₂, nitrogen hyperfine splitting constant (in Gauss) $a^N=12.7$.

The samples that contained DEPMPO or Fe(II)(DTCS)_2 complexes in water but were not irradiated by plasma did not exhibit EPR spectra at room temperature. However, after plasma irradiation (1.6 W power, 9 min exposure) the samples that contained the DEPMPO spin trap, gave rise to an EPR spectrum shown in Fig. 1a. By spectral simulation (Fig. 1b) it was determined that this spectrum is composed of two components, one that arises from the DEPMPO/H adduct (Fig. 1c) and the other that arises from the DEPMPO/OH adduct (Fig. 1d). From additions of the simulated spectra (Figs. 1c and d), we could estimate the contributions of adducts to be $\sim 50\%$ for DEPMPO/H and $\sim 50\%$ for DEPMPO/OH. It should also be noted that the same EPR signal was observed even after 2-10 min irradiation with powers less than 1.6 W. In the experiments with Fe(II)(DTCS)_2 , it was observed that after plasma irradiation (1.6 W power, 9 min exposure), an EPR signal characteristic of the Fe(NO)(DTCS)_2 is detected (Fig. 2a). This indicates that NO^* is produced during plasma irradiation as well.

3.2 Sterilization of planktonic samples of *E. faecalis* and *P. aeruginosa*

The plasma needle was used for sterilization of several species of Gram positive and Gram negative bacteria. Here, we will present results of plasma treatments of *E. faecalis* ATCC 29212 and *P. aeruginosa* ATCC 27853 in more detail. The samples for both bacterial strains were prepared in a form of suspensions of four different concentrations. Apart from concentration of bacteria in suspensions, we have varied flow of buffer gas, power delivered to the plasma and time of treatment. In Figs. 3a, 3b results of sterilization of *E. faecalis* for the helium flows 0.5 and 1 sLm are shown. We can see that better results were obtained for the higher flow of 1 sLm (Fig. 3b). This could be explained by higher input of the radicals created in the plasma into the suspension that contains bacteria. As expected, the reduction in colonies increased with the increase in treatment time. In case of the lowest concentration we have obtained complete sterilization of the samples, i.e., after the treatment no bacterial colonies were formed. In all other cases reduction was several orders of magnitude.

In Figs. 4a, 4b results for the longest treatment time are shown comparing different powers and initial concentrations. We can see that for the lowest power we did not observe any significant reduction in bacterial colonies compared to the untreated positive control. On the other hand, with an increase of the power delivered to the plasma, sterilization effect increases. As expected,

