

Antimicrobial Efficacy of Two Surface Barrier Discharges with Air Plasma against *In Vitro* Biofilms

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Abstract

The treatment of infected wounds is one possible therapeutic aspect of plasma medicine. Chronic wounds are often associated with microbial biofilms which limit the efficacy of antiseptics. The present study investigates two different surface barrier discharges with air plasma to compare their efficacy against microbial biofilms with chlorhexidine digluconate solution (CHX) as representative of an important antibiofilm antiseptic. *Pseudomonas aeruginosa* SG81 and *Staphylococcus epidermidis* RP62A were cultivated on polycarbonate discs. The biofilms were treated for 30, 60, 150, 300 or 600 s with plasma or for 600 s with 0.1% CHX, respectively. After treatment, biofilms were dispensed by ultrasound and the antimicrobial effects were determined as difference in the number of the colony forming units by microbial culture. A high antimicrobial efficacy on biofilms of both plasma sources in comparison to CHX treatment was shown. The efficacy differs between the used strains and plasma sources. For illustration, the biofilms were examined under a scanning electron microscope before and after treatment. Additionally, cytotoxicity was determined by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay with L929 mouse fibroblast cell line. The cell toxicity of the used plasma limits its applicability on human tissue to maximally 150 s. The emitted UV irradiance was measured to estimate whether UV could limit the application on human tissue at the given parameters. It was found that the UV emission is negligibly low. In conclusion, the results support the assumption that air plasma could be an option for therapy of chronic wounds.

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Introduction

Physical plasmas under atmospheric conditions, operated near room temperature, can be used to inactivate microorganisms successfully and are discussed as possible treatment method in health care [1,2]. Their development has generated a new field of research, the so-called plasma medicine [3]. Chronic wounds, device related infections as well as inflammations of implants are often associated with microbial colonisations [4,5]. The formation of biofilms protects the microorganism against antiseptic treatment and host defences. Additionally, the biofilms prolong the inflammation processes in chronic wounds. The efficacy of antiseptics is limited by tissue toxicity [6,7,8]. Additionally, chronically infected wounds increase therapy costs, they are painful and impair the patients quality of life [9]. Moreover, sometimes the wound does not heal despite correct treatment [10]. Therefore alternative treatment methods are required. The treatment of chronically infected wounds by tissue tolerable plasma (TTP) is an interesting field of investigation [11]. Investigations carried out in this respect with a TTP plasma jet [12] – the so-called kinpen 09 [13] – resulted in antibiofilm effects

[14,15], inactivation of drug resistant bacteria [16] as well as tissue activation [17] and improvement of tissue regeneration, which has meanwhile been confirmed on real wounds of humans and dogs [18,19].

A review of different plasma sources for medical applications including skin and wound treatment, and the relevant physical and biological mechanisms has already been given by Park et al. [20].

Often, the efficacy of many different plasma sources for medical use was investigated on bacteria spread on nutrient agar plates. Those practices falsify *in vivo* conditions, because bacteria mostly live in biofilms, also in chronic wounds [21]. Investigations of antimicrobial effects on *in vitro* biofilms with different plasma sources are of high interest for potential wound treatment.

The plasma chemistry and the interaction with living systems are very complex and currently under investigation by many research groups [22,23]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are discussed as main effectors for antimicrobial mechanisms of plasma [24], UV radiation and pH variations seem to be supportive [25]. To enhance the understanding of interactions between plasma and microorganisms and the development of suitable plasma devices, the

Table 1. Operational parameters of SBD-A and SBD-B.

	SBD-A	SBD-B
properties		
U_{SS} (kV)	13	8
applied frequency (kHz)	20	30
plasma mode	burst (2 pulse/ms) = 800 pulse in 400 ms	burst (30 pulse/ms) = 7500 pulse in 250 ms
working mode	400 ms on, 1200 ms off	250 ms on, 750 ms off
dissipated electrical energy (mJ/puls)	0.6	0.44
mean power/area (mW/cm²)	0.03	0.18
mean power/area (mJ/cm² per minute)	1.8	10.88
treatment area (cm²)	10	18
electrode form	circular	rectangular
gas flow in slm	0	0.5

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antimicrobial efficacy of different plasma sources on biofilms is to be investigated and compared. Surface dielectric barrier discharge (SBD) plasmas could be suitable for wound treatment because the generated plasma spreads over a large area, does not need the substrate as second electrode [3] and the physical parameters can be modified to generate a plasma with tissue tolerable properties.

In this study, the antimicrobial efficacy of two different SBD plasma sources was investigated for different exposure times on *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* as biofilm forming organisms. A high antimicrobial efficacy on biofilms was expected due to the high amount of released ROS [26,27]. *Pseudomonas aeruginosa* is ubiquitous in wet habitats and frequently identified in chronic wounds. *Staphylococcus epidermidis* is a common skin flora organism and often isolated from implants or catheter associated infections and chronic wounds [28,29].

The results of both plasma sources were compared to the antimicrobial efficiency of chlorhexidine digluconate (CHX). CHX as an important antiseptic is regarded as gold standard for dental biofilm treatment [30] and also used in wound dressings [31]. Additionally, UV emission was measured and cytotoxicity on a fibroblasts cell line was examined to evaluate the potential applicability of the generated plasmas of both SBD plasma sources on living tissue.

Materials and Methods

Plasma Sources

Two different types of surface barrier discharges were used (neoplas GmbH, Greifswald, Germany): a structured electrode planar SBD (SBD-A) and a wire electrode SBD (SBD-B). They were both developed and described by Leibniz Institute for Plasma Science and Technology (INP, Greifswald, Germany) [3]. The specifications and physical parameters of both plasma sources are shown in table 1. The dissipated electrical power in both electrode arrangements was measured via the Lissajous method [32]. A Lissajous figure is the x - y plot of the charge dissipated into the plasma and the applied voltage. If the plasma is switched on, this plot will yield a parallelogram, whose area resembles the electrical energy E_{el} dissipated into the plasma per duty cycle of the applied voltage. The output power is given by the product of frequency f and dissipated electrical energy. SBD-A is made of a 1.5 mm thick printed circuit board (epoxy glass fiber bulk material), which acts

as dielectric barrier, having etched copper electrode structures on its surface (Figure 1). SBD-B has a grounded metallic layer electrode and a high voltage electrode consisting of seven wires of 0.6 mm thickness. Here, silicone insulations around each wire act as dielectric barrier (Figure 2). Both SBD sources are operated with ambient air.

Additionally, SBD-B had applied a flow of compressed air of 0.5 standard litres per minute (slm) through perforations in the grounded electrodes along the border of the insulated powered wire electrodes. The gas flow was controlled by a mass flow controller (MKS Instruments, Germany). The gas temperature of both discharges is close to room temperature at the surface of the substrate [3].

