The role of humidity and UV-C emission in the inactivation of *B. subtilis* spores during atmospheric-pressure dielectric barrier discharge treatment

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Abstract

Experiments are performed to assess the inactivation of *Bacillus subtilis* spores using a non-thermal atmospheric-pressure dielectric barrier discharge. The plasma source used in this study is mounted inside a vacuum vessel and operated in controlled gas mixtures. In this context, spore inactivation is measured under varying nitrogen/oxygen and humidity content and compared to spore inactivation using ambient air. Operating the dielectric barrier discharge in a sealed vessel offers the ability to distinguish between possible spore inactivation mechanisms since different process gas mixtures lead to the formation of distinct reactive species. The UV irradiance and the ozone density within the plasma volume are determined applying spectroscopic diagnostics with neither found to fully correlate with spore inactivation. It is found that spore inactivation is most strongly correlated with the humidity content in the feed gas, implying that reactive species formed, either directly or indirectly, from water molecules are strong mediators of spore inactivation.

Keywords: dielectric barrier discharge, inactivation experiments, spectroscopy, atmospheric pressure, controlled atmosphere, *Bacillus subtilis* spores

(Some figures may appear in colour only in the online journal)

1. Introduction

A variety of physical and chemical means, such as heat, chemical vapor or radiation, offer the possibility to achieve the inactivation of harmful microorganisms. However, the accompanying material damage of most conventional sterilisation methods cannot be neglected [1]. In particular, any application designed to reduce bacterial load on living tissue must demonstrate minimal risk for patients while showing clinical effectiveness in the inactivation of harmful microorganisms. Due to their low gas temperatures, non-thermal plasmas produced at atmospheric pressure are a promising approach for such
applications. These plasmas produced in air and other gas mixtures have been demonstrated to cause inactivation of microorganisms on treated surfaces and in human wounds [2–8]. Significant effort has been made in recent years to understand the mechanisms of deactivation. For example, the work of Krewing et al used E. coli mutants to identify 87 genes that confer a certain level of resistance against treatment using the effluent of a He/O₂ plasma jet [9]. By exposing these mutants to individual stressors expected to be present during plasma exposure, the authors were able to conclude that most genes acted to protect bacteria from hydrogen peroxide, superoxide and/or nitric oxide. These results provide a strong indication that these species are important mediators of plasma induced effects under the conditions studied by the authors. In addition, using a combination of UV radiation measurements, under different plasma conditions and inactivation experiments, Reineke et al were able to demonstrate that UV radiation played an important role in the inactivation of B. subtilis spores exposed to an Ar/O₂/N₂ plasma jet [10]. Moreover, Klämpfl et al showed that different spores can also be inactivated using an atmospheric surface microdischarge plasma source in a closed environment, suggesting that the inactivation might be related to ozone [11]. However, despite a large number of studies demonstrating the inactivation of microorganisms as a result of plasma treatment, a complete picture of which plasma-produced components are important under a particular operating condition is still to be formed. This is in large part due to the wide range of reactive components delivered by non-thermal plasmas and the difficulty in measuring all relevant components for a given treatment condition. This issue is compounded by the wide variety of plasma sources used for such studies that each deliver different quantities of reactive components.

With respect to the effects on living tissues, it has been shown that plasma-produced molecules, such as nitric oxide, are capable of promoting wound healing [12, 13]. Contact-free and painless application of these plasmas to human tissue has also been demonstrated in clinical studies [14]. The possibility to induce favourable effects in tissues while also exerting antimicrobial activity means that non-thermal plasmas have a wide variety of potential applications in biomedicine. However, the widespread and effective use of these devices in biomedical applications requires further knowledge of their mechanisms of interactions with biological systems so that the plasma properties can be optimised for applications. This view is supported by the recent meta-analysis of clinical studies of non-thermal plasmas for chronic wound healing by Assadian et al [15]. This analysis concluded that the use of non-thermal plasmas for chronic wound healing has been demonstrated to be safe, however, no statistically significant clinical benefit in terms of reduction in wound size or bioburden has yet been demonstrated compared to standard chronic wound treatments. To overcome this challenge, additional fundamental research on the mode of action of plasma sources in medical applications is needed to point towards favourable plasma regimes to maximise beneficial effects, such as a reduction in bacterial load.

The Gram-positive spore-forming bacterium Bacillus subtilis is one of the most extensively characterised microorganisms. Several bacteria are able to form endospores to protect their DNA in case of severe outer stressors, such as UV emission, heat, lack of nutrition or chemicals. Bacillus subtilis is able to form dormant endospores under extreme environmental stress, such as nutrient and water deprivation. In their spore form, bacteria do not show metabolism, but do show significantly increased resistance to stressors compared to their vegetative form. Briefly, this is achieved by discarding irrelevant components from the vegetative cell and forming several protective layers called the spore coat. In addition, DNA is further protected by small acid-soluble proteins (SASP), in a spore-specific calcium dipicolinic acid (Ca-DPA) environment at the core. The core and spore coat are separated by the cortex which exhibits two membranes to enable nutrient transport into the spore, if the surroundings are favorable again. Dormant spores harbor the most efficient protection mechanisms among bacteria and have therefore served several studies as a model to verify sterilization and inactivation methods, biodosimetry, and are also used as the gold standard to regularly evaluate and test standard sterilization procedures [16]. Furthermore, the non-pathogenic nature of B. subtilis makes it an ideal model organism for many scientific and industrial purposes [16–18]. Detailed information on the spore structure and protection mechanisms can be found in [19–21].