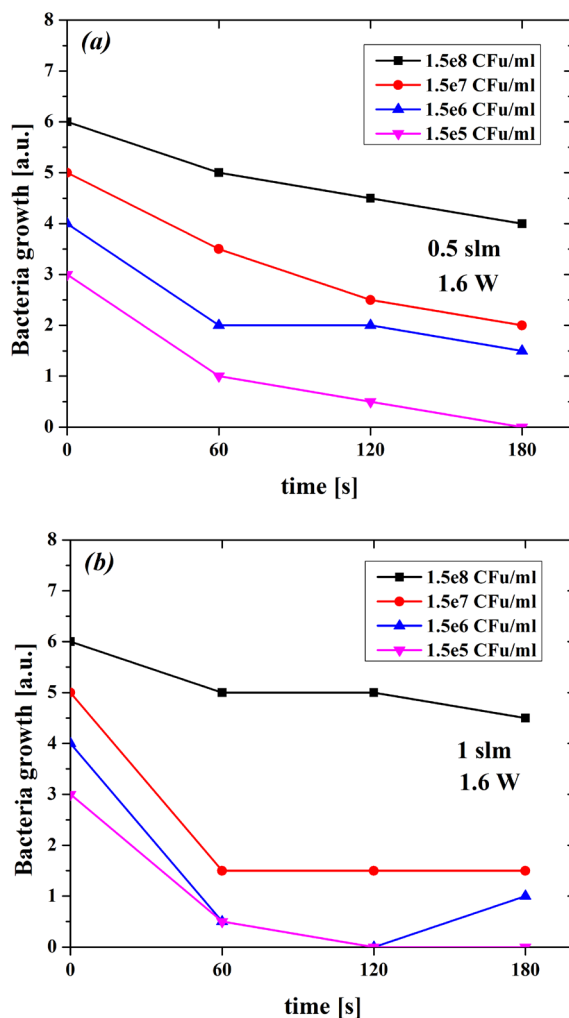


Figure 3: Sterilization of *Enterococcus faecalis* ATCC 29212. The samples contained four different concentrations of bacteria. Treatment power was 1.6 W. (a) He flow 0.5 sLm; (b) He flow 1 sLm. Values for 0 s correspond to the values of positive untreated control.

the higher power resulted in better sterilization effects. However, the final result was highly dependent on the initial concentration of bacteria. We can see that for the highest concentration of 1.5×10^8 CFU mL^{-1} the reduction was only one to two orders of magnitude. Again, higher flow of 1 sLm yielded better results.

The second strain of bacteria used in experiments was gram negative *P. aeruginosa* ATCC 27853. In Figs. 5a, 5b we show the results of plasma treatments of four different concentrations for two flows of buffer gas. Presented results were obtained for the highest power delivered to the plasma. As with *E. faecalis*, a better sterilization was achieved with the higher flow of helium. We can see that for the concentrations of 1.5×10^6 CFU mL^{-1} and 1.5×10^5 CFU mL^{-1} a complete sterilization of samples was

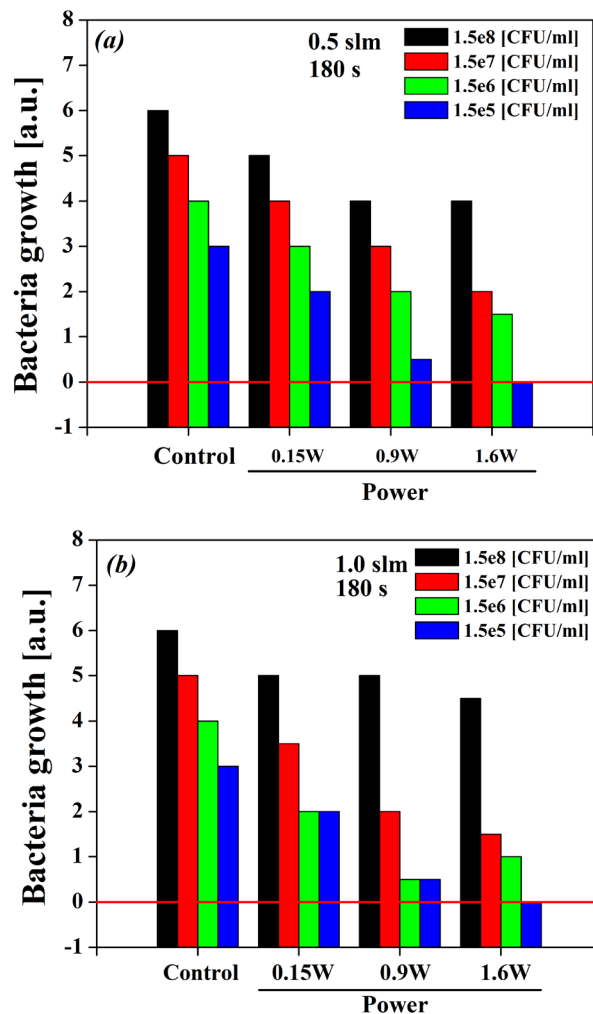


Figure 4: Sterilization of *Enterococcus faecalis* ATCC 29212. The samples contained four different concentrations of bacteria. The untreated control values for bacterial growth are $6(1.5 \times 10^8 \text{ CFU mL}^{-1})$, $5(1.5 \times 10^7 \text{ CFU mL}^{-1})$, $4(1.5 \times 10^6 \text{ CFU mL}^{-1})$ and $3(1.5 \times 10^5 \text{ CFU mL}^{-1})$. Treatment time was 180 s. (a) He flow 0.5 sLm; (b) He flow 1 sLm. The red line represents complete sterilization of the sample.

already achieved for the treatment time of 60 s. For higher concentrations the reduction in bacteria was 4-5 orders of magnitude. The dependence of the sterilization on power delivered to the plasma is shown in Figs. 6a, 6b. With an increase in power delivered to the plasma we achieved a better sterilization.

