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ORIGINAL PAPER

The persistent microbicidal effect in water exposed to the corona discharge

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Abstract This article describes and particularly explains a new phenomenon of persistent microbicidal effect of water previously exposed to the low-temperature plasma, which cannot be attributed to the acidification only. The direct microbicidal action of plasma is well documented, being mediated by number of reactive particles with a short lifetime. However, we observed the microbicidal effect also in exposed water stored for a month, where it must be mediated by stable particles. In water and in phosphate-buffered saline, the formation of NO_x and corresponding acids, H_2O_2 and O_3 was confirmed after exposition to the low-temperature plasma generated in air by DC negative glow corona and positive streamer discharge. The time course of acidification, H_2O_2 and O_3 formation were deremined. Except uncertain traces of HCN, SIFT-MS analysis of exposed liquids reveals no additional reactive compounds. The microbicidal effect persists almost unchanged during 4 weeks of storage, although O_3 completely and H_2O_2 almost disappears. *Staphylococcus epidermidis* and *Escherichia coli* were inactivated within 10 min of incubation in exposed liquids, *Candida albicans* needs at least 1 h. The solutions prepared by artificial mixing of reactive compounds mimic the action of exposed water, but in lesser extent. The acid milieu is the main cause of the microbicidal effect, but the possibility of still unidentified additional compound remains open.

Introduction

The low-temperature plasma displays many effects on biological objects including living organisms. Its use in medicine was described e.g. in [1,2] or [3]. The majority of previous papers were devoted to microbiological applications (for review, see [4]), describing various techniques of non-thermal plasma generation, their use for

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decontamination or sterilization including their limitations and mechanisms. Our previous studies in this field dealt with the microbicidal effects of low-temperature plasma generated by DC corona in the point-to plane and point-to point experimental arrangement [5,6]. During the study devoted to the inactivation of prions [7], we observed an interesting phenomenon: the cell culture susceptible for prion infection was killed after addition of small volume of water previously exposed to the positive stream discharge. We also observed and tentatively reported a similar inhibition effect after the addition of exposed water to bacterial suspensions [8]. At the beginning, we called this killing water by a nickname "water of death", although this term is not perfect: in fairy tales, it denotes the magic water used "to glue the cut parts together" [9]. Rather than a mystery, we believe that the killing of cell or microbes is mediated by reactive particles persisting in the exposed water.

The nature of reactive particles responsible for the bactericidal effect of low-temperature plasma is very complex task and was previously reported in many papers, e.g. [10–16]. Their abundance depends on many parameters including the nature of the discharge or the composition and humidity of the atmosphere around the discharge. Atomic oxygen O and nitrogen N, other radicals and ions ($O_2^{\bullet-}$, $OH^{\bullet}$, OH^-, $O_2H^{\bullet}$ etc.) or singlet oxygen $^1O_2$ were reported most frequently. All of them are instable (e.g., the life times of $OH^{\cdot}$ and $O_2^{\bullet-}$ are 200 μs and 5 s, respectively) and cannot persist in solution longer than seconds. Among the stable particles, the formation of NO_x, hydrogen peroxide and ozone were often reported. The former one responsible for acidification of target solutions due to the formation of HNO_3 and HNO_2, the formation of ozone may be recognized by its smell.

The production of some reactive particles in water was recently studied in detail. Liu et al. [17] studied the inactivation of *Staphylococcus aureus* in suspension using a plasma microjet device with air as the working gas. During exposure, pH decreased rapidly to 3.2 in pure water and to ca. 4.5 in buffered culture medium. The concentrations of NO_2^- and NO_3^- reached 37 and 21 mg l^{-1} respectively and the presence of reactive oxygen compounds ($HOO^{\cdot}$, H_2O_2, O_3) was presumed but not determined. The inactivation of *S. aureus* with plasma jet started under pH 4.5, but the acidity alone (HNO_3 at pH 4.2) was not able to inhibit this bacterium effectively.

Ikawa et al. [18] used the plasma jet to produce reactive particles by low frequency/high voltage (LF/HV) pulses in a stream of helium, and in a closed system in a stream of He and air. They observed a significant inactivation of *Escherichia coli* and an acidophilic *Leuconostoc citreum* bacteria within 120 s, but only if the acidity (caused by NO_x formation) decreased under the critical value of pH 4.7. The inactivation was attributed to the presence of reactive superoxide $O_2^{\bullet-}$ and hydroperoxide $O_2H^{\cdot}$, whose activity may be break down by added superoxiddismutase. Neither the presence of H_2O_2 in acid nor the UV radiation had sterilization effect. They also added bacteria to the solution 10 min after it was exposed to plasma, but despite the sufficient concentrations of NO_x and H_2O_2, no inactivation was observed.

Oehmigen et al. [19] thoroughly investigated water disinfection by plasma treatment using a surface dielectric barrier discharge (DBD) in air, generated at 10 kV and 20 kHz. They determined the kinetics of NO_x and subsequent nitrous HNO_2 and nitric acid HNO_3 formation, responsible for pH changes: the decrease of pH to values between 2 and 3 was observed in physiological saline within 30 min, whereas no acidification occurred in phosphate buffered saline (PBS). Simultaneously, H_2O_2 was generated up to concentrations of 3 mg l^{-1}–18 mg l^{-1}. The concentrations of all components depended on the volume of treated liquid and were lower in 10 ml than in 5 ml. Regarding direct inactivation of microorganisms by DBD plasma, *E. coli* in non-buffered physiological saline was completely inactivated after 5–15 min of exposure, but there was nearly no effect in the buffered suspension. Similar results were found with *S. aureus*. A slight *Bacillus atrophaeus* spores inactivation was found in 1.5 ml physiological saline only, whereas in higher volumes as well as in PBS no inactivation occurred. To test the role of acidification in antimicrobial plasma activity, microorganisms were incubated in hydrochloric acid and nitric acid: for *S. aureus* and *B. atrophaeus* spores, no antimicrobial effect could be found. *E. coli* was inactivated, but only in strong HCl and HNO_3 solutions at pH 2 (after 30 and 60 min of incubation, respectively). It was stated that neither an acidic environment alone nor the additional action of nitrate ions could induce inactivation of microorganisms comparable to plasma treatment. The effect of UV radiation was also excluded. The possible role of reactive oxygen and nitrogen species, namely superoxide O_2^-, hydroperoxyl HOO and peroxynitrite $ONOO^-$ is discussed.

