Inactivation of bacterial endospores on surfaces by plasma processed air

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Abstract

Aims: In case of biological hazards and pandemics, personal protective equipment of rescue forces is currently manually decontaminated with harmful disinfectants, primarily peracetic acid. To overcome current drawbacks regarding supply, handling and disposal of chemicals, the use of plasma processed air (PPA) represents a promising alternative for surface decontamination on site. In this study, the sporicidal efficiency of a portable plasma system, designed for field applications, was evaluated.

Methods and Results: The developed plasma device is based on a dielectric barrier discharge (DBD) and operated with ambient air as process gas. PPA from the plasma nozzle was flushed into a treatment chamber (volume: 300 l) and bacterial endospores (Bacillus subtilis and Bacillus atrophaeus) dried on different surfaces were treated under variable conditions. Reductions in spores by more than 4 log10 were found within 3 min of PPA exposure. However, the presence of endospores in agglomerates or in an organic matrix as well as the complexity of the respective surface microstructure negatively affected the inactivation efficiency. When endospores were embedded in a dried protein matrix, mechanical wiping with swabs during exposure to PPA increased the inactivation effect significantly. Gaseous ozone alone did not provide a sporicidal effect. Significant spore inactivation was only obtained when water vapour was injected into the PPA stream.

Conclusion: The results show that endospores dried on surfaces can be reduced by several orders of magnitude within few minutes in a treatment chamber which is flushed with PPA from a DBD plasma nozzle.

Significance and Impact of the Study: Plasma processed air generated on site by DBD plasma nozzles could be a suitable alternative for the disinfection of various surfaces in closed rooms.

Introduction

Biological agents like pathogenic bacteria, viruses or toxins represent serious threats for human health. In case of biological hazards and pandemics, rescue forces such as firefighters, civil defence and disaster control or medical staff, must be protected from possible contamination, on the one hand by wearing suitable personal protective equipment and on the other hand by efficient, but user-compatible decontamination measures of their protective equipment. In biological worst-case conditions, the disinfection of personal protective equipment suspected to be contaminated with hazardous biologically agents should be highly effective within short time, easy to use and compatible with the environmental conditions (Lemmer et al. 2012). Peracetic acid (PAA) is used as a cold sterilization agent in many areas and it is currently mostly used as sporicidal agent for the disinfection of personal protective equipment of rescue forces. Although it represents a potent disinfectant with sporicidal activity, there are several drawbacks, including the necessity of rescue teams to transport hazardous chemicals, the
required proper disposal of the chemicals as well as the need for elaborate scrubbing of the personal protective equipment with brushes.

The use of cold atmospheric plasma (CAP) as sporicidal agent for the surface disinfection of protective suits could be a suitable approach to overcome these detriments. Plasma is referred to the fourth state of matter, a partially ionized gas composed of various reactive species including electrons, ions, free radicals, atoms and molecules in the ground or excited state as well as electromagnetic radiation (Bourke et al. 2017). Cold atmospheric plasma is not in thermal equilibrium since the temperature of its electrons is much higher than the temperature of ions and neutrals (Müller et al. 2018). The microbicidal activity of cold plasma has long been known and was demonstrated in various research studies over the last two decades. For disinfection and sterilization purposes it is most commonly created by dielectric barrier discharge (DBD) and atmospheric pressure plasma jets (Scholtz et al. 2015; Bourke et al. 2017). Various parameters are known to affect the sterilization efficiency, including the reactor geometry as well as the operating conditions like the gas pressure, the type of gas mixture, the gas flow and the frequency and power of plasma excitation. Plasma generated from air comprises reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as atomic oxygen (O) and singlet oxygen (\(^{1}\text{O}_2\)), superoxide anion (\(\text{O}_2^{-}\)), ozone (\(\text{O}_3\)), hydroxyl radicals (\(\text{HO}^\cdot\)), atomic nitrogen N, excited nitrogen N\(_2\) (A), nitric oxide (NO) or NO\(_2\) (Scholtz et al. 2015). These oxygen- and nitrogen-based reactive species have strong oxidative effects on microbial structures like lipids, proteins and DNA (Laroussi 2005). The microbicidal effect of CAP depends on various factors, especially the mixture of generated reactive species and the operational mode. Regarding the operational mode, it is decisive whether the surface to be disinfected is in direct contact to the plasma glow or indirectly treated by remote mode. The sporicidal action of atmospheric plasma in direct application mode is believed to be dominated by spore erosion due to a surface action of radicals and internal oxidative irreversible damage, subsequent to the diffusion of the radicals deeply inside the spores. UV photons causing DNA lesions can also be dominating the spore inactivation, depending on the operating conditions (Boudam et al. 2006). Direct plasma treatment of surfaces within the glow discharge is known to be highly efficient but its suitability for the disinfection of large areas with complex geometries appears to be limited (Müller et al. 2018). If exposed remotely, the surface to be disinfected is spatially separated from the plasma volume or in an adjacent chamber (Laroussi 2005). Although usually requiring longer treatment times, remote plasma offers a more gentle treatment of complex targets (Müller et al. 2018). Charged particles then play a minor role for disinfection since they recombine before arriving at the substrate, which is likewise the case for short-lived neutral reactive species (Laroussi 2005; Misra et al. 2011). In a few studies, the surface to be disinfected was placed in the flowing afterglow of the plasma source (Boudam et al. 2006; Moisan et al. 2014; Müller et al. 2018). The sporicidal effect of plasma processed air (PPA) which is flushed into a separated chamber of a larger volume has rarely been studied. In order to disinfect three-dimensional structures (e.g. protective suits) the entire surface must be exposed to the reactive species in order to enable an even and fast process. This could be achieved by use of plasma nozzles where the afterglow stream is directed into decontamination chambers, for example, tents. The use of such a reactive gas mixture appears to be a suitable alternative in case of biological hazards in order to enable an on-site decontamination of surfaces without chemicals.

