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Decontamination Effects of Low-temperature Plasma Generated by Corona Discharge Part II: New Insights

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Abstract: The second part of our paper presents the results of experiments with the decontamination of surfaces by low-temperature plasma generated by corona discharge in air at atmospheric pressure. A simple device is described and the effects of the corona discharge on model microorganisms, *viz*. the yeast *Candida albicans*, Gram-negative bacteria *Escherichia coli, Enterobacter aerogenes*, *Neisseria sicca*, *Stenotrophomonas maltophilia*, Gram-positive bacteria *Deinococcus radiodurans*, *Enterococcus faecium*, *Staphylococcus epidermidis*, *Streptococcus sanguinis,* and vegetative and spore forms of *Geobacillus stearothermophilus* are discussed. A similar microbicidal effect after about one-minute exposure was observed in all vegetative forms of the microorganisms. Measurement in growth inhibition zones on a semisolid medium was used to determine the dependence of the microbicidal effect on exposure time and the distance between electrodes. Counting of colonies served to assess the microbicidal effect of the discharge on contaminated inert surfaces observable after more than 1 min exposure. *Geobacillus stearothermophilus* spores were found to have several times lower susceptibility to the action of the discharge and the microbicidal effect was observed only after an 8 min exposure. Reaction with the iodide reagent did not unambiguously demonstrate the difference between ozone and singlet oxygen as presumed active components of the corona. The area distribution of reactive oxygen species was determined; it was found to differ from the Wartburg law depending on exposure time. Qualitative evidence was obtained on the penetration of the reactive oxygen species into the semisolid medium.

Introduction

Literature references relating to the subject of this study and their assessment are given in part I of this article. This part, formulated as an experimental study, contains a brief description of the methods used, new results and their evaluation. A more extensive and detailed description can be found in [1].

Materials and Methods

Apparatus

We used a simple apparatus of an open air type. It is based on a source of direct current high voltage HT 2103 (Utes, Brno, Czech Republic) that enables set the voltage up to 10 kV and current up to 0.5 mA. Negative point-to-plane corona discharge was generated on the point electrode represented by the tip of a syringe needle 0.7 × 35 mm (Chirana, Stará Turá). The needle was fixed in a vertical position in a laboratory stand above the horizontal surface of a plane electrode and its distance from the anode surface was set by a micrometer screw. The anode, connected to the positive pole of the source, was in different experiments realized by the surface of an ion-conducting semisolid cultivation medium, aluminium plate or water surface. The apparatus is shown in Figure 1.

Microorganisms

The microorganisms under study included "wild" strains of the following species isolated at the Institute of Immunology and Microbiology: *Candida albicans*, *Escherichia coli*, *Staphylococcus epidermidis*. The other bacteria were defined type strains obtained from the Czech Collection of Microorganisms, Brno (CCM), namely *Deinococcus radiodurans* (CCM 1700), *Enterobacter aerogenes* (CCM 2531), *Enterococcus faecium* (CCM 3609), *Geobacillus stearothermophilus* (CCM 4395), *Neisseria sicca* (CCM 4404), *Stenotrophomonas maltophilia* (CCM 4764), and *Streptococcus sanguinis* (CCM 4047). The collection strains were revived according to the supplier's instructions; all strains were propagated by cultivation in a liquid medium, the cells were suspended in sterile water with the addition of 10 % glycerol, the cell concentration was determined by plating and counting of colonies and the strains were stored in a fridge.

Sporulated forms of *Geobacillus stearothermophilus* were prepared by maintaining a stock suspension for 14 days at 20 °C. This led to an incomplete spontaneous sporulation. Immediately before the exposure the suspension was warmed up to 75 °C for 15 min. This treatment inactivated all vegetative forms of the bacteria and the spore forms were at the same time activated to germination to the vegetative form. Since this transition takes several hours, the inoculum could be considered to contain the spores only throughout the exposure period.

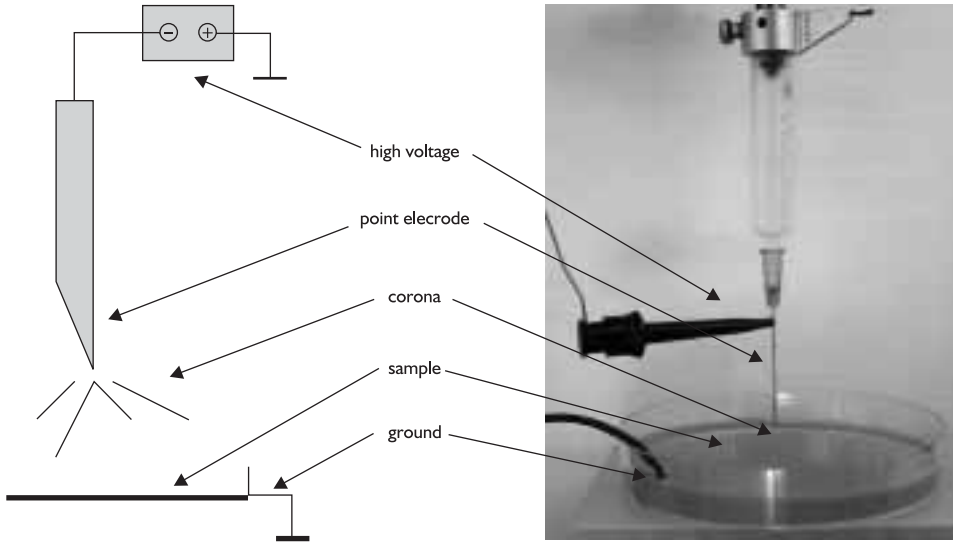

Figure 1 – Scheme of the apparatus for corona discharge generation. Negative point-to-plane corona discharge was generated on the tip of a needle fixed in a vertical position above a horizontal surface of the plane anode and its distance from the anode surface was adjusted by a micrometric screw.