In this contribution, we attempt to study the production of stable reactive species by plasma produced by positive and negative DC discharges in air, and the persisting microbicidal effect in solutions exposed to these discharges.

Material and methods

Plasma generation

The non-thermal plasma (NTP) was generated using the simple apparatus of an open-air type [20], no control of surrounding atmosphere composition or humidity was applied. Briefly, the negative glow corona or positive streamer discharge were generated on the point electrode represented by the tip of a syringe needle and connected to the source of direct current high voltage via the stabilizing 20 MΩ resistor. The plane electrode was realized by the surface of liquid, connected with the source by an immersed glass sealed platinum wire. A micrometer screw set the distance of the point electrode from the water surface (ca. 4 mm) and thus the current passing the corona. The used source HT 2103 (Utes, Brno) made it possible to set a variable voltage up to 10 kV and current up to 0.5 mA. The experimental arrangement is depicted in Fig. 1.

Exposure of samples

Deionized water prepared in Milli-Q apparatus (Millipore, Molsheim) and PBS (phosphate buffered saline, 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2PO_4) were

exposed to positive and negative discharge under the following conditions: positive streamer discharge at $U = 9$ kV, $I = 300$ μA, calculated power $P = 0.9$ W, and negative glow corona at $U = 9$ kV, $I = 320$ μA, $P = 0.83$ W. The volume of exposed liquids was in both cases 1, 3, 5 and 10 ml pH measurements were performed in 2 min intervals up to 30 min, then after 60 min of exposure. H_2O_2 and O_3 content were measured after 5 min and further in 10 min intervals up to 60 min of exposure. All samples exposed for 60 min were stored in a refrigerator at 4 °C for 4 weeks and then measured and tested again. Samples of 1 ml of deionized water and PBS exposed for 30 min and stored in a refrigerator up to 1 week were analyzed by SIFT-MS.

Microbial cultures

A Gram-positive bacterium *Staphylococcus epidermidis*, a Gram-negative *E. coli* and a yeast *Candida albicans*, isolated and identified at Institute of Immunology and Microbiology, were used as testing microbes. Bacteria were propagated by cultivation in the liquid Brain Heart Infusion medium (Oxoid Ltd, Basingstroke), the yeast was propagated in the Sabouraud medium (Merck KGaA, Darmstadt). The cultures were suspended in sterile water with the addition of 10% glycerol as a preservative and their concentration, expressed as the number of colony forming units (cfu) ml^{-1}, was determined by plating and counting of colonies. These stock suspensions were stored in a fridge. To test the exposed water or PBS, 10 μl of stock microbial suspension was added to 0.5 ml of the sample to achieve concentration of 10^5—10^7 cfu ml^{-1} and incubated for 10 min, 1 h and 24 h at 20 °C. After incubation, the suspensions were further diluted six times decimally, all dilutions were plated and the best dilution was evaluated by counting colonies. As a control, the spontaneous inactivation caused by lysis in unexposed water and PBS was also determined.

pH measurement

pH was measured using the pH-meter Level 1 (inoLab, Wellheim) apparatus with the glass microelectrode.

Hydrogen peroxide determination

H_2O_2 was determined semiquantitatively using Quantofix Peroxide 25 and Quantofix Peroxide 100 strips (Macherey—Nagel, GmbH). Both strip types were used to measure a particular sample, samples containing more that 25 mg H_2O_2 l^{-1} were diluted and measured again. This simple semiquantitative method seems to be sufficient, cheaper and less laborious than the sophisticated ones, e.g. photometry with titanyl sulphate.

Ozone determination

O_3 was determined using the Nanocolor Chlorine/ozone 2 kit (Macherey—Nagel, GmbH), based on the colorimetric measurement of color product of the ozone reaction with N,N-diethyl-1,4-phenylene diamine (DPD)/potassium iodide. Due to the small volume of exposed samples, the procedure recommended by the manufacturer was slightly adopted: the reagent DPD was dissolved in 4 ml of water and 1 ml of this solution was added immediately to 0.5 ml of the sample together with a drop of Reagent 2. The absorbance A was then measured on the S.750I (SECOMAM, Domont-Cedex) spectrophotometer at 540 nm, optical path length 10 mm. The color development appeared to be time-dependent, so that the reaction time of 2 min was held strictly. Because we did not know the absorbance coefficient of the color product, the ozone content is further expressed as the values of absorbance A_{540}, making possible the comparison of particular samples rather than absolute concentrations of O_3.

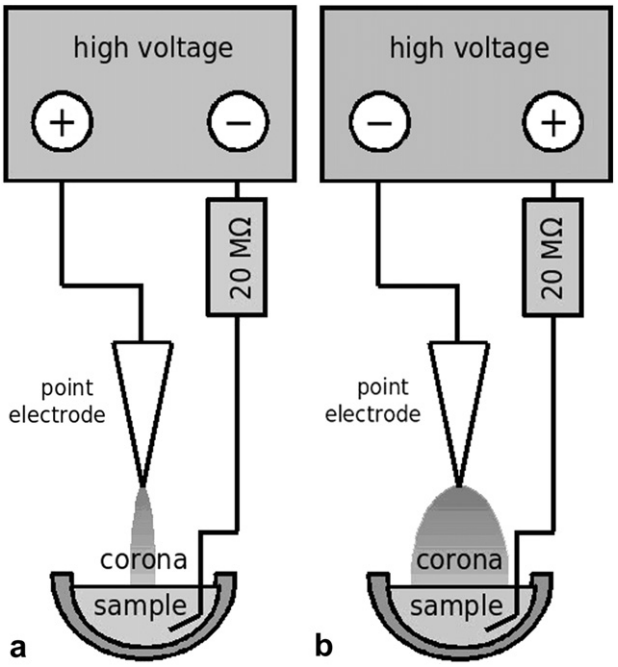

Figure 1 Experimental arrangement of exposure to positive streamer discharge (a) and negative glow corona (b).

