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Arthur Salmon, Gabi Daniel Stancu, Christophe O Laux. Decontamination Of Endospores By Plasma Sources On Dried Surfaces: A Review Of Key Parameters And Inactivation Results. *Frontiers in Physics*, 2021, 9, pp.677971. 10.3389/fphy.2021.677971 . hal-04563753

HAL Id: hal-04563753

<https://hal.science/hal-04563753v1>

Submitted on 7 May 2024

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Decontamination Of Endospores By Plasma Sources On Dried Surfaces: A Review Of Key Parameters And Inactivation Results

A Salmon*, G. D Stancu and C. O Laux

Laboratoire EM2C, CNRS, UPR 288, CentraleSupélec, Université Paris Saclay, 8-10 rue Joliot Curie, F-91190 Gif-sur-Yvette, France

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*Correspondence:

A Salmon
arthur.salmon@centraliens.net

Specialty section:

This article was submitted to
Plasma Physics,
a section of the journal
Frontiers in Physics

Received: 08 March 2021

Accepted: 10 May 2021

Published: 26 May 2021

Citation:

Salmon A, Stancu GD and Laux CO
(2021) Decontamination Of
Endospores By Plasma Sources On
Dried Surfaces: A Review Of Key
Parameters And Inactivation Results.
Front. Phys. 9:677971.
doi: 10.3389/fphy.2021.677971

The efficiency of plasmas sources for the decontamination of heat-sensitive devices has been proven for more than 20 years, but commercial plasma-based sterilizers still have a narrow range of applications. This can be partially explained by difficulties to determine reliable bio-indicators and standardized microbiological test procedures required by industrial uses. In this paper, we examine the influence of environmental factors on the inactivation rate of microorganisms deposited on surfaces and treated by plasma sources. In addition, we present a literature review showing that several in-discharge and afterglow plasma sterilizers offer shorter treatment times than conventional low-temperature sterilizers to reduce the concentration of endospores on contaminated surfaces by 6-log. Finally we make a few recommendations for future plasma decontamination standards.

Keywords: plasma, endospores, decontamination, sterilization, non-thermal

1 INTRODUCTION

Low-temperature decontamination methods are increasingly used for the treatment of heat-sensitive materials in devices exposed to biohazards, such as endoscopes in hospitals [1], containers in the food industry [2], spacecraft components [3] or contaminated equipment in areas exposed to war acts [4]. Standard non-thermal sterilization methods suffer from limitations related to their toxicity, high cost, low material compatibility, and/or long sterilization cycles (several hours). An alternative approach consists in using atmospheric pressure nonequilibrium plasmas produced by electric discharges. The use of plasma sources for low-temperature decontamination has been widely studied for more than 20 years [5–7]. Various biocidal mechanisms of plasmas have been demonstrated, including cell lysis induced by charged species [8, 9], DNA inactivation by radiation from excited molecules and atoms [8, 10], and alteration of cell components by chemical products such as reactive oxygen and nitrogen species (RONS). This combination of inactivation mechanisms is attractive because a broad range of microorganisms can be treated, namely bacteria, viruses [11] including Sars-CoV2 [12], fungi [13, 14] and prions [15]. However, the intensities of the mentioned biocidal mechanisms strongly depend on the type of plasma source (discharge kind, gas flow, power, . . .), working distance between the plasma and the contaminated surface, and operating conditions (surface nature, humidity, . . .). Therefore, it is difficult to compare decontamination results using plasma sources because the inactivation results are highly dependent on the operating conditions. Comparing the results requires to define standard microbiological test conditions, and thus the major parameters that need to be controlled.

TABLE 1 | Standard sterilization methods and microorganisms commonly used as bio-indicators for these methods, modified from [6].

Method	Microorganism	Form	Standard
Wet heat	<i>G. Stearothermophilus</i>	Spore	ISO 11138-3
Dry heat	<i>B. Atrophaeus</i>	Spore	ISO 11138-4
Radiation (γ , X, e-beam)	<i>B. Pumilus</i>	Spore	ISO 11137
O ₃	<i>G. Stearothermophilus</i>	Spore	
C ₂ H ₄ O	<i>B. Atrophaeus</i>	Spore	ISO 11138-2
CH ₂ O	<i>G. Stearothermophilus</i>	Spore	ISO 11138-5
H ₂ O ₂	<i>B. Atrophaeus</i>	Spore	ISO 11138-6