The bacteria were cultivated on Nutrient Agar (Živný agar No. 2, Imuna Michal'any, Slovakia), yeast on Sabouraud Dextrose Agar (BBL). The media were prepared according to manufacturers' instructions and dispensed into Petri dishes. Preliminary experiments were also performed with the use of a common blood agar and Endo agar.

Prior to the inoculation onto the culture medium, the stock suspensions of the bacteria were diluted to the required concentration expressed as the number of colonies or colony forming units (cfu) per 1 cm^2 agar grown after inoculating the suspension. Cell concentrations were either those yielding 20 to 100 cfu cm^{-2} (low concentration), i.e. ensuring the growth of countable isolated colonies, or a high concentration of ca. 10^6 cfu cm^{-2} which provided a continuous bacterial coat on the surface of the agar. In the latter case, the cell concentration was determined after a dilution of the inoculated suspension.

Decontamination on culture media

One-ml aliquots of suspension of the vegetative forms of the organisms under study in appropriate concentration were plated on the whole surface of the semisolid culture medium in a Petri dish. Immediately after absorption of the inoculum, they were exposed to the corona discharge with an initial current of 0.05 mA at a variable inter-electrode distance of 2, 4, 8 and 10 mm for 1, 2, 4, 8 and 16 min. Spores were prepared and exposed under the same conditions but the concentration of the inoculated suspension was lower and the exposure lasted 2, 4, 8, 16 and 32 min. The state of the cultures was assessed by counting the produced colonies; alternatively, the growth inhibition zones were extrapolated to an elliptical shape, their diameter was measured and their surface area calculated. The experimental procedure is schematically illustrated in Figure 2.

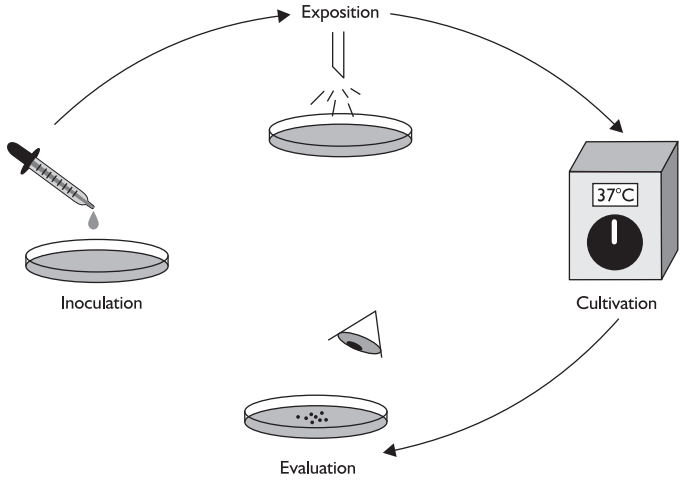

Figure 2 – Scheme of decontamination tests. Bacteria in a selected concentration were plated on culture medium; the sample was exposed to the corona discharge, cultivated and evaluated.

Decontamination on inert surfaces

In-depth decontamination properties of the corona discharge were studied on inert surfaces excluding a potential reaction of the discharge with the culture medium. These experiments were performed with *Escherichia coli* suspensions spread either on Teflon-laminated rubber cylinders 1 cm in diameter and 2 mm high, or on 2 × 2 cm cotton wool fabric squares 0.5 mm thick. These materials were placed on the plane electrode represented by an aluminium plate 5 cm in diameter. A drop of the bacterial suspension was placed on the Teflon surface; it retained its compact shape due to the hydrophobic Teflon surface. The same suspension, when applied dropwise on the fabric, was uniformly adsorbed into it, forming a spot with a radius of about 0.5 cm. Immediately after dropping or adsorption of the suspension, the point electrode was positioned 1 cm above the samples, initial current was set at 0.05 mA and the samples were exposed to the discharge for 0.5, 1, 2, 4 and 8 min. After the exposure the Teflon surface and both the upper and lower surface of the fabric were stamped onto the surface of the agar medium, which was cultivated for 18–20 hours at 37 °C and the resulting colonies were counted.

Comparison of the bactericidal effect of reactive particles and UV light

To compare the effect of reactive particles, whose amount corresponds to the current distribution in the discharge, with the effect of UV light generated in the discharge, *Staphylococcus epidermidis* and *Escherichia coli* were exposed to it in a separate experiment. The bacteria were inoculated on the culture medium and exposed to a corona discharge generated as described in the section Decontamination on culture media. However, in this case the inter-electrode distance was $d = 10$ mm and the exposure periods were 1, 2, 4, 8, 16 and 32 min. The inhibitory effect in this case was expressed as the diameter of growth inhibition zones. The acquired values were compared with the theoretical course of dependence of discharge components on exposure time, calculated from the following equations: The theoretical dependence of the zone diameter x irradiated by the UV radiation is given by the relation

$$x = M \,.(I_0 \,.\, t/C - d^2)^{0.5}$$

The dependence of zone diameter x on the time correspond to the theoretical current density is given by an analogous relation derived from the Wartburg law:

$$x = M.d \tan(\arccos(K/t.j_0)^{1/5})$$

where x is the theoretical inhibition zone diameter, C and K are fit coefficients expressing the "bacterial resistance", M is the fit coefficient expressing other causes, I_0 is the total intensity of UV radiation by the source, t is exposure time, d is the distance of the electrodes and j_0 is the corona axis current density.

Determination of reactive oxygen species

Reactive oxygen species were determined in a reaction with the iodide reagent of the following composition: KH_2PO_4 (0.05 mol l^{-1}), NaOH (0.08 mol l^{-1}), KI (0.12 mol l^{-1}), $(NH_4)_2MoO_4$ (0.01 mol l^{-1}) [2].