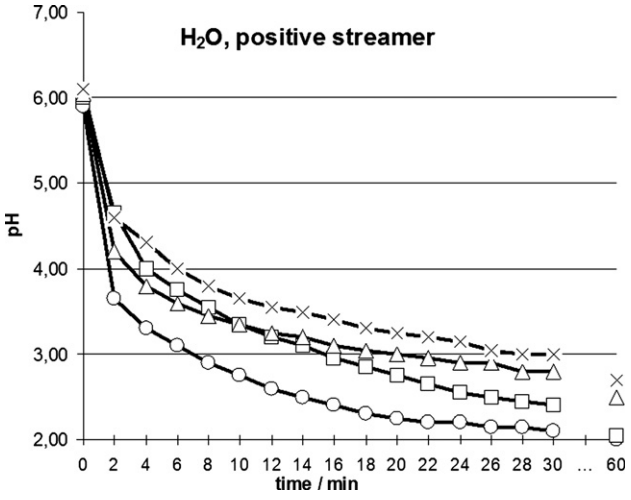

Figure 2 pH of deionized water exposed by the positive streamer. Exposed volume: ○ 1 ml; □ 3 ml; △ 5 ml; × 10 ml.

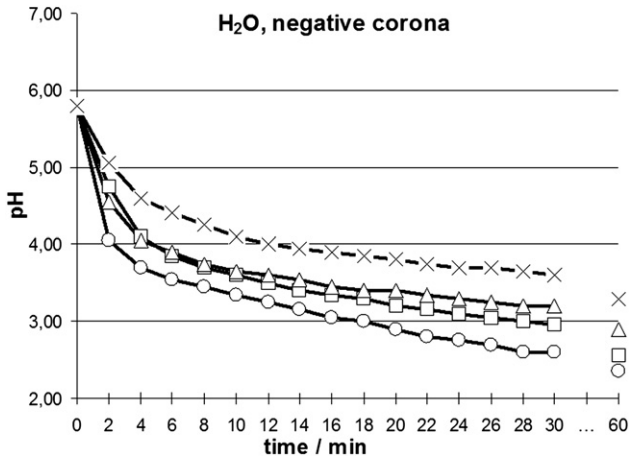

Figure 3 pH of deionized water exposed by the negative corona. Exposed volume: ○ 1 ml; □ 3 ml; △ 5 ml; × 10 ml.

Ozone production

The ozone was produced using the HG-1500 Ozone Generator (Ozone Solutions, Inc.). A stream of dry air at ca. 100 ml min^{-1} was fed into the apparatus set at maximal ozone output, bubbled into 100 ml of water and the resulting concentration of ozone was determined.

Artificial "water of death" preparation

An ozone solution in water was prepared as specified above. The highest concentration achieved after 120 min of bubbling corresponded to $A_{540} = 0.360$. 30% H_2O_2 was added to final concentration of 300 mg l^{-1} and adjusted with conc. HNO_3 to pH 2.5. This solution corresponds to small volumes of water exposed to positive streamer for 60 min. Another solution at the same pH, containing 50 mg H_2O_2 l^{-1} and no ozone was also prepared, corresponding to 1 ml water exposed to positive streamer for 60 min and stored for 4 weeks.

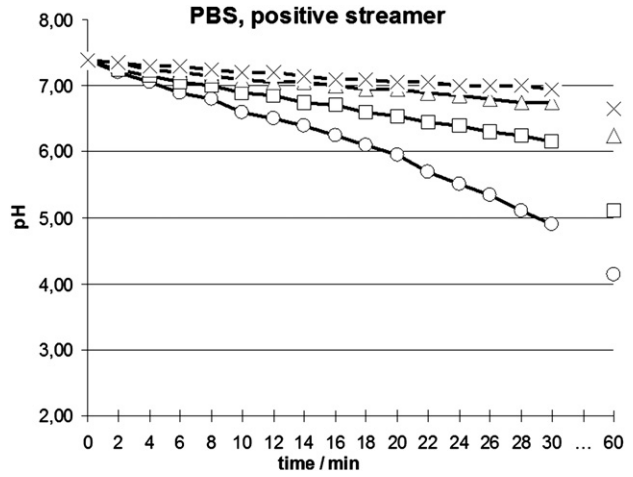

Figure 4 pH of PBS exposed by the positive streamer. Exposed volume: ○ 1 ml; □ 3 ml; △ 5 ml; × 10 ml.

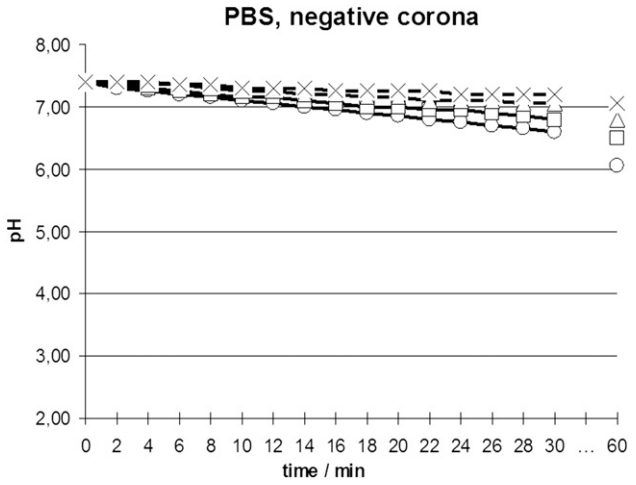

Figure 5 pH of PBS exposed by the negative corona. Exposed volume: ○ 1 ml; □ 3 ml; △ 5 ml; × 10 ml.

SIFT-MS

The selected ion flow tube mass spectrometry (SIFT-MS) is a sophisticated analytical method convenient for the detection of small molecules down to ppb levels. Its detailed descriptions is given in [21] or [22], its use for determination of volatile bacterial metabolites is described in [23]. In this work, 1 ml of water and PBS exposed in to positive discharge for 30 min were placed in 4 ml vials and stripped by the stream of air at ambient temperature. Volatiles were fed into the TransSIFT (Trans Spectra Ltd, Newcastle) apparatus, ionized with H_3O^+ or O_2^+ primary ions and the mass spectra of product ions compared with those from unexposed water and PBS, respectively.