In this study, the sporicidal efficiency of a newly designed portable plasma system based on a DBD and operated with ambient air was investigated. Since spores represent the most resistant form of micro-organisms, they are commonly used to assess the efficiency of sterilization processes. This is also a prerequisite for a later approval of this technology by the relevant authorities (e.g. Robert Koch Institute). Bacillus spores were used to simulate worst-case conditions and served as surrogates for any potentially relevant pathogenic bacteria, for example, Bacillus anthracis, which is of high relevance in the area of civil security. Plasma processed air from a plasma nozzle was introduced in a closed treatment chamber with a volume of approximately 300 l. The inactivation of bacterial endospores dried on different surfaces was assessed under various conditions. The complexity of the surface structure, the way the endospores are distributed on the surface, the impact of an organic matrix as well as the combination of PPA exposure with mechanical wiping was assessed. To the best of our knowledge, the sporicidal action of PPA has not been investigated in a comparable way yet.

Materials and methods

Plasma source and treatment conditions

A portable plasma system developed by the company Plasmametreat GmbH (Germany) was used for all test trials. Plasma processed air was generated with a CD40 plasma nozzle based on a DBD equipped with an air cooling system. Ambient air at a pressure of 6 bar was used as process gas (7.5 l min\(^{-1}\)). The input power was approximately 200 W and the discharge frequency was 13 500 Hz.
previous trials, it has been found that the introduction of water strongly improves the microbicidal action of PPA. Therefore, if not stated otherwise, the plasma afterglow was humidified by introducing water (1 ml min⁻¹) via a carrier gas stream (0.5 l air min⁻¹) and an evaporator (150°C). The water vapor was directed into the afterglow at the outlet of the plasma nozzle approximately 10 cm from the plasma discharge. A HPLC-pump Gynkotek M 300 (Gynkotek, Munich, Germany) was used for water injection. The water vapour was directed into the afterglow at the outlet of the plasma nozzle approximately 10 cm from the plasma discharge. A HPLC-pump Gynkotek M 300 (Gynkotek, Munich, Germany) was used for water injection.

The CD40 plasma nozzle was centrally placed on the bottom of a Glovebox made from acrylic glass (depth: 0.5 m; height: 0.6 m; width: 1 m). The Glovebox was equipped with a valve to avoid overpressure, ozone-resistant gloves PIEC16750Y106/10 (Piercan, Bondy Cedex, France) and a positioning system to introduce and remove samples. The experimental setup is schematically shown in Fig. 1.

Test strains, spore and sample preparation

Two different endospore-forming bacteria belonging to the genus Bacillus were used as test micro-organisms. B. subtilis DSM 4181 and B. atrophaeus DSM 675 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The two strains are recommended for the microbiological validation of aseptic filling machines operated by hydrogen peroxide and PAA according to the Industry Association for Food Processing Machinery and Packaging Machinery (VDMA 2016). Preparation of spore suspensions was done as described previously by Muranyi et al. (2007) using manganese sulphate for induction of sporulation. After harvesting, spore suspensions were stored at 5°C.

PET-films and Tychem® F protective suits made from Polyethylene (Tyvek with coated polymers) (DuPont, Neu-Isenburg, Germany) were used as substrates for surface disinfection trials. Pre-cut parts of 4 × 4 cm were wiped with ethanol and four pieces (samples) were placed together in quadratic Petri dishes respectively. Inoculation was done by either spraying or spot inoculation. Spray inoculation provides a more homogeneous distribution of spores on the respective surface, thereby avoiding the formation of agglomerates. In dependency of the endospore density of the applied suspension, the sprayed volume and other process parameters, it has been shown that monolayers of spores can be obtained (Muranyi et al. 2007). Spot inoculation on the other hand leads to the formation of spore agglomerates and multilayers, which may retard the inactivation. Furthermore, the application of small spots was conducted according to a method of the German Federal Office for Civil Protection. The distribution of endospores on PET samples was examined microscopically.

For this purpose, spores were washed two times with deionized water and finally diluted to approximately 5 × 10⁷ CFU per ml either in deionized water or in an aqueous solution of 1–10 g l⁻¹ bovine serum albumin (BSA) (Biomol, Hamburg, Germany). BSA was applied to investigate the sporicidal efficiency of the plasma gas when endospores are embedded in an organic matrix. In case of spraying, 5–7 µl of a prepared spore suspension was sprayed on an area of 2 × 2 cm with a two-substance nozzle (Schlick, Untersiemau, Germany) using nitrogen (2 bar) as process gas. The applied method was previously described by Muranyi et al. (2007). In case of spot inoculation, 5 × 2 µl of the prepared spore suspension was deposited on the sample surface respectively (Lemmer et al. 2012). Inoculated samples were immediately dried in a laminar flow safety cabinet for 10 min. Endospores did not germinate under these conditions, which was checked by phase-contrast microscopy.