This reagent dissolved in H_2O or in D_2O was exposed in a Petri dish to a corona discharge at a starting current of 0.05 mA and inter-electrode distance 6 mm for 15, 30, 45, 60, 75 and 90 s. After each exposure, the reagent was transferred into a silica cuvette with 1 cm optical path and its absorbance was measured in the range of 300 – 600 nm on a Unicam UV 300 instrument. The resulting concentration c of the triiodide anion was calculated according to the Lambert--Beer law $c = A/\varepsilon$, where A is the absorbance at 351 nm and extinction coefficient at this wavelength is $\varepsilon = 2.47.10^4$ l $mol^{-1}cm^{-1}$.

In another experiment, Petri dishes filled with the reagent dissolved in H_2O and supplemented with 4 % agar were used as plane electrodes. These samples were exposed to a corona discharge at an initial current of 0.05 mA and inter-electrode distance of 6 mm for 1, 2, 4, 8 and 16 min and the resulting yellow zones were photographed by the Fujifilm FinePix S5500 digital camera. An agar strip 1 mm thick was then cut out of the middle of the exposure zone with a lancet and photographed from a side to record the penetration of reactive oxygen species into the semisolid solution. Our own software was used to obtain from the digital photographs the area and depth distribution of the yellow colour intensity (or loss of blue component in RGB colour representation) corresponding to the amount of I_3^- formed in the sample.

Results

Control exposure of culture media

Prior to the start of the actual experiments, a control experiment was conducted to verify the growth of the microorganisms on culture media previously exposed to the corona discharge. The discharge could affect the composition of the media and make it potentially unsuitable for the growth of the microorganisms. No difference was found between the growth on exposed and unexposed media if the exposure was shorter than ca. 60 min. After longer exposures, the medium was

Table 1 – Time dependence of the current passing through the corona discharge at different inter-electrode distances

		I [μA]					
d [mm]	U [kV]	t = 0 min	t = 1 min	t = 2 min	t = 4 min	t = 8 min	t = 16 min
2	2.8	50	47	44	39	28	19
4	4.6	50	48	47	44	38	34
6	6.8	50	50	49	48	46	43
10	9.8	50	50	50	49	48	47

partially deteriorated and the growth of bacteria was limited, probably due to a gradual medium dehydration.

Effects caused by particle streaming called ion wind, and described previously in [3], have been observed on agar media. During the exposure, the ion drift carries with it relatively considerable amount of air from the point electrode to the plane electrode and the streaming air dilutes the products of the corona and its pressure gradually deforms the agar medium. The surface of the agar is therefore corrugated to form a pit whose depth increases with progressing exposure. This effect was more conspicuous at lower initial distances of the needle tip from the agar surface. Due to the bending, the surface of the agar (i.e. the place electrode) moved away from the tip of the point electrode; this caused a drop in the current passing through the discharge at a constant inter-electrode voltage. The dependence of the voltage U and the passing current I on exposure time t at different initial distances d between the electrodes is given in Table 1. The inter-electrode voltage at the beginning of the exposure was always set so that a current of 50 μA passed between the electrodes.

In experiments with the low concentration of inoculated bacteria (50 to 100 cfu cm^{-2}), the inhibition zones caused by the action of the corona discharge were not sharply delineated and the number of colonies of surviving microorganisms gradually increased in the outward direction. These experiments were therefore evaluated only qualitatively by stating that, under given conditions and exposure times up to 16 min, the microbicidal effect is sufficiently perceptible. The standard conditions for quantitative evaluation of the microbicidal properties of the corona discharge included inoculation with high concentrations of the microorganisms (of the order of 10^6 cfu cm^{-2}) that give sharply delineated zones (Figure 3).

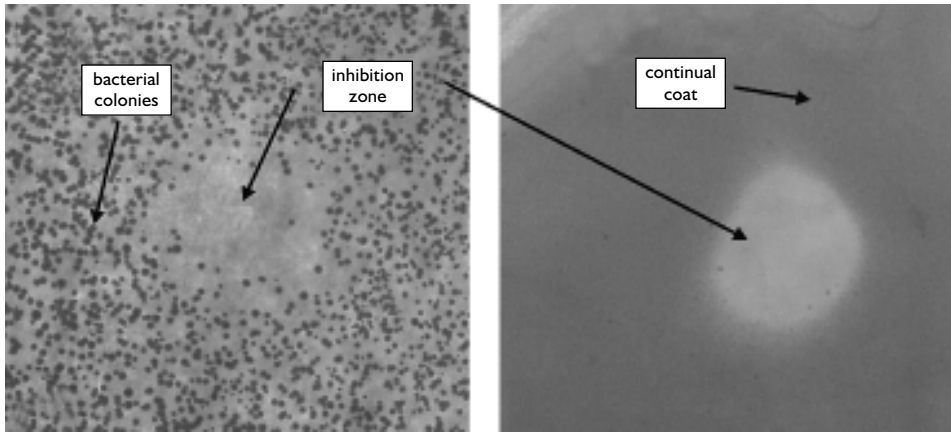

Figure 3 – Appearance of inhibition zones for (a) low (ca. 100 cfu cm^{-2}) and (b) high (ca. 10^6 cfu cm^{-2}) inoculum concentration. At low inoculum concentration, the inhibition zone is not sharply delineated and the number of colonies gradually increases towards the zone periphery. At high inoculum concentration, the zone is sharply delineated and approximately circular.

The independence of the size of the inhibition zones of the concentration of inoculated bacteria was verified in an experiment in which stock suspensions of *Enterobacter aerogenes* and *Neisseria sicca* diluted 1:10 and 1:100 were plated on the culture medium before the exposure. As seen in Table 2, the size of the inhibitory zones for different inoculum dilutions did not change.

Microbicidal effects of the corona discharge on vegetative forms of microorganisms
The inhibition zone areas *S* obtained for individual microorganisms inoculated on the culture media in given concentrations and exposed to the corona discharge for different times *t* at a variable inter-electrode distance *d* are summarized in Tables 3–12. These data have in part been published in [4].

As seen from the data in these tables, the inhibition zone size increased with exposure time. An exception was the cessation of zone size increase observed with *Streptococcus sanguinis* after 4–8 min.

