ORIGINAL PAPER

BIOEFFECTS OF ATMOSPHERIC PLASMA DISCHARGE ON GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

IULIANA MOTRESCU[1], ANTONIEA POIATA[2], ANDREI VASILE NASTUTA[3,4], DORINA CREANGA[4], GHEORGHE POPA[4]

Manuscript received: 23.07.2015; Accepted paper: 29.08.2015;

Published online: 30.09.2015.

Abstract. *The impact of accelerated particles as well as of UV photons generated in low temperature plasma discharge on pathogen bacterial germs was studied. An experimental setup of a dielectric barrier discharge in atmospheric air was used to treat agarized cultures of Gram-negative and Gram-positive bacteria for two distances between the high voltage electrode and the grounded one. The diameters of the growth inhibition areas were higher for the shortest distance between the electrodes – 2.5 cm for all tested germs which can be attributed to the synergistic action of accelerated electrons, ions, and reactive oxygen species, as well as UV photons. For higher distance between the electrodes – 3.5 cm, only the Gram-negative bacteria were still killed, fact that can be related to the higher UV-generated ozone level. The more efficient action of ozone on the proteins present in the cellular wall of Gram-negative bacteria is supposed to explain this behavioral trend different from Gram-positive bacteria response.*

Keywords*: B. cereus, S. lutea, P. aeruginosa, helium plasma discharge, growth inhibition areas*

1. INTRODUCTION

The combined effects of ultraviolet radiation and reactive species from plasma discharges were investigated as efficient factors in bacteria destroying at room temperature - this sterilization alternative method representing the only available way to preserve the integrity of temperature sensitive medical utensils and biomedical material samples with microorganism loading. Even if, it is assumed that UV photons can mainly kill the bacterial cells at surface, while only the radicals are able of in depth action, UV-generated ozone may dissolve and diffuse in aqueous media, producing bactericidal effects Thus only the synergistic actions of all these plasma discharge components can ensure the success and efficiency of plasma treatment. Lackmann et al. 2012 [1] have found that in atmospheric plasma discharge UV-generated ozone reaches significant concentrations. In [2] Lackmann et al., 2015 concluded that cold atmospheric-pressure plasmas represent efficient inactivators of microorganisms due to the combined action of photons and accelerated particles that interact with air and water from the environment generating various free radicals able to attack

[1] "Ion Ionescu dela Brad" University of Agronomical Sciences and Veterinary Medicine, Department of Sciences, Iasi, Romania
[2] "Grigore T. Popa" University of Medicine and Pharmacy, Iasi, Romania
[3] "Grigore T. Popa" University of Medicine and Pharmacy, Faculty of Medical Bioengineering, Biomedical Science Department, Iasi, Romania
[4] "Alexandru Ioan Cuza" University, Physics Faculty, Iasi, Romania, e-mail: dorina.creanga@gmail.com

simultaneously different cellular targets. Liu et al. 2010 [3] highlighted that, considering the extensions of the inhibition zones around the spot where plasma jet comes directly in contact with bacterial medium, the cells distant from the jet axis are killed due to radial diffusion of reactive oxygen species formed by UV photons along plasma jet trajectory in air.

The main role of VUV and UV photons from atmospheric plasma discharge in the inactivation of vegetative *E. coli* bacteria was evidenced by Schneider et al., 2012 [4]. In [5], Poiata et al., 2010 the bactericidal plasma effect on *E. coli, C. albicans, B. subtilis*, and *S. aureus* was discussed based on the complex composition of atmospheric plasma jet action. In the present study we present the evidenced bactericidal effects of dielectric barrier discharge (DBD) on other Gram-positive and Gram-negative pathogen germs aiming to reveal the influence of UV generated ozone in the plasma jet.

2. MATERIALS AND METHODS

2.1. MATERIALS

The test germs – two Gram-positive and one Gram-negative bacteria - were standard collection strains (ATCC) preserved in lyophilized form until the time of the experiment. The microorganism inoculums were prepared in normal physiological sterile saline to a density of approximately 3.10^8 cells/ml based on calibration curves. Equal aliquots from each inoculum (0.2 ml) were added to 10 ml Mueller-Hinton (Oxoid) molten agar being further cooled to 45 °C, in sterile plastic Petri plates (100 mm diameter) and mixed thoroughly.