Measurement of UV Radiation and Ozone

The UV irradiance was measured within a range of 200–400 nm in $\mu\text{W}/\text{cm}^2$ by optical emission spectroscopy using a fiber optics coupled spectrometer (Avantes AvaSpec-3648, Apeldoorn, Netherlands) at the same distance as applied for antimicrobial plasma treatment (2 mm). The UV exposition in mJ/cm^2 was determined in accordance with ICNIRP Guidelines [33]. The representation of the graph of UV irradiation and the area below the curve was calculated by using Origin 7.0 (OriginLab Corporation, Northampton, USA). The VUV radiation emission was investigated by means of an apparatus used to determine the VUV radiance [34].

The ozone concentration of both plasma sources for the used treatment conditions was determined by the ozone analyzer (APOA-360, HORIBA, Germany). The detection limit of the ozone analyzer is 16 ppm. Here, the analyzed gas was diluted with a controlled argon gas flow (200 sccm or 400 sccm) to calculate the maximal generated ozone density, which exceeded 16 ppm.

Cultivation and Evaluation of Biofilms

The Gram-negative strain *Pseudomonas aeruginosa* SG81 (*P. aeruginosa*) and the Gram-positive *Staphylococcus epidermidis* RP62A (*S. epidermidis*) were used. Bacterial cultivation and preparation were performed as described elsewhere [14,35]. For biofilm cultivation 10% fetal bovine serum (Gibco, Life Technologies) was added to the MEM cell culture medium (Minimum Essential Medium Eagle with L-glutamine, PAA Laboratories, Germany), corresponding to the composition of an artificial

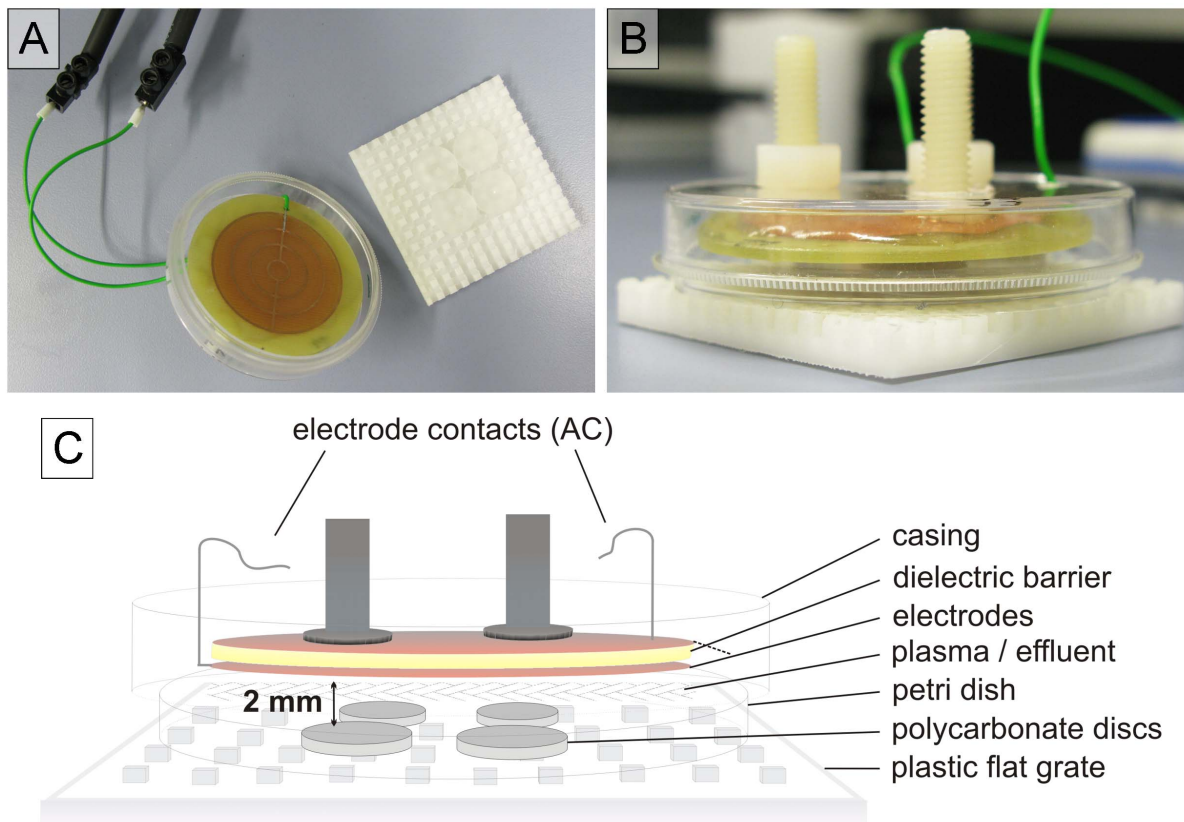


Figure 1. Experimental setup of the SBD-A plasma source. A: Electrode and discs with biofilms on plastic flat grate. B: Configuration of the electrode in action mode. C: Schematic representation of the experimental setup of SBD-A. doi:10.1371/journal.pone.0070462.g001

wound fluid [6] [36], was used and inoculated with the test bacteria at a final concentration of 10^7 colony forming units (CFU)/ml. The biofilms were grown on sterile polycarbonate discs (\varnothing 13 mm, height 3 mm, Arthur Krueger KG, Barsbüttel, Germany), positioned into wells of microplates (Sarstedt AG & Co., Nümbrecht, Germany) and were covered with 800 μ l of batch medium. The medium was replaced after 4 h and 24 h. After 24 h discs were turned around to assure similar conditions for both sides of the discs. The biofilms were grown for 48 h at 37°C aerobically and washed once with phosphate buffered saline solution (PBS) to remove unattached bacteria before antiseptic treatments were performed. After treatment, each disc was transferred into sterile wells of microplates and filled with 1 ml PBS. The biofilms were dispensed for 20 min by ultrasound (130 W, Branson 2510 Ultrasonic Cleaner, EMERSON Technologies GmbH & Co. OHG, Dietzenbach, Germany). The antimicrobial effect was determined as difference in the number of the colony forming units (CFU) by microbial culture, as described earlier [14].

Plasma Treatment

Plasma sources and experimental setup are shown in figures 1 and 2. The prepared biofilm-covered discs were transferred onto sterile plastic flat grates. The plasma sources were positioned 2 mm above the discs. The plasma exposure times were 30, 60, 120, 300 or 600 s, each, on the upper and bottom side of the discs (for this, the discs were turned).

Treatment with Chlorhexidine Digluconate (CHX)

The prepared biofilm-covered discs were positioned in sterile microplate wells filled with 0.8 ml of 0.1% CHX solution (20% in H₂O, Fagron GmbH & Co KG, Barsbüttel, Germany) and incubated for 600 s at room temperature. Antiseptic activity was stopped by replacing CHX with 1 ml of inactivation solution (40 g/l Tween 80, 30 g/l saponine, 4 g/l lecithin, 10 g/l sodium dodecyl sulphate, 1 g/l sodium thioglycolate [Serva, Heidelberg, Germany]) and left for 600 s. The efficacy of the inactivation solution was verified according to DIN 1040 [37].

