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Proceeding Paper

Investigation in the Antimicrobial Impact of Cold Atmospheric Plasma on Wet and Dry Microorganisms †

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Abstract: This study aims to investigate the antimicrobial effect of cold atmospheric plasma on microbial contaminated liquid films and dried surfaces. Additionally, the contribution of the plasma generated UV radiation to the total inactivation is assessed. The experiments were performed with the nearfield module of the relyon piezobrush PZ3 plasma pen on *Staphylococcus carnosus*, *Pseudomonas fluorescens*, *Streptococcus vestibularis* and *Candida auris*. It was observed that in the liquid there seems to be no obvious general sensitivity differences between gram-positive and gram-negative bacteria. However, all bacteria in liquids were clearly more susceptible to the plasma than the yeast. In contrast, there was no significant difference between *C. auris* and *S. carnosus* on dried surfaces. The plasma emissions exhibited strong UVA and UVB radiation and only weak emissions in the UVC range. The antimicrobial impact of the UVA und UVB emissions were negligible. However, an estimation based on two simplifications revealed that the UVC radiation probably contributed considerably to the inactivation properties of the plasma. This might be a special feature of the near-field plasma application on thin liquid samples.

Keywords: cold atmospheric plasma; disinfection; *Staphylococcus carnosus*; *Pseudomonas fluorescens*; *Streptococcus vestibularis*; *Candida auris*; *UVA*; *UVB*; *UVC*

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1. Introduction

More than 25 years ago, it was first observed that so-called cold or nonthermal plasma can inactivate microorganisms without damaging materials and some biological tissues [1]. This plasma is (partially) ionized gas or air, in which the free electrons have a high temperature, while the heavier gas molecules are relatively cold. The plasma emits UV radiation and visible light, but the observed antimicrobial effect of cold atmospheric plasma is not attributed to UV, but mainly to reactive oxygen and nitrogen species such as O, $^1O_2$, O_2^-, O_3, OH, NO, NO_2, … [2–5]. The reactive species attack cell structures and lead to the death of the cell, whereby different cells or microorganisms can have different sensitivities. For example, it is assumed that gram-negative and gram-positive bacteria differ in their sensitivity and that fungi are generally less sensitive than bacteria [6–9].

Plasma is applied in the cleaning and disinfection of surfaces and liquids, e.g., in the health and food sector [5–8,10]. Often dry surfaces are examined, but also liquids, with different volumes.

In the here presented study, the inactivation of gram-positive and gram-negative bacteria will be investigated in comparison to a yeast. Surrogates of significant pathogens are selected for this purpose. The specimen will be microorganisms in thin liquid samples, such as liquid layers on wounds or washed hands or food, which will be compared to

dried microorganisms. In addition, the importance of UV emissions from the plasma will be assessed. Therefore, microorganisms whose UV sensitivity is known are selected.

2. Materials and Methods

2.1. Microbiology

For the inactivation experiments different microorganisms were cultivated. The yeast *Candida auris* (DSM 21092) was selected as a fungi representative. *C. auris* was cultivated in liquid YEPG (yeast extract peptone glucose) and on M129 agar plates. As a gram-negative bacterium *Pseudomonas fluorescens* (DSM 4358) was chosen and as gram-positive bacteria *Staphylococcus carnosus* (DSM 20501) and *Streptococcus vestibularis* (DSM 5636). *P. fluorescens* was cultivated in M535, *S. carnosus* in M92 and *S. vestibularis* in BHI (brain heart infusion) medium at a temperature of 37 °C, with the exception of the 30 °C cultivation of *P. fluorescens*. The description of all media can be found here [11].

After reaching the mid-exponential phase of the cultivation procedure, samples were taken from each culture and centrifuged at 7.000× *g* for 5 min. The resulting pellet was then resuspended with PBS (phosphate buffered saline) and the centrifugation was repeated. The washed samples were diluted until a population density of 8×10^6 to 1×10^8 colony-forming units per ml (CFU/mL) was reached.

2.2. Plasma Source and Sample Treatment

For the inactivation experiments, the piezobrush PZ3 pen of relyon plasma GmbH (Regensburg, Germany) was equipped with its nearfield nozzle and it generated a cold atmospheric air plasma. The plasma emission was measured with a spectrophotometer CAS 140D of Instruments Systems (Munich, Germany) by placing the plasma pen and a grounded wire directly in front of the aperture of an integrating sphere as illustrated in Figure 1. The distance between pen and wire was about 2 mm. Together with published log reduction doses and an antimicrobial action spectrum the plasma emission was applied to assess the UV contribution to the total antimicrobial impact of the plasma.