Results

pH measurements

The results are summarized in Figs. 2—5. A sudden drop of pH was observed in all volumes of deionized water at short exposure times, followed by its continual decrease. The positive streamer was slightly more efficient: pH reached

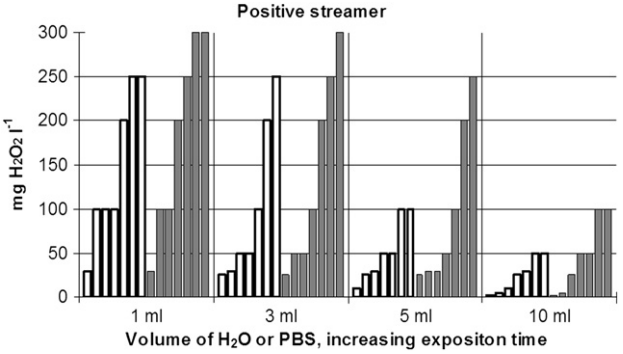

Figure 6 H_2O_2 content (in mg l^{-1}) in deionized water and PBS exposed by the positive streamer. Empty bars: deionized water, filled bars: PBS. Exposure 5, 10, 20, 30, 40, 50 and 60 min, exposed volumes are indicated below the *x* axe.

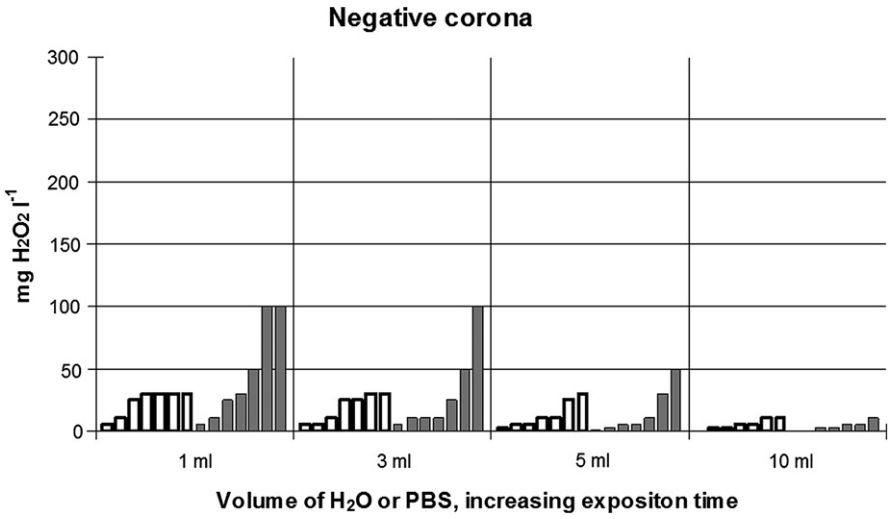

Figure 7 H_2O_2 content (in mg l^{-1}) in deionized water and PBS exposed by the negative corona. Empty bars: deionized water, filled bars: PBS. Exposure 5, 10, 20, 30, 40, 50 and 60 min, exposed volumes are indicated below the *x* axe.

the value of 2.00 ± 0.05 (i.e., $[H_3O^+] = 10^{-2}$ mol l^{-1}) in 1 ml after 60 min, as compared to pH 2.35 ± 0.05 (i.e., $[H_3O^+] = 4.5.10^{-3}$ mol l^{-1}) under negative corona. The course of curves was similar for various volumes, which differed no more than 1 pH unit.

No sudden drop in pH occurred in PBS. A significant decrease was apparent in small volumes, reaching after 60 min of exposure to positive streamer pH 4.15 ($[H_3O^+] = 7.1.10^{-5}$ mol l^{-1}) and 5.10 ($[H_3O^+] = 7.9.10^{-6}$ mol l^{-1}) in 1 and 3 ml, respectively. pH under negative corona remains almost unchanged with minimal pH 6.05 ($[H_3O^+] = 8.9.10^{-7}$ mol l^{-1}) in 1 ml.

As expected, no significant changes in pH were observed in all exposed samples after the 4 weeks storage.

Hydrogen peroxide determination

The results are summarized in Figs. 6 and 7. The H_2O_2 content increased in all samples with increasing exposure time, up to 300 mg l^{-1} under the positive streamer in 1 ml of PBS. In deionized water, the maximal values were somewhat lower. Under the negative corona, the highest H_2O_2 content was 100 mg l^{-1} only. The H_2O_2 content decreased in all cases with the increasing volume of exposed sample.

In samples exposed for 60 min and stored for 4 weeks in a refrigerator (Figs. 8 and 9), the H_2O_2 content decreased markedly, but still remained at 50 mg l^{-1} in 1 ml of exposed water. In PBS, the amount of remaining H_2O_2 was lesser than the corresponding amount in water. Only negligible amounts of H_2O_2 remained in water exposed by the negative corona and no H_2O_2 in PBS.

Ozone determination

The results are summarized in Figs. 10 and 11. In most samples, the absorbance A_{540} increased during the first 20—40 min of exposure and then decreased; this effect was

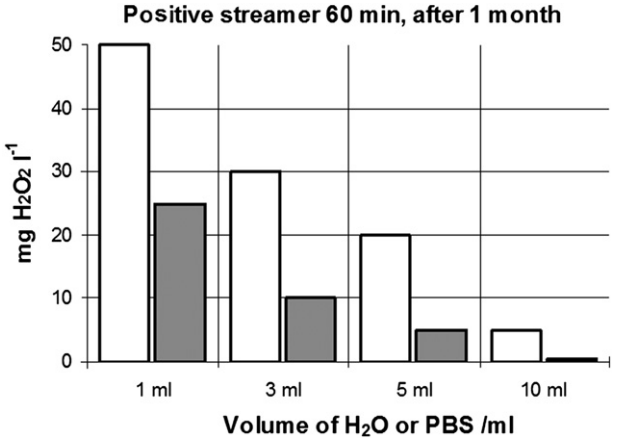

Figure 8 H_2O_2 content (in mg l^{-1}) in deionized water and PBS exposed by the positive streamer for 60 min and stored for 4 weeks. Empty bars: deionized water, filled bars: PBS. Exposed volumes indicated below the *x* axe.