For the cytotoxicity test the 0.1% CHX solution was prepared in culture medium (identical with biofilm growth medium) medium.

Statistical Analysis

Counted CFU were transformed to \log_{10} (CFU/cm²). The colony reduction factor (CRF) is defined by the formula:

$$CRF = \log_{10} - (\text{mean CFU of untreated samples}) \\ - \log_{10} \hat{Z} (\text{CFU of treated samples})$$

Standard deviations (SD), confidence intervals (CI) and p values were calculated based on \log_{10} (CFU/cm²) values. Statistical differences between the different treatment times, applied plasma sources and test organisms were analyzed with the Kruskal-Wallis test, followed by a Mann-Whitney *U*-test using statistical analyses system SAS® Enterprise Guide® 4.1 (SAS Institute GmbH,

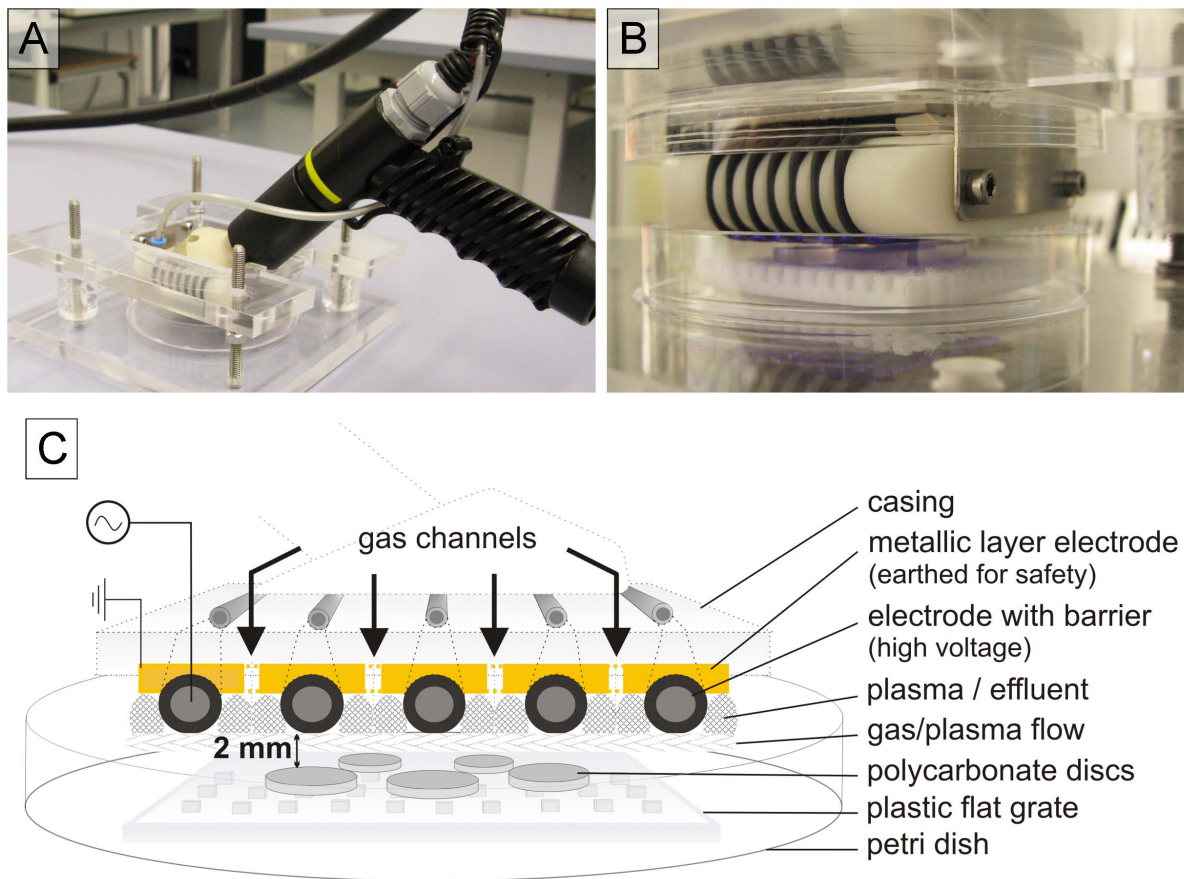


Figure 2. Experimental setup of the SBD-B plasma source. A: Overview of the experimental setup. B: Near focus of the electrode in action mode above the discs with biofilms. C: Schematic representation of the experimental setup of SBD-B in cross section.
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Heidelberg, Germany), where applicable. For the analyses of the cytotoxicity additionally the Bonferroni correction was used.

Scanning Electron Microscopic Images

For the scanning electron microscopy, the biofilms of *S. epidermidis* and *P. aeruginosa* were prepared in different ways. Discs with *S. epidermidis* biofilms were fixed in 2.5% glutaraldehyde (Sigma-Aldrich, Munich, Germany), solved in 5 mM HEPES (pH 7.4), for 1 h at room temperature and subsequently at 4°C before treatment with amino acid-sucrose-solution (2% arginine, 2% glycine, 2% glutamate, 2% sucrose) followed by guanidine-tannin-solution (2% guanidine, 2% tannin; Sigma-Aldrich, Munich, Germany) for the first 5 min in a microwave and then extended to 1.5 h at room temperature for each solution, 1% osmium tetroxide for 2 h, and 2% uranyl acetate (Plano GmbH, Wetzlar, Germany) for 1.5 h with washing steps in between. After that, samples were dehydrated in a graded series of aqueous ethanol solutions (10–100%) and then critical point-dried via amylacetate and CO₂.

Discs with *P. aeruginosa* biofilms were prepared as follows. After a fixation step (1 h in 1% glutaraldehyde, 4% paraformaldehyde (Science Services GmbH, Munich, Germany), 100 mM cacodylate buffer [pH 7.4], 1 mM CaCl₂, 1 mM MgCl₂, and 50 mM NaN₃ at room temperature, then 4°C over night), the samples were treated with 2% tannic acid for 1 h, 1% osmium tetroxide for 2 h and after a washing step with 1% osmium tetroxide overnight, and with 2% uranyl acetate for 2 h with washing steps in between. The

samples were dehydrated in a graded series of aqueous ethanol solution (10–100%) and then critical point-dried via amylacetate and CO₂.

Finally, samples of both microorganisms were mounted on aluminium stubs, sputtered with gold/palladium and examined in a scanning electron microscope EVO LS10 SEM (Zeiss, Oberkochen, Germany).