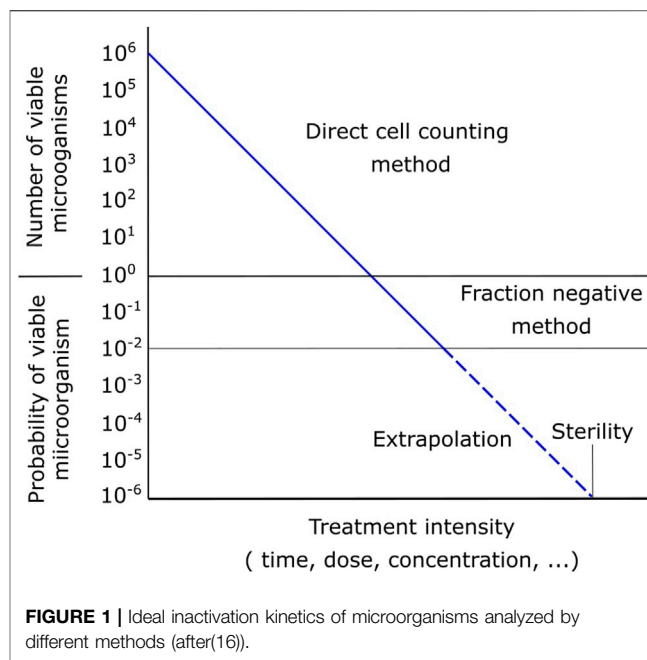
O₃: ozone, C₂H₄O: ethylene oxide; CH₂O: formaldehyde; H₂O₂: hydrogen peroxide

The present paper first aims to identify the influence of several environmental parameters that are not systematically mentioned in publications. Second, the decontamination efficiency of various plasma sources is assessed on the basis of the treatment time required to obtain a 6-log reduction of endospores, which are commonly used as bio-indicators (BI) to validate the efficiency of sterilizers. These results are classified according to the gas pressure and to the working distance between the plasma and the treated sample because the biocidal effects of plasmas are reduced as the working distance increases. In addition, the working distance is an important parameter for certain applications, particularly the decontamination of long tubes, where sensitive surfaces cannot be treated directly by the plasma. The observed trends are discussed and compared with inactivation results obtained against the same microorganisms using standard low-temperature sterilizers.

The paper is organized as follows. The first part introduces the definitions of the microbiological quantities used in the article, namely log-reduction, D-value, and bio-indicators. The second part shows the influence of various environmental factors on the inactivation of microorganisms treated by plasma sources or by chemicals. The third part compares and discusses literature results on the basis of the key environmental factors introduced in part 2.

2 DEFINITIONS OF THE PARAMETERS USED TO ASSESS DECONTAMINATION METHODS

The efficiency of decontamination methods is usually assessed by direct cell-counting methods that are based on the following procedure. First, a suspension of microorganisms is prepared, generally in sterile water. The concentration of the suspension is adjusted by subsequent dilution. Second, a fraction of the suspension is deposited onto two carriers and dried. In general, about $\sim 10^6$ microorganisms are deposited on the carriers so as to follow ISO 11138 series and ISO 14161 standards. Third, one of the carriers is treated while the other is used as a control. Fourth, the microorganisms are extracted from the carriers and counted using a plate count method. To this end, the suspension of recovered microorganisms is diluted, spread on plates, and incubated. The number of Colony

**FIGURE 1** | Ideal inactivation kinetics of microorganisms analyzed by different methods (after[16]).

Forming Units (CFU) on the plate is finally counted. If N is the number of CFU after the decontamination treatment, and N_0 the number of CFU on the control sample, the log-reduction factor (RF) is defined as follows:

$$RF = \log \frac{N_0}{N}$$

For an initial load of 10^6 microorganisms on a surface, the maximum reduction factor measurable by direct cell counting is 6-log. On the basis of a statistical analysis, a reduction factor as low as 8-log can be determined by a fraction negative method [16].

The ISO 11138 series and ISO 14161 standards define sterility as a Sterility Assurance Level (SAL) of 10^{-6} . The SAL denotes the probability that only one viable microorganism survives the treatment. Sterility cannot be verified in practice but can be extrapolated from the kinetics of inactivation of the most resistant microorganism, named the bio-indicator (BI). Table 1 lists some of the commonly used bio-indicators. It should also be noted that other standards, e.g. EN 13697, are used in industrial applications for disinfection purposes.