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![Figure 1](wileyonlinelibrary.com)
Exposure of test micro-organisms to plasma processed air

Prior to each inactivation trial, the treatment chamber (Glovebox) was flushed with PPA for 20 min. The ozone concentration inside the chamber was measured with a GM-6000-RTI Ozomat (Anseros, Germany). After 20 min, the ozone concentration reached a maximum of 1.8 ± 0.2 g m⁻³ when 1 ml of water min⁻¹ was injected into the plasma afterglow and 2.1 ± 0.2 g m⁻³ without humidification. The humidity and temperature inside the chamber was measured with a Basetech BTTH-1014 Thermo-/Hygrometer (Conrad Electronics, Hirschau, Germany). The humidity inside the treatment chamber increased from 25 ± 5% to 90 ± 10% RH when 1 ml of water min⁻¹ was pumped into the plasma afterglow. The temperature increased from 21 ± 1 to 25 ± 1°C. After the plasma nozzle had been turned off, the ozone concentration inside the chamber decreased by about 50% (at 90% RH and 25°C) within 15 min. For the inactivation trials, four individual samples placed in one quadratic Petri dish were introduced into the Glovebox through a positing system and placed on lifting plates centrally located in the treatment chamber. In order to assure an indirect treatment, the turbulent PPA stream from the outlet of the plasma nozzle was not directed towards the sample surface (Fig. 1). The samples were treated inside the glovebox for different times up to a maximum of 10 min. Longer treatment times were not considered since they are not of practical relevance for the decontamination of personnel equipment of rescue forces. As long as not otherwise stated, the plasma system was in operation throughout the whole treatment duration. The impact of the parameters on the sporicidal efficiency were studied under the conditions indicated in Table 1.

In case of additional mechanical wiping, endospores (B. atrophaeus) were dispersed in 10 g l⁻¹ BSA, spot inoculated (5 × 2 µl) on the Tychem F protective suit and finally dried. The endospore concentration of the inoculation suspension was adjusted to 10⁷ CFU per ml, resulting in endospore counts of 10⁷ CFU per sample. While being exposed to the PPA for either 1 or 10 min in total, each sample surface was individually wiped with a swab with bristles (Master Amp Buccal Swab Brush; Epicentre Technologies, Madison, WI, USA) for 1 min. For this purpose, 10 µl of either sterile deionized water or plasma processed water (PPW) were used to resolve the dried protein matrix with embedded spores during wiping. PPW was obtained by collecting condensate in a flask at the nozzle outlet. The resulting pH value of the condensate was ~1. PPW was used immediately. Four individual replicate samples were treated respectively and the swab as well as the sample surface (Tychem F) were regarded as individual samples. The number of colony-forming units present on the swabs and the corresponding Tychem F samples was therefore determined separately. Untreated reference samples (four replicate samples) were manually wiped as well in order to determine the distribution of spores after wiping. For this purpose, the number of CFU on the Tychem F samples and on the swabs after wiping was likewise separately determined. The trials including mechanical wiping were performed twice in analogous manner.

Sample handling and enumeration of colony-forming units

Directly after exposure to PPA in the glovebox, the samples were transferred to 50 ml of sterile 1/4 strength ringer solution supplemented with 0.1% Tween 80 as detergent (Sigma Aldrich, St. Louis, MO, USA) in stomacher bags. All samples were manually rubbed for 1 min to detach spores from the sample surface and to suspend them homogeneously. The number of colony-forming units of the sample suspensions was determined by the pour plate technique using tryptic soy agar. 1 ml of undiluted or serially (10-fold) diluted sample suspension in 1/4 strength ringer solution was transferred to Petri dishes in triplicates (3 × 1 ml) respectively. After solidification, all plates were incubated at 30°C for 48 h before the number of colony-forming units was counted manually. Colony counts were calculated as CFU per sample on the basis of mean values from the three replicate plates and the respective dilution factor. Results are presented as mean values (n = 4 or n = 8) with standard deviations and respective log₁₀ reductions (log₁₀ (N₀ N⁻¹)). The errors of the log₁₀-reductions were derived from Gaussian error propagation. Where indicated, significant differences of mean values were assessed by t-testing using SIGMAPLOT 12.5 (Systat Software Inc., San Jose, CA, USA) on a significance level of P < 0.05.