Cytotoxicity Determination of Plasma

Mouse fibroblasts of the cell line L929 (NCTC clone 929, ATCC CCL-1, USA) were cultivated at 37°C and 5% CO₂ on round cover glasses (Ø 15 mm, Menzel GmbH, Braunschweig, Germany) in wells of microplates by using 1.5 ml culture medium (identical with biofilm growth medium) per well. The colorimetric MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Munich, Germany) assay in accordance with DIN EN ISO 10993-5 [38] was used to determine the cytotoxic effects of CHX and the generated plasma (parameters as used for biofilm treatment, see above) respectively. The MTT assay was based on the selective ability of living cells to reduce the salt MTT to formazan by dehydrogenases [39].

For plasma treatment, discs with cells were positioned on plastic flat grates in a petri dish at a distance of 2 mm to the electrodes of the plasma sources. The edges of the discs were surrounded with medium to have wet conditions to avoid drying. For CHX treatment, discs were transferred into a separate 24-well-microplate and the cells were covered with 300 µl of 0.1% CHX

Table 2. Antiseptic treatment of *Pseudomonas aeruginosa* SG81 biofilms.

treatment	SBD-A				SBD-B				p
	n	CRF ± SD	95% CI limits		n	CRF ± SD	95% CI limits		
			lower	upper			lower	upper	
plasma - 30 s	20	1.44±0.41 ^{c*}	1.25	1.63	26	1.16±0.60 ^{c*}	0.92	1.40	0.0037
plasma - 60 s	24	1.78±0.70	1.48	2.07	20	1.32±0.41 ^c	1.13	1.51	0.0267
plasma - 150 s	22	2.60±0.77 ^c	2.26	2.94	20	2.06±0.66 [*]	1.76	2.37	0.0136
plasma - 300 s	23	4.83±2.19 ^{c*}	3.88	5.77	20	2.98±0.96 ^{c*}	2.53	3.43	0.0050
plasma - 600 s	16	7.11±1.17 ^{c*}	6.48	7.73	12	3.81±1.51 ^c	2.85	4.77	0.0001
p		0.0001				0.0001			0.0001
CHX 0.1%	35	1.72±0.46	1.56	1.88					
control	38	0.00±0.15	-0.05	0.05					
p		0.0001				0.0001			0.0001

The analytical results by the Number of samples (n), Colony reduction factor (CRF) in \log_{10} (CFU/cm²) ± Standard Deviation (SD), lower and upper 95% confidence limits (CI) after exposure to air plasma for 30–600 s treatment time respectively and 0.1% CHX after 600 s exposure time and untreated control of *Pseudomonas aeruginosa* SG81 biofilms [p-values of omnibus tests (Kruskal-Wallis) and two-sample tests (Whitney U); statistical significance: $\alpha=0.05$].

^csignificantly different from CHX.

^{*}significantly different from the respective treatment time of *Staphylococcus epidermidis* RP62A.

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solution for 30, 60, 150, 300 and 600 s, respectively. Then, the discs were washed two times with fresh cell culture media.

The cytotoxicity test was carried out after 48 h of cell cultivation (4 samples with plasma and 3 samples with CHX, respectively). After plasma treatment, 5 µg/ml gentamicin and 0.25 µg/ml amphotericin B were added to the medium to inactivate possible bacterial contamination during treatment. Three samples were used as untreated control, on open air beside the plasma treatment and 6 samples incubated with fresh media only for the CHX treatment.

For the MTT assay the discs were transferred into a new microplate. The wells were filled with 0.5 ml MTT media (500 µg MTT/ml culture media) and incubated at 37°C with 5% CO₂ for 3 h. Next, MTT media was replaced by 0.04 M HCl in 2-

propanol (97%, Merck KGaA, Darmstadt, Germany) for 1 h at room temperature on an agitator at 300 rpm (Polymax, Heidolph, Germany) to elute the formazan crystals. 200 µl of the eluate of each well were transferred into wells of a 96-well microplate and measured by spectrometry at 540 nm (PowerWave HT, BioTek Instruments GmbH, Bad Friedrichshall, Germany). Besides the untreated control, a positive control with TritonTM X-100 (10% in H₂O, Sigma-Aldrich, Munich, Germany) was used to verify the test.

Results

Antimicrobial effects were shown for all treatment variations (Tables 2 and 3) and were statistically significant compared to the

Table 3. Antiseptic treatment of *Staphylococcus epidermidis* RP62A biofilms.

treatment	SBD-A				SBD-B				p
	n	CRF ± SD	95% CI limits		n	CRF ± SD	95% CI limits		
			lower	upper			lower	upper	
plasma - 30 s	20	0.66±0.64 ^{c*}	0.36	0.97	12	0.57±0.45 ^{c*}	0.28	0.85	0.8763
plasma - 60 s	22	1.55±1.04	1.09	2.01	20	1.23±0.40	1.05	1.42	0.5795
plasma - 150 s	22	2.32±1.19 ^c	1.79	2.85	20	1.45±0.56 ^{c*}	1.19	1.71	0.0219
plasma - 300 s	22	2.77±1.27 ^{c*}	2.20	3.33	20	2.04±0.58 ^{c*}	1.77	2.31	0.0698
plasma - 600 s	16	3.38±0.87 ^{c*}	2.92	3.85	12	2.69±0.98 ^c	2.07	3.31	0.0459
p		0.0001				0.0001			0.0001
CHX 0.1%	32	1.14±0.73	0.88	1.41					
control	30	0.00±0.30	-0.11	0.11					
p		0.0001				0.0001			0.0001

The analytical results by the Number of samples (n), Colony reduction factor (CRF) in \log_{10} (CFU/cm²) ± Standard Deviation (SD), lower and upper 95% confidence limits (CI) after exposure to air plasma for 30–600 s treatment time and 0.1% CHX after 600 s exposure time and untreated control of *Staphylococcus epidermidis* RP62A biofilms [p-values of omnibus tests (Kruskal-Wallis) and two-sample tests (Whitney U); statistical significance: $\alpha=0.05$].

^csignificantly different from CHX.

^{*}significantly different from the respective treatment time of *Pseudomonas aeruginosa* SG81.

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control. The efficacy depended on treatment time and differed between SBD-A and SBD-B. Generally, SBD-A showed a higher CRF than SBD-B and the CRF increased with increasing treatment time. The difference between both plasma sources is statistically significant for every treatment applied to *P. aeruginosa*, irrespective of the duration, and within a treatment time of 150 and 600 s for *S. epidermidis*. The range of CRF of SBD-A electrode was $1.4 \log_{10}$ (CFU/cm²) up to $7.1 \log_{10}$ (CFU/cm²), and of SBD-B electrode $1.1 \log_{10}$ (CFU/cm²) up to $3.8 \log_{10}$ (CFU/cm²) for *P. aeruginosa*, and $0.7 \log_{10}$ (CFU/cm²) to $3.4 \log_{10}$ (CFU/cm²) as well as $0.6 \log_{10}$ (CFU/cm²) to $2.7 \log_{10}$ (CFU/cm²) for *S. epidermidis*.