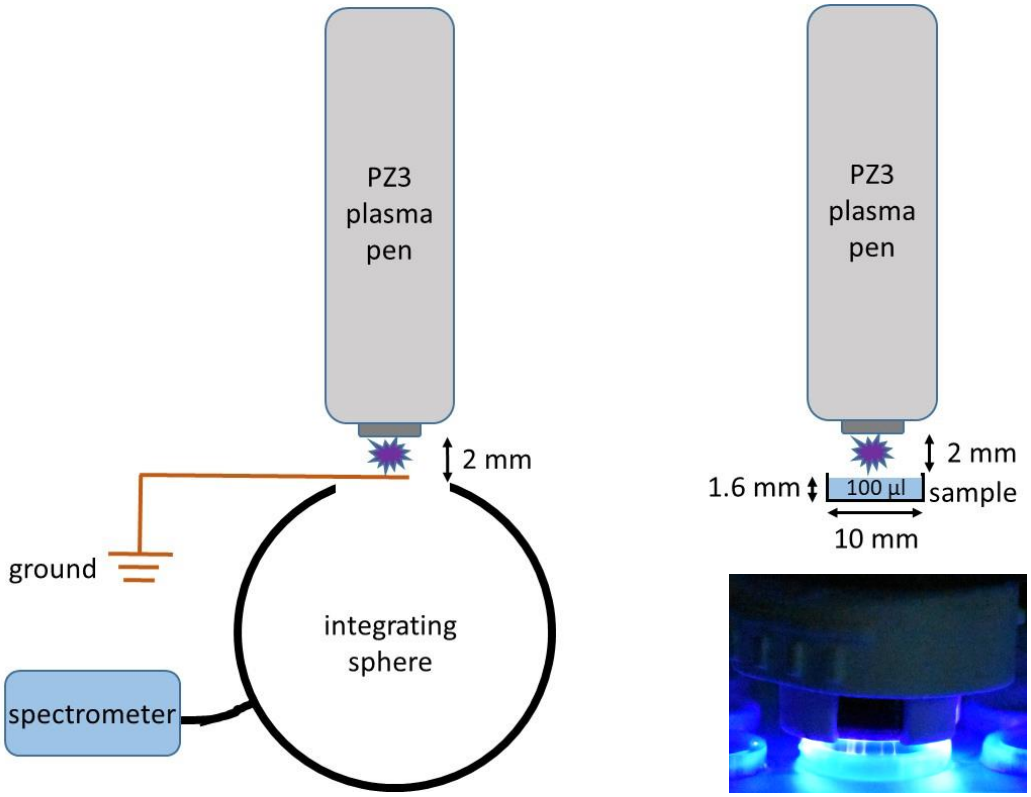

Figure 1. Left: schematic setup of the measurement of the plasma emission; right: schematic setup of the plasma treatment of samples in 10 mm wells. (Both unscaled representations!) Right bottom: photograph of the plasma treated sample.

For the plasma treatment of thin liquids an array of wells with a diameter of 10 mm and a height of 2 mm was printed in resin type BioMed Clear Resin of Formlabs (Berlin, Germany). Samples with a volume of 100 µL (sample height 1.6 mm) could be placed in it beforehand. To perform the suspension experiments, the liquid sample was treated directly with the cold plasma. To perform experiments with a contaminated dry surface, the suspension was dried for three hours, prior to the plasma treatment.

The plasma was applied for periods of up to 7 min for liquid samples and up to 2 min for dried ones. For liquid samples the temperature was measured with an infrared thermometer type Raynger MX of Raytek (Berlin, Deutschland) and ozone and pH with test stripes type Water Test of Tytlyworth (unknown origin) and Dosatest pH test stripes pH 4.5–10.0 of VWR (Darmstadt, Germany), respectively.

The respective liquid samples were directly removed after the plasma treatment and spread on agar plates in different dilutions. For recovery of the dried sample, 100 µL of PBS was added to the well containing the dried microorganisms. Then, the sample volume was agitated with the pipette tip for 15 s before the sample was removed and plated out again. Incubation was carried out at the same temperature as during cultivation and after 48 h, the grown colonies were counted and evaluated. The determined log reduction values referred to a starting value, which was an untreated sample that was filled in a well and recovered as were all the plasma treated samples.

3. Results

The experiments revealed that all microorganisms (liquid and dried samples) were reduced by several orders of magnitude within a few minutes. The required plasma application durations for a log reduction can be found in Table 1. In the semi-logarithmic representation in Figure 2 the measured data lie more or less well on a straight line, which is indicative of an approximate exponential reduction of the microorganisms. The measured physical and chemical properties in the liquid samples are listed in Table 2 as a function of time. They are not assumed to have a large influence on the bacteria and yeast reduction.