Table 2 – Dependence of the inhibition zone areas *S* on inoculum dilution. Stock suspension of bacteria, undiluted and diluted 1:10 and 1:100, discharge parameters *d* = 4 mm and *t* = 8 mm

	S [mm^2]		
bacterium / dilution	undiluted	1:10	1:100
Enterobacter aerogenes	15	15	15
Neisseria sicca	27	27	27

Table 3 – Areas *S* of inhibition zones in the yeast *Candida albicans* (concentration 2.10^6 cfu cm^{-2})

	S [mm^2]				
d [mm]	*t* = 1 min	*t* = 2 min	*t* = 4 min	*t* = 8 min	*t* = 16 min
2	5	10	15	25	40
4	3	10	10	28	63
6	0	0	0	8	63
10	0	0	0	0	40

Table 4 – Areas *S* of inhibition zones in the bacterium *Deinococcus radiodurans* (concentration 3.10^6 cfu cm^{-2})

	S [mm^2]				
d [mm]	*t* = 1 min	*t* = 2 min	*t* = 4 min	*t* = 8 min	*t* = 16 min
2	3	9	10	31	56
4	3	6	10	33	48
6	3	3	10	28	45
10	0	0	3	13	25

Depending on the inter-electrode distance, the zone size had a generally rising trend. The frequent exceptions from this trend observed with most of the bacteria were perceptible as an increase in the zone size for medium inter-electrode distances (4–6 mm). These anomalies, however, can be taken to represent a drop in the efficiency of the discharge at the lowest distance of 2 mm. For long exposures, in particular 16 min, the drop in the zone size

Table 5 – Areas *S* of inhibition zones in the bacterium *Enterobacter aerogenes* (concentration 2.10^6 cfu cm^{-2})

	S [mm^2]				
d [mm]	*t* = 1 min	*t* = 2 min	*t* = 4 min	*t* = 8 min	*t* = 16 min
2	3	3	5	9	15
4	3	3	16	16	20
6	3	3	7	16	20
10	0	0	0	7	20

Table 6 – Areas *S* of inhibition zones in the bacterium *Enterococcus faecium* (concentration 3.10^6 cfu cm^{-2})

	S [mm^2]				
d [mm]	*t* = 1 min	*t* = 2 min	*t* = 4 min	*t* = 8 min	*t* = 16 min
2	8	13	20	28	38
4	8	13	28	28	38
6	8	8	13	28	38
10	0	0	3	13	28

Table 7 – Areas *S* of inhibition zones in the bacterium *Escherichia coli* (concentration 9.10^6 cfu cm^{-2})

	S [mm^2]				
d [mm]	*t* = 1 min	*t* = 2 min	*t* = 4 min	*t* = 8 min	*t* = 16 min
2	7	7	9	16	50
4	3	7	9	20	44
6	3	4	7	16	28
10	0	0	3	13	33

Table 8 – Areas *S* of inhibition zones in the vegetative forms of *Geobacillus stearothermophilus* (concentration 2.10^6 cfu cm^{-2})

	S [mm^2]				
d [mm]	*t* = 1 min	*t* = 2 min	*t* = 4 min	*t* = 8 min	*t* = 16 min
2	3	3	5	8	13
4	3	3	5	8	20
6	0	3	3	3	20
10	0	0	0	3	13

with increasing inter-electrode distance was in many cases no longer perceptible or the zone actually mildly increased in size. At large inter-electrode distances (usually 10 mm) and short exposures (usually 1–2 min), the microbicidal effect of the discharge was no longer observed. An exception was *Stenotrophomonas maltophila*, which exhibited marked zones even under these conditions.

Table 9 – Areas *S* of inhibition zones in the bacterium *Neisseria sicca* (concentration 12.10^6 cfu cm^{-2})

	S [mm^2]				
d [mm]	*t* = 1 min	*t* = 2 min	*t* = 4 min	*t* = 8 min	*t* = 16 min
2	3	10	15	33	38
4	8	10	20	38	43
6	0	8	13	28	50
10	0	0	8	15	50

Table 10 – Areas *S* of inhibition zones in the bacterium *Staphylococcus epidermidis* (concentration 8.10^6 cfu cm^{-2})

	S [mm^2]				
d [mm]	*t* = 1 min	*t* = 2 min	*t* = 4 min	*t* = 8 min	*t* = 16 min
2	7	7	9	16	56
4	3	6	10	20	44
6	3	3	7	20	28
10	0	0	3	13	28

Table 11 – Areas *S* of inhibition zones in the bacterium *Stenotrophomonas maltophilia* (concentration 5.10^6 cfu cm^{-2})

	S [mm^2]				
d [mm]	*t* = 1 min	*t* = 2 min	*t* = 4 min	*t* = 8 min	*t* = 16 min
2	10	10	24	28	44
4	10	15	28	31	44
6	10	15	24	44	44
10	3	13	15	28	38

Table 12 – Areas *S* of inhibition zones in the bacterium *Streptococcus sanguinis* (concentration 1.10^6 cfu cm^{-2})

	S [mm^2]				
d [mm]	*t* = 1 min	*t* = 2 min	*t* = 4 min	*t* = 8 min	*t* = 16 min
2	10	13	20	20	20
4	10	10	20	33	33
6	8	8	20	33	33
10	0	0	10	8	38

In general, it can be concluded that the smallest inhibition zones were observed for short exposure times and large inter-electrode distances. The largest inhibition zones were observed to occur after long exposures and the largest, or, on the other hand, smallest inter-electrode distances; in some microorganisms (*Geobacillus stearothermophilus*, *Candida albicans*), large zones were observed also at medium inter-electrode distances. These anomalies could be partly due to an error in measuring the inhibition zone diameters that could exceed the arbitrarily defined value of ± 1 mm.