The scanning electron micrographs in figure 3 show untreated (Figure 3 A and B) as well as plasma treated biofilms of *P. aeruginosa* (Figure 3 C and D) and *S. epidermidis* (Figure 3 E and F). Untreated biofilms of *P. aeruginosa* show stable rod-shaped and *S. epidermidis* spherical cell morphology (Figure 3 A and B). After plasma treatment the micrographs showed that the biofilms were covered by a flat layer (Figure 3 E) or by conglomerated cells (Figures 3 C, D and F). Figure 4 shows destructed but non-conglomerated cells in different morphology for *P. aeruginosa* and *S. epidermidis*. Cells of *P. aeruginosa* show mushy (Figure 4 A) and *S. epidermidis* show split shape (Figure 4 B).

The maximum UV irradiation measured between 200–400 nm was $2.5 \mu\text{W}/\text{cm}^2$ by SBD-B during 300 s of exposure time. Here, the emission spectrum is shown in Figure 5. The UV radiation of SBD-A and the measured VUV radiation from 115 to 200 nm of both plasma sources is below the detection limit.

The measured ozone density of SBD-A increased to a maximum of 34.7 ppm and SBD-B of 18.8 ppm within 80 s plasma exposure time.

The cytotoxicity of both plasma sources determined by the MTT-assay is similar (Table 4). Compared to the untreated control, the viability exceeds in this case 50% after plasma treatment times from 30 s to 150 s, whereas it decreases below 50% after 300 s and 600 s treatment time. The gas flow of SBD-B showed no devitalising effects. Compared to the control values, the viability after the cytotoxicity test of 0.1% CHX was less than 50% at treatment times between 30 to 600 s (Table 5). The differences between plasma of SBD A for 600 s treatment time and CHX treated cells for 30 to 600 s treatment time to the untreated control as well as the differences between the plasma treated cells to the CHX treated cells for both plasma sources at any treatment time were statistically significant.

Discussion

Currently, no standard model for testing of antiseptics against microorganisms in biofilms on various substrates is defined in literature. The used biofilm model with incubation times of 48 h at 37°C and early replacement of medium showed optimal results for stable and standardized biofilms in previous experiments. The used test microorganisms *Pseudomonas aeruginosa* SG81 and *Staphylococcus epidermidis* RP62A are often used for biofilm analyses and tests [40,41,42,43,44]. They are relevant biofilm forming pathogens in chronic wounds or device related infections [29,45]. Furthermore, both organisms have been partly isolated from clinical cases as drug-resistant strains [46,47]. The number of infections with drug-resistant microorganisms is increasing worldwide [48] which underlines the necessity to develop new eradication strategies.

The medium contained 10% fetal bovine serum (corresponding to the composition of an artificial wound fluid [6] [36]) and was used as growth medium to investigate biofilms under more realistic wound-like conditions. For the experiments, polycarbonate discs

as substrate were used because polycarbonate is widely used in medicine [49] and is also suited to culture biofilms [50]. The biofilms of the untreated controls had to result in more than $6 \log_{10}$ (CFU/cm²) after dispersion by ultrasound to ensure a sufficient biofilm growth. The untreated controls in the study showed mean values at $7.89 \pm 0.38 \log_{10}$ (CFU/cm²) for *P. aeruginosa* and $6.85 \pm 0.41 \log_{10}$ (CFU/cm²) for *S. epidermidis* and were used to calculate the CRFs after antiseptic treatment. The antiseptic CHX was used as positive control and as control of the homogeneity of the biofilm sensitivity to antiseptic treatment, because 0.1% CHX showed comparable or higher antimicrobial efficacy against *in vitro* biofilms than the standard wound antiseptic polyhexanide [14] and CHX is also used as antiseptic in wound dressing [31]. Furthermore, 0.1% CHX is also recommended for biofilm inhibition in the mouth cavity [51] and was often used as positive control for plasma treatments on biofilms [14,15,52].

Biofilm covered discs used for plasma treatment were transferred onto a plastic flat grate in order to keep the contact area minimal, since both sides of the discs were exposed to the plasma for the respective treatment time in order to treat the whole colonised area. A distance of 2 mm between the discs and electrode had been determined as optimal for treatment by the microbial agar test method [35] by pre-tests.

The two investigated SBD plasma sources were selected because large area discharges could be suitable to treat homogeneously superficial infected wounds on skin. Additionally, the shape of SBD-B electrode can be customized to different surface structures and application forms for possible plasma treatment on skin or wounds [53]. Furthermore, the temperature of the electrodes during plasma generation was equal to the adjacent room temperature for SBD-A and close to body temperature for SBD-B (fluoroptic temperature measurement), and the maximally generated UV radiation at $2.5 \mu\text{W}/\text{cm}^2$ corresponded to an UV-exposition of less than $0.05 \text{ mJ}/\text{cm}^2$ which is extremely low and non-hazardous according to the ICNIRP Guidelines on limits of exposure to ultraviolet radiation [33]. The graphical peaks were NO at 297 nm and excited N₂ at 314, 316.7, 338, 354.5, 358.5, 376.5 and 381 nm. The irregular peaks between 200 and 225 nm are due to increased noise caused by the detection limit of the spectrometer used. Both plasma sources are operated with or in air. Therefore VUV radiation is quickly absorbed in this atmosphere [33] and was too low for detection in the used application setting.

Advantages of air as working gas are that oxygen and nitrogen for ROS and RNS generation are continuously available, no separate gas tanks or gas connections are necessary, thus it is easier applicable and cheaper than commonly used inert gases. The gas flow of SBD-B was applied to enhance the homogeneous distribution of the reactive plasma components.

Both SBDs showed different inactivation efficacies (Tables 2 and 3). SBD-A is more effective than SBD-B for all treatment times on *P. aeruginosa* ($p < 0.027$) and after 600 s on *S. epidermidis* biofilms ($p = 0.046$). The difference was obvious, in particular for *P. aeruginosa* where the maximum CRF of SBD-A was $7.1 \log_{10}$ (CFU/cm²) but only $3.8 \log_{10}$ (CFU/cm²) by SBD-B. However, for *S. epidermidis* the maximum CRF of SBD-A was $3.4 \log_{10}$ (CFU/cm²) and $2.7 \log_{10}$ (CFU/cm²) by SBD-B.

