Pulsed dielectric barrier discharge for *Bacillus subtilis* inactivation in water

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Abstract. The inactivation of *Bacillus subtilis* bacteria in water has been experimentally studied by means of a pulsed dielectric barrier discharge (PDBD) in a coaxial reactor endowed with an alumina dielectric. The plasma source is capable of operating at atmospheric pressure with gas, water or hybrid gas–liquid media at adjustable 25 kV pulses, 30 µs long and at a 500 Hz frequency. In order to evaluate the inactivation efficiency of the system, a set of experiments were designed on the basis of oxygen flow control. The initial data have showed a significant bacterial rate reduction of 10³-10⁷ CFU/mL. Additional results proved that applying an oxygen flow for a few seconds during the PDBD treatment inactivates the *Bacillus subtilis* population with 99.99% effectiveness. As a reference, without gas flow but with the same exposure times, this percentage is reduced to ~90%. The analysis of the relationship between inactivation rate and chemical species in the discharge has been carried out using optical emission spectroscopy as to identifying the main reactive species. Reactive oxygen species such as atomic oxygen and ozone turned out to be the dominant germicidal species. Some proposed inactivation mechanisms of this technique are discussed.

1. Introduction
Bacteria are omnipresent. They are found in all natural habitats and some species have been found in boiling hot spring as well as extreme cold condition [1]. A number of aerobic endospore-forming bacterial species are opportunistic pathogens of animals (including humans). These bacteria are widely distributed in the environment where their habitats are soils of all kinds, ranging from alkaline to acid, cold to hot, as well as bottom deposits of water. Thus, the term “aerobic endospore-forming bacteria” is used to embrace *Bacillus* species and related genera, capable of forming resistant endosporers. *Bacillus subtilis* (*B. subtilis*) is an endospore-forming Gram-positive bacterium, extremely dormant metabolically. This dormancy is the key to their resistance to many agents, including heat, radiation, pressure, desiccation and chemicals, hence their survival over long periods [2].

Some pathogen bacteria contaminate solid or liquid food, and particularly drinking water, originating diseases to the point of being the main cause of human death [3]. Clearly, applying some proportionately effective disinfection methods becomes indispensable. Conventional water disinfection techniques generally involve the use of hazardous chemicals that lead to large waste streams that must be eventually treated, in order to avoid adverse environmental impacts. By contrast, non-thermal plasmas used for inactivation of harmful bacteria and pollutants are recently drawing
increasing attention in the study of biological and chemical decontamination [4]. There have been an increasing number of reports on the capability of non-thermal plasmas to inactivate a wide range of microorganisms, including Gram-negative and Gram-positive bacteria [5,6]. Most of the early investigations have used arc or corona discharges where the abrupt release of energy through a narrow plasma channel causes a so-called electrohydraulic shock wave. Apart from these high pressure waves, discharges in aqueous solutions initiate a number of effects that cause microbial inactivation: formation of chemical reactive radicals by electron impact ionization, photo dissociation as well as direct interaction of UV photons with the DNA of living microorganism [7]. In this case, the presence of dielectrics plays an important role in arcing prevention generating a uniform room pressure plasma discharge.

The *B. subtilis* species has received particular attention due to its highly resistive nature, endospore-forming capability and are believed to be among the most resistant microorganism. As a result, this type of bacteria has often been chosen for inactivation experiments due to its comprehensive characterization as a model organism for laboratory studies. Evidence of inactivation and/or complete removal of *B. subtilis* in water with $10^3$, $10^5$ and $10^7$ bacterial cells/mL has been found in the present study, using pulsed dielectric barrier discharge (PDBD) plasma at atmospheric pressure, with oxygen (O$_2$) as work gas, thus proving the efficacy of this process to inactivate bacteria.

2. Methods

2.1. Pulsed dielectric barrier discharge system

Efforts have been specifically focused on bacterial inactivation by direct plasma application in a water environment via PDBD at atmospheric pressure. Figure 1 shows the experimental layout including the adjustable 100-1000 Hz power supply whose pulse width is 1-50 μs, operating at 1-30 kV. The reactor has been made out of a stainless steel tube of 1.25 mm inner diameter, operating as a cathode, and a 0.1 mm diameter tungsten wire which has been adapted as anode. The wire has been covered with alumina ceramic as dielectric. Total length of the reactor is 300 mm, including a work gas inlet.

![Figure 1. Experimental layout for pulsed dielectric barrier discharge in a water environment.](image)

Voltage, current and power are measured using an oscilloscope (Tektronix TDS-2024) connected to a voltage divider (Tektronix P6015) and a current transformer (Stangenes 2-0.1W). Main plasma
diagnostics include spectroscopy of the light emission from the plasma (Jaz OceanOptics) and ozone monitoring (Teledyne Instruments 460L) interfaced by means of a personal computer.

2.2. **Bacteria culture**

5 mL of Tryptone Soya Broth (TSB) solution was inoculated with a strain of *B. subtilis* and incubated for 24 h at 37°C until the bacterial concentration reached approximately 10⁷ bacterial cells/mL. Then, the solution was twice centrifuged for 10 minutes at 5000 rpm. The sedimented bacteria were put in a 5 mL suspension of distilled and sterilized water. The final concentration was determined by direct phase contrast microscopy and then diluted to concentrations of 10⁴, 10⁵ and 10⁷ bacteria/mL.