In this work, the inactivation of B. subtilis spores by a dielectric barrier discharge (DBD) is studied. To distinguish and evaluate the impact of possible mechanisms by which atmospheric-pressure plasmas lead to the inactivation of bacterial spores the composition of the process gas was changed in a controlled manner. The influence of the gas composition on the absolute UV irradiance and ozone (O₃) concentration, two prominent biologically active components of the plasma, was determined using emission and absorption spectroscopy, respectively. This approach offers the ability to determine whether or not these components play a prominent role in spore inactivation.

The manuscript is structured in the following way: in section 2, the experimental setup, as well as the handling protocol of the spores, is described in detail. Additionally, the experimental setups used for optical emission and absorption spectroscopy are introduced and the geometrical factor needed to determine the absolute UV irradiance on any treated biological sample is established. In section 3, the results of the inactivation experiments as well as the absolute UV irradiance and ozone concentrations are described. Finally, the results are discussed in section 4, with a focus on potential inactivation mechanisms.

2. Experimental procedures

2.1. Experimental setup

The DBD used in this work consists of a cylindrical copper electrode covered by aluminium oxide (Al₂O₃) and driven by a self-built pulsed power supply [22]. The driven electrode has
a diameter of 10 mm, including the dielectric, and the distance between the dielectric and the treated sample is kept constant at 1 mm for all experiments. In figure 1, a sketch of the experimental setup of the DBD is presented. The DBD is driven by damped sine wave pulses with a frequency of around 500 kHz as depicted in figure 1 (right). The pulse repetition frequency can be varied between 100 Hz and 4 kHz and the high voltage amplitude can be set within the range of 0 kV pp to 28 kV pp.

For the determination of the power deposited in the plasma, an electrical characterisation of the discharge is carried out. This is done according to Kogelheide et al. [22]. Voltage and current measurements are conducted using a capacitive voltage divider (P6015A, Tektronix, USA), a current monitor (Model 2877, Pearson Electronics, USA) and a digital oscilloscope (LeCroy 9459 DUAL 350 MHz, LeCroy, USA). In figure 1 (right), the current-voltage characteristics of the discharge are displayed after subtraction of the displacement current [22].

The DBD is placed inside a vacuum vessel made of aluminium which is mounted to a miniature flow passage and a vacuum pump (Trivac D16BCS-PFPE, Leybold, Germany) in order to operate the DBD in a defined atmosphere. The total gas flow is adjusted via two mass flow controllers (El-Flow Prestige, Bronkhorst, Germany) and amounts to 2 slm. In this study, different nitrogen/oxygen (purity 5.0, Air Liquide, Germany) gas mixtures, namely N$_2$:O$_2$ 99.9%:0.1%, 80%:20%, 50%:50%, 20%:80% and 0.1%:99.9%, are chosen as the process gas. For experiments carried out in synthetic air (N$_2$:O$_2$ 79%:21%) in combination with different humidity content, the feed gas is partly passed through a gas washing bottle filled with distilled water. The relative humidity as a function of the fraction of the feed gas passed through the gas washing bottle is measured inside the vacuum vessel prior to the experiments using a humidity probe (Testo 435, Testo SE & Co KGaA, Germany). The pressure inside the vessel is set to 1 bar using an adjustable valve and a vacuum pump. To control and monitor the pressure inside the vessel, a pressure gauge (Baratron 750-C, MKS, USA) and pressure controller (Model 250, MKS, USA) are used. All inactivation measurements are carried out by placing the spore covered sample on a grounded aluminium plate underneath the driven electrode inside the vacuum vessel.

2.2. Bacterial strains, sporulation and spore purification

Spores of B. subtilis (strain PY79, a prototrophic laboratory strain with wild-type DNA repair and spore protection capabilities) are obtained by the cultivation of vegetative cells under vigorous aeration at 37 °C for 7 days in double-strength liquid Schaeffer sporulation medium [23]. Afterwards, the spores are purified and stored as described in [24,25]. The prepared spores consisted of a suspension of single spores with no detectable clumps, and were free (> 99%) of growing cells, germinated spores, and cell debris, as verified using a phase-contrast microscope [26].

2.3. Preparation of spore samples

To ensure a homogeneous and reproducible treatment of the samples, the spores are deposited on glass substrates with a diameter of 11 mm by spray deposition. The glass slides used were chosen to be larger in diameter than the driven electrode of the DBD to ensure that the discharge does not interact with the rough edges of the glass substrates making the plasma less homogeneous. The disadvantages of this arrangement, such as the decaying photon flux and radical densities towards the edges, are discussed in section 4. A self-built spray device described in [27], based on a similar device presented in [28], is used. A spore solution of 5 × 10$^9$ spores ml$^{-1}$ is sprayed for 150 ms using 1.3 bar nitrogen (purity 5.0, Air Liquide, Germany) as the carrier gas. To verify the homogeneity of the sprayed solution and to check if the spores are present in a monolayer on the substrate, scanning electron microscopy (JSM6510, JEOL, Eching, Germany) is performed.

2.4. Treatment conditions

The samples are placed on a sample holder made specifically for the glass slides used to ensure that every sample is placed directly underneath the electrode in a reproducible way.