Results

Endospores are known to be among the most resistant micro-organisms towards various chemical and physical stressors, including CAP (Bourke et al. 2017). Therefore, endospores from the two different bacterial species B. subtilis (DSM 4181) and B. atrophaeus (DSM 675) were used as surrogates for pathogenic micro-organisms. Both strains are recommended by the VDMA for microbiological validations of aseptic filling machines operated with PAA or hydrogen peroxide (VDMA 2016). Preliminary tests have shown that these two strains are slightly more resistant to PPA than B. pumilus DSM 492 and B. thuringiensis DSM 350 (data not shown). Latter is usually used as a surrogate strain for Bacillus anthracis.
When endospores were either spot inoculated or sprayed onto PET films and exposed to PPA without humidification of the plasma afterglow, the sporicidal effect was negligible in both cases (Table 2). Subsequently, different flow rates of water were applied in order to investigate the impact of water which is delivered to the evaporator connected to plasma nozzle (Table 3). For this purpose, a rather low spore density was adjusted by spray inoculation on smooth PET films in order to avoid any interfering matrix effects coming from agglomerate formation or complex surface microstructures as far as possible. A high sporicidal effect of PPA (reduction of the spore count by more than 3 log10 within 2 min) was obtained with flow rates of 0.5 or 1 ml water min\(^{-1}\). The humidity increased to more than 90% RH in both cases. The injection of a sufficient amount of water vapour into the plasma afterglow therefore proved to be crucial in order to obtain a high sporicidal effect. Based on these results, a flow rate of 1 ml water min\(^{-1}\) was used for all further trials. It was furthermore investigated, whether a significant sporicidal effect can also be obtained when the plasma nozzle is switched off after flushing the treatment chamber with humidified PPA. For this purpose, *B. atrophaeus* endospores were spray inoculated on PET films and treated in the chamber after the plasma nozzle and the vaporizer (1 ml water min\(^{-1}\)) had been running for 20 min. A maximum reduction by only 0.63 log10 was found after a treatment for 10 min whereas a reduction by more than 4.25 log10 was reached within 5 min when the nozzle and the vaporizer were operating during the treatment (Table 4). These findings showed that the plasma nozzle as well as the vaporizer necessarily need to be in operation during sample treatment in the chamber in order to obtain a high sporicidal effect.

In a next step, the inactivation efficiency of PPA on surfaces with different complexity of the microstructure was determined. In this context, the impact of the distribution of spores on the respective surface was assessed as well. For this purpose, the spores were either spray inoculated (Table 4) or spot inoculated (Table 5) and then dried on either PET films or Tychem F samples. Spores were either evenly distributed without interfering matrix effects coming from agglomerates or organic loads (spraying) or present spore multilayers and clusters (spot inoculation) which was likewise confirmed microscopically. Table 4 shows the inactivation of endospores on PET films and the Tychem F protective suit when samples were spray inoculated and exposed to PPA in the treatment chamber for different times. On PET films, the detection limit was reached after 5 min which corresponded to a reduction in the initial load by 4.24 log10 (*B. atrophaeus*) and 4.25 log10 (*B. subtilis*) respectively. The endospore reduction on the Tychem F protective suit was considerably lower. In this case, reductions in the initial load by 3.69 log10 (*B. subtilis*) and 3.54 log10 (*B. atrophaeus*) were reached after PPA exposure for 10 min. The presence of spores in agglomerates distinctively affected the inactivation efficiency compared to spray inoculation on both surfaces (Table 5). After the maximum treatment time of 10 min, reductions in the initial endospore load by 2.72 log10 (*B. subtilis*) and 2.47 log10 (*B. atrophaeus*) were found on the Tychem F samples. The inactivation efficiency on PET film was again higher, resulting in count reductions by 4.04 log10 (*B. subtilis*) and 4.41 log10 (*B. atrophaeus*) after 10 min. In addition, a lower flow rate of 0.5 ml water min\(^{-1}\) which is vapourized and injected into the afterglow stream was applied under these experimental conditions (Table 6). The determined colony counts on the Tychem F surface or the PET films were not significantly different to those obtained at a flow rate of 1 ml water min\(^{-1}\), which confirmed that the sporicidal effect is not affected by the water supply within a flow range between 0.5 and 1 ml min\(^{-1}\).
B. Kramer et al. on behalf of Society for Applied Microbiology

Sporicidal action of plasma processed air

Table 2
Inactivation of endospores from Bacillus subtilis and Bacillus atrophaeus dried on PET-films (spray and spot inoculation) in a volume of 300 l flushed with PPA without injection of water vapour into the plasma afterglow ($\lambda_{ex} = 4 \mu m$).

| Spore species | Treatment time (min) | Colony count (CFU per sample) | Log$_{10}$ (water) | Log$_{10}$ (PPW) |
|---------------|---------------------|-------------------------------|-------------------|------------------|
| B. subtilis   | 0                   | $4.15 \pm 2.34 \times 10^4$   | $-0.00 \pm 0.05$  | $-0.05 \pm 0.05$ |
|               | 5                   | $3.55 \pm 1.47 \times 10^4$   | $-0.02 \pm 0.04$  | $-0.06 \pm 0.06$ |
|               | 10                  | $3.65 \pm 1.31 \times 10^4$   | $-0.00 \pm 0.04$  | $-0.06 \pm 0.06$ |

The impact of an organic matrix, in which the endospores are embedded, was investigated using BSA. Endospores were suspended in BSA solution and subsequently spot inoculated and dried on the sample surfaces. The protein matrix should serve as a model for practically relevant organic contaminations on protective suits like, for example, blood, vomit or dirt. A concentration of $10 \text{ g} \text{l}^{-1}$ almost completely prevented the sporicidal action of PPA for both species within 10 min (Table 7). The BSA concentration was therefore distinctly higher when PPW was applied (1-46 log$_{10}$ after 10 min on Tychem F samples (Table 7). These results showed that even comparably low concentrations of protein may inhibit the sporicidal effect of PPA.