Microbicidal effects of the corona discharge on bacterial spores

No marked inhibition of *Geobacillus stearothermophilus* spores inoculated to a high concentration of ca. 10^6 cfu cm^{-2} was observed for exposure times shorter than 30 min. Although inhibition zones were formed at longer exposure times, it was not clear if they were due to an actual spore inhibition or to a partial degradation of the culture medium at very long exposures. The following experiments were therefore performed at a low spore concentration of ca. 100 cfu cm^{-2}; after inoculation, the spores were exposed for 2, 4, 8, 16 and 32 min at an inter-electrode distance of 2, 4, 6 and 10 mm. This treatment gave rise to approximately circular inhibition zones, which were not sharply demarcated. In this case, the inhibition zone was taken to be the approximately circular area free of all colonies. To compare bacterial spores with vegetative cells, the same exposure was used for samples of the nonsporulating *Staphylococcus epidermidis* inoculated at the same low concentration. The resulting inhibition zone areas *S* are given in Tables 13 and 14. We can state that spore inhibition appeared after an

Table 13 – Areas *S* of inhibition zones in the sporulated bacterium *Geobacillus stearothermophilus* (concentration 80 cfu cm^{-2})

	S [mm^2]				
d [mm]	*t* = 1 min	*t* = 2 min	*t* = 4 min	*t* = 8 min	*t* = 16 min
2	0	0	0	1	3
4	0	0	14	14	7
6	0	0	8	18	18
10	0	0	7	16	42

Table 14 – Areas *S* of inhibition zones in the bacterium *Staphylococcus epidermidis* (concentration 80 cfu cm^{-2})

	S [mm^2]				
d [mm]	*t* = 1 min	*t* = 2 min	*t* = 4 min	*t* = 8 min	*t* = 16 min
2	16	38	63	95	155
4	7	38	38	115	255
6	0	0	0	30	255
10	0	0	0	0	155

8 min exposure at the earliest. When attempting a quantitative comparison, it can be said that, for different inter-electrode distances, zones of comparable size (of the order of 10 mm^2) appeared with the vegetative forms after a 2–16 min exposure whereas a 16–32 min exposure was needed with the spores. Hence, the efficiency of action of the discharge on spores can be roughly estimated to be 4 to 8 fold less than with vegetative forms. The dependence of zone size on inter-electrode distance is also different for different bacterial forms; for a 16 min exposure it is similar in spores and in vegetative forms while for a 32 min exposure the zone size conspicuously increases with the inter-electrode distance.

Measurement of decontamination effects on inert surfaces

The dependence of the numbers of *Escherichia coli* colonies formed by stamping the materials under study onto culture medium on the time of exposure *t* is given in Table 15. These data have been published in [5]. The number of surviving bacteria decreased with exposure time and the bacteria were completely killed with exposures longer than 1 min. With the cotton wool fabric, significantly different numbers of surviving bacteria were found on the top surface of the fabric that is directly exposed to the corona discharge and on the fabric bottom surface, which is shielded from the discharge by the mass of the fabric. The dependence of

Table 15 – Number of *Escherichia coli* bacteria surviving on contaminated and exposed materials

	number of surviving bacteria					
material	t = 0 min	t = 0,5 min	t = 1 min	t = 2 min	t = 4 min	t = 8 min
teflon	200	80	10	0	0	0
fabric top	200	50	13	0	0	0
fabric bottom	41	31	20	0	0	0

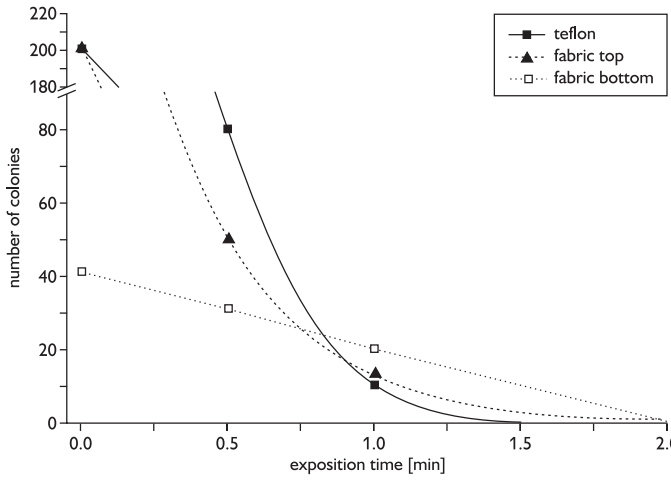

Figure 4 – Dependence of the number of Escherichia coli bacteria surviving on contaminated materials after the exposure.

the number of surviving bacteria on exposure time is also markedly different for the two cases: The decrease of the number of bacteria on the topside of the fabric is approximately exponential like with the Teflon surface, but on the bottom side of the discharge-exposed fabric, the decrease is linear (see Figure 4).

Comparison of the bactericidal effect of reactive particles and UV light
The inhibition zone sizes determined for *Staphylococcus epidermidis* and *Escherichia coli* and expressed as colony diameters in mm are shown in Table 16. The theoretical dependences of the diameters of zones irradiated by UV light with those exposed to the stream of particles given by the current density and calculated from the formulas given in Materials and Methods, are given in Figures 5 and 6. The graphs contain at the same time the measured diameters of inhibition zones along with the measurement errors estimated at ± 0.5 mm, determined for both bacteria, and presented in Table 16. These results have already been published in [6].