2.3. **Plasma treatment**

The plasma treatment of the culture was carried out placing 15 mL of water containing 10⁴, 10⁵ and 10⁷ bacteria/mL controlled concentrations into the reactor, namely, between the dielectric and the cathode. The power supply was adjusted to 25 kV, 500 Hz and to a ~30 µs pulse width. Experiments were conducted in water during periods dependent on each concentration and in two modes: with and without gas flow. In the second case, the reactor gas inlet was closed generating a discharge in a heterogeneous media of air and water. In the first case, and in order to produce reactive oxygen species (ROS), oxygen gas was injected at 0.5 L/min flow rate in continuous mode for each treatment period, producing gas bubbles and allowing a continuous generation of ozone. Data acquisition of ozone concentration and analysis by means of optical emission spectroscopy (OES) along the process was performed in every experiment, after which, the microbiological analysis was completed.

Immediately after the PDBD treatment, the samples were removed from the reactor and transferred to sterile test tubes. A standard culturing technique was followed in order to practice the microbiological evaluation of the *B. subtilis* survivability. Culture in Petri dishes containing Tryptone Soya Agar (TSA) was implemented where a three dish set per sample was compounded by inoculating 100µL aliquots that were taken from the serially diluted bacterial suspension. The inoculated dishes, once labeled, were incubated for 24 h periods at 37°C. After incubation, colony forming units (CFU) were counted, and concentrations of CFU/mL were determined by colony counts and plotted as a function of the treatment time. The surviving fraction is determined by comparison between the number of surviving CFUs from the treated samples and those of the untreated sample.

3. **Results**

The electric discharges produce physical phenomena and reactive chemical species acting synergistically and thereby accelerating the elimination rate of microorganisms. In particular, molecular breaking down in water and gas media can generate powerful oxidation species such as the hydroxyl radical (OH) with a high potential to inactivate the bacteria [8], [9]. In order to investigate this possibility, we identify the chemical species present in the discharge applying OES.

Figure 2 shows the discharge emission bands with and without oxygen flow in the wavelength range of 270-870 nm. Figure 2(a) displays the respective emission spectra produced by the PDBD in H₂O without O₂ flow. The main products appear as follows: hydroxyl radical (OH) in the 306.3 nm range of 270-870 nm. Figure 2(a) displays the respective emission spectra produced by the PDBD in H₂O without O₂ flow. The main products appear as follows: hydroxyl radical (OH) in the 306.3 nm range of 270-870 nm. Figure 2(b) shows the effects of the addition of O₂. The emission intensities of H₂ (434 nm, 5d²D→2p²P'), H₂ (486.1, 4d²D→2p²P'), H₂ (656.3 nm, 3d²D→2p²P'), O (777.4 nm, 3p²P→3s²S⁰), and the OH radical in the band of 613.7 nm (X₂Π, 5-0) with a significant difference from the oxygen flow free case.

The ozone concentration was monitored during each experiment using an ultraviolet (UV) absorption ozone meter (Ozone Monitor model 460L, Teledyne Instruments™) tuned in to 253.7 nm. Data acquisition from the ozone monitor was processed on LabView™ software and plotted versus
treatment time. Similar curves of ozone concentration in ppm were obtained from all experiments as shown in figure 3. The ozone concentration was saturated within 140-155 ppm in continuous oxygen flow while the ozone level, measured in the process without gas flow, remained under 10 ppm.

![Figure 2](image1.png)

**Figure 2.** Optical emission spectroscopy of the in PDBD in a water environment: (a) without oxygen gas flow and (b) with continuous oxygen gas flow into the reactor.

![Figure 3](image2.png)

**Figure 3.** Curves of ozone concentration without oxygen flow and with continuous oxygen flow.

The aim of the experiment has been to determine the way in which the gas flow mode or the concentration of the ROS should shorten the inactivation time. A characterization of the inactivation phases of *B. subtilis* during the plasma treatment versus time was carried out. Initial experiments of *B. subtilis* inactivation were performed at $10^3$ CFU/mL for different treatment periods with and without oxygen flow. The quantitative results are presented in figure 4.

The survival curves in figure 4 indicate that the gas flow increased the efficacy of the process. In particular, figure 4(a) shows the survivability percentage of *B. subtilis* at a $10^3$ CFU/mL, where the pure discharge yields, without gas flow, a 6.785% of survivability after 120 s, whereas the addition of O$_2$ leads, over the same time interval, to 0.08% of survivability equivalent to a 99.92% inactivation. By contrast, without gas flow, the inactivation percentage is only 99.85% after 360 s. Figure 4(b) shows the survivability percentage at a $10^5$ CFU/mL concentration. In this case, 99.99% inactivation
was achieved at 360 s in the gas flow mode, while 90.38% of inactivation was obtained in the same time and 99.30% after 600 s, both, without gas flow in the reactor. Samples at elevated concentrations as high as $10^7$ CFU/mL reached 99.85% inactivation after 1200 s with oxygen flow whereas 90% of inactivation was obtained during a similar period without gas flow, as shown in figure 4(c).