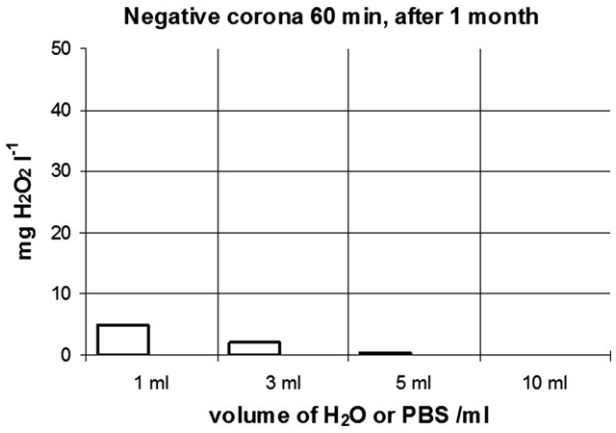

Figure 9 H_2O_2 content (in mg l^{-1}) in deionized water and PBS exposed by the negative corona for 60 min and stored for 4 weeks. Empty bars: deionized water, filled bars: PBS. Exposed volumes indicated below the *x* axe.

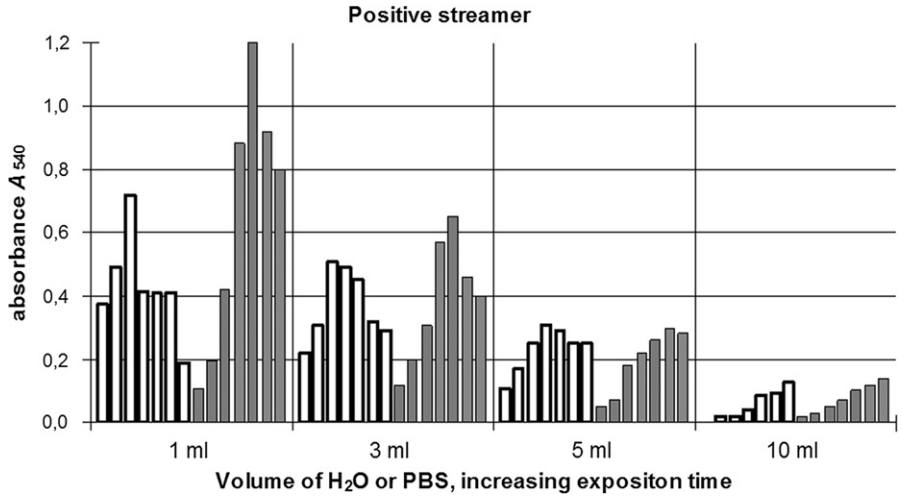

Figure 10 O_3 content (expressed as A_{540}) in deionized water and PBS exposed by the positive streamer. Empty bars: deionized water, filled bars: PBS. Exposure 5, 10, 20, 30, 40, 50 and 60 min, exposed volumes are indicated below the *x* axe.

less pronounced in PBS and almost disappeared in 10 ml samples. As the manufacturer declares the used test as reliable at pH greater than 3, this effect may be attributed to acidification of samples at longer exposure times and decomposition of colour product. This corresponds to the rapid bleaching of these samples.

In samples exposed for 60 min and stored for 4 weeks in a refrigerator, the absorbance $A_{540} > 0.05$ and thus the negligible O_3 content were found.

SIFT measurements

NO_x and corresponding acids were found in the headspace over the samples. After subtraction of background, NO (mass-to-charge ratio m/z 30) and NO_2 (m/z 46) in O_2^+ ionization mode, and HNO_2 (m/z 48), HNO_3 (m/z 64) and HCN (m/z 28) in H_3O^+ ionization mode appeared in the SIFT-MS spectra. The quantitative abundance of these particles in stripping air (in ppb) is summarized in Table 1.

Microbicidal effect of exposed and stored liquids

As a representative example, the microbicidal effect of water exposed to positive streamer for 60 min and stored for 4 weeks is shown in Figs. 12—14. The complete inactivation of *E. coli* and *S. epidermidis* was observed after 10 min (approx. 0.2 h) of incubation in 1 ml of exposed water, this time increased with increasing volume of water. The complete inactivation of *C. albicans* needed 24 h of incubation. The lane "control" shows the spontaneous inactivation of organisms caused mainly by lysis in non exposed water.

Similar graphs were plotted also for other experimental conditions (stored and fresh, water and PBS, exposed by positive and negative dischage). The complete results are very voluminous (24 graphs in total) and are thus summarized in tables. PBS exposed to positive streamer exhibited the complete inactivation in the 1 ml only, no inactivation was observed in larger volumes (Table 2). The exposure to

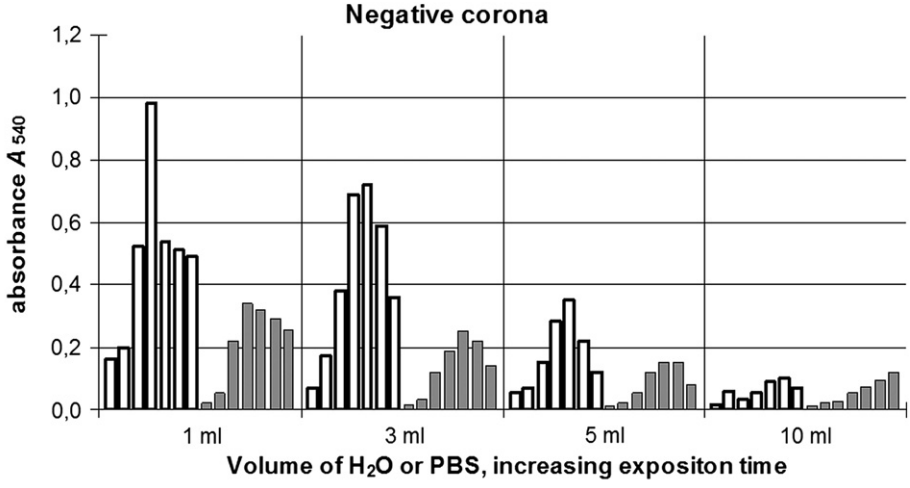

Figure 11 O_3 content (expressed as A_{540}) in deionized water and PBS exposed by the negative corona. Empty bars: deionized water, filled bars: PBS. Exposure 5, 10, 20, 30, 40, 50 and 60 min, exposed volumes are indicated below the *x* axe.