Figure 1 shows the inactivation kinetics on a semi-logarithmic scale, and the methods used to analyze it. Direct cell counting methods are based on measurements the number of surviving microorganisms. Fraction negative methods, e.g. Stumbo-Murphy-Cochran method and Spearman-Kärber method, are statistical techniques applied in the region of the inactivation curve where only a fraction of the treated items have no viable microorganisms after the treatment, i.e. the so-called quantal zone. The technique allows to demonstrate SAL up to 10^{-2} and to determine the D-value from a reduced range of the inactivation curve. Demonstrating lower SAL ($SAL < 10^{-2}$) is difficult because of the large number of tests required, so extrapolation of the inactivation curve is necessary.

For sterilization methods such as steam heat, the inactivation kinetics follow an ideal exponential decay, and the inactivation curve can be easily extrapolated to determine the conditions for which sterility can be guaranteed. The slope of the semi-logarithmic line is termed the *D-value* and is widely used to assess the efficiency of a sterilization process. It corresponds to the time required to reduce the population of one type of microorganism by one order of magnitude.

In the case of non-thermal decontamination, a multi-step decay of microorganisms is regularly reported, e.g. [17–20]. The multi-step decay is generally attributed to a clumping of microorganisms that reduces the number of microorganisms attainable by the antimicrobial agents [5, 21] or to space-varying mechanisms of inactivation [16, 22] possibly related to an inhomogeneous distribution of the plasma agents across the test sample. Such multiphase decay prevents the determination of accurate D-values and thus does not allow plasma decontamination methods to reach the SAL of 10^{-6} required for sterilization. However, appropriate preparation and deposition of bioindicators may allow to observe a monophasic inactivation curve.

3 ENVIRONMENTAL FACTORS

In this section, we review preparation methods and operating conditions that can significantly modify the inactivation results of plasma decontamination processes.

Effect of the preparation method

The initial suspension of microorganisms is generally prepared in sterile water. However, in realistic conditions, the bioburden is surrounded by organic and inorganic matter (soil). The soil can be well-removed by a preliminary cleaning using water (lowest bio-decontamination level) according to the guidelines of Rutala et al. [23]. However inorganic residues may still persist after cleaning. These residues can be simulated in microbiological treatments by adding salt or serum to the initial suspension of microorganisms. Such additives are known to strongly reduce the efficiency of most decontamination methods [24]. In particular, it has been shown that a high concentration of crystalline-type materials provides a greater protection to spores than serum with high protein content [23]. Using a surface microdischarge (SMD), Klämpfl et al. [25] observed by scanning electron microscopy (SEM) that *Clostridium Difficile* (NCTC 13366) endospores prepared with 0.03% BSA (bovine serum albumine), deposited and dried on stainless steel substrates, form clusters with surrounding saline structures. This barrier decreases the number of spores attainable by plasma species. As a result, the inactivation rate was lowered by 3-log when BSA was added to the suspension. Thus the inactivation results are highly sensitive to the method of preparation, which must be specified to make useful comparisons between decontamination systems.

Effect of the deposition method

Two main techniques are commonly used to deposit microorganisms on substrates: the spot and spray methods.

The spot technique is more common and consists in placing a droplet of the prepared suspension of microbes on the inoculation surface. This method does not allow an accurate control of the local concentration of microorganisms, which readily form clusters and multilayered structures. In contrast, the spray method allows to deposit a monolayer of microorganisms onto the surface, thus ensuring a homogeneous and controllable surface concentration of microorganisms [26–28]. For this reason, the spray deposition method is interesting for standardized inoculation on substrates [29]. Raguse et al. [29] compared the reduction factor for 5×10^7 *B. Subtilis* spores deposited by spray and liquid spot methods on a glass substrate exposed to a low-pressure argon plasma. After 90 s of exposure, a 0.8-log reduction was measured on the substrate inoculated using the spot method while a 4.8-log-reduction was achieved using the spray method. The slower inactivation observed using the spot method can be attributed to the slower diffusion of germicidal agents inside the multi-layered cell structures. According to Shintani et al. [21], [30], the characteristic penetration depth of plasma species is about ~ 10 nm, and ~ 1 μm for hydrogen peroxide. Since, the dimension of a microbe is typically ~ 1 μm , chemical sterilants readily diffuse deeply through multiple layers of cells while plasma species only access the first layer.

As a result, the method of microorganism deposition strongly influences the decontamination efficiency of non-penetrating surface agents such as plasma species, but also UV radiation [31]. However, Raguse et al. [29] showed that highly-penetrating agents such as X-rays were not influenced by the deposition technique.