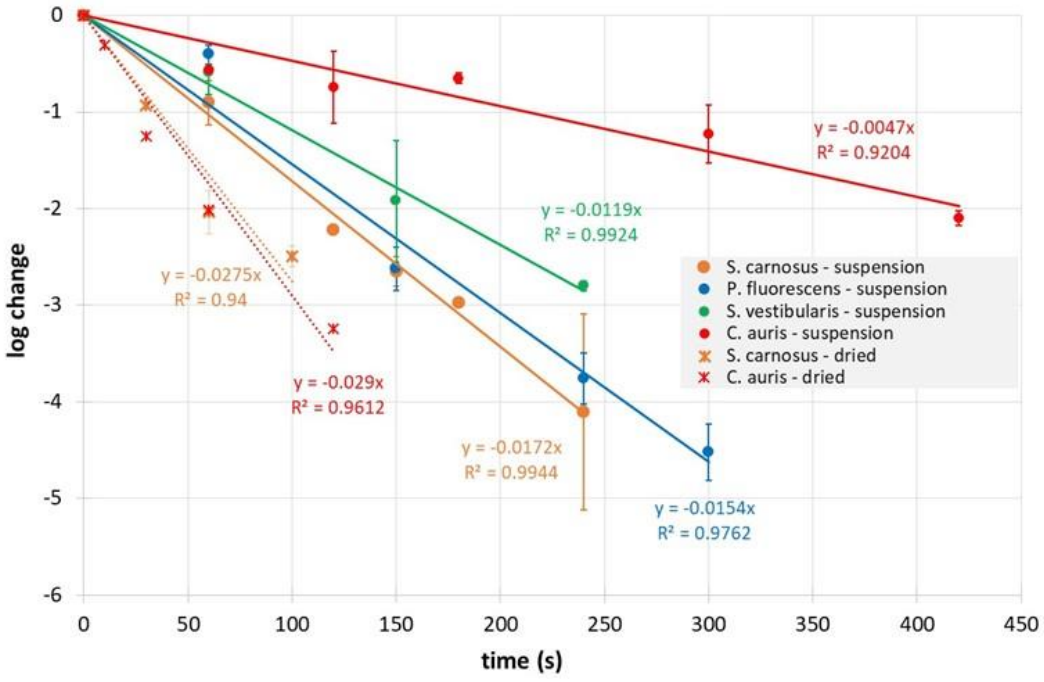

Figure 2. Log change for the microbial concentrations for the different microorganisms and conditions as a function of time and fitted trend lines.

Table 1. Plasma log reduction times for the different microorganisms and conditions.

Microorganism	Log Reduction Time for Microorganism in Suspension	Log Reduction Time for Dried Microorganism
S. carnosus (gram +)	58.1 s	36.4 s
P. fluorescens (gram -)	64.9 s	-
S. vestibularis (gram +)	84.0 s	-
C. auris (yeast)	212.8 s	34.5 s

Table 2. Ozone concentration, pH and temperature in liquid samples as a function of plasma application duration.

Plasma Application Duration [s]	Ozone [mg/L]	pH	Temperature [°C]
0	<4	7.0	23.8
60	<4	7.0	30.7
120	<4	7.0	35.1
180	<4	7.0	37.2
240	<4	6.7–7.0	38.5
300	<4	6.7	40.0
360	<4	6.4–6.7	40.2
420	<4	6.4–6.7	41.3

Figure 3 gives the short wavelength plasma emission spectrum. In the UV region, UVA (315–400 nm) is the strongest with 28.4 µW, followed by UVB (280–315 nm) with 7.0 µW and UVC with 5.4 µW. However, if the antimicrobial action spectrum [12] is considered, the photoinactivation impact of the UVC emission is about 11x times higher than for the UVB and 111x times higher than for the UVA plasma emissions. Therefore, UVB and UVA do not play a significant role in the antimicrobial impact of the plasma.