Table 1 Abundance of nitrogen compounds in the headspace over exposed water and PBS (in ppb).

	Water [ppb]	PBS [ppb]
NO	7609	15630
NO_2	2192	5485
HNO_2	10	10
HNO_3	11	22
HCN	8	26

the negative corona caused the persistent effect in smaller volumes of water, whereas a small effect of exposed PBS was observed for *E. coli* only (Table 3).

Microbicidal effect of fresh exposed liquids

The microbicidal effect of exposed liquids measured immediately after exposure was almost the same as summarized in the previous paragraph; thus, the results are not repeated here in details. The differences were apparent in a few cases only; they consist mainly of the shift to lower values of incubation time, conspicuous for *E. coli* exposed to positive corona in PBS. Pertinent values are given in Table 2 and Table 3 in parentheses. Slight but probably not significant differences appeared occasionally also in the detailed course of cfu/time dependencies and are not presented here.

Microbicidal effects of artificial "water of death"

The comparison of microbicidal effect of water exposed to the positive streamer with the water prepared by artificial mixing of components detected in 1 ml of exposed water is summarized in Figs. 15—17. The incubation of *E. coli* and *S. epidermidis* in water exposed to the discharge caused complete inactivation of both bacteria within 10 min, regardless of the storage of exposed water (cf. Figs. 12 and 13 and Table 2). On the other hand, the artificially prepared solution mimicking the exposed water inactivated these

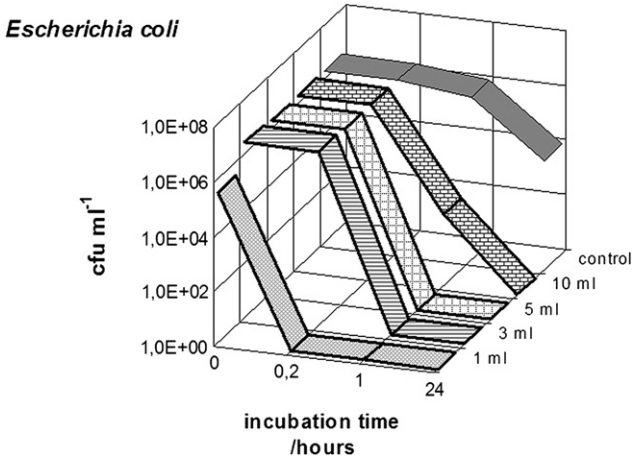

Figure 12 Inactivation of *Escherichia coli* in exposed and stored water as compared with the control (unexposed water). Approximate value of 0.2 h on *x* axe stands for 10 min.

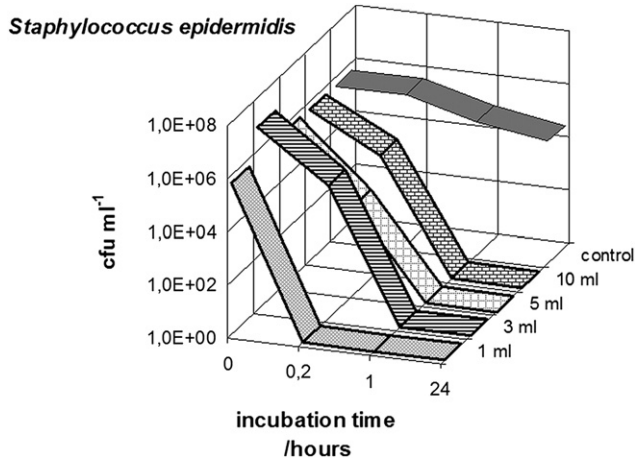

Figure 13 Inactivation of *Staphylococcus epidermidis* in exposed and stored water as compared with the control (unexposed water). Approximate value of 0.2 h on *x* axe stands for 10 min.

bacteria within 24 h. The courses of inactivation in artificial solutions mimicking the fresh and stored exposed water were similar, except somewhat more rapid inactivation of *E. coli*, in fresh and somewhat slower inactivation of *S. epidermidis* in old solutions. Different results were obtained for *C. albicans*: its inactivation in exposed water occurred within 1 h and 24 h in fresh and old exposed water, respectively (cf. Table 2 and Fig. 14). It was also inactivated in the "fresh" artificial mixture, but the stored "old" mixture was not able to inactivate it completely even after 24 h.

Discussion

The course of acidification was different in water and PBS: whereas a drop of ca. 2 pH units occurred during the first 2 min of exposure in water, pH of PBS remains almost unchanged and its decrease was apparent in small exposed

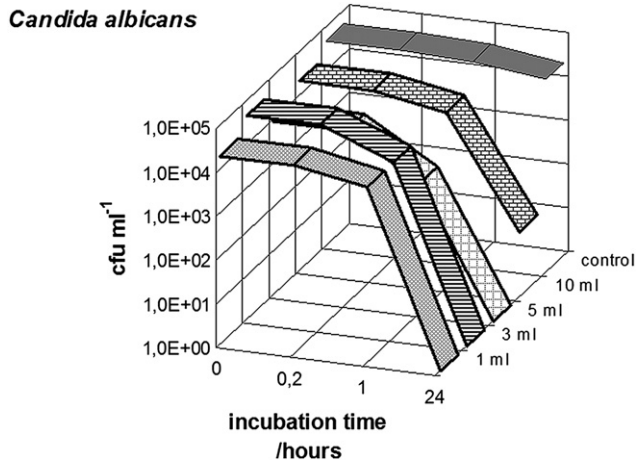

Figure 14 Inactivation of *Candida albicans* in exposed and stored water as compared with the control (unexposed water). Approximate value of 0.2 h on *x* axe stands for 10 min.

Table 2 Microbicidal effect of H_2O and PBS exposed by positive streamer and stored for 4 weeks. The times of incubation causing the complete inhibition (if occurred) are given; "decrease" indicates the lowering in two orders of magnitude. The different results obtained for freshly exposed H_2O and PBS are given in parentheses.