Effect of surface concentration

The surface concentration of microorganisms is defined as the ratio of the initial microorganisms load to the inoculation area. Increasing the surface concentration of microorganisms enhances the formation of multi-layered structures preventing the diffusion of plasma species in the case of microorganisms deposited by a spot method [32]. Thus, decreasing the initial load of microorganisms or increasing the inoculation area may lead to higher inactivation rates. In Ref. [33], different dilutions of *E. Coli* on Agar were treated by surface microdischarges (SMD). A difference of 4-log in the initial load caused a difference of about 1 log in the inactivation rate after 10 s of treatment. A similar behaviour was observed in Ref. [34] with a corona discharge.

Effect of the type strain

Variations in the strains of a single microorganism species are referenced in collections –e.g. American Type Culture Collection (ATCC), National Collection of Type Culture (NCTC). The susceptibility of different strains of *E. Coli* (ATCC 25922 and NCTC 12900) to air DBD exposure was studied in Ref. [35]. After 30 sec of air plasma treatment, it was shown that the strain ATCC 25922 was reduced by 3.4 log whereas the strain NCTC 12900 (corresponding to collection n° ATCC 700728) was only reduced by 1.8 log. The different efficiencies are due to intrinsic DNA variations within the same type of microorganism. It is important

TABLE 2 | Different names of some bacillus strains of bacteria (after [63])

Microorganism	DSM collection number	References	Previous names	References number in other collections
B. Atrophaeus	DSM 675		<i>Bacillus Globigii</i> 'red strain' → <i>Bacillus Subtilis</i> var. <i>Niger</i> → <i>Bacillus Subtilis</i>	ATCC 9372, NCIB 8058, CIP 77.17, NRS 121A IFO 13721, NCDO 738
B. Atrophaeus	DSM 2277		<i>Bacillus Globigii</i>	ATCC 51189, NCTC 10073, NCIB 8649, CIP 103406
B. Atrophaeus	DSM 7264 ^T		→ <i>Bacillus Subtilis</i> <i>Bacillus Subtilis</i> var. <i>Niger</i>	ATCC 49337 ^T NRRL-NRS 213 ^T , ATCC 6051 ^T , CCM 2216 ^T , NCIB 3610 ^T , NCTC 3610 ^T , IFO 12210 ^T , NBRC 13719 ^T
B. Subtilis	DSM 10 ^T			NCIB 10106, NBRC 111470
B. Subtilis	DSM 402			ATCC 27142, CIP 77.25, NCTC 10327, NCIB 10692
B. Pumilus	DSM 492			ATCC 12980, NBRC 12950, CCM 2062
G. Stearothermophilus	DSM 22			ATCC 7953
G. Stearothermophilus	DSM 5934			

CCM: Czech Collection of Microorganisms; CIP: Collection de l'Institut Pasteur; DSM, DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen; IFO: Institute for Fermentation, Osaka; NCDO: National Collection of Dairy Organisms; NCIB: National Collection of Industrial Bacteria; NCTC: National Collection of Type Cultures; NRRL: Northern Regional Research Laboratory; NRS: Nathan R. Smith, NBRC: Biological Resource Center NITE. T superscript refers to the type strain.

to note also that the name of microorganisms is subject to adjustments. **Table 2** gives the correspondance of DSM (German Collection of Microorganisms and Cell Cultures) collection numbers with other collection numbers of some bacillus strains of bacteria, as well as previous designations that may also be encountered. It should also be noted that microorganisms available from culture collections are typically less resistant than their real-world counterparts, because the latter have been subjected to environmental stresses and have evolved to adapt over countless generations.

Effect of the relative humidity

High relative humidity (RH) is known to cause the swelling of endospores [36], which enhances the efficiency of many decontamination methods –e.g. EtO and formaldehyde [37] – because of the higher water content in the spore core allowing the formation of aggressive chemicals, such as OH, inside the spore. In Ref. [37], the moisture content (hydration) of *B. Subtilis* was measured for different RH. It was shown that the moisture content slightly increases from 0 to 20% when the RH is varied from 0 to 75%, and strongly increases at higher RH (from 20 to 70% between 75 and 95% RH). At RH > 75 %, the strong increase of the water content causes spore swelling.