We can see that the two main parameters in sterilization of planktonic samples of bacteria are power delivered to the plasma and duration of treatment. The flow of buffer gas had some influence on the results of sterilization, but not that pronounced as power or treatment time. The importance of power delivered to the plasma can be explained by chemistry in the discharge.

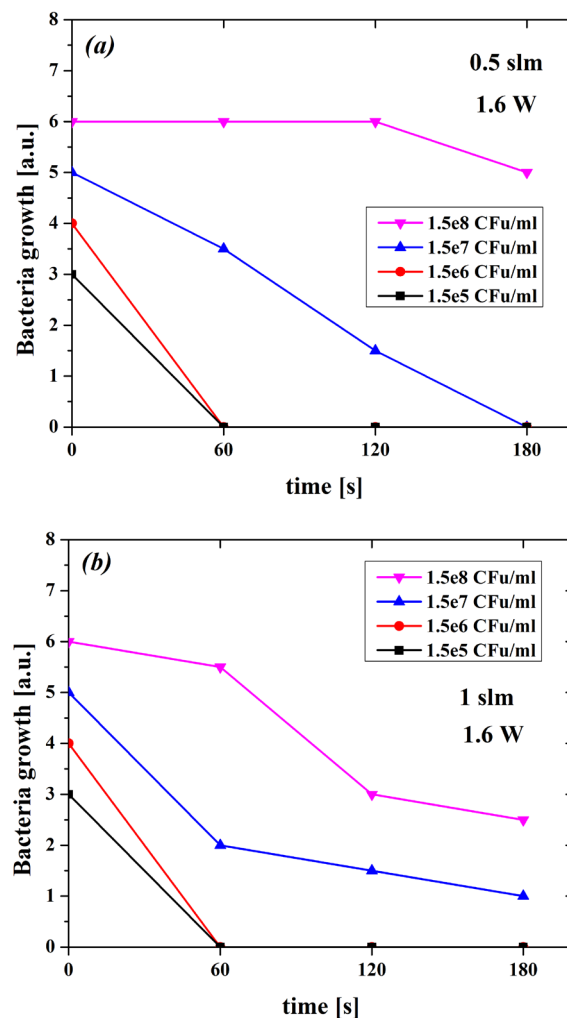


Figure 5: Sterilization of *Pseudomonas aeruginosa* ATCC 27853. The samples contained four different concentrations of bacteria. Treatment power was 1.6 W. (a) He flow 0.5 sLm; (b) He flow 1 sLm. Values for 0 s correspond to the values of positive untreated control.

If more power is delivered to the plasma, reactive species responsible for the sterilization are produced more effectively and in higher concentrations (unless there is some chemical reaction promoted at higher densities of other constituents). Similarly, the duration of treatment is responsible for the total amounts of radicals and other species involved in process of sterilization delivered to the samples. The combination of these two parameters provides a possibility for a wider range of optimal sterilization conditions. One can reduce the treatment time and increase the power delivered to the plasma or increase the duration of treatments and work with lower power and still have a similar sterilization effect.