Since the protein matrix severely affected the sporicidal effect, PPA exposure was also tested in combination with mechanical wiping using swabs with bristles. In this case, a high inoculation density was chosen ($10^7$ CFU per sample), together with the highest protein concentration applied ($10 \text{ g} \text{l}^{-1}$) and spot inoculation on the more complex surface of the Tychem F protective suit. These conditions ought to represent a worst case scenario. After manual wiping of untreated reference samples for 1 min, it was found that the distribution of endospores in the swab or on the Tychem F surface was rather equal. Approximately 50% of the spores initially dried in the protein matrix were found in the swabs, while about 50% of the endospores were found on the Tychem F surface after wiping. Exposure of samples to PPA for 10 min without mechanical wiping did not cause any sporicidal effect at all (data not shown). However, when the organic matrix was resolved mechanically by manual wiping with a swab and 10 $\mu l$ of liquid (water or PPW) for 1 min, the inactivation of spores on the Tychem F samples was significantly increased (Fig. 2). Bacillus atrophaeus endospores were reduced by 4.59 log$_{10}$ within a treatment time of 10 min when 10 $\mu l$ of water were used. In case of wiping with PPW, the spore count was reduced to the detection limit corresponding to a reduction by more than 5.64 log$_{10}$. When samples were exposed to PPA for only 1 min, the reduction of the initial loads was 3.1 log$_{10}$ (water) and 3.53 log$_{10}$ (PPW). The spore reduction was therefore distinctively higher when PPW was applied instead of water. In contrast to the Tychem F surface, the inactivation of endospores present on the swabs was considerably lower. Reductions by 0.84 log$_{10}$ (wiping with water) and 3.26 log$_{10}$ (wiping with PPW) were found after 10 min treatment. When the treatment time was only 1 min, spore reductions by 0.66 log$_{10}$ (water) and 0.67 log$_{10}$ (PPW) were determined on the swabs (Fig. 2).
Discussion

Sporicidal effect of plasma processed air

The sporicidal action of different CAP systems has been demonstrated with different setups (Boudam et al. 2006; Bourke et al. 2017; Hertwig et al. 2018). It is well accepted that the highest sporicidal effect can be achieved when spores are directly exposed to the plasma discharge (Moisan et al. 2014). This is due to the incidence of UV photons as well as presence of highly reactive short-lived species, including accelerated ions and electrons, but also uncharged particles, such as excited atoms, molecules and radicals (Moisan et al. 2001). In the present study, significant inactivation of endospores has also been found with a remote operational setup where the target is not directly in the plasma discharge but spatially separated from the plasma source (Fig. 1). UV-irradiation was therefore not involved in the spore inactivation. Similar findings have already been reported in previous studies (Moisan et al. 2014; Müller et al. 2018) but it is yet difficult to attribute the endospore inactivation of remote plasma applications to certain reactive species (Müller et al. 2018). The plasma afterglow contains relatively few charged particles, being essentially comprised of neutral atoms, radicals and molecules, some of which are in an excited state (Herrmann et al. 1999; Moisan et al. 2001). When the gas exits the discharge volume, ions and electrons are rapidly lost by recombination (Herrmann et al. 1999) so only comparably stable species like, for example, ozone are likely to reach the target. An ozone concentration of about 2 g m$^{-3}$ close to the sample surface has been proven in the present study. The sporicidal effect of reactive components which are self-quenching, with a relatively short half-life, might depend on the respective time of flight to the target (Misra et al. 2011). Since the plasma discharge in the present study was at least 1 m away from the target surface, highly reactive components most likely did not reach the target. Using a circulating plasma afterglow generated by a surface microdischarge with humid air, Müller et al. (2018) reported about the inactivation of B. atrophaeus endospores by remote plasma in different treatment volumes of 0.54, 1.8 and 2.6 l by 2.6 $\log_{10}$ within 10, 20 and 30 min respectively. Their approach is comparable to our study, apart from the plasma source and the volume of the treatment chamber being more than 100-fold larger in the present study. Moisan et al. (2013) reported about the sporicidal effect of a flowing afterglow from a reduced-pressure N$_2$-O$_2$ discharge, where reductions in the initial load of B. atrophaeus, B. pumilus and Geobacillus stearothermophilus by approximately 3–4 $\log_{10}$ were reached within 15 min in a treatment chamber with a volume of 5.5 l (Moisan et al. 2013). Furthermore, Schnabel et al. (2019) found inactivation rates of B. atrophaeus endospores up to 2.74 $\log_{10}$ within 5 min in a treatment chamber of 4.5 l which was flushed with PPA derived from a microwave discharge. Similar spore reduction rates were obtained in the present study but the treatment chamber volume of 300 l was considerably larger compared to those mentioned in the above-cited studies. This shows that the DBD-plasma nozzle exhibits a high potential for the disinfection of surfaces even when operated in remote application mode.

In addition, our findings indicate that primarily water-based reactive species are responsible for the sporicidal effect. Ozone alone cannot be the cause for the sporicidal action. When no water vapour was injected into the plasma afterglow, no inactivation of spores was found within 10 min although the resulting ozone concentration in the treatment chamber was equal. A rather slow sporicidal effect of ozone has been demonstrated previously. Mahfoudh et al. (2010) reported that dry ozone at a concentration of about 8 g m$^{-3}$ causes a comparably slow inactivation of B. atrophaeus endospores. Two phases in the survival curves were found, exhibiting $D$-values of 130 and 360 min respectively. At 82% RH, significantly shorter $D$-values of 11 and 128 min were determined.