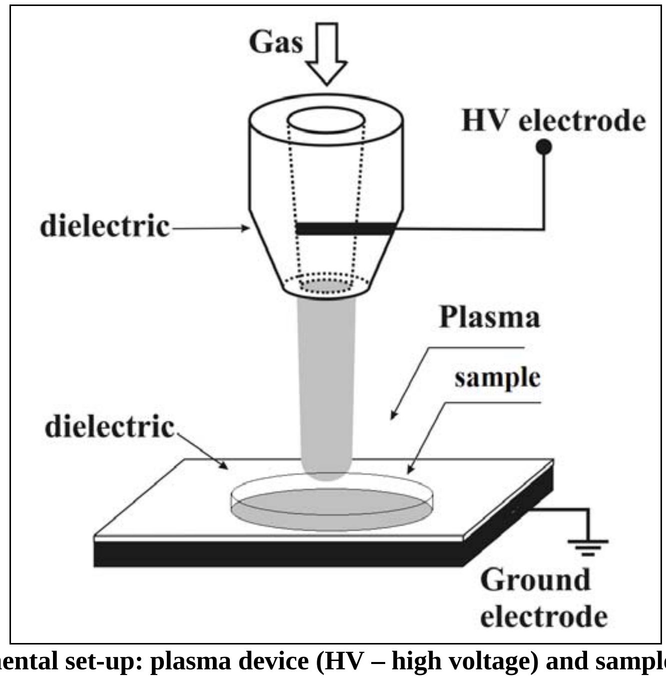


Figure 1. Experimental set-up: plasma device (HV – high voltage) and sample (Petri plates with inoculated bacteria) placed on the grounded electrode.

Helium plasma jet generated at atmospheric pressure by asymmetric dielectric barrier discharge was produced at a gas flow rate of 0.15 l/min 9kV - peak to peak value, at 1.6 kHz frequency [5, 6]. The scheme of the experimental set-up is presented in Fig. 1.

2.2. METHODS

Optical Emission Spectroscopy (OES) was used to analyze the radiative states of the species produced in the gaseous plasma discharge using a Triax 550 (Horiba Jobin Yvon) device with a CCD (Symphony Horiba Jobin Yvon) detector type. Aiming to investigate the global emission spectrum of the discharge in the area where the sample exposure occurred, the optical fiber was placed above the ground electrode. The plasma exposure configurations were arranged for two distances between the high voltage electrode and the grounded one: 2.5 and 3.5 cm.

The Petri dishes containing bacteria cultures were placed on the dielectric superposed on the plate ground electrode. The array of exposure time was the same for both plasma jet lengths: 25; 35; 50; 75, and 100 seconds. After bacteria incubation for 24 h at 37.0±0.5 °C, the circular growth inhibition zones were readily seen by direct visual inspection, their diameters being measured with 0.5 mm precision usual ruler. Four repetitions of every exposure type were carried out on every Petri plate.

3. RESULTS AND DISCUSSION

3.1. RESULTS

The optical analysis of the helium plasma discharge in atmospheric conditions at room temperature was accomplished for the two spatial arrangements of the experimental study: 2.5 cm and 3.5 cm distance between the high voltage electrode and the ground one. The OES results and the band assignment to atmospheric oxygen and nitrogen species are shown in Fig. 2 and Table 1.