![Figure 1. Sketch of the experimental setup of the DBD inside a vessel (left) and the corresponding voltage and conduction current characteristics of the DBD for a pulse repetition frequency of $f = 1000$ Hz and a peak-to-peak voltage of 24 kV pp (right).](image-url)
All samples are handled in the same way: first, the sample is placed inside the vessel on the sample holder. Then, the vessel is evacuated down to approximately 1 mbar in order to minimise the impact of impurities during the plasma treatment. In order to ensure stable conditions during the plasma treatment, the composition of the process gas, as well as the pressure, is adjusted thereafter. Finally, the sample is treated with the DBD.

In this study, the samples are treated with DBD produced in different mixtures of nitrogen/oxygen/humidity and power densities. Unless stated otherwise, all experiments were carried out with a pulse repetition frequency of 300 Hz and a maximum voltage amplitude of 24 kVpp.

2.5. Spore survival assay

After the plasma treatment, each sample is covered with 60 μl of 10% polyvinyl alcohol (PVA) solution to remove more than 95% of the spores from the glass slides [29]. The PVA is applied to an area with a radius of 10 mm to exclude spores at the edge of the sample carriers for two reasons: firstly, the edges of the sample carriers show small fractures from the manufacturing process. Spores lying in cavities or trenches would likely be shielded from UV emission and radicals which would influence the reproducibility of the samples. Secondly, as shown in the results section, the flux of reactive plasma components changes significantly from the center of the electrode to the side. To reduce the influence of this effect only spores located in the plasma region are taken into account for the analysis. The PVA coated samples were dried on a clean bench until the PVA layer was completely solidified. The dried PVA film is then removed from the substrate and dissolved in 120 μl water. This procedure makes it possible to recover spores without affecting their viability [18]. Afterwards, serial dilutions of the recovered samples are plated on LB agar plates as described previously in [27]. Plates are then incubated overnight at 37 °C, after which, colonies are counted. Plates with a number of colonies between 1 and 1000 are included in the calculation of the survival rates. For higher numbers, overlapping or merging of the colonies is observed. All data shown are expressed as averages (±) standard deviations (n = 3, 10).

2.6. Optical emission spectroscopy

For the determination of the UV irradiance experienced by the biological samples, absolute measurements were performed using an echelle spectrometer (ESA 4000, LLA Instruments, Germany). The spectrometer has a spectral range between 200 nm and 800 nm with a spectral resolution of 0.015 nm < Δλ < 0.06 nm. To calculate the UV irradiance absolutely calibrated intensities have to be obtained. The calibration procedure as well as the determination of absolute intensities is described in detail in [30]. As a result, the number of photons s⁻¹ nm⁻¹ at the fibre entrance L_{abs}(λ) is known. To calculate the UV irradiance experienced by the biological samples, an aperture is used to define the plasma volume (V_{plasma}) seen by the optical fibre. Therewith, the average emissivity of the plasma ε(λ) in photons s⁻¹ nm⁻¹ cm⁻³ can be determined. This procedure assumes a homogeneous plasma emission in the whole volume which was demonstrated for N₂ emission using a CCD camera in [31]. However, as the sample carrier in contact with the discharge has the same dimension as the electrode and the plasma volume, the irradiance experienced by the spores strongly depends on their position on the sample carrier. This is the case as each emitting particle in the plasma volume radiates homogeneously in 4π and the fraction of light reaching the sample depends on the distance between the emitting particle and the sample. Figure 2 shows a sketch of the plasma volume with the electrode at the top and the sample at the bottom of the plasma cylinder. The particles in the volume r d φ dr dz irradiate their emission on a surface of a sphere of 4πr² with ρ = √r² + z² being the sphere radius. If the radiation reaches the surface tilted, the effective area of the surface seen by the particle decreases and has to be corrected with the cosine of the angle between surface normal and photon yielding

\[
\cos(\arctan(r/z)) = \frac{z}{\sqrt{r^2 + z^2}}.
\]

Thus, the geometrical factor to calculate the irradiance at the center of the sample carrier G₀ generated by the whole plasma volume is given by

\[
G_0 = \int_0^{r_{max}} \int_0^{\varphi_{max}} \int_0^{2\pi} \frac{1}{4\pi r^2} \frac{1}{\sqrt{(\xi)^2 + 1}} \ r \ d \varphi \ dr \ dz.
\]  

To determine the photon flux on the sample at positions off the center, the position on the sample carrier has to be shifted by r₀. Due to the rotational symmetry the resulting value is
valid on a circle with radius \( r_0 \). By shifting the point of irradiance, the distance \( \rho \) to the emitting particle changes. As the distance in \( z \)-direction is not affected by the change of \( \rho \), \( \rho \) only depends on the shift in the \( r - \varphi - \) plane. Figure 2 depicts the resulting projection of \( \rho \) on the sample plane \( \rho_{r\varphi} \). As \( r, r_0 \), and \( \varphi \) are known, \( \rho_{r\varphi} \) can be determined using the cosine rule:

\[
\rho_{r\varphi}^2 = (r - r_0 \cos(\varphi))^2 + (r_0 \sin(\varphi))^2 \tag{2}
\]