	E. coli	*S. epidermidis*	*C. albicans*
1 ml H_2O	10 min	10 min	24 h (1 h)
3 ml H_2O	1 h	1 h	24 h
5 ml H_2O	1 h	1 h	24 h
10 ml H_2O	24 h	1 h	decrease
1 ml PBS	10 min	24 h (10 min)	24 h
3 ml PBS	decrease (24 h)	decrease	no inhibition (24 h)
5 ml PBS	no inhibition (24 h)	no inhibition (decrease)	no inhibition
10 ml PBS	no inhibition (24 h)	no inhibition	no inhibition

volumes after a long exposure, after exhausting the phosphate buffer capacity. In both cases, the positive discharge was more powerful than the negative one despite the negligible difference in their power. During storage, no changes in pH appeared. In comparison with previous works [16,18], where a microjet was used for plasma generation, lower minimal values of pH were achieved by both positive and negative corona, comparable with those of [19] using the DBD discharge. In contrast to this work, significant pH decrease was observed also in PBS under positive discharge.

The nature of acidification was confirmed by SIFT-MS, detecting NO_x and corresponding acids. Their formation was approx. two times higher in PBS than that in pure water; we have no plausible explanation of this effect. On the other hand, the higher concentration of nitrite ions in PBS samples may be the reason of more rapid decomposition of H_2O_2 by its reduction (see Figs. 8 and 9). No additional compounds were detected by this method, except traces of HCN; due to its low amount, its actual presence is dubious and its significance remains unclear. In general, however, the SIFT-MS is applicable to the volatile compounds released to the stripping gas only and the detection limit of SIFT-MS is the mass-to-charge ratios $m/z < 200$.

The H_2O_2 content increased regularly with the exposure time in all volumes. The H_2O_2 production was higher in PBS than in pure water. Again, the positive discharge was at least three times more powerful than the negative one. The H_2O_2 decomposed during storage more rapidly in exposed PBS than in pure water; its reduction by higher amounts of HNO_2 is the probable reason of this effect. After four weeks in water, H_2O_2 decomposed to less than the one third of the original concentration, in samples exposed to negative corona almost no H_2O_2 remained. In comparison with [19], much higher H_2O_2 amounts were produced under positive and in lesser extent under negative discharge.

The O_3 determination may be regarded as semi-quantitative only. In small volumes at longer exposure times, the acidification of samples occurred simultaneously and the used test becomes unreliable, which makes the presented values dubious. Nevertheless, it is apparent that: 1) The content of O_3 is higher in small volumes as compared to greater ones. 2) The content of O_3 is higher under the positive streamer as compared to the negative corona. 3) The content of O_3 seems to be higher in PBS as compared to the deionized water under the positive streamer, whereas an adverse trend appeared under the negative corona. The well-known antimicrobial properties of ozonated water were reviewed [24], but it works in freshly prepared ozonated water only. The complete decomposition of O_3 during four weeks is apparent, so that this compound cannot contribute to microbial inactivation in stored samples of exposed liquids.

Previous papers [17,18] also mentioned the bactericidal effect of acids during exposure to plasma, the critical borderline values of pH 4.5 and 4.7, respectively, were stated. These values roughly correspond with our results: in liquids previously exposed to plasma, significant decrease of cfu occurred under pH of 5, i.e., in exposed water, in lesser extent also in exposed PBS. Nevertheless, slight effect occurred also in small volumes of PBS at pH 6 (cf. Tables 2 and 3 and Figs. 2—5). The cidal effect was different for various microbes: surprisingly, *S. epidermidis* protected by a thick bacterial cell wall, appeared to be slightly more sensitive than *E. coli* with a thin cell wall. *C. albicans* with no cell wall was more resistant than bacteria.

Table 3 Microbicidal effect of H_2O and PBS exposed by negative corona and stored for 4 weeks. The times of incubation causing the complete inhibition (if occurred) are given; "decrease" indicates the lowering in two orders of magnitude. The different results obtained for freshly exposed H_2O and PBS are given in parentheses.

	E. coli	*S. epidermidis*	*C. albicans*
1 ml H_2O	10 min	1 h	24 h
3 ml H_2O	24 h	1 h (24 h)	decrease
5 ml H_2O	decrease	24 h	decrease
10 ml H_2O	decrease	24 h	no inhibition
1 ml PBS	decrease (24 h)	no inhibition (decrease)	no inhibition
3 ml PBS	decrease	no inhibition (decrease)	no inhibition
5 ml PBS	no inhibition	no inhibition	no inhibition
10 ml PBS	no inhibition	no inhibition	no inhibition

This implies a conclusion that the structure of microbial envelope is not crucial for the sensibility of microbes to acidity.

Concerning the published effects of artificially prepared acidic solutions, the acidity alone (HNO_3 at pH 4.2) was not able to inhibit *S. aureus* effectively [17], and only *E. coli* but not *S. aureus* was inactivated in strong HCl and HNO_3 solutions at pH 2 [19]. In this work, we observed complete inactivation of both bacterial species after incubation for 24 h in solutions containing HNO_3 at pH 2.5, but in presence of small amounts of H_2O_2 (50 mg l^{-1}) in stored, or H_2O_2 and O_3 in fresh solutions. Surprisingly, the fresh and stored solutions exhibited almost equal inactivation potency, although strong disinfectants O_3 completely and H_2O_2 almost disappeared during storage. Small differences may be the slower inactivation of *E. coli*, more rapid inactivation of *S. epidermidis* and incomplete inactivation of *C. albicans* in "stored" solutions.

The most surprising phenomenon is the persistent microbicidal effect in water exposed to low-temperature plasma, i.e., their ability to kill microbes even one month after exposure to the positive streamer discharge; this effect was preliminary reported in [8]. Ikawa et al. [18] added bacteria to the solution 10 min after it was exposed to plasma, but despite the sufficient concentrations of NO_x and H_2O_2, no inactivation was observed. In our experiments with eucaryotic cell cultures [7], we observed the killing of cells after addition of liquid (protein suspension) previously exposed with positive discharge. Only small volume of this water (25 μl into 0.5 ml, i.e. dilution 1:20) was added to the cell culture in buffered cultivation medium, so that its pH remained unchanged; thus, the cidal effect cannot be caused by acidification only. The elution of an unknown killing compound from vessel materials was excluded by the exposure conducted in vessels from polystyrene, polymethylmethacrylate, borosilicate glass and aluminium with the same effect in all cases. Liquids exposed with other discharge types did not exhibit this effect. The detailed study of eucaryotic cell killing is in progress and will be published in proper time.