Table 3 Inactivation of endospores from Bacillus atrophaeus dried on PET-films (spray inoculation) in a volume of 300 l flushed with PPA with injection of different flow rates of water into the nozzle outlet. Treatment time: 2 min ($n = 4$)

| Water flow rate (ml min$^{-1}$) | Colony count (CFU per sample) | $\log_{10}$ ($N_0, N^{-1}$) |
|-------------------------------|-------------------------------|----------------------------|
| 0.00                          | $1.7 \times 10^5 \pm 5 \times 10^4$ | 0.25 $\pm$ 0.15           |
| 0.10                          | $9.6 \times 10^4 \pm 1.4 \times 10^4$ | 0.60 $\pm$ 0.14           |
| 0.37                          | $4.2 \times 10^4 \pm 2.2 \times 10^4$ | 3.76 $\pm$ 0.28           |
| 0.50                          | $2.9 \times 10^4 \pm 1 \times 10^4$ | 3.66 $\pm$ 0.39           |
| 1.00                          | $3.8 \times 10^4 \pm 3 \times 10^4$ |                             |

*Inoculated surface.
with 8 g m\(^{-3}\) ozone, which indicated a strong dependency of the sporicidal effect from the humidity (Mahfoudh et al. 2010). A relatively slow sporicidal effect of gaseous ozone was also reported by Aydogan and Gurol (2006) who likewise found that the inactivation of endospores depends on the respective humidity. A 3 log\(_{10}\) reduction of *B. subtilis* endospores dried on different surfaces was consequently found within 4 h at a concentration of 3 g m\(^{-3}\) of gaseous ozone under high humidity (90% RH) while at 70% RH, the spore reduction was <2 log\(_{10}\) within 4 h (Aydogan and Gurol 2006). According to these previous findings, an increased humidity appears to increase the sporicidal effect of ozone. However, the sole combination of ozone with a high humidity cannot be seen as the cause for the spore inactivation found in the present study, since significant endospore reductions were only found when the plasma nozzle and the vapourizer were in operation during the treatment. In contrast, almost no inactivation of *B. atrophaeus* was found when the treatment chamber was initially flushed with PPA for 20 min before the plasma nozzle and the vaporizer were switched off and the samples subsequently placed in the treatment chamber. Therefore, the combination of ozone and high humidity (>90% RH), being also present when the nozzle was not in operation during the treatment, was not the cause for the spore inactivation in this study.

In case of high humidity, H\(_2\)O\(_2\), OH\(^-\), \(\cdot\)OH radicals or H\(_2\)O\(_2\) are generated in nonthermal gas plasmas (Scholtz et al. 2015). Hydroxyl radicals are known to be highly reactive ROS species formed in air plasmas (Hähnel et al. 2010). Our previous work (Muranyi et al. 2008) has shown that highly oxidizing hydroxyl radicals derived from elevated humidity in a DBD are likely to be involved in the destruction of spore structures. Patil et al. (2014) showed that *B. atrophaeus* endospores can be reduced by more than 6 log\(_{10}\) within 1 min inside a sealed package with a DBD. A preferably high humidity proved to be a critical factor for the pronounced sporicidal effect, contributing to the formation of reactive species other than ozone (Patil et al. 2014). Using surface micro-discharge plasma, Jeon et al. (2014) reported about an increased inactivation of *G. stearothermophilus* endospores with increasing humidity. Their findings were attributed to a higher generation of water related species under high humidity such as hydrogen peroxide and hydroxyl radicals. The sporicidal effect of ozone was in contrast negligible but may have contributed to chemical reactions that formed water-related sporicidal species such as \(\cdot\)OH and H\(_2\)O\(_2\) (Jeon et al. 2014). The same authors furthermore assumed that condensation of plasma activated water on the sample surface may contribute to the inactivation of spores (Jeon et al. 2014). This effect could also have been relevant in the present study since the humidity in the
**Table 5** Inactivation of endospores from *Bacillus subtilis* and *Bacillus atrophaeus* dried (spot inoculation) on either PET films or pre-cuts of a Tychem® F protective suit in a volume of 300 l flushed with plasma processed air humidified with 1 ml water min$^{-1}$ ($n = 4$)

| Treatment time (min) | Spot inoculated/spores dried in water/PET$^*$ | Spot inoculated/spores dried in water/Tychem$^*$ |
|----------------------|---------------------------------------------|-----------------------------------------------|
|                      | *B. subtilis DSM 4181*                      | *B. subtilis DSM 4181*                        |
|                      | Colony count (CFU per sample) $\log_{10}$ ($N_0 \times N^{-1}$) | Colony count (CFU per sample) $\log_{10}$ ($N_0 \times N^{-1}$) |
| 0                    | 4.1 E+5 ± 2.4 E+4                          | 4.2 E+5 ± 1.1 E+5                            |
| 5                    | 8.0 E+3 ± 6.9 E+3                          | 5.5 E+4 ± 4.2 E+4                            |
| 10                   | 3.7 E+1 ± 2.1 E+1                          | <1.7 E+1                                     |

$^*$Inoculated surface.