Therefore, the geometrical factor to calculate the irradiance at distance \( r_0 \) to the center \( G(r_0) \) is

\[
G(r_0) = \int_0^{\rho_{r\varphi}^2} \int_0^{\rho_{r\varphi}^2} \int_0^{2\pi} \frac{1}{4\pi(\rho_{r\varphi}^2 + z^2)} \left( \frac{\rho_{r\varphi}^2}{2} \right) \frac{1}{\sqrt{(\rho_{r\varphi}^2)^2 + 1}} r \, d\varphi \, dr \, dz. \tag{3}
\]

Due to the symmetry of the system, \( G(r_0) \) is also valid for the upper electrode with the aluminium oxide cover which has a reflectivity of around 90% in the UV range [32, 33]. Thus, to determine the total irradiance reaching the sample, the reflection from the electrode must also be taken into account. For this, a diffuse reflection of the irradiance \( G(r') \) at any point \((r', z_{max})\) on the aluminium oxide is assumed (note that the upper radial vector is chosen to \( r' \) and the radial vector on the sample carrier to \( r_0 \)). Each point is a radiating surface emitting into a half sphere. The half sphere radius is the distance between the reflection point \( r', z_{max} \) to the point on the sample \((r_0, 0)\). As in the first case, the \( z \)-component is constant and the change in the radius is determined by the projection of the distance vector on the \( r - \varphi - \) plane \( \rho_{r\varphi}' \), which is again calculated with the cosine rule:

\[
\rho_{r\varphi}'^2 = (r' - r_0 \cos(\varphi))^2 + (r_0 \sin(\varphi))^2. \tag{4}
\]

In addition, the effective area of the reflecting upper electrode and of the sample carrier have to be taken into account using a cosine correction as the line-of-sight from the emission point \((r_0', z_{max})\) to the observation point \((r_0, 0)\) is not parallel to the normal of both surfaces. Thus, the geometrical factor to calculate the reflected irradiance from the upper electrode to a point on the sample carrier \( G_{\text{refl}}(r_0) \) is determined with

\[
G_{\text{refl}}(r_0) = \frac{1}{\pi(\rho_{r\varphi}^2 + z_{max}^2)} \left( \frac{\rho_{r\varphi}^2}{2} \right) \frac{1}{\sqrt{(\rho_{r\varphi}^2)^2 + 1}} r_0 \, dr_0 \, d\varphi'. \tag{5}
\]

Thereupon, the total geometrical factor to determine the irradiance at a point on the sample carrier \( G_{\text{tot}}(r_0) \) assuming a homogeneous discharge with constant emissivity and a reflectivity of the upper electrode of 90% is

\[
G_{\text{tot}}(r_0) = G(r_0) + G_{\text{refl}}(r_0). \tag{6}
\]

By taking the constant emissivity \( \varepsilon(\lambda) \) and the photon energy \( \frac{hc}{\lambda} \) of a photon with wavelength \( \lambda \), the Planck constant \( h \) and the speed of light \( c \) into account, the UV energy flux at a position \( r_0 \) on the sample carrier \( [UV](r_0) \) in Joule \( s^{-1} \) \( cm^{-2} \) or the total UV energy flux on the sample carrier \([UV]\) in Joule \( s^{-1} \) is calculated:

\[
[UV](r_0) = \int_{[UV]} \varepsilon(\lambda) \frac{hc}{\lambda} G_{\text{tot}}(r_0) \, d\lambda \tag{7}
\]

\[
[UV] = 2\pi \int_{0}^{\rho_{r\varphi}^2} [UV](r_0) \, r_0 \, dr_0. \tag{8}
\]

### 2.7 Optical absorption spectroscopy

Absolute densities of ozone are determined by applying absorption spectroscopy within the range of \( \lambda = 200–320 \) nm. A deuterium lamp (L9518, Hamamatsu Photonics K.K, Japan) and a broad-band spectrometer (QE65000, Ocean Optics, Germany) are used, as well as two parabolic mirrors (effective focal length = 25 cm), in order to establish a collimated light.
beam. The light emitted from the deuterium lamp is collimated by a mirror into a parallel beam which is directed through the plasma volume onto another mirror. This second mirror is used to focus the light beam onto the entrance of the optical fibre connected to the spectrometer. The absolute densities of ozone are determined applying the Beer–Lambert law.

\[ I_T(\lambda) = I_0(\lambda) \cdot \exp \left( -\sigma_{253nm}(\lambda) \cdot n_{O3} \cdot l \right). \]  \hspace{1cm} (10)

In equation (10), \( I_T(\lambda) \) denotes a measured signal that is transmitted through the medium, \( I_0(\lambda) \) denotes the second measured signal without absorption but along an identical path, \( \sigma_{253nm}(\lambda) \) and \( n_{O3} \) denote the absorption cross section and the density of the absorbing medium, and \( l \) denotes the length of the absorption path. Dividing by \( I_0(\lambda) \), applying the natural logarithm, and rearranging for \( n_{O3} \) yields the density. To correct the incident and the transmitted spectral intensity \( I_0(\lambda) \) and \( I_T(\lambda) \) for the background signal as well as the plasma emission, four measurements are needed:

\[ \frac{I_T(\lambda)}{I_0(\lambda)} = \frac{I_{\text{plasma on, lamp on}}(\lambda) - I_{\text{plasma on, lamp off}}(\lambda)}{I_{\text{plasma off, lamp on}}(\lambda) - I_{\text{plasma off, lamp off}}(\lambda)}. \]  \hspace{1cm} (11)