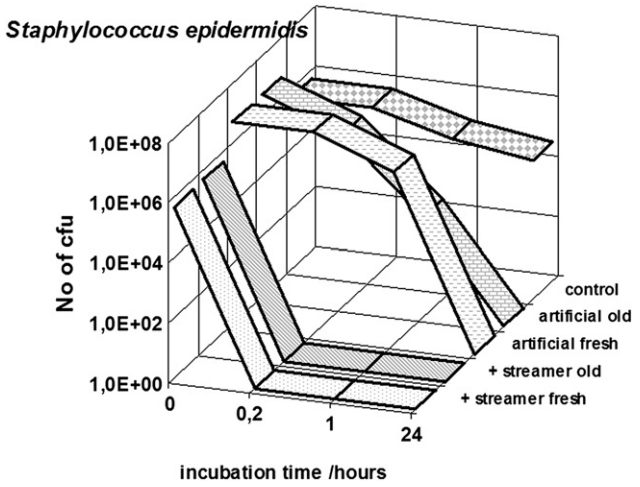

Figure 16 Inactivation of *Staphylococcus epidermidis* in 1 ml fresh and stored (old) water exposed to the positive discharge and in adequate artificially prepared solutions. Approximate value of 0.2 h on *x* axe stands for 10 min.

We attempted to simulate the persistent microbicidal effect by mixing the known components of exposed water in artificial solutions. In all cases, artificially prepared solutions were less powerful than adequate solutions prepared by plasma action. Different results were obtained for bacteria and for yeast: bacteria were inactivated slowly but completely in fresh and stored solutions, whereas for *C. albicans* a significant lowering of inactivation potency was observed in stored solutions. This implies that the inactivation of yeast may be mediated by the presence of ozone and hydrogen peroxide, not present in sufficient concentrations in stored solutions; these compounds thus are of lesser significance for bacterial inactivation. Nevertheless, the crucial difference in the action of exposed and artificial

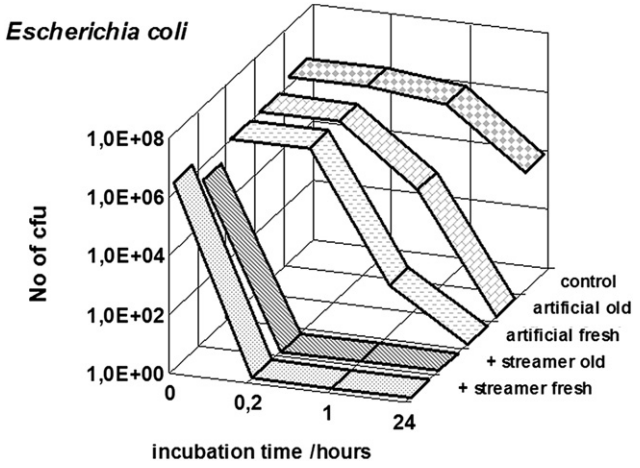

Figure 15 Inactivation of *Escherichia coli* in 1 ml fresh and stored (old) water exposed to the positive discharge and in adequate artificially prepared solutions. Approximate value of 0.2 h on *x* axe stands for 10 min.

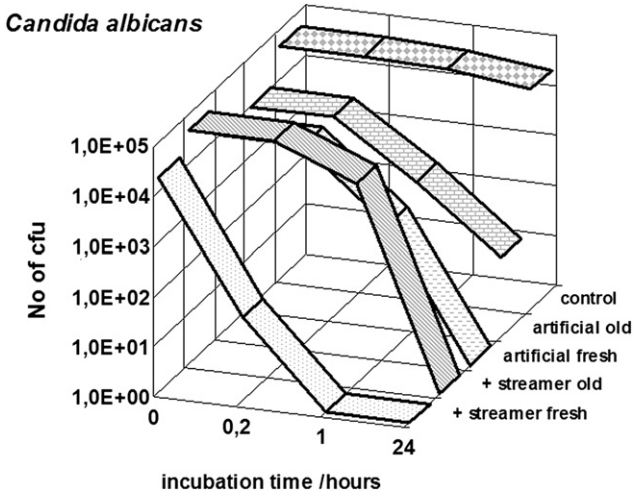

Figure 17 Inactivation of *Candida albicans* in 1 ml fresh and stored (old) water exposed to the positive discharge and in adequate artificially prepared solutions. Approximate value of 0.2 h on *x* axe stands for 10 min.

solutions, as well as the nature of a hypothetic stable microbicidal compound causing the persistence of microbicidal effect long time after the exposition remains unclear. A possible explanation may be the lack of nitrous acid HNO_2 in artificial solutions, which may contribute to inactivation in exposed liquids by oxidative degradation. Considering these facts, the nature of persistent bactericidal effect is only partially explained here.

Conclusions

The persistent microbicidal effect was observed in water and PBS exposed to low-temperatrure plasma. From its more thorough examination presented here, it may be concluded that: 1) The positive discharge is more powerful in its production than the negative one. 2) This effect is more pronounced in pure water than in the buffered one, probably due to the lesser acidity. 3) Its efficiency differs for various organisms. 4) It may be supported by the presence of H_2O_2 but not by O_3, which completely disappears during storage for 4 weeks. 5) The additional action of H_2O_2 and O_3 is probably important in freshly prepared solutions, namely in PBS, where more rapid inactivation occurs. 6) It cannot be imitated in full by artificially mixed solutions. 7) We did not found any additional compound which may contribute to this effect. Thus, the microbicidal effect is undoubtedly mediated by acid milieu in exposed liquids, may be supported by the presence of H_2O_2 and HNO_2, but speculations about an unknown killing factor remain open.

Conflict of interest

The authors have no conflict of interest.

Acknowledgements

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