Determination of reactive oxygen species
The optical spectrum of one of the liquid samples exposed to the corona discharge is shown in Figure 7 as an example. For each exposure time, the least squares method

Table 16 – Inhibition zone diameters (in mm) of *Staphylococcus epidermidis* and *Escherichia coli* after exposure to the corona discharge from a 10 mm distance

exposition time [min]	*Staphylococcus epidermidis*	*Escherichia coli*
1		4
2	11	6
4	19	9
8	29	12
16	33	14
32		16

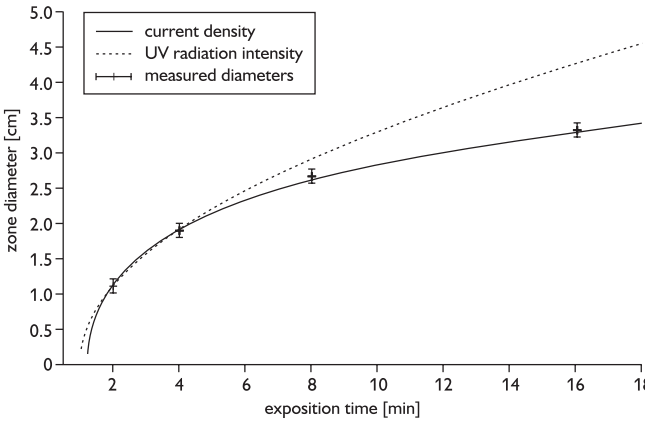

Figure 5 – Dependences of the size of inhibition zones on exposure time calculated for the microbicidal action of UV light and current densities in the corona discharge. Inter-electrode distance 10 mm; experimental points give inhibition zone diameters determined with Staphylococcus epidermidis, vertical bars correspond to ± 0.5 mm measurement error.

was used to calculate from ten parallel measurements a regression line for the dependence of I_3^- concentration on exposure time for samples in H_2O and in heavy water D_2O. The ensuing regression coefficients were in both cases higher than 0.99. These dependences are given in Figure 8.

Determining the total amount of reactive oxygen species penetrating into the semisolid solution

Exposure of the iodide reagent solidified with agar gave rise to yellow zones (spots) on agar surface, whose size and intensity increased with exposure time. Although the computer processing of the spot photographs was done, the yellow colour had a low contrast and low intensity and the results therefore exhibited large noise. Despite this, the dependence of colour intensity on the distance from the spot centre for individual exposure times could be read and interpolated by polynomial functions. The relevant formula equations are not given in detail here but the functions are plotted in the graph in Figure 9. The intensity of the yellow colour was expressed in arbitrary units (a.u.) defined as a relative deviation in the blue channel in RGB colour representation, multiplied by 255. The medium intensity of the photograph was taken as the reference level. Beside the intensity dependences, the graph contains also the theoretical current density calculated from the Wartburg law.

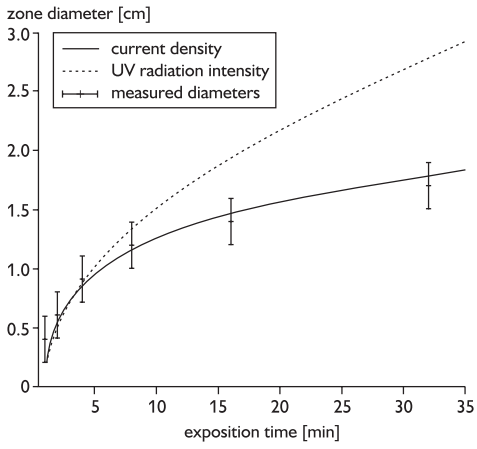

Figure 6 – Dependences of the size of inhibition zones on exposure time calculated for the microbicidal action of UV light and current densities in the corona discharge. Inter-electrode distance 10 mm; experimental points give inhibition zone diameters determined with Escherichia coli, vertical bars correspond to ± 0.5 mm measurement error.

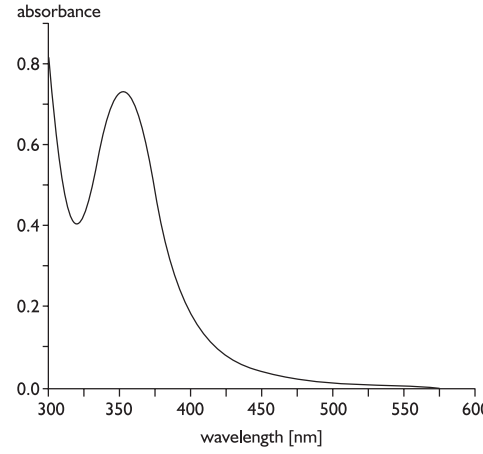

Figure 7 – Example of absorption spectrum of the triiodide anion I_3^- recorded in a 300 to 600 nm wavelength range.

Inspecting the strips of exposed agar, it could be qualitatively stated that the yellow colour extended into a depth of ca. 3 mm depending on exposure time; this corresponds to the depth into which reactive oxygen species have penetrated. In this case, we also attempted to carry out quantitative statistical evaluation of the dependence of colour intensity on the depth of penetration. However, the calculated data displayed large scatter due to the low colour intensity and comparable magnitude of noise, and could not be satisfactorily assessed.

Discussion

The apparatus used in this study for examining the decontamination properties of low-temperature plasma generated by a negative corona discharge at atmospheric

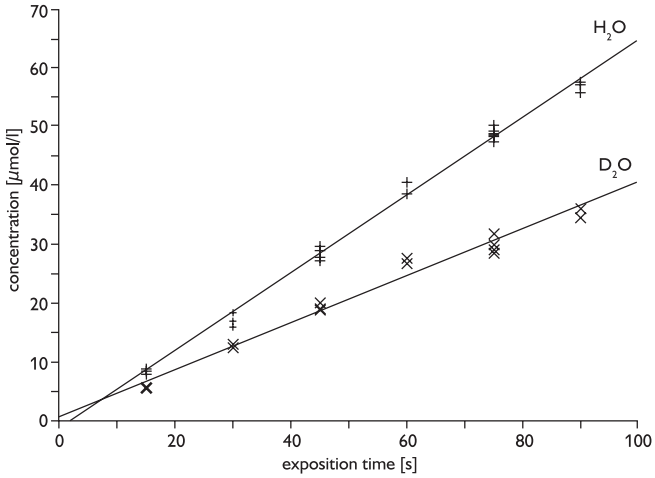

Figure 8 – Dependence of concentration of the triiodide anion I_3^- on exposure time for samples in light water H_2O and heavy water D_2O.