**Table 6** Inactivation of endospores from *Bacillus subtilis* and *Bacillus atrophaeus* dried (spot inoculation) on either PET films or pre-cuts of a Tychem® F protective suit in a volume of 300 l flushed with plasma processed air humidified with 0.5 ml water min$^{-1}$ ($n = 4$)

| Treatment time (min) | Spot inoculated/spores dried in water/PET$^*$ | Spot inoculated/spores dried in water/Tychem$^*$ |
|----------------------|---------------------------------------------|-----------------------------------------------|
|                      | *B. subtilis DSM 4181*                      | *B. subtilis DSM 4181*                        |
|                      | Colony count (CFU per sample) $\log_{10}$ ($N_0 \times N^{-1}$) | Colony count (CFU per sample) $\log_{10}$ ($N_0 \times N^{-1}$) |
| 0                    | 3.9 E+5 ± 6.4 E+4                          | 5.7 E+5 ± 3.8 E+4                            |
| 5                    | 8.8 E+3 ± 6.2 E+3                          | 2.5 E+4 ± 2.4 E+4                            |
| 10                   | 2.4 E+2 ± 1.5 E+2                          | <1.7 E+1                                     |
Table 7  Inactivation of endospores from *Bacillus subtilis* and *Bacillus atrophaeus* on PET films (spot inoculation) in a volume of 300 l flushed with plasma processed air. Spores were either dried in water or in 10 g l⁻¹ bovine serum albumin (n = 4)

| Treatment | Colony count (CFU per sample) | Log₁₀ | Colony count (CFU per sample) | Log₁₀ |
|-----------|-------------------------------|-------|-------------------------------|-------|
|           | (N₀, N⁻¹)                     |       | (N₀, N⁻¹)                     |       |
| 0         | 4.7 E+5 ± 8.9                 |       | 5.9 E+5 ± 2.1                 |       |
| 0         | E+4                           |       | E+4                           |       |
| 5         | 3.3 E+5 ± 0.09                | 0.16  | 3.7 E+5 ± 3.7                 | 0.20  |
| 5         | E+4                           |       | E+4                           |       |
| 10        | 2.7 E+5 ± 0.11                | 0.25  | 4.2 E+5 ± 2.6                 | 0.15  |
| 10        | E+4                           |       | E+4                           |       |

*Inoculated surface.

### Impact of PPA

The surface topography of PET has been shown to have general and irregularities where endospores might be protected. The Tychem F surface exhibits crevices and irregularities where endospores might be protected. The smooth effect of PPA was in general lower on the Tychem F surface compared to the PET film even though Tychem F surface adhesion was comparable. The complexity of the Tychem F surface topography therefore had significant impact on the spore inactivation. The Tychem F surface adhesion was comparable. The complexity of the Tychem F surface topography therefore had significant impact on the spore inactivation.

### Sporicidal action of plasma processed air

Based on the presented results as well as the above cited findings, further studies are needed in order to clarify which water related species are exactly responsible for the sporicidal effect of PPA, and in which way they may be meriated with other components.

The Tychem F surface compared to the PET film even though Tychem F surface adhesion was comparable. The Tychem F surface topography therefore had significant impact on the spore inactivation. The Tychem F surface adhesion was comparable. The complexity of the Tychem F surface topography therefore had significant impact on the spore inactivation.
implications on the inactivation efficiency of atmospheric plasma. In case of complex food surface structures which can be found, for instance, on fresh produce like lettuce, the microbicidal action is commonly affected by cavities or crevices which provide shelter for micro-organisms (Bourke et al. 2017). Butscher et al. (2016) reported about the impact of the surface structure of the substrate to be disinfected. The inactivation of *G. stearothermophilus* by a DBD was more efficient on polypropylene than on wheat grains due to their rough surface and the deep ventral furrow. While on polypropylene granules, 10 min of treatment yielded spore reductions by about 5 log₁₀, the maximum spore inactivation on wheat grains was 3 log₁₀ units after 60 min of treatment (Butscher et al. 2016).
et al. 2016). Hertwig et al. (2015) reported about a significantly lower sporicidal (B. subtilis) action of CAP on peppercorns compared to flat glass or spherical glass beads. This was explained by the more complex surface structure of peppercorns which is characterized by cracks, grooves and pits, which might cause shadow effects for the different generated components of the plasma (Hertwig et al. 2015). Regarding the use of PPA for the disinfection of surfaces like, for example, the protective equipment of rescue forces, the topography (smoothness) of the respective devices or protective suits needs to be considered and the treatment parameters adjusted accordingly.

Impact of agglomerates and organic loads

It is known that with the exception of gamma and e-beam irradiation, more or less all sterilization techniques are to some degree affected by two major aspects, which limit the accessibility of micro-organisms. The presence of micro-organisms in agglomerates or multiple layers as well as their presence in organic matrices like proteins or polysaccharides (Moisan et al. 2014). The results of this study are in accordance with previous findings and show that matrix effects due to microbes present in agglomerates as well as embedment in organic loads are absolutely decisive for the achievable inactivation of endospores by PPA. When spot inoculation was applied, endospores were partly present in agglomerates, which significantly affected the sporicidal efficiency of PPA compared to spray inoculation, where only monolayers were present. The inactivation effect was negligible when endospores were embedded in a protein matrix, which showed that matrix effects due to organic loads are presumably even more critical than agglomeration of spores. Accordingly, the penetration depth of the reactive species seemed to be rather limited. A high fraction of endospores which are not directly accessible are likely to survive longer treatment periods. Müller et al. (2018) likewise reported that the presence of spore clusters can affect the sporidal efficiency of remote plasma due to shielding effects. Deng et al. (2005) previously investigated the impact of the microbial load (B. subtilis endospores) on substrates (polycarbonate membranes) treated with atmospheric-pressure glow discharges. They found that increasing inoculation densities over four orders of magnitude led to significantly higher D-values. This was attributed to stacked endospores forming multilayered structures when more than $6 \times 10^7$ spores were present on a filter of $0.78 \text{ cm}^2$. The top layers of endospores (even if inactivated) might form a physical barrier to shield those beneath them from gas plasma penetration and hence contribute to increased survival (Deng et al. 2005).