An explanation could be the formation of differently dense cell detritus layers on top of the biofilms which inhibits deeper plasma effects on biofilm. Scanning electron micrographs of *P. aeruginosa* biofilms after 300 s of exposure to plasma (Figure 3 C, D) showed that the plasma treatment seems to produce different dense detritus layers. Maybe, for SBD-A, the reactive plasma-gas compound could work in deeper biofilm regions of *P. aeruginosa* than SBD-B despite of gentle gas flow, because the reactive

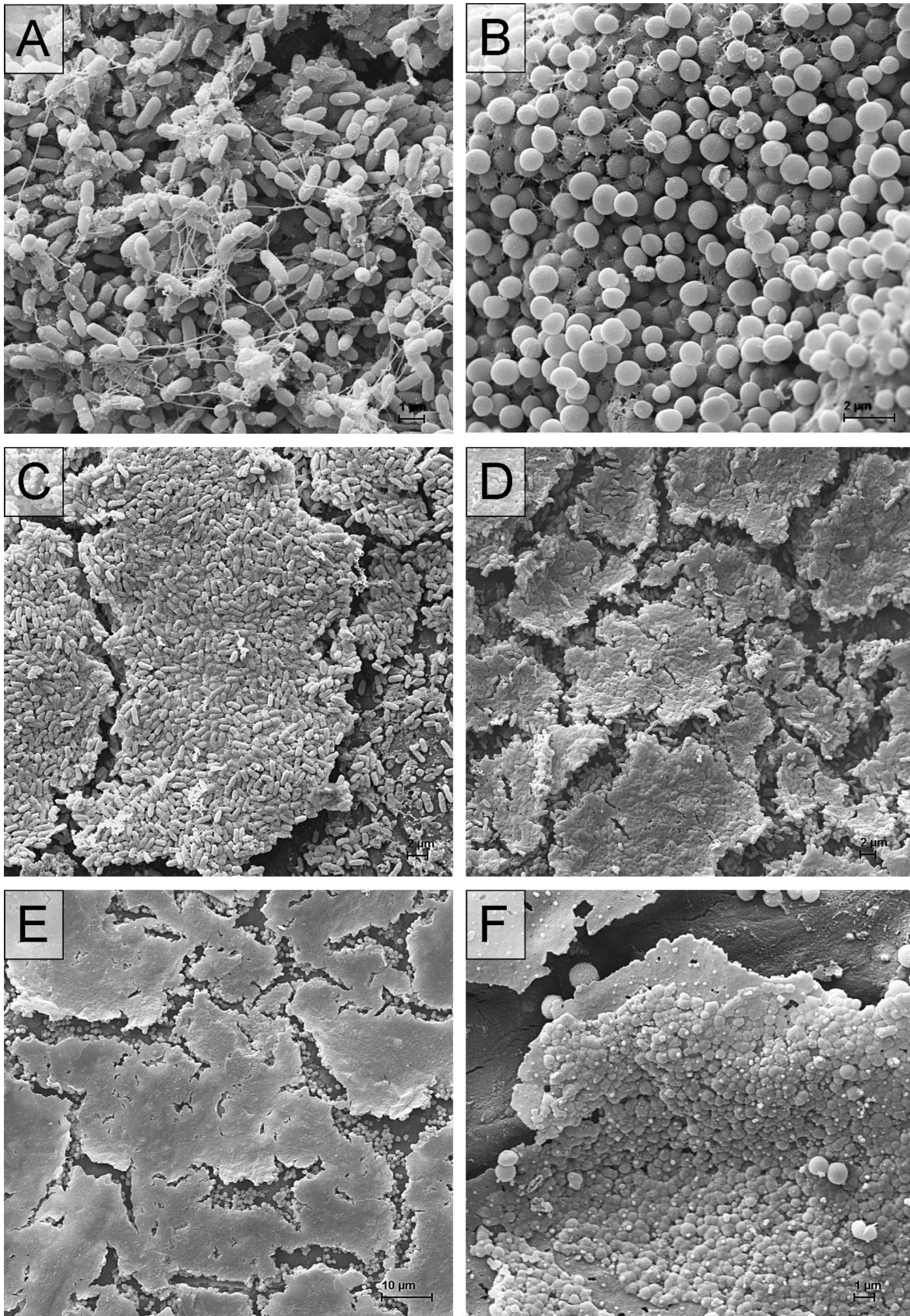


Figure 3. Scanning electron micrographs of untreated and air plasma treated biofilms on polycarbonate discs. A) untreated biofilm of *Pseudomonas aeruginosa* SG81 (5000-fold), B) untreated biofilm of *Staphylococcus epidermidis* RP62A (5000-fold), C) *Pseudomonas aeruginosa* SG81

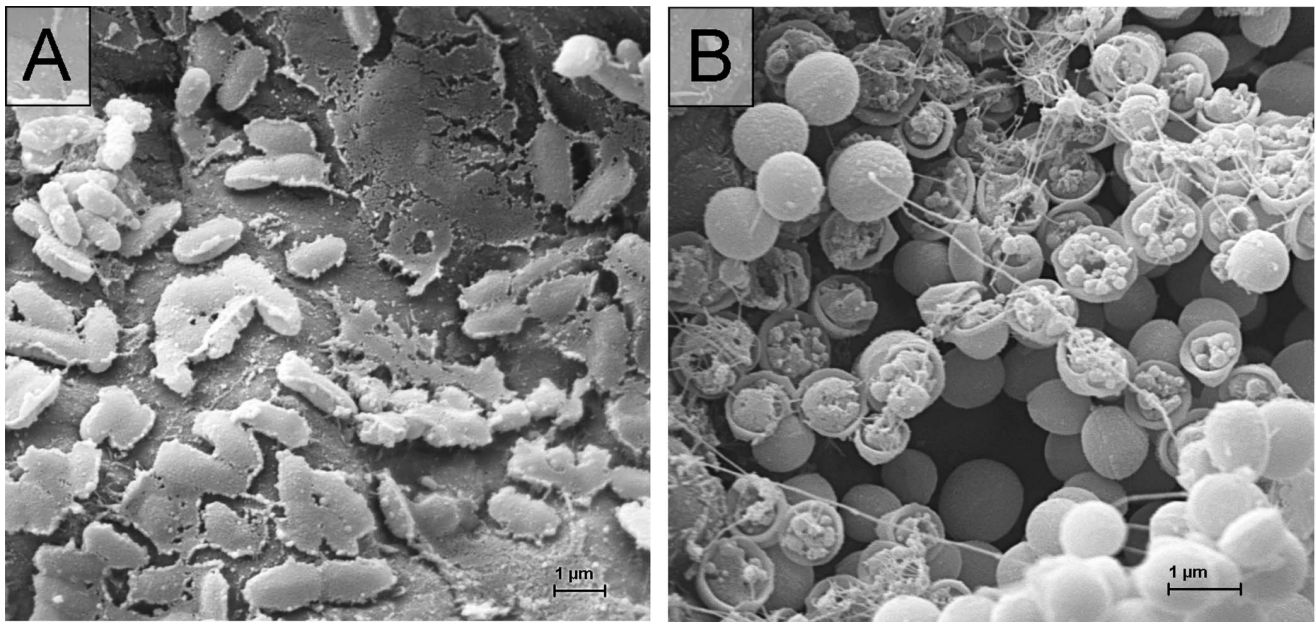


Figure 4. Scanning electron micrographs of cell morphology damaged biofilm bacteria on polycarbonate discs after 300 s of air plasma treatment with A) *Pseudomonas aeruginosa* SG81 by SBD-B (7000-fold) and B) *Staphylococcus epidermidis* RP62A by SBD-A (10000-fold).