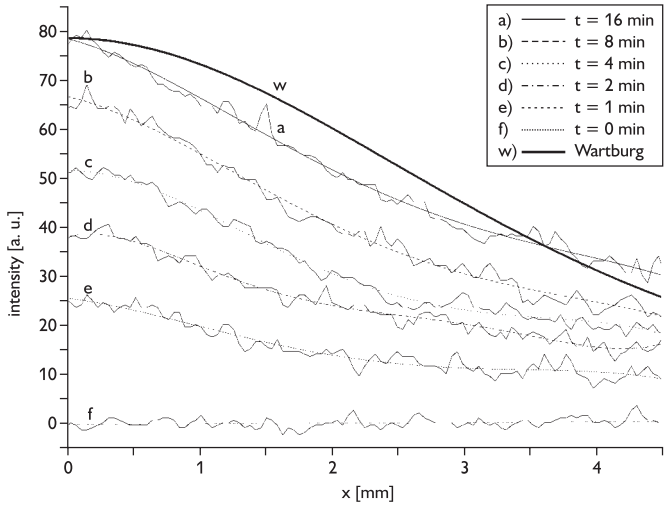

Figure 9 – Dependence of the area distribution of yellow color intensity (in arbitrary units, a.u.) on the distance from the spot centre x. Experimental values determined for exposure times 1, 2, 4, 8 and 16 min are plotted together with the polynomial interpolation. Theoretical current density profile calculated by means of the Wartburg law (curve W) has been adjusted to the maximum intensity of the yellow color in the centre of the spot.

pressure is very simple. In contrast to other studies published so far, which used other types of electric discharge and required the construction of complicated devices, the device described here has the advantage of being easy-to-construct, requiring low investment costs and operating at negligible costs.

The microbicidal effect of the corona discharge and the plasma generated by it was quantitatively measured by a method analogous to the disc diffusion test for determining the sensitivity to antibiotics, i.e. by measuring the growth inhibition zones arising in the continuous microbial lawn on the surface of a semisolid agar medium. A complete growth inhibition took place inside these zones. Quantitative assessment was carried out of the increase in zone size, and thus the increasing microbicidal effect, with extending exposure time; the dependence of zone area on the inter-electrode distance decreased in an irregular manner. The irregularities of this dependence included most often the appearance of relatively small zones at the lowest inter-electrode distances. This effect can be attributed to the fact that the area on the agar plane electrode within which the microbicidal effect takes place is given by the area in which the approximately conical drift region of the corona discharge penetrates the surface of the agar. At a low distance between the electrodes, the diameter of the drift cone is smaller than at large inter-electrode distances and the discharge, although it is very effective due to the small distance between the electrodes, acts only over a small area.

The sensitivity of all vegetative forms of the bacteria under study to the microbicidal effect of the corona discharge is about comparable – no significant differences were for instance observed between Gram-positive and Gram-negative bacteria, or between cocci and rods. Despite these small differences, we attempted to classify the microorganism under investigation according to their sensitivity. On using as the sensitivity criterion the area of the inhibition zone observed after a 16-min exposure, the following order of sensitivity was obtained:

1. *Candida albicans* (63 mm^2)
2. *Deinococcus radiodurans* (56 mm^2)
3. *Staphylococcus epidermidis* (56 mm^2)
4. *Neisseria sicca* (50 mm^2)
5. *Escherichia coli* (50 mm^2)
6. *Stenotrophomonas maltophilia* (44 mm^2)
7. *Enterococcus faecium* (38 mm^2)
8. *Streptococcus sanguinis* (38 mm^2)
9. *Enterobacter aerogenes* (20 mm^2)
10. *Geobacillus stearothermophilus* vegetative form (20 mm^2).

An order based on other parameters is slightly different. For instance, *Stenotrophomonas maltophilia* would appear to be the most sensitive because it exhibited inhibition zones at the shortest exposures and the largest inter-electrode

distances. This finding can have a potential practical significance since a similar sensitivity can be presumed also in its close relative, the pathogenic *Pseudomonas aeruginosa*. An interesting feature is also the high sensitivity of *Deinococcus radiodurans*, which is known for its high resistance to intensive ionizing radiation [7, 8] but a low resistance against the action of, e.g., $^1O_2$ [9]. This finding supports the hypothesis of the low importance of UV radiation in the decontamination by the corona discharge.

The sporulating bacterium *Geobacillus stearothermophilus* is a species used routinely for the control of correct performance and validation of sterilization procedures. As expected, its spores displayed a markedly lower sensitivity to the action of the corona discharge and the plasma generated by it when compared with the vegetative forms of bacteria; a significant microbicidal effect, although perceptible, was observed only after longer exposures and when lower initial cell concentration was used. When compared with vegetative forms exposed under the same conditions, the sensitivity of the spores was about 4 to 8 times lower. The dependence of the zone size on the inter-electrode distance, which is considerably different for spores and for vegetative forms, implies that the mechanism of the microbicidal effect on spores differs from that taking place in vegetative forms.

Escherichia coli on the surface of inert materials were found to succumb to a significant microbicidal effect at exposure times longer than a mere 1 min. An in-depth microbicidal effect was also observed; it was observed in a drop of exposed bacterial suspension and also on the adverse side of a contaminated fabric where the bacteria were shielded from the direct effect of the radiation. The lowering of the killing effect in the depth of the material corresponds to the decrease in the number of reactive particles during their diffusion through water or the fabric; however, the effect on the surface of the material can include a certain contribution of the non-penetrating UV radiation.

The theoretical dependences of the sizes of zones impacted by UV light and those struck by the stream of particles generated by the corona discharge on exposure time differ in their course. The dependence of the actually determined sizes of inhibition zones agrees well with the calculated course of the current density; this indicates that the inhibitory or microbicidal effect is mediated predominantly by the strain of reactive and/or charged particles rather than by UV radiation.