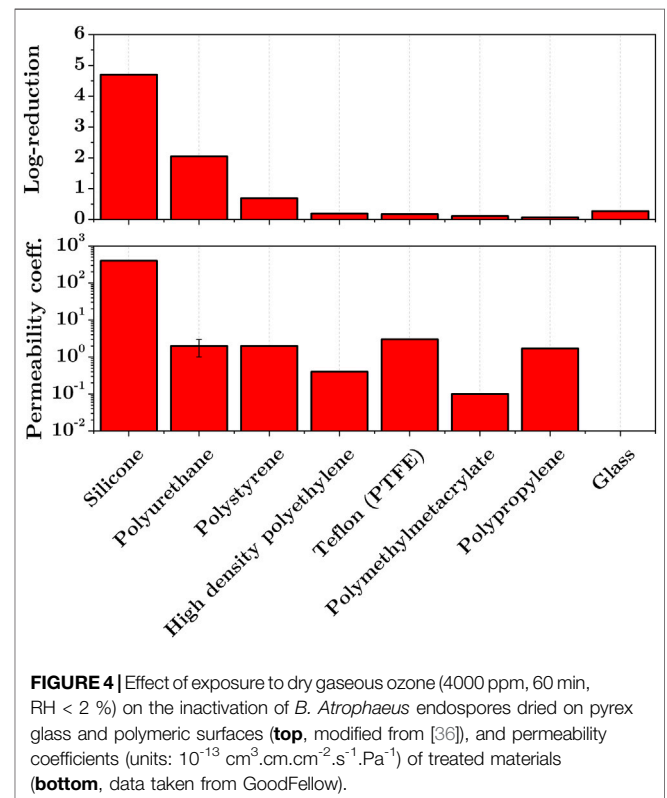
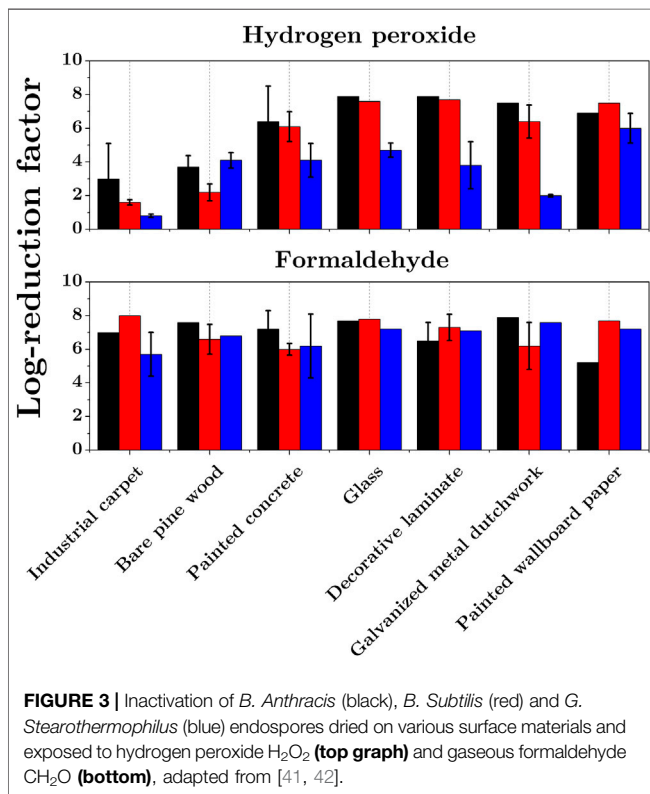
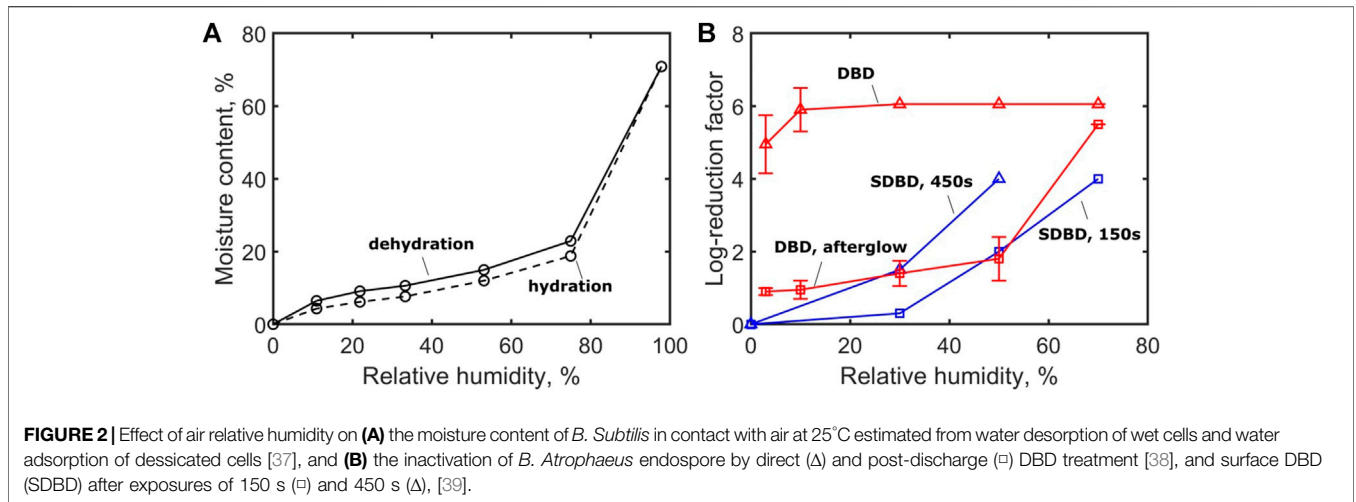
The relative humidity (RH) also affects the gas-phase plasma chemistry [36]. **Figure 2** shows the influence of the RH on the