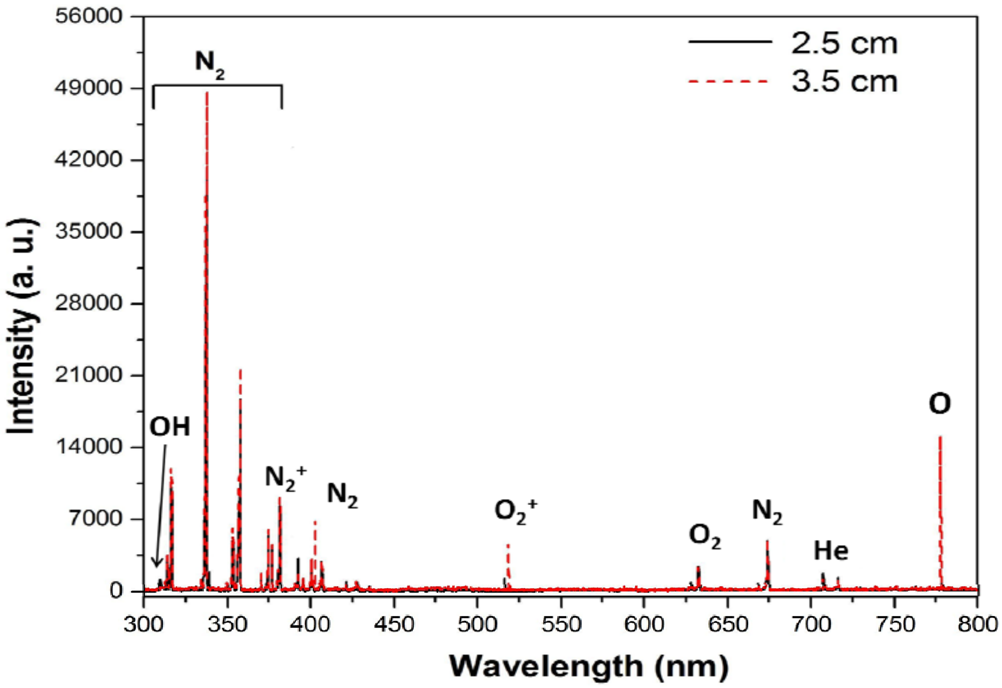


Figure 2. The OES recorded data for DBD plasma discharge (a.u.-arbitrary units).

The bactericidal effects were measured and discussed based on the inhibition of the bacteria growth that appeared as circular areas of relatively more transparent discs – compared to the opaque background corresponding to the growth of non-exposed bacteria (Fig. 3). In Fig. 4 a, b the graphical representations of the average values of inhibition areas are given for each exposure.

Figure 3. Bacterial growth inhibition areas for *P. aeruginosa* and *B. cereus*.

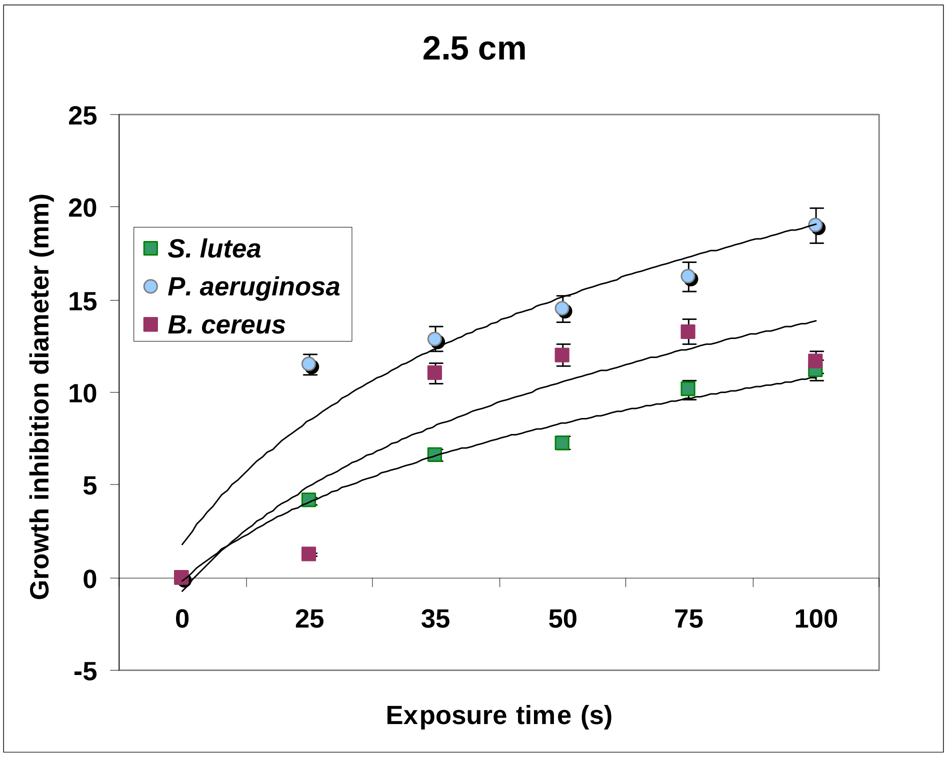


Figure 4 a. The correlation of bacteria growth inhibition and plasma exposure time for 2.5 cm plasma jet length.