\( I_{\text{plasma on, lamp on}}(\lambda) \) represents the intensity of the light source with the plasma source ignited and \( I_{\text{plasma on, lamp off}}(\lambda) \) the plasma emission which has to be taken into account. The intensity of the light source \( I_{\text{plasma off, lamp on}}(\lambda) \) is corrected for the background signal \( I_{\text{plasma off, lamp off}}(\lambda) \). The absorption cross section of ozone \( \sigma_{253nm} \) at \( \lambda = 253.65 \text{ nm} \), the maximum of the Hartley absorption band [34], is used. The head of the Hartley absorption band is used because this part of the band mainly represents the absorption due to ozone molecules in the ground state while the edges of the band are slightly more sensitive to vibrationally excited ozone molecules which are sensitive to the gas temperature [35]. The temperature dependence is accounted for via a polynomial fit derived from the data presented in [35].

3. Results

3.1. Spore sample preparation

The influence of the power density on the CFU count is depicted in figure 4 (right). The power density is controlled by changing the repetition frequency between 300 Hz and 1000 Hz and the voltage amplitude between 18 \( \text{KV}_{pp} \) and 24 \( \text{KV}_{pp} \). The fraction of surviving spores were determined after 1 min and 2 min treatment time. In general, the reduction of CFU correlates with the power density with CFU count after 2 minute treatments decreasing by approximately 1.5 log from the lowest to the highest power density.
3.3. Inactivation of spores in humidity controlled nitrogen/oxygen atmospheres

The following results were obtained using a sealed vessel to control the atmospheric conditions under which the inactivation experiments were performed. Figure 5 (left) depicts CFU counts after B. subtilis spore treatment in dry atmosphere with varying nitrogen/oxygen content. In general, the results of treatments using gas mixtures with an oxygen content $\geq 20\%$ are comparable, resulting in a small reduction in CFU count of approximately one log after 10 min. Interestingly, in a nitrogen atmosphere with oxygen residuals ($\leq 0.1\%$) the CFU count decreases more quickly showing a two log decrease in the same treatment time. However, this reduction is already achieved after 5 min suggesting that prolonged treatment times have only a minor effect. An oxygen fraction of $\sim 0.1\%$ corresponds to the impurities in the vacuum vessel during the inactivation experiments using pure nitrogen as process gas. This impurity content was estimated applying optical emission spectroscopy by comparing the spectrum of the DBD obtained during the inactivation experiments, with only nitrogen gas fed into the chamber, to spectra of the DBD ignited in nitrogen with distinct admixtures of oxygen. While this approach does not allow for the measurement of a precise impurity content, it does allow for the order of magnitude of the oxygen impurities to be assessed.

The influence of the relative humidity content on the inactivation efficiency of B. subtilis spores in synthetic air (N$_2$:O$_2$ 79%:21%) is depicted in figure 5 (right). Treatment times of 1 min are compared for 0%, 25%, 40%, 65%, and 80% relative humidity. The addition of water vapour strongly increases the inactivation efficiency from less than one order of magnitude in dry air up to approximately two orders of magnitude at 80% relative humidity. The rise in efficiency scales nearly exponentially with the relative humidity increase.

3.4. Ozone density in humidity controlled nitrogen/oxygen atmosphere

Figure 6 depicts the volume averaged ozone density produced by the DBD in dry nitrogen/oxygen mixtures and with varying relative humidity in synthetic air. In a dry atmosphere, the ozone density is highest at an oxygen content of 80% reaching a value of $1.3 \times 10^{17}$ cm$^{-3}$. In nearly pure oxygen and with 50% nitrogen the density of ozone is similar at $1.1 \times 10^{17}$ cm$^{-3}$. With increasing nitrogen content the ozone density...
strongly decreases to $6.2 \times 10^{16} \text{ cm}^{-3}$ at 80% nitrogen content and below the detection limit ($n_{O_3} = 3.0 \times 10^{13} \text{ cm}^{-3}$) in nearly pure nitrogen atmosphere. In synthetic air ($N_2$:O$_2$ 79%:21%) the addition of water into the gas phase significantly reduces the ozone concentration in the discharge. In a dry atmosphere the ozone density is $7.0 \times 10^{16} \text{ cm}^{-3}$ and decreases to nearly a third at 80% relative humidity.

### 3.5. UV dose in humidity controlled nitrogen/oxygen atmosphere

The diameter of the Al$_2$O$_3$-covered, driven electrode and the sample carrier are nearly identical which results in an inhomogeneous irradiation of the spores even in the case of a homogeneous discharge below the whole electrode. The irradiance profile, determined with equation (7), as a function of radial distance from the center of the sample carrier is shown in figure 7 as well as the exemplary UV irradiance profile in a N$_2$:O$_2$ mixture of 80%:20% calculated with equation (9). The calculations show that spores in a circle of 0.4 cm radius in the center of the discharge experience a similar irradiance. However, with increasing distance the photon flux is significantly reduced, down to 50% at the edge of the driven electrode at $r_0 = 0.5$ cm and to 10% at $r_0 = 0.6$ cm (outside of the active plasma zone). Thus, the UV irradiance changes significantly depending on the position of the spore on the sample carrier and has to be taken into account in the interpretation of the inactivation results. To compare the UV irradiance for different gas mixtures, the photon flux, separated into different wavelength ranges, at the center of the sample carrier is given in figure 8 for each treatment condition.