spore moisture content and the log-reduction of spores treated by atmospheric cold plasma (ACP) [38] and dielectric barrier discharge (DBD) [39]. The results indicate that a higher RH is always correlated with higher sporocidal activity, probably because of the formation of water-related biocides, such as OH, H₂O₂, HNO₂, HNO₃. Similar conclusions were obtained in Ref. [26]

Effect of the surface material

The effect of the material and the structure of the treated surface on the antimicrobial agent efficacy is complex. Sigwarth et al. [40] studied the reduction of *G. Stearothermophilus* (ATCC 7953) endospores deposited on various surface materials after exposure to hydrogen peroxide. Up to a 3-log difference in the reduction was measured on different materials, but the authors saw no clear correlation between the material properties and the shift in the resistance of endospores.

Figure 3 shows the inactivation of endospores *B. Anthracis*, *B. Subtilis* and *G. Stearothermophilus* on different substrates exposed to formaldehyde and hydrogen peroxide [41, 42]. With formaldehyde, the inactivation results are fairly similar for all materials. In contrast, for hydrogen peroxide exposure, the inactivation results strongly depend on the substrate material as shown in Ref. [40]. These results suggest that endospores are less likely inactivated by H₂O₂ when they are deposited on porous

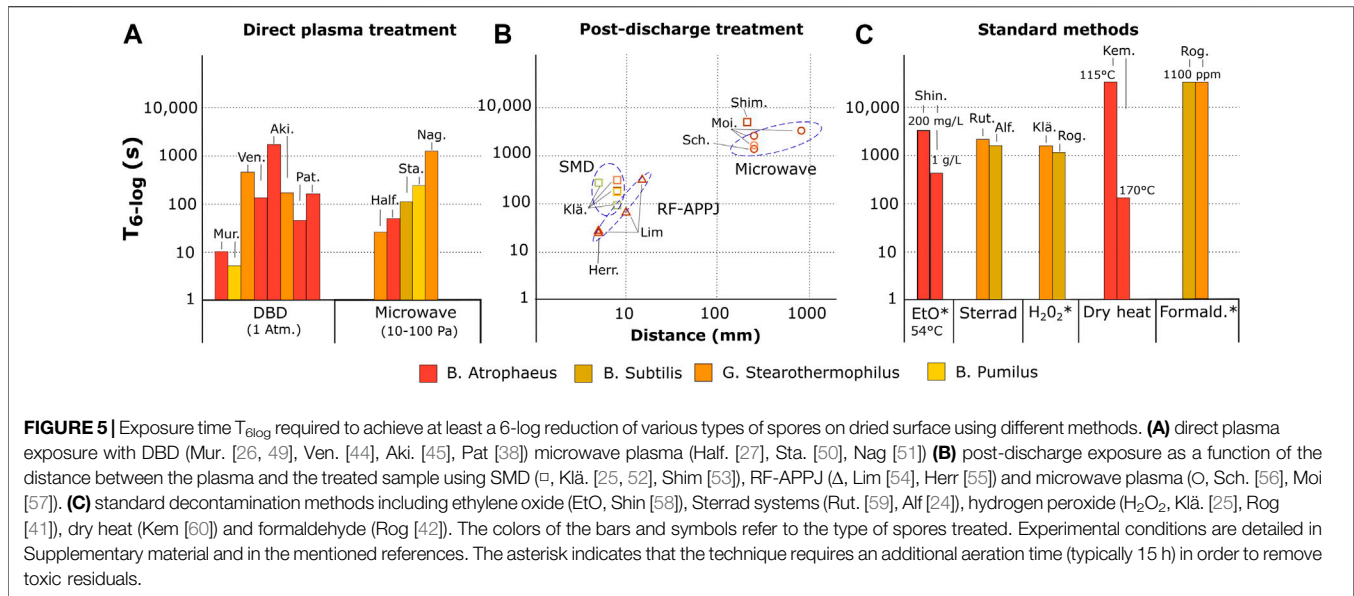


materials. According to the authors, a possible penetration of spores inside porous substrates precludes interaction of H_2O_2 with a significant fraction of spores because of the comparatively small penetration depth of H_2O_2 [23].

The effect of material permeability can be further understood from the work of Mahfoudh et al. [36] who studied the effect of dry gaseous ozone on the inactivation of endospores deposited on different polymeric surfaces. Figure 4 shows the results obtained by exposing the samples contaminated by *B. Atrophaeus* to 4000 ppm of dry ozone for 1 hour. The log-reductions are shown

together with the permeability coefficients of the materials studied (values taken from GoodFellow). The results show a correlation between the permeability of the surface material and the achieved inactivation rate with a maximum 4.6-log reduction obtained on a highly permeable silicone surface. Similar results were obtained in decontamination using ethylene oxide (EtO) in Ref. [37].

In direct plasma decontamination, additional interactions between the plasma and the surfaces increase the influence of the surface material. Interactions between the surface material



and the gas phase species were observed by Levif et al. [43]. Operating at reduced pressure, the authors demonstrated that *B. Atrophaeus* spores deposited on polystyrene petri dishes were more resistant than on a glass surface, because of the interaction between the plasma and the surface material. As a result, treating microorganisms on porous surfaces significantly increases the inactivation rate. A possible reason for this is the penetration of spores inside the material, thus preventing clumping and shielding of microorganisms at the surface. In the case of direct plasma treatment, interaction of the plasma with the surface material influences the gas phase chemistry and thus the inactivation mechanisms.