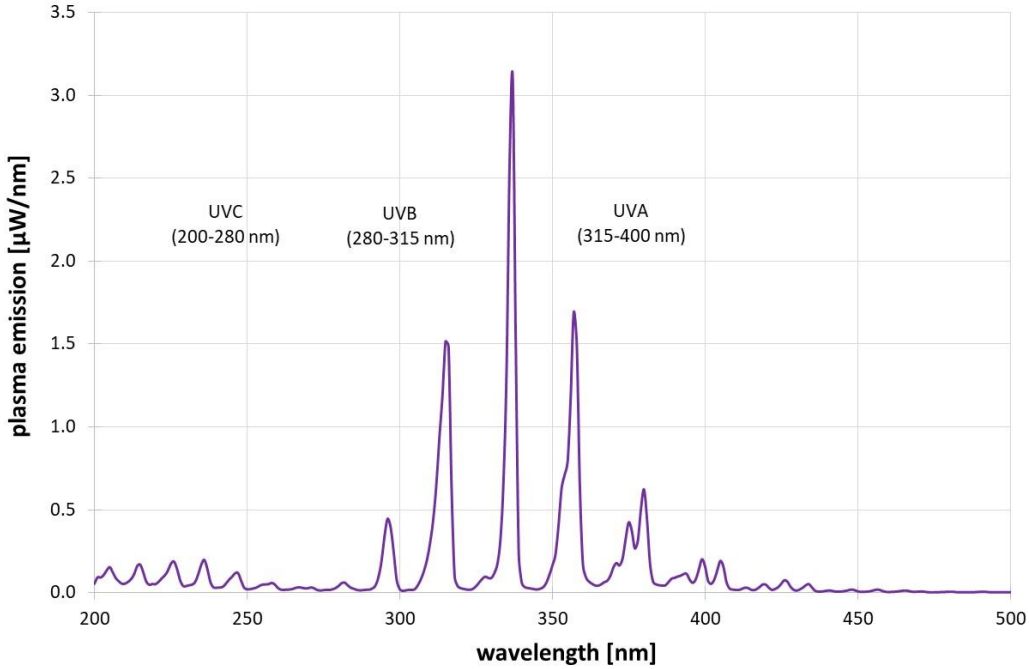

Figure 3. Measured short wavelength plasma emissions.

For assessing the contribution of the UVC plasma emission the average UVC irradiation intensity inside the 10 mm well is determined to be 6.9 μW/cm². The applied UVC doses during the plasma log reduction time (90% reduction) is given in Table 3 together with known UVC (254 nm) log reduction doses. Also given is the microbial reduction that would be expected by the UVC application only with the simplification that the UVC photosensitivities can be approximated by published 254 nm values.

Table 3. Applied UVC dose during plasma log reduction times (see Table 1) for different microorganism, published UVC (254 nm) log reduction doses, and assumed reduction just based on UVC irradiation.

Microorganism	Applied Dose during log Reduction Time [mJ/cm²]	Published UVC Log Reduction Doses [mJ/cm²]	Expected Reduction Just by UVC [%]
S. carnosus (gram +)	0.40	1.26 [13]	52
P. fluorescens (gram -)	0.44	1.59 [13]	47
S. vestibularis (gram +)	0.57	1.7 [14]	54
C. auris (yeast)	1.45	13.2 [15]	22

4. Discussion

The cold atmospheric plasma of the piezobrush PZ3 reduced all three bacteria and the yeast *C. auris* within minutes by orders of magnitude, and the decrease appears to be approximately exponential. A three phase behavior as suggested in [3] or [5] was not observed. In the liquid samples, the yeast was clearly more resistant than the bacteria, but in the dried state *C. auris* and *S. carnosus* appear to be equally susceptible.

The observed differences between the bacteria in liquids were small. The gram-negative representative lay between the two gram-positive bacteria in its sensitivity to plasma. Therefore, in this study, there is no evidence of general differences in plasma sensitivities due to bacterial cell wall structure (gram +/gram -).

Reactive oxygen and nitrogen species, which attack cell structures, are usually cited as the most important mechanism in the antimicrobial action of cold plasma. UV radiation is assumed to play a very minor role at atmospheric pressure. Although this study confirms that UVA and UVB radiation do not cause any relevant inactivation, the UVC emissions from the plasma could account for a large contribution to the antimicrobial effect of the plasma in this particular nearfield application on thin liquid samples. However, this quantitative statement is not certain, because two assumptions were implied that affect the calculated strength of the UVC effect.

First, for simplicity, it was assumed that the photosensitivities of each microorganism in the spectral range 200–280 nm does not vary substantially from its photosensitivity at 254 nm. Second, the plasma emissions were not measured over the sample wells as in the real application, but in a special arrangement in front of the integrating sphere of the spectrometer in combination with a grounded wire. Therefore, the UV emissions in this setup might have been higher than in inactivation experiments. On the other hand, the here measured UV intensities of the piezobrush PZ3 with nearfield nozzle seem to be in the range of the results of Timmermann et al. [9]. This statement is based on the UVA and UVB emissions only as Timmermann et al. published no data below 280 nm.

Both assumptions may lead to an overestimation of the UVC influence, but the error will probably not be an order of magnitude and thus it can be assumed that the UVC emissions from the plasma contributes clearly to the antimicrobial effect of the plasma, at least under these particular conditions.

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