Only in nitrogen with residual oxygen a significant amount of UV-C emission is produced resulting from the formation of NO and emission from the NO$_x$ band (NO(A-X)) [36]. This can be observed in the emission spectra shown in figure 9, for the DBD operated in a N$_2$:O$_2$ mixture of 99.9%:≤0.1% and 80%:20% at 300 Hz and 24 kV$_{pp}$. The wavelength ranges corresponding to UV-A, -B and -C are marked. Under both conditions the second positive (N$_2$(C-B)) system and the first negative (N$_2^+$ (B-X)) system of nitrogen are observed in the UV-A range. This emission dominates the UV spectrum under most conditions studied.

### 4. Discussion

The discussion of the results will be divided into two parts: first, the time and power variation experiments in...
ambient air will be discussed which serve as a proof-of-concept to demonstrate the possibility of inactivating spores of *B. subtilis* with an atmospheric-pressure DBD. Afterwards, the results of the experiments in defined atmospheres at intermediate power (f = 300 Hz, \( V_{pp} = 24 \text{ kV}_{pp} \)) will be examined regarding different inactivation mechanisms.

### Time and power variation in ambient air

The results of the time and power variation (figure 4) measurements demonstrate that *B. subtilis* spores can be inactivated by the DBD used in this study under ambient conditions. The time variation reveals a fast inactivation in the first three minutes and strong decrease of the inactivation rate for longer treatment times. In low-pressure plasmas, this effect is normally observed in the case of stacked spores where the lower spores are protected from the plasma components by the upper ones [28]. However, the sample preparation analysis shows that the treated spores are not stacked but are assembled as monolayers on the glass slides. Therefore, this effect must be a result of the plasma itself and is most-likely connected to the spatial distribution of the flux of plasma components on the sample. In the case of photons, the emission profile on the sample carrier (figure 7) shows a significant drop in the photon flux at distances above 4 mm from the center if a homogeneous emission in the plasma volume is assumed. Taking the area of the sample carrier into account, approximately 50% of the spores are located in a region where the photon flux is strongly decreasing. The same effect holds true for radical species. Previous measurements reveal that the ozone density varies approximately 3.5-fold from the center to the edge of the plasma. Regarding the density of atomic oxygen, a 6-fold reduction has been observed [31]. The implication is that the spores present in the centre of the plasma region where UV fluxes and reactive species densities are high are inactivated quickly and account for the fast decrease in CFU count at short treatment times. The slow decrease at longer treatment times can then be related to inactivation of spores in regions of lower UV irradiance and reactive species concentration at the edges of the active plasma region. This has to be kept in mind during the interpretation and discussion of the results.

Since the plasma filaments are relatively homogeneously distributed, the UV fluxes as well as the radical concentrations take on a diffuse profile on the long time scales used in this study. This can also be observed in the previous profile measurements of the O and O\(_2\) densities [31] as well as in the phase-resolved electron density distributions of the discharge published in [22].

Increasing the power by using higher peak voltages and/or frequencies results in faster inactivation. Table 1 lists the power densities and surviving CFU after 1 minute and 2 minutes treatment. In general, the inactivation rate scales quite well with power density taking into account the variation of the biological results. This suggests that increasing radical and/or photon fluxes leads to a faster inactivation, but has to be confirmed by future measurements with varying power.

### Controlled dry atmosphere

Although experiments in ambient air show a reduction of *B. subtilis* spores by several orders of magnitude in a few minutes depending on the power density, the mechanisms leading to the inactivation cannot be easily analysed due to changing conditions from day to day, especially in the case of humidity. Furthermore, the role of the different photon or radical species cannot be differentiated as the gas mixture is fixed and controlled changes in the plasma chemistry are not possible. By performing inactivation experiments inside a sealed chamber the gas mixture as well as the humidity can be controlled, leading to a controlled change in plasma-produced components. In the absence of humidity in the gas phase, a decrease in the O\(_2\) content from \( \approx 99\% \) to 20\% (increase in N\(_2\) content) leads to a reduction in the ozone concentration and an increase in total UV emission. When the O\(_2\) content is less than 0.1\% the ozone concentration drops below the detection limit (\( 3 \times 10^{13} \text{ cm}^{-3} \)) and the total UV emission increases strongly, including a substantial increase in UV-C emission from nitric oxide (see figures 6, 8 and 9). Considering first the range of O\(_2\) content between 20\% and 99\% no clear correlation between the inactivation of *B. subtilis* spores and the density of ozone can be observed. In this range, a small reduction of CFU count with time is observed that is rather independent of the ozone density. This is in strong contrast to [11] where full inactivation of different spores with a base contamination of \( 1 \times 10^7 \) to \( 1 \times 10^8 \) is achieved after 5 min to 10 min treatment time using an atmospheric surface microdischarge plasma source. That system was used in a closed environment with ambient air and generated an ozone concentration of 9.3 ppm which is approx. 4.7 times greater than that measured in the DBD used in this work when ignited in dry synthetic air. As both systems show similar ozone densities but different inactivation results using spores of *B. subtilis*, this effect is most-likely attributed to the relative humidity in the system. Possible inactivation mechanisms will be discussed in the respective section.