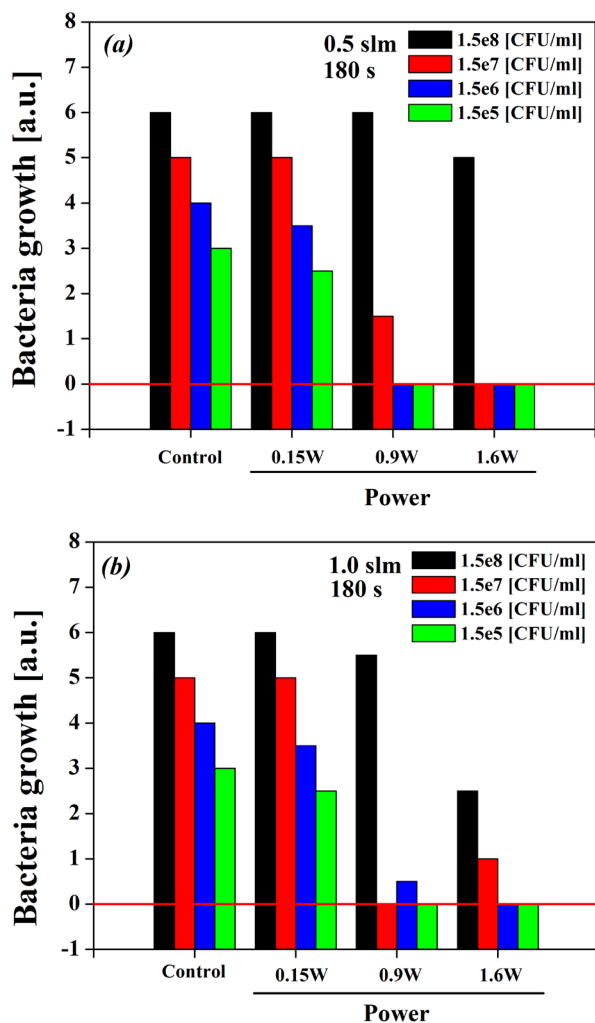


Figure 6: Sterilization of *Pseudomonas aeruginosa* ATCC 27853. The samples contained four different concentrations of bacteria. The untreated control values are $6(1.5 \times 10^8 \text{ CFU mL}^{-1})$, $5(1.5 \times 10^7 \text{ CFU mL}^{-1})$, $4(1.5 \times 10^6 \text{ CFU mL}^{-1})$ and $3(1.5 \times 10^5 \text{ CFU mL}^{-1})$. Treatment time was 180 s. (a) He flow 0.5 sLm; (b) He flow 1 sLm. The red line represents complete sterilization of the sample.

Apart from these two important parameters, the effect mostly depends on concentration of bacteria and on the bacterial strain. Broadly speaking, there are two types of bacteria, Gram positive and Gram negative. This division is due to the difference in the cell wall structure. In Gram positive bacteria the cell wall consists of multiple layers of peptidoglycan, forming a thick, rigid structure. Gram negative cell wall contains only a thin layer of peptidoglycan and an outer membrane composed of lipopolysaccharide. In Figs. 7a, 7b we have shown results of plasma treatment of two Gram negative (*P. aeruginosa*, *Escherichia coli*) and two Gram positive (*E. faecalis*, *Staphylococcus aureus*) strains of bacteria. We can see

that better results were obtained in the treatments of Gram negative bacteria. It seems reasonable to assume that either the thinner wall or the chemical composition of the wall of the Gram negative bacteria leaves them more exposed to the extracellular influence. The main conclusion that can be drawn here is that major actors in sterilization mechanism are radicals coming from the plasma. Also, the sterilization mechanism is closely connected to the interaction of these radicals (NO , OH , $^1\text{O}_2$, H , H_2O_2 etc.) with the lipopolysaccharides in the outer membrane of the bacterial cell wall. The absence of the lipopolysaccharides in the cell walls of the Gram positive bacteria, on the other hand, may be responsible for lower sterilization effect. Although we obtained the sterilization effect of low temperature atmospheric plasma generated by a plasma needle source on Gram positive bacteria, it was not that pronounced as the sterilization effect on Gram negative bacteria. The difference in susceptibility to the cold plasma treatment between Gram positive and Gram negative bacteria has already been reported [5,19]. However, a number of studies demonstrated excellent effects of low temperature atmospheric plasma against Gram positive bacteria [4,8], including eradication of their extremely resistant biofilms [7,9]. Nevertheless, one has to take care when generalizing the conclusion of the plasma influence on all strains in one type of bacteria (for example Gram negative). Even within the same type of bacteria there are differences in the results of sterilization treatments. We can see in Fig. 7 that much better results are obtained for *E. coli* as compared to the results obtained for *P. aeruginosa*. This implies that in addition to the general properties of the cell wall other characteristics of bacteria also have an important role in plasma-cell interaction.