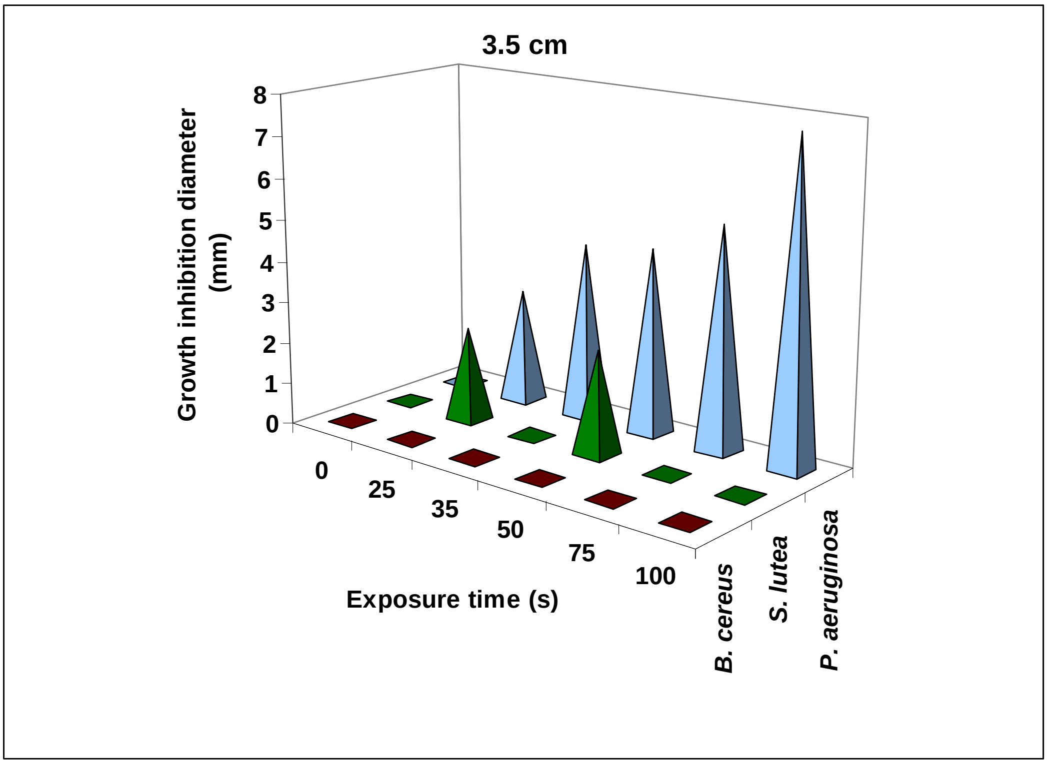


Figure 4 b. The response of bacteria to 3.5 cm plasma jet length.

In Fig. 4 a the situation corresponding to the plasma jet length of 2.5 cm is presented - standard deviations of 5-7% are calculated for the four repetitions of each exposure time. In the case of 3.5 cm plasma jet length, the areas of the bacteria growth inactivation are considerably smaller (Fig. 4 b) compared to the case of 2.5 cm plasma jet; for *B. cereus* and *S. lutea* most of the transparent disc diameters were zeroed – proving quite low response to the physico-chemical agents applied in this experiment; for *P. aeruginosa* the diameter of growth inhibition discs diminished of about 2.5 times.

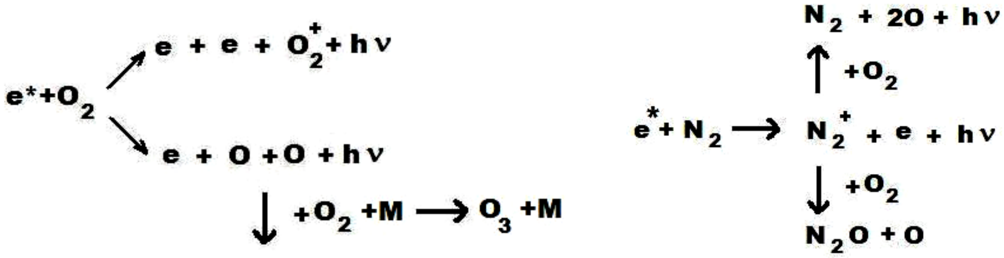
3.2. DISCUSSION

The numerical data corresponding to the OES recordings can be seen in Table 1 representing the intensity of different emission lines measured for the two studied configurations. Beside the quantitative differences consisting in relatively higher intensity of the bands recorded for 3.5 cm in comparison to the case of 2.5 cm distance between the two discharge electrodes, the main qualitative change is represented by the appearance of the peak at 777 nm, present only for 3.5 cm configuration.