For a gas mixture of \( 99.9\% \leq 0.1\% \) N\(_2\):O\(_2\), UV-C emission is present, without ozone, and a decrease in CFU of two orders

### Table 1. Influence of the power density on the inactivation of *B. subtilis* spores in ambient air.

| \( V_{pp} \) (kV) | \( f \) (Hz) | power density (W cm\(^{-1}\)) | CFU | ratio to lowest power | inverted ratio to lowest power |
|------------------|--------------|-------------------------------|-----|-----------------------|---------------------------------|
| 18               | 300          | 0.05                          | 2.6 \( \times \) 10\(^6\) | 5.9 \( \times \) 10\(^5\) | ---                             |
| 24               | 300          | 0.2                           | 6.2 \( \times \) 10\(^5\) | 1.3 \( \times \) 10\(^5\) | 4                               |
| 18               | 1000         | 0.25                          | 7.7 \( \times \) 10\(^6\) | 4.9 \( \times \) 10\(^5\) | 5                               |
| 24               | 1000         | 0.7                           | 1.3 \( \times \) 10\(^5\) | 1.2 \( \times \) 10\(^5\) | 14                              |

| CFU\(_{1\text{min}}\) | CFU\(_{2\text{min}}\) |
|-----------------------|-----------------------|
| 4                     | 4.1                   |
| 3.3                   | 4.5                   |
| 1.2                   | 20                    |
| 49                    |                       |
of magnitude in 5 minutes is observed. This is substantially faster than for the higher O₂ content cases where ozone is present in higher concentrations, which provides further indication that ozone is not the main driver of inactivation. In this case, the photon fluxes in the UV-C range are comparable to low pressure air plasmas that show fast deactivation. The results indicate that neither UV-A nor UV-B emission play a significant role in the inactivation of *B. subtilis* spores in this study. This finding is in accordance with inactivation experiments of Munakata *et al* applying monochromatic synchrotron radiation to *B. subtilis* spores [37]. Munakata *et al* defined an inactivation rate constant, \( k \), which correlates the photon flux at a specific wavelength to the spore inactivation. By applying the same formula to a broader wavelength band (e.g. UV-C), the applicability of the inactivation rate constant of the DBD marked as blue triangle. It can be seen that the calculated inactivation rate constant of the DBD marked as blue triangle.

\[
k = -\ln\left(\frac{N}{N_0}\right) \frac{1}{\Gamma_{\text{photons}}}.
\]

with \( N \) as the number of CFU after plasma treatment, \( N_0 \) indicating the initial spore load and \( \Gamma_{\text{photons}} \) representing the photon dose per m². Since only the spectrum of the gas mixture of 99.9%:≤0.1% N₂:O₂ is distributed throughout the UV-C range as depicted in figure 9, only this gas mixture is suitable for calculating the inactivation rate constant. The inactivation rate constant of the gas mixture 99.9%:≤0.1% N₂:O₂ and those calculated by Munakata *et al* are presented in figure 10. It can be seen that the calculated inactivation rate constant of this study correlates well with the data of Munakata *et al*. The calculated inactivation rate constant is an integral value since all values in the UV region above 200 nm were taken into account in contrast to the data of Munakata *et al* who treated the biological samples at a specific wavelength. However, the data correlates well indicating that the inactivation efficiency of the broadband plasma emission in 99.9%:≤0.1% N₂:O₂ is comparable to the inactivation efficiency of monochromatic light. This allows the assumption that synergistic effects do not lead to the inactivation of spores in this specific gas mixture and UV-C emission is the dominant inactivation mechanism under these conditions.

For treatment times between 5 and 10 minutes the CFU count was not found to decrease further. This effect can be attributed to the inhomogeneous photon flux on the sample carrier mentioned above (see figure 7). However, the flux profile is only valid for a homogeneous emission below the whole area of the driven electrode. Since the UV-C emission is emitted by nitric oxide it will exhibit a density profile, likely similar to that of ozone, with a maximum at the center and a decrease to the edges of the electrode [31]. This would further increase the UV flux gradient from the center to the side of the sample carrier and would prolong the time required to inactivate spores located further from the center of the sample holder. Thus, a potential explanation for the inactivation kinetics observed in a gas mixture of N₂:O₂ 99.9%:0.1% is that spores in the center receive much higher fluxes of UV-C emission and NO radicals than in the outer parts and are inactivated on significantly shorter time scales.

To verify the assumption that the inactivation of *B. subtilis* spores depends on the flux profiles of the UV radiation and radicals underneath the driven electrode, spores sprayed on glass substrates were transferred via stamping onto LB agar plates after plasma treatment. Afterwards, the plates were incubated overnight at 24 °C and for 6 h at 37 °C to visualize spore revival and outgrowth in dependence of the treated sample carrier geometry. In figure 11 the results are depicted. It can be seen that colonies grow stochastically distributed within the whole area of the stamped control. After 2 min and 10 min of plasma treatment a ring-structure can clearly be seen which correlated with the treatment time. These results indicate that the inhomogeneous emission below the whole area of the driven electrode due to different flux profiles of plasma-generated species has an impact on the inactivation efficiency of the experimental setup.