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products which are responsible for the antimicrobial effect in deeper layers could possibly generated at different concentrations. The micrographs additionally showed that the detritus layer on *S. epidermidis* biofilm after plasma treatment with both plasma sources was very dense (Figure 3 E, F). The scanning electron micrographs

revealed non-destroyed bacteria visible between the clods of the disrupted biofilms. Those bacteria are probably not inactivated by plasma. It can be deduced that the detritus layer on the biofilm surface restricts the plasma efficiency. This could be an explanation for the increased standard deviation after treatment

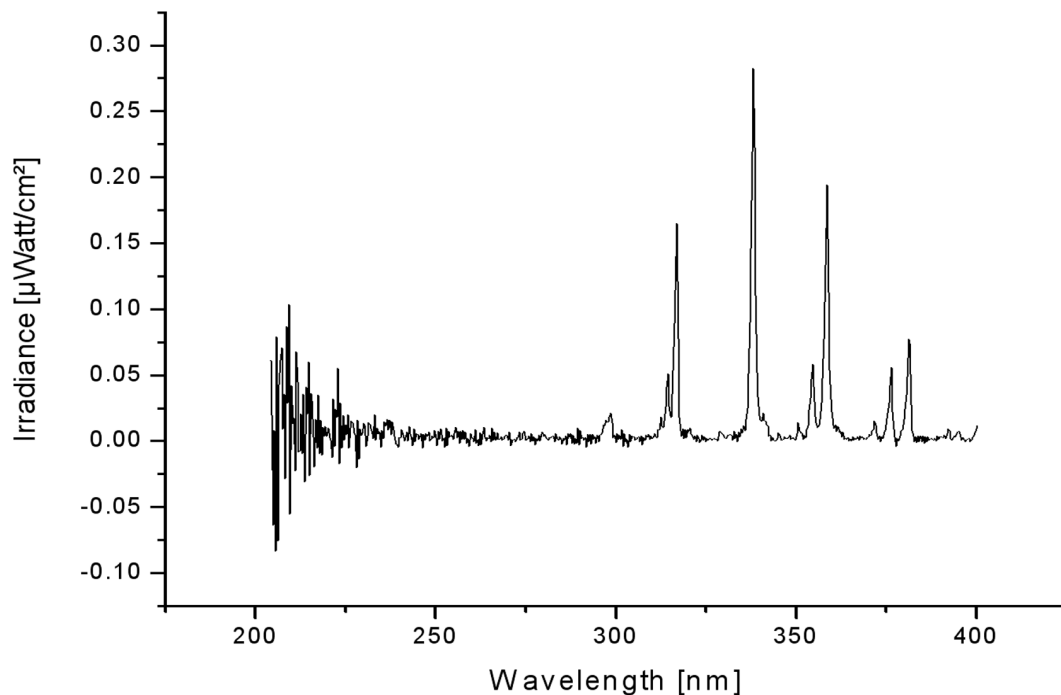


Figure 5. Spectrometric graph of irradiance by SBD-B generated air plasma within 300 s of exposure time between 200 and 400 nm.

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Table 4. Cytotoxicity of air plasma by SBD-A and SBD-B on L929 cell line (mouse fibroblasts).

treatment	n	SBD-A		n	SBD-B	
		mean ± SD	ratio to control (%)		mean ± SD	ratio to control (%)
plasma - 30 s	3	0.090 ± 0.022	89.0	2	0.094 ± 0.004	92.6
plasma - 60 s	4	0.096 ± 0.005	94.1	4	0.075 ± 0.004	73.9
plasma - 150 s	4	0.075 ± 0.022	74.1	4	0.085 ± 0.014	83.3
plasma - 300 s	4	0.036 ± 0.024	35.0	4	0.044 ± 0.032	43.3
plasma - 600 s	4	0.004 ± 0.003	4.2	4	0.043 ± 0.028	42.6
gas flow - 300 s				2	0.088 ± 0.010	86.7
control	4	0.102 ± 0.011				

Measured values of MTT-Assay after 30–600 s treatment time of air plasma by SBD-A and SBD-B with the Number of samples (n), mean, standard deviation (SD), and the cell viability as ratio in comparison to the control in percent.

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of *P. aeruginosa* biofilms on the one hand and the limited antimicrobial effect against *S. epidermidis* on the other hand.

The detritus layer seems to be the result of coagulated biofilm matrix as well as cytoplasm after bacterial cell wall disruption by reactions with the generated plasma products. Differences in disrupted cell morphology were visible between the Gram-negative *P. aeruginosa* and the Gram-positive *S. epidermidis*, whereas *S. epidermidis* as a Gram-positive strain with a cell wall containing a thick peptidoglycan layer is opened and *P. aeruginosa* as a Gram-negative strain with an outer cell membrane and only a thin peptidoglycan layer loses the three-dimensionality to a flat irregular morphology (Figure 4) after longer treatment time. That corresponds to observations of other authors [54,55]. Similar cell wall disruption of *S. epidermidis* was shown for *Candida albicans*, a unicellular fungi with a glycan rich cell wall, in a former study [52]. That supports the supposition of a cell wall influenced dependence of the antimicrobial mechanism of plasma [55]. For comparison, figure 3 shows untreated biofilms of *P. aeruginosa* and *S. epidermidis*. The “clods” of disrupted biofilms visible in scanning electron micrographs of figure 3 C–F were probably caused by drying due to the plasma treatment or the drying steps by SEM procedure.

Also, a different structure of biofilm matrix between *S. epidermidis* and *P. aeruginosa* could explain the different results of SBD-B treatment on both test organisms but not the different results between both plasma sources against *P. aeruginosa* biofilms. The

higher power per area of SBD-B at 0.18 mW/cm² compared to SBD-A electrode at 0.03 mW/cm² cannot be decisive for antimicrobial results. Here, effluent distribution over the substrate or different plasma-gas compounds by different plasma generation seems to influence the biological effects perceptibly. To clarify the different efficacy, the generated ozone concentration of both devices was determined. The results obtained using the experimental setup showed a higher share of O₃ generated by SBD-A. Moore et al. [56] showed a higher susceptibility of Gram-negative bacterial strains to O₃ which could explain the unexpected high antimicrobial effect of SBD-A on biofilms of *P. aeruginosa*. Additionally, a study of Zhang et al. [57] suggests that O₃ play a minor role for inactivation of the Gram-positive *Staphylococcus aureus*. However, other reactive species in the gas or different secondary products on the substrate cannot be excluded to be decisive for the observed effect.