Table 1. The intensity (in arbitrary units) of the optical emission bands characterizing helium plasma discharge in open atmosphere

(nm)	315	337	353	394	407	520	631	661	747	777
2.5cm	10,000	38,500	17,500	8,700	1,000	1,100	2,500	5,000	1,200	-
3.5cm	12,000	48,800	21,000	8,800	6,800	5,200	2,600	5,300	1,270	15,000

One may suppose that the longer pathway of the accelerated electrons through the atmospheric air could be the cause of the increased concentration of excited species of oxygen and nitrogen and consequently of the increased intensity of the corresponding de-excitation bands from Fig. 2 and Table 1. The accelerated electrons that hit oxygen molecules may lead to the formation of oxygen reactive species and release of visible photons with further ozone generation, according to the reaction scheme proposed by Choi et al., 2006 [7]:



where e^* is an energetic electron while M is either O, O_2 or already generated ozone (O_3).

The scheme also shows the UV optical emission between 320-420 nm (Table 1) associated with radiative processes of excited states of N_2 and N_2^+ which are the results of the collisions with accelerated electrons and interaction with oxygen molecules – according to [7].

The discussion of the plasma bioeffect in the test gems was accomplished by means of the average values of transparent disc diameters in correlation with the exposure time durations as presented in Figs. 4 a, b. We observe a saturation tendency of the bactericidal effect with the increase of the plasma exposure time toward 100 s evidenced in Fig. 4 a in the case of 2.5 cm plasma jet length, especially for *B. cereus* culture. Moreover, in this case a slight decrease of the inhibition growth diameter from 13.5 mm (for 75 s) to 11.08 mm (for 100 s) was evidenced. The largest inhibition growth areas – up to19.0 mm were noticed for Gram- negative *P. aeruginosa*, that seems to be the most sensitive germ to the plasma treatment (among those discussed here), while for Gram-positive *S. lutea* and *B. cereus,* similar but lower responses were noticed – maximum inhibition growth areas were of about 12 mm.

The most appropriate mathematical fitting of the experimental data graphs for all investigated bacteria was a logarithmic one with high correlation coefficients (Table 2).

Table 2. The mathematical approach of the experimental data for 2.5 cm plasma jet (*y*-diameter of growth inhibition areas, in mm; *x*- the exposure time, in s)

Mathematical fitting	Correlation coefficient	Bacteria
y=9.62 Ln x+1.79	0.966	*P. aeruginosa*
y=6.10 Ln x-0.14	0.990	*S. lutea*
y=8.43 Ln x-2.14	0.912	*B. cereus*

Additionally, the average percentage of surviving bacteria colonies within the transparent areas was counted; it was found to range between 0% for *P. aeruginosa* and 27% - 25% in the case of *B. cereus* and *S. lutea* respectively, for 2.5 cm - so that the sterilization efficiency could be assessed as being between 100%, and 73%, respectively.

For 3.5 cm plasma jet length (Fig. 4 b) *S. lutea* responded randomly – the inhibition growth areas being either of about 2 mm or zero– with standard a deviation of about 50%. This gives no statistical significance to the data. In the same time *B. cereus* plasma treatment resulted in non-measurable inhibition growth effect. The bactericidal effect remained important for *P. aeruginosa* the diameter of growth inhibition area increasing as the plasma exposure time increased, with maximum values of 10 mm and standard deviations of less than 8%. The average percentage of bacteria surviving colonies on the transparent areas of growth inhibition was of 32% for *P. aeruginosa* corresponding to a sterilization efficiency of 68%. The other two bacteria strains proved insignificant inhibition growth area for 3.5 cm plasma jet length.

One explanation for the difference in the results presented in Fig. 4 a and 4 b. can be given if we consider the plasma components at its impact on the bacteria culture for the two plasma jet lengths tested here First, we suppose that the physical impact of corpuscular radiation (ions, electrons, radicals) diminished for 3.5 cm compared to the case of 2.5 cm due to the atmospheric air attenuation, which could explain the reduced response of all analyzed germs, but especially of the two Gram-positive strains (*B. cereus* and *S. lutea*) that were insignificantly affected .

Then, the OES results led to the conclusion that UV generated ozone made a difference between the results corresponding to the two distances between the discharge electrodes, the spectral emission line at 777 nm, present only for 3.5 cm plasma jet, indicates the supplementary ozone contribution to the bacteria inactivation which didn't existed in the case of 2.5 cm plasma jet. We assume that the longer pathway of UV photons in the air resulted in a higher probability of ozone generation, and, consequently, led to some bactericide action in the case of 3.5 cm experiments with Gram-negative bacteria.

Therefore, the sensitivity to ozone of *P. aeruginosa* could be invoked to explain the growth inhibition of these microorganisms for longer plasma jet. In this case the ozone generation is more intensive due to increased UV absorption in higher number of oxygen molecules along the UV photon longer trajectory in air. The relatively high sensitivity of Gram-negative microorganisms to ozone was reported by several authors that studied bactericidal effects of environment ozonization. The sensitivity of Gram-negative bacteria in aqueous media ozone was noticed by Restaino et al., 1995 [8], who concluded that the Gram-negative bacteria were substantially more sensitive to ozonated water than the Gram-positive ones both in the absence or in the presence of added organic material.