**Controlled humidity**

To analyze the effect of humidity on the inactivation of *B. subtilis* spores the humidity content in synthetic air (N₂:O₂ 80%:20%) is controlled and the samples are treated for 1 min. By adding humidity to the gas phase the inactivation can be increased from approx. 0.5 orders of magnitude with 0% relative humidity to 2.5 orders of magnitude at 80% relative humidity. At the same time, the ozone density decreases by a factor of 4, no UV-C emission is present, and the emission in the UV-B and UV-A does not correlate with spore inactivation.
Inactivation mechanisms

The results demonstrate that neither UV-B/UV-A emission nor ozone play a significant role individually in the inactivation of \textit{B. subtilis} spores in this study. In contrast, UV-C emission (and possibly the flux of nitric oxide which emits the UV-C radiation) is effectively inactivating spores if enough photons reach the sample. This is in agreement with experiments in the low-pressure regime [27] and also known from the literature using UV-C lamps [19, 21, 29]. The strong inactivation effect due to humidity suggests a crucial influence of water-related products, such as OH, H$_2$O$_2$, or gas-phase acids such as HNO$_2$, HNO$_3$, or H$_2$O$_2$NO, which are expected to be produced in humid air plasmas [38]. Furthermore, ozone seems to be of minor importance as the inactivation of \textit{B. subtilis} spores is uncorrelated to the ozone density. The importance of humidity has also been demonstrated by Ishizaki et al [39] by inactivating different \textit{Bacillus} spores using ozone in the gas phase (250–1500 ppm) in controlled relative humidity (50%–90%). The inactivation was drastically reduced with decreasing water content and even stopped for one strain below 54%. A correlation between relative humidity (70%–95%) and inactivation efficiency of \textit{B. subtilis} spores in ozone containing atmosphere (500–5000 ppm) was also observed by Aydogan et al [40]. In both studies the time needed for 1-log reduction varied between a few minutes to several hours. Furthermore, this is a possible explanation why inactivation of spores seems to be much faster using atmospheric pressure plasmas in humid air (e.g. [41]) because of the faster generation of water related species like H$_2$O$_2$.

This assumption is also supported by known inactivation mechanisms of \textit{B. subtilis} spores from the literature: while the spore coat layers are of major importance in resisting oxidizing agents, such as ozone or peroxyxinitrite, they are only of minor importance regarding the resistance of the spore to hydrogen peroxide [19, 42, 43]. For most oxidizing agents the layers seem to act as a ‘reactive armor’ which detoxifies the chemicals at the outer layers of the spore coat and prevent damaging of the inner membrane, for example [42, 44]. Damaging the inner membrane may cause it to rupture during germination and result in spore death. However, the precise mechanism of the process is not known [42]. Therefore, the strong inactivation in the presence of water in the gas phase compared to ozone might be connected to the different reactions of the molecules with the \textit{B. subtilis} spore coat layers preventing the ozone from reaching the inner membrane while hydrogen peroxide, for example, leads to a significant damage. In addition, a few chemicals, such as formaldehyde or nitrous acid (HNO$_2$), are known to cause spore death by inducing DNA damage [19, 42, 45] and might play a role in the inactivation of spores if they are generated during the plasma-spore interaction. Although the damage can be observed, it is not clear how the chemicals penetrate the spore core, but a connection to the permeability of the inner membrane is assumed [42]. However, this is not the case for hydrogen peroxide as small, acid-soluble proteins (SASPs) protect the DNA [42]. Furthermore, the efficiency of water-related products might be directly connected to UV emission (UV-B, UV-A) or generated species, such as ozone, which do not have a significant effect on their own but might be necessary in the reaction pathway for spore inactivation.

5. Conclusion

Inactivation experiments are performed using \textit{B. subtilis} spores and a non-thermal atmospheric pressure discharge, namely a dielectric barrier discharge, as well as spectroscopic measurements to determine the ozone density and the UV irradiance. The experiments are carried out in ambient air, as well as in a vacuum vessel, in order to control the atmospheric conditions, the relative N$_2$:O$_2$ mixture and humidity content. This approach offers the possibility to execute inactivation experiments with the focus on the differentiation of possible inactivation mechanisms. It can be concluded that neither UV-A and UV-B irradiance nor ozone alone contribute significantly to the inactivation of \textit{B. subtilis} spores. Comparable with experimental results in the low-pressure regime the UV-C irradiance seems to play a role in spore inactivation under specific conditions. This conclusion is drawn due to the results obtained from the spectroscopic measurements regarding the UV irradiance as well as the ozone density. The strongest inactivation effect is evoked by humidified synthetic air, with spore inactivation directly correlating with humidity content. No positive correlation between UV irradiance or ozone concentration and spore inactivation is observed for
the humidity variation. This implies that other reactive species formed from water molecules, such as OH, H₂O₂, HNO₂, HNO₃, or H₂O₂, play an important role in spore inactivation. As such, consideration should be given as to how to properly control humidity content in applications in order to ensure reproducible outcomes for sterilisation processes.

In addition, the kinetics of spore inactivation demonstrate two timescales with high inactivation rates at short times, and low inactivation rates at longer times. This has been observed in previous experiments as a result of spore stacking. However, the spore spraying approach used for deposition of spores on surfaces in this work avoids this issue. In this case, two timescales with high inactivation rates at short times, and low inactivation rates at longer times. This has been observed in previous experiments as a result of spore stacking.

In this work, the spore spraying approach used for deposition of spores on surfaces avoids this issue. In this case, two timescales with high inactivation rates at short times, and low inactivation rates at longer times. This has been observed in previous experiments as a result of spore stacking.

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