4 Conclusion

We have presented the results of plasma sterilization of Gram positive and Gram negative strains of bacteria. The sterilization was performed by using atmospheric pressure plasma device and treated samples were in form of a suspension. Electron paramagnetic resonance was used in order to detect radicals deposited in the solution. We have detected H , OH and NO radicals deposited in the solution after the plasma treatment. In this case the most important radical is NO which is a potent antimicrobial agent effective against a range of Gram-negative and Gram-positive bacteria [21]. In experiments we have used four initial concentrations of bacteria (10^5 - 10^8 CFU mL^{-1}). For higher concentrations we have observed reduction of several orders of magnitude and for the lower ones

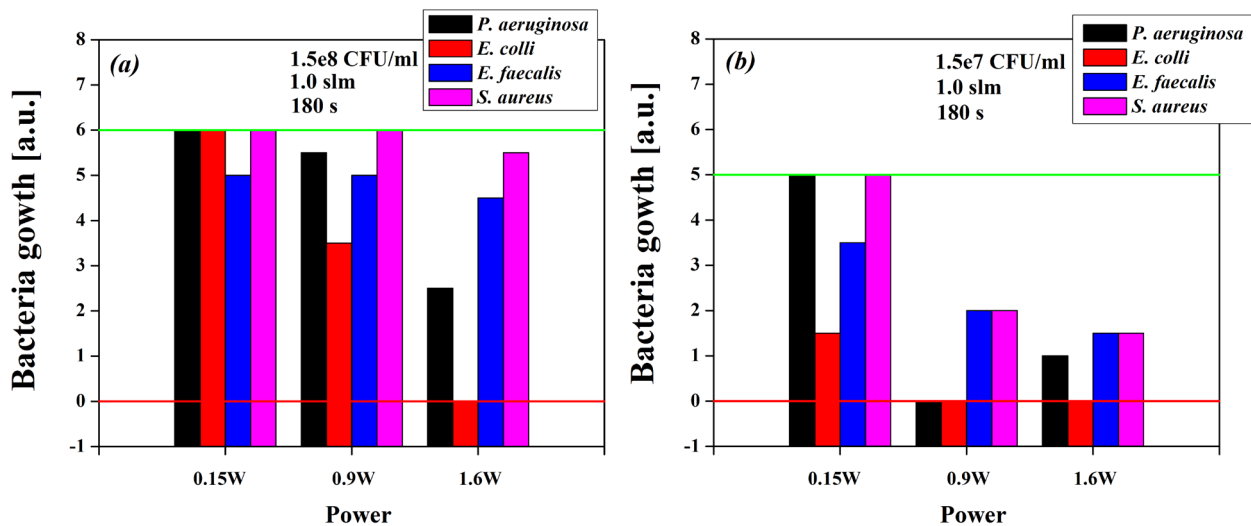


Figure 7: Sterilization of four different strains of bacteria: *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. More detailed data for the *Escherichia coli* and *Staphylococcus aureus* are given in [20]. Here, a part of the data from [20] are given for clearer comparison between plasma effect on Gram positive and Gram negative bacteria. The flow of helium was 1 slm and the treatment time 180 s. Green line represents untreated control values and red line represents complete sterilization of the sample. (a) concentration 1.5×10^8 CFU mL⁻¹; (b) concentration 1.5×10^7 CFU mL⁻¹.

a complete eradication of the bacteria. It was shown that power delivered to the plasma and duration of the treatment are closely interlinked. One can reduce the treatment time and increase the power delivered to the plasma or increase the duration of treatments and work with lower power and still have a similar sterilization effect. The sterilization effect also depends on the type of bacterial strain. Gram negative bacteria appear more sensitive to the plasma treatment, which can be partially explained by their thinner wall and/or presence of lipopolysaccharides in their envelopes.

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