The scale of the sensitivity of many bacterial strains to ozone was published into a web site synthesis [9] There we can see the relatively higher sensitivity of *E. coli* from water and feces to the concentration of ozone in water compared to *B. cereus*, while *Pseudomonas* species are considered among the most susceptible microorganisms to ozone effect.

Thanomsub et al., 2002 [10] pointed out that the main difference between the two types of microorganisms, i.e. the higher content of proteins from the cellular wall of the Gram-negative germs could support the experimental findings regarding their higher sensitivity to ozone – based on the hypothesis of Komanapalli et al., 1998 [11] who assumed that ozone reacts readily with proteins than lipids.

The atmospheric plasma sensitivity evidenced in this experiment for the Gram-negative germs – otherwise known for their complex cellular wall and higher resistance to chemical agents - is consistent with the fact that the physical treatment based on low temperature plasma jet could be still efficient for the inactivation of those bacteria that usually can be killed with certain difficulties by purely chemical treatments (drugs or disinfection chemicals).

4. CONCLUSIONS

DBD plasma discharge was found efficient in the inactivation of some bacteria. Gram-positive bacteria were better sterilized for 2.5 cm distance between the two discharge electrodes, with diameters of the inhibition growth areas up to about 11 cm (for *B. cereus*), and 13 cm (for *S. lutea*), respectively, the main factor being plasma generated reactive species penetrating into the bacteria culture media. Surviving bacteria percentage for 2.5 cm plasma jet ranged between 0% for *P. aeruginosa* and 27% for *B. cereus*, so that test germ killing efficacy was 100%, and 73%, respectively.

For the 3.5 cm plasma jet length the Gram-negative bacteria were still sensitive to plasma electro-optical effects when the physical impact of plasma generated reactive species diminished but the UV-generated ozone level was increased - compared to the Gram-positive germs that had no significant response: the diameters of the growth inhibition zones were up to 8 mm in *P. aeruginosa* while the inactivation was of about 68%.

REFERENCES

[1] Lackmann, J.-W., Schneider, S., Narberhaus, F., Benedikt, J., Bandow, J. E., in *Plasma for bio-decontamination, medicine and food security* (eds Machala Z., Hensel K., Akishev Y.), pp. 17–29. Heidelberg, Germany: Springer, 2012.
[2] Lackmann, J.-W., Schneider, S., Edengeiser, E., Jarzina, F., Brinckmann, S., Steinborn, E., Havenith, M., Benedikt, J., Bandow, J. E., *Journal of Royal Society Interface*, **10**: 20130591, 2015.
[3] Liu, D. X., Rong, M. Z., Wang, X. H., Iza, F., Kong, M. G., Bruggeman, P., *Plasma Processes and Polymers*, **7**, 846, 2010.
[4] Schneider, S., Lackmann, J.-W., Ellerweg, E., Denis, B., Narberhaus, F., Bandow, J. E., Benedikt, J., *Plasma Processes and Polymers*, Special Issue: Plasma Sterilisation and Decontamination, 9, 561, 2012.
[5] Poiata, A., Motrescu, I., Năstută, A., Creanga, D., Popa, G., *Journal of Electrostatics*, **68**, 128, 2010.
[6] Dumitrascu, N., Topala, I., Popa, G., *IEEE Transactions on Plasma Science*, **33**, 1710, 2005.
[7] Choi, J. H., Han, I., Baik, H. K., Lee, M. H., Han, D. W., Park, J. C., Lee, I. S., Song, K. M., Lim, Y. S., *Journal of Electrostatics*, **64,** 17, 2006.
[8] Restaino, L., Frampton, E.W., Hemphill, J. B., Palnikar, P., *Applied Environmental Microbiology*, **61,** 3471, 1995.
[9] http://www.ozoneapplications.com/info/ozone_bacteria_mold_viruses.htm.
[10] Thanomsub, B., Anupunpisit, V., Chanphetch, S., Watcharachaipong, T., Poonkhum, R., Srisukonth, C., *Journal of General and Applied Microbiology*, **48,** 193, 2002.
[11] Komanapalli, I. R., Lau, B. H. S., *Applied Microbiology and Biotechnology*, **49,** 766, 1998.