4 OVERVIEW OF ENDOSPORE DECONTAMINATION RESULTS ON DRIED SURFACES

In several demonstrations of plasma treatments of spores, the D-values could not be determined. In these cases, the inactivation curve cannot be extrapolated and the sterility cannot be guaranteed according to the sterility assurance level criteria (SAL of 10^{-6}). Rather than speaking of “sterilization”, von Woedtke et al. [16] proposed the microbiological safety qualification “proof of antimicrobial efficiency on the highest experimentally accessible level” as the highest possible qualification for plasma decontamination. Here we report the experimental results for which a 6-log reduction of bacterial spores (most common BIs) dried on surfaces was demonstrated according to the von Woedtke definition. The time required to ensure a 6-log reduction of BIs is noted $T_{6\text{-log}}$.

Figure 5 shows an overview of spore inactivation results on dried surfaces by atmospheric and reduced pressure plasmas, as a function of the distance between the plasma source and the sample. **Figure 5** also shows typical $T_{6\text{-log}}$ values for standard decontamination methods. We only plot the experimental results

for which a 6-log reduction of spores was measured or extrapolated from a single D-value. The experimental conditions of the reported results are detailed in Supplementary material (**Supplementary table S1, S2**) and in the cited references. An additional table (**Supplementary table S3**) provides a review of the experimental results that could not be included in **Figure 5**.

Figure 5A. shows that two types of plasma sources demonstrated a 6-log reduction of endospores on dried surfaces in the plasma phase: microwave (MW) sources operating at reduced pressure, and dielectric barrier discharges (DBD) operating at atmospheric pressure. The lowest $T_{6\text{-log}}$ value was reported by Muranyi et al. on *B. Pumilis*, *B. Atrophaeus* and *A. Niger* spores using a cascaded-DBD (CDBD) in atmospheric pressure air. The reported $T_{6\text{-log}}$ is about 10 seconds whereas other DBDs required about 100 seconds [38, 44, 45]. This difference can be partially explained by the fact that Muranyi et al. sprayed the initial microbial load of 10^6 CFU on a dried polyethylene terephthalate (PET) sample of 16 cm^2 , so that a homogeneous concentration of spores of about 6×10^4 spores/ cm^2 was obtained. In the other reports, the concentration was typically 10^6 spores/ cm^2 , thus increasing the risk of formation of multiple layers of spores, and preventing the contact of plasma agents with the spores. This example shows that standardized microbiological test procedures are needed to better compare the efficiencies of different plasma sources.