The efficacy of both plasma devices is comparable to the results of other studies. An argon plasma jet (kinpen 09), for instance, induced a reduction of 5.4 log₁₀ for *P. aeruginosa* and 3 log₁₀ for *S. epidermidis* after 300 s exposure time [58]. A helium plasma jet caused a reduction of 4 log₁₀ of *P. aeruginosa* after 240 s exposure time [59]. Otherwise, only approximately 1 log₁₀ reduction were reached by 600 s blowing of a DBD generated air plasma (in a separate box) on biofilm embedded Staphylococci [60] or 2 log₁₀ reduction against *S. epidermidis* after 600 s by a sprayed gliding discharge plasma of humidified air [61]. Here, the distance between the electrodes and the target was extended. Those differences of biological effectiveness underline the important influence of the distance between the plasma generating electrodes and bacteria.

Generally, in biofilms *P. aeruginosa* SG81 was more sensitive than *S. epidermidis* RP62A to air plasma in that study, which could be due to the oxidation of membrane lipids by ROS of the Gram-negative *P. aeruginosa* strain [62,63]. However, a previous study showed that other conditions or plasma setups can result in different effects between Gram-positive and Gram-negative bacteria spread on agar plates by treatment with air plasma [64]. In comparison to the untreated control all antiseptic treatments exhibited a superior efficacy towards the used strains (p<0.001) which is statistically significant. In comparison to 600 s of CHX treatment, the CRFs of plasma had shown a statistically higher value already after 150 s of treatment for both strains (all p<0.028), except for SBD-B at 150 s treatment time for *P. aeruginosa* (p = 0.057).

Additional, results of the cytotoxicity test by MTT assay showed that the average viability of the cells did not decrease below 50%

Table 5. Cytotoxicity of 0.1% chlorhexidine digluconate solution on L929 cell line (mouse fibroblasts).

CHX (0.1%)			
treatment	n	mean ± SD	ratio to control (%)
CHX - 30 s	3	0.046 ± 0.029	17.3
CHX - 60 s	3	0.021 ± 0.017	7.7
CHX - 150 s	3	0.069 ± 0.013	25.9
CHX - 300 s	3	0.016 ± 0.011	6.0
CHX - 600 s	3	0.007 ± 0.002	2.5
control	6	0.267 ± 0.076	

Measured values of MTT-Assay after 30–600 s treatment time with 0.1% of chlorhexidine diuconate solution (in culture media) with the Number of samples (n), mean, standard deviation (SD), and the cell viability as ratio in comparison to the control in percent.

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until 150 s of exposure to plasma for both plasma sources. The 50% limit was chosen based on the XTT assay, which is similar to the MTT assay, described in ISO 10993-5. Here, a loss of the cell viability between 70% to 50% compared to the control is described as moderate cytotoxicity [38]. That suggests an acceptable tissue tolerability within individual limited treatment times. Here, the antimicrobial efficacy on biofilms was approximately $1.5 \log_{10}$ (CFU/cm²) for *S. epidermidis* and 1.8 to 2 \log_{10} (CFU/cm²) for *P. aeruginosa*. That is low but reaches the antimicrobial effect of CHX on biofilms. Additionally, the cell viability of 0.1% CHX was clearly less than 50% compared to the control for 30 s to 600 s treatment time while the antimicrobial efficacy with a CRF at 1.1 \log_{10} (CFU/cm²) for *S. epidermidis* and 1.7 \log_{10} (CFU/cm²) for *P. aeruginosa* is lower or comparable to the air plasma treatment times for cytotoxicity acceptable doses.

Beside the antimicrobial effect on biofilms, the stimulation of wound healing processes is necessary to heal chronic wounds. An enhanced cell growth by air plasma was reported by other authors [22,65]. Plasma supported wound healing was also demonstrated in clinical or preclinical practice [66,67,68]. These facts could promote a therapeutic application form of the investigated plasma sources as support for wound healing processes with slight antimicrobial effect (in tissue tolerable doses).

For future therapy options, it is indispensable to consider the limitations of the presented plasma application, i.e., side effects like the observed cell detritus. Also, the treatment time must be correctly chosen as plasma treatment could turn toxic if applied too long.

The present study recommends short treatment times (<60 s) on biofilms to reduce possible dense cell detritus layers which complicate a whole biofilm inactivation and to minimize the toxic risks. Maybe combination of plasma with antiseptic solutions could be a treatment option. Promising results for combinations of chemical antiseptics with plasma were presented [19].

The study has some limitations. We used 48-h-old monospecies *in vitro* biofilms grown under controlled conditions to investigate the antimicrobial efficacy of plasma, however natural and wound multi-species biofilms *in vivo* are more complex than our monospecies biofilms [21]. The plasma treatment is to be realised under defined exposure distances from the specimen for standardization, while biological surfaces to be treated often have variable structures. In order to allow the comparability, the cytotoxicity test was carried out with a standard murine fibroblast cell line for *in vitro* cytotoxicity tests [38] and not with human epithelial cells.

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Consequently, these results are only for orientation. Additional, plasma effects on real wounds are influenced by individual wound secret and local immunologic cells, so more complex reactions and influences than under laboratory conditions are expected.

Conclusion

A high antimicrobial effect on biofilms with *P. aeruginosa* and *S. epidermidis* is confirmed for both plasma sources used. Here, SBD-A is more effective than SBD-B. Taking into consideration the cytotoxicity, it is recommended to limit the application time to 60 s for SBD-A and 150 s for SBD-B. Within these exposure times both plasma sources meet important preconditions of tissue tolerability such as temperature, UV exposure and cytotoxicity. Their antimicrobial efficacy is comparable to the efficacy of 0.1% chlorhexidine digluconate solution, the antiseptic gold standard for dental biofilm treatment, however with significantly lower cytotoxicity of the used plasma. In addition, if these plasma sources prove suitable to expedite wound healing processes, they are likely to be successfully applied in wound care management, too.

Formation of bacterial cell detritus on biofilm surface can limit the antimicrobial effect which depends on plasma source, plasma exposition time and treated type of microorganism. Here, the Gram-negative bacterium was more sensitive to air plasma than the Gram-positive strain. Future investigations into the antimicrobial mechanisms of plasma could elucidate the differences of both generated “types” of air plasma by SBD-A and -B.

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Author Contributions

Conceived and designed the experiments: RM. Performed the experiments: RM RS. Analyzed the data: RM RS RB. Contributed reagents/materials/analysis tools: RM RS RB. Wrote the paper: RM CB RS IK RB SR JL KDW AK. Physical consultation: RB SR.

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