According to Moisan et al. (2014), there are two phases in the spore inactivation kinetics when layers of spores are present. The first phase corresponds to isolated spores or those on top of a stack while spores underneath a stack are inactivated in a second phase. The inactivation of B. atrophaeus endospores with a flowing late afterglow of a N$_2$-O$_2$ microwave discharge (200 W of microwave power at 2450 MHz) in a 50 l treatment chamber exhibited two different D-values. The first phase resulted in a $5 \log_{10}$ reduction after 15 min ($D_1 = 3 \cdot 1 \min$) which is comparable to the spore inactivation obtained in the present study. The second phase between 15 and 60 min indicated a D-value of 33 min (Moisan et al. 2013; Moisan et al. 2014). It was therefore assumed, that matrix effects due to spores within a stack, aggregated, located in cavities or crevices, or covered by some bio-product/debris primarily have impact on the inactivation efficiency. In contrast, phenotypic variations (e.g. more resistant subpopulations) are likely to play a minor role (Moisan et al. 2013). Furthermore, the microbicidal efficiency of cold atmospheric gas plasma is also known to be affected when bacteria are protected in biofilms (Scholtz et al. 2015; Bourke et al. 2017). In order to overcome such shielding effects, the combination of PPA exposure with mechanical disintegration of matrices may synergistically improve the sporicidal effect.

Impact of mechanical wiping and PPW during PPA exposure

When high loads of endospores were dried in a protein matrix and treated with PPA in combination with mechanical wiping using either small amounts of deionized water or PPW (condensate), a high inactivation of endospores on the Tychem F surface by up to more than $5 \log_{10}$ was achieved. In contrast, there was no inactivation at all without mechanical wiping. It is reasonable to assume, that the previously embedded spores were distributed on the sample surface after resolving the matrix during wiping and therefore became exposed to PPA. Using 10 µl of PPW (condensate) instead of water to resolve the matrix further improved the sporidal effect significantly (Fig. 2). Plasma processed water (PPW) is a known disinfectant comprising a complex mixture of various chemical species belonging to ROS and RNS. The use of oxygen, nitrogen and water as parent molecules in PPW production will result in various primary species produced in the plasma discharge which continue to react to form more stable secondary species (Thirumdas et al. 2018). Being characterized by a typically high oxidation-reduction potential, increased conductivity and low pH due to the formation of nitric and nitrous acid, significant antimicrobial activity of PPW was found in many
studies, mainly against vegetative bacterial cells (Thirumdas et al. 2018). The presence of nitrogen oxides (NOx) and corresponding acids, hydrogen peroxide (H₂O₂), ozone (O₃) and peroxynitrite (ONOO⁻) are assumed to be responsible for the microbicidal activity (Scholtz et al. 2015). The sporicidal effect of PPW has not been studied in detail to date. In the present study, PPW (collected condensate) alone did not provide any sporicidal effect within 10 min, neither when endospores were dried on the Tychem F surface and submerged in PPW nor when spores were directly suspended in PPW (data not shown). Similar findings have already been published previously. In fact, Sun et al. (2012) found a strong sporicidal (B. subtilis) effect of PPW using a Cold Atmospheric-Pressure Air Plasma Microjet but only when spores were already present in the water during PPW generation. They assumed that short-lived species were most likely responsible. However, when B. subtilis endospores were treated in previously generated PPW, Sun et al. (2012) did not observe any inactivation. Long lived species therefore did not provide a sporicidal effect within the applied treatment time of 20 min (Sun et al. 2012). Oehmigen et al. (2010) likewise did not prove any sporicidal effect when B. atrophaeus was treated for up to 30 min in physiological saline which was activated by a DBD. The same accounts for Schnabel et al. (2019) who did not find a sporicidal effect of PPW derived from a microwave discharge at a frequency of 2.45 GHz and an input power in the range of 1-1 kW. Nevertheless, as stated above, in the present study it was found that the endospore inactivation on the Tychem F samples was more pronounced when PPW was used instead of water during mechanical wiping, which points to a synergistic effect between PPW and PPA. Previous studies already speculated that the acidity and the plasma derived reactive species are interconnected. A lower pH may enhance the penetration of reactive species to penetrate cell walls while the presence of reactive species may reduce the resistance to an acidic environment (Oehmigen et al. 2010; Sun et al. 2012). A synergistic effect of PPA and PPW for the inactivation of various vegetative bacteria as well as B. atrophaeus endospores was indeed recently reported by Schnabel et al. (2019) when PPA and PPW were applied serially. However, more detailed studies are necessary to confirm a synergistic sporicidal effect of PPW combined with PPA during mechanical wiping.

Overall, it can be concluded that the developed portable plasma system is suitable to reduce microbial loads on surfaces which are placed in a treatment chamber of large volume (here: 300 l) and flushed with a humidified afterglow of the DBD plasma nozzle. The reactive species in PPA with respect to ROS and RNS remain to be identified in future studies as well as the exact composition of plasma-activated water (PPW). PPW may serve as an additional on-site generated disinfectant, which synergistically improves the sporicidal action of PPA.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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