In the post-discharge (see **Figure 5B**), surface micro-discharges (SMD), radiofrequency atmospheric pressure plasma jets (RF-APPJ) and reduced pressure MW plasma sources were also able to reach 6-log reduction. The $T_{6\text{-log}}$ values range from about 20 seconds at a few millimeters from the discharge using RF-APPJ to several thousands of seconds at 1 meter using MW plasmas. As expected, increasing the distance from the plasma source lowers the dose of biocidal agents, but provides lower material

degradation and higher treatment area. This configuration is promising for the decontamination of long, small diameter tubes such as the lumens of endoscopes [19, 46], for which the diffusion of the sterilant along the whole length of the lumen is still a major concern [24], particularly in the presence of residual inorganic and organic soil [47, 48].

All reported results showed that a 6-log reduction can be achieved in less than a few thousands of seconds, which is comparable with the typical performances of standard low temperature sterilizers (see **Figure 5C**).

5 CONCLUSIONS

Plasma decontamination has various advantages compared to other methods including:

- i/ compatibility with heat-sensitive devices,
- ii/ ability to penetrate into narrow devices (e.g. endoscopes),
- iii/ absence of toxic waste,
- iv/ no shelf life of the sterilization products
- v/ onsite operational characteristic.

To date, however, the role of plasma is minor in commercial sterilizing processes. For instance, the efficiency of hydrogen peroxide plasma (e.g. Sterrad sterilization system from Johnson & Johnson) is mainly due to hydrogen peroxide exposure, while the plasma phase is employed to eliminate toxic residues [61]. Nonetheless one application of low-pressure plasma has been clearly identified recently for packaging sterilization in the pharmaceutical industry [62]. Its efficiency against endospores typically used as bio-indicators shows that plasma is a promising candidate as a sterilization method. Yet, multiphase inactivation curves indicate that the biocidal agents are not distributed uniformly (low penetration depth and inhomogeneous plasma) thus preventing efficient contact with the microorganisms and making it difficult to compare with literature results. In order to avoid this, standardized microbiological test procedures are needed, and the following recommendation can be made. First a **monolayer of spores** should be used to obtain reproducible results. To this end, the concentration of spores should be low and the **spray deposition** method should be employed in order to prevent the formation of clusters. Second, a **small inoculation area** should be used to ensure that the distribution of plasma agents is homogeneous across the test sample. Third, **non-porous and inert substrate material** (e.g. glass holder) should be used, except if a specific application is considered (e.g. decontamination of polymer surfaces). In particular, we expect that a high permeability of the substrate allows diffusion of spores inside the material, thus preventing homogeneous exposure of the spores to the plasma, and increasing the risk of incomplete collection of the spores before counting.

The literature review indicates that the relative humidity (RH) increases the sporicidal activity of the plasma phase through the swelling of spores. We suggest that RH should be considered as a parameter to optimize the process, keeping in mind that high RH may be detrimental for the treatment of moisture-sensitive devices such as electronics.

We gave a summary of plasma inactivation results for which a 6-log reduction of endospores dried on surfaces was achieved. We found that several types of plasma sources operating in various gases and pressures can achieve a 6-log reduction in less than one minute, which is comparable with the processing time of standard low-temperature sterilization methods. From the literature review, we could not identify a single relevant bioindicator for plasma sterilization, because this depends on the dominant biocidal mechanism, which is specific to each plasma source.

In conclusion, plasma sources have demonstrated to be competitive sterilizing techniques. A number of biocidal plasma mechanisms are now well-known, but inadequate preparation and control of the environmental conditions does not allow a fair comparison of the biocidal efficiencies of different plasma sources. We recommend that future studies carefully consider the preparation and deposition of bioindicators in order to accurately determine the processing time required for sterility, and thus provide a means to rigorously compare the efficiency of different plasma devices. In summary, we recommend using:

- monolayer of spores for reproducibility
- spray deposition method to prevent the formation of clusters
- inoculation area smaller than size of the plasma to ensure homogeneous treatment
- inert standard deposition material with low permeability to prevent the diffusion of spores inside the material (good candidate substrates could be glass holder)

AUTHOR CONTRIBUTIONS

AS, GDS, COL, have contributed equally to this manuscript.

FUNDING

This research was supported by DGA grant DESDEMONA (RAPID contract EJ nr. 2101 241 872).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphy.2021.677971/full#supplementary-material>

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- Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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