Attempts at determining the reactive oxygen species were motivated by the effort to document the participation of singlet oxygen whose generation by the corona discharge in an oxygen atmosphere can be safely presumed. As expected, the presence of active oxygen in the corona discharge was successfully proved and determined by the iodide reagent. An attempt at differentiating and better determining the forms of active oxygen was done by parallel determination in light and heavy water. In the presence of singlet oxygen, the yield of triiodide in D_2O should be higher than in H_2O, in the absence of singlet oxygen it should be the

same. Surprisingly, we found a lower yield of triiodide in D_2O than in H_2O; we did not succeed in interpreting this result. The insensitivity of I_3^- production to the isotope exchange in the detection reagent indicates that the dominant oxidizing particle is not $^1O_2$ but very probably ozone, which is undoubtedly present in the discharge. However, the experiments performed so far did not allow us to confirm or disprove the presence of lower concentrations of $^1O_2$ in the corona discharge and its participation in the decontamination. We therefore intend to determine $^1O_2$ by using substrates or reagents selectively oxidizable with $^1O_2$, e.g. uric acid.

Determination of the area distribution of active oxygen on the surface of the medium helped us to define approximately its dependence on exposure time, which should correlate with the theoretical distribution of the current density according to the Wartburg law. However, the experimental data qualitatively differ from the theoretical distribution since the theoretical current density curve drops less steeply than the measured yellow colour intensity up to the distance of ca. 3.5 mm from the maximum in which the theoretical value begins to be below the measured one. This phenomenon can be attributed to the ion wind, which carries charged particles out of the drift region; as a result, their number in the centre decreases while that on the periphery increases.

Similarly to the experiments with decontamination of inert surfaces, attempts at determining the in-depth distribution of reactive oxygen species in the semisolid medium showed the penetration of reactive oxygen species below the surface of the medium, which is a prerequisite for in-depth decontaminating action of the discharge. We did not succeed in evaluating these experiments quantitatively, i.e. in determining the concentration of reactive oxygen species as dependent on depth, since the low intensity of the triiodide stain gave rise to considerable noise and scatter of measured values. We therefore plan to develop a reagent containing starch whose colour should be much more intensive and should permit an exact quantitative evaluation. At any rate, the qualitative assessment implies that the corona discharge can decontaminate not only smooth surfaces but also porous and wet surfaces, and even liquids to a certain depth. The corona discharge could therefore be an effective tool for killing encapsulated bacteria, e.g. virulent pneumococci.

Conclusion

We constructed a simple device for the study of decontamination effects of corona discharge. These effects were studied on a set of microorganisms including Gram-negative, Gram-positive sporulating and nonsporulating bacteria of different cell shapes and one species of yeast.

The action of the corona discharge does not affect the properties of common semisolid culture media at exposures shorter than ca. 60 min except for causing medium dehydration. In vegetative forms on culture media, the microbicidal effect is perceptible after a mere 1 min exposure. Its efficiency, measured by the size of

the growth inhibition zone, does not appreciably differ in different bacteria, rises regularly with exposure time, and drops in an irregular manner with increasing inter-electrode distance. On inert materials, the killing effect is perceptible after more than 1 min. Its efficiency, measured by the number of colonies, differs depending on whether the colonies are directly exposed to the drift region of the discharge or are shielded from it. The killing effect on spores is perceptible only after an exposure lasting at least 8 min. Its efficiency, measured as the size of the inhibition zone, is several-fold less than in vegetative forms.

Comparison of the microbicidal effect of the corona discharge on directly exposed and optically shielded surfaces, and comparison of the determined magnitude of the microbicidal effect with theoretically calculated values imply that the killing effect is mediated predominantly by the action of reactive particles with a possible smaller contribution of UV light.

Determination of reactive oxygen species by the iodide reagent in H_2O and D_2O did not enable us to distinguish ozone from singlet oxygen. Determination of the area distribution of active oxygen on the surface of the semisolid medium was used to determine its dependence on exposure time, which, probably due to the action of the ion wind, differs from the theoretical distribution according to the Wartburg law. Determination of the in-depth distribution qualitatively documented the penetration of corona discharge particles into the semisolid medium.

References

1. SCHOLTZ V.: Optimalizace korónového výboje za atmosférického tlaku pro dekontaminaci povrchů. Thesis, ČVUT, Praha 2007.
2. MOSINGER J., MOSINGER B.: Photodynamic s*ensitizers assay: rapid and sensitive iodometric measurement. Experientia (Cell. Mol. Life Sci.)* 51: 106–109, 1995.
3. SIGMOND R. S., KURDELOVA B., KURDEL M.: Action of corona discharge on bacteria and spores. *Cz. J. Phys.* 49: 405–420, 1999.
4. JULÁK J., KŘÍHA V., SCHOLTZ V.: The study of bactericidal effects of corona discharge at atmospheric pressure. *Europhysics Conference Abstracts* 301: P–4.007, 2006.
5. JULÁK J., KŘÍHA V., SCHOLTZ V.: Germicidal effects of atmospheric corona discharge studied on *Escherichia coli* bacteria. Proceeding of the XXVII International Conference on Phenomena in Ionized Gases, Eindhoven 2005, p. 04–177, http://www.icpig2005.nl.
6. SCHOLTZ V.: The distribution of bactericidal effects of negative point to plane corona discharge. Poster 2006, 10th International Student Conference on Electrical Engineering. CVUT FEL, Praha, May 18, 2006.
7. COX M. M., BATTISTA J. R.: *Deinococcus radiodurans* – the consummate survivor. *Nature Revs. Microbiol.* 3: 882–892, 2005.
8. SCHOLTZ V.: *Deinococcus radiodurans* – podivná guľôčka prežívajúca rádioaktívne žiarenie. *Aldebaran Bull*. 5: 3, 2007.
9. SCHÄFER M., SCHMITZ C., FACIUS R., HORNECK G., MILOW B., FUNKEN K.-H., ORTNER J.: Systematic study of parameters influencing the action of rose bengal with visible light on bacterial cells: Comparison between the biological effect and singlet-oxygen production**.** *Photochem. Photobiol.* 71: 514–523, 2000.