![Figure 4](image-url)

Figure 4. Survivability percentage of *B. subtilis* at (a) $10^3$, (b) $10^5$ and (c) $10^7$ CFU/mL versus exposure time.

We estimate that the pure discharge survival curve reflects inactivation by UV photons, mainly since no enough radicals from the gaseous phase are available. In contrast, when adding O$_2$, which partially dissociates to yield O, O$_2^+$, O$_3$, then both UV and ROS contribute to the inactivation. A logarithm plot of the number of surviving microorganisms as a function of exposure time is presented in figure 5.

According to figure 5, the process with O$_2$ gas flow injection raises the elimination efficacy in all the cases. Thus, a reduction $\sim 4 \log_{10}$ is attained at concentrations $10^3$ CFU/mL, and similar reductions are achieved at $10^5$ CFU/mL. At $10^7$ CFU/mL, a $\sim 3 \log_{10}$ reduction is obtained when O$_2$ gas is present. Finally, if concentrations of $10^7$ CFU/mL are chosen, around $1 \log_{10}$ reduction demands 1200 s of treatment without oxygen gas flow. As expected, higher reduction requires even longer treatment periods.

4. Discussion
The inactivation of *B. subtilis* can be caused either by an individual or synergistic effect of plasma components on the microorganism. We believe that the difference between processes presented above is due to the effect of the reactive species generated by the addition of O$_2$ to the discharge. The reactive species can be atomic and molecular radicals, for instance O and O$_3$, and excited molecules or ions, for example, the O$_2^*$. Then, the subsequent gas discharge radicals, O$_3$, high-energy electrons, and UV radiation act synergistically to inactivate the bacteria. On its part, in the absence of reactive species, only intrinsic photodesorption and rupture of the cytoplasmic membrane due to the electrostatic force can take place. Therefore, a longer treatment time is required for bacterial inactivation, according to the mechanism presented in [7].
Figure 5. Inactivation kinetics curve of the *Bacillus subtilis* by PDBD at: (a) $10^7$, (c) $10^5$, and (e) $10^3$ CFU/mL without gas flow; (b) $10^7$, (d) $10^5$, and (f) $10^3$ CFU/mL in continuous oxygen flow.

The experimental results suggest that the addition of O$_2$ increased the effect of inactivation by ROS albeit generated OH radicals have an inactivation effect too. In order to understand this, we observed the difference between spectra in figure 2 in order to discuss the production processes of O and OH radicals under the added oxygen condition. The additional O$_2$ can be dissociated through reactions (1)-(3), while considerable proportions of O (1D) and O atoms can be produced according to [8]:

\[
e + O_2 \rightarrow O^- + O \quad (1)
\]

\[
e + O_2 \rightarrow e + O + O (1D) \quad (2)
\]

\[
e + O_2 \rightarrow O^+ + O + 2e \quad (3)
\]

The main pathway leading to OH radical formation by discharge is thought to be the result of the direct dissociation of water molecules by electron impact, leading to the generation of H and O as:

\[
e + H_2O \rightarrow e + H + OH \quad (4)
\]

\[
e + H_2O \rightarrow 2H + O \quad (5)
\]

However, it has also been reported that the generation of OH radicals increases along with the concentration of O$_2$ molecules [9]. Likewise, a lot of OH radicals can be produced by the interaction of O (1D) and H$_2$O:

\[
O (1D) + H_2O \rightarrow 2OH \quad (6)
\]

O$_3$ is the primary species produced by an electrical discharge occurring in either air or oxygen. It is the only active species that can diffuse into the liquid phase and react with aqueous contaminants that are susceptible to degradation by direct attack [10], e.g., as molecular O$_3$. Ozone can also act indirectly, via radical-intermediates formed during O$_3$ decomposition in the aqueous media. OH radical is the most important species formed during O$_3$ decomposition:

\[
H + O_3 \rightarrow OH + O_2 \quad (7)
\]
These findings can be interpreted as active chemical species, being formed under gas flow, reacting with the environment and interacting with the cytoplasmic membrane of {\textit{B. subtilis}}. Similar observations have been reported by Sato et al. [11] and Chen et al. [12]. Likewise, ROS and OH radicals are suggested to play a fundamental role in the bacterial inactivation and to cause physical destruction of the cytoplasmic membrane by oxidation after plasma exposure.

5. Conclusions
This report has proven that the pulsed dielectric barrier discharge technique can be successfully used in the inactivation of microorganisms in water, and, in particular, that its application is lethal to \textit{Bacillus subtilis} even at high concentrations. The obtained results revealed the possible physical and chemical mechanisms of bacterial inactivation. Through optical emission spectroscopy and inactivation kinetics studies, it has been established that PDBD is effective to produce radicals in such a way that observed bacteria inactivation was most probably induced by the radicals, UV photons, and the electric field, acting synergistically. According to this, a relationship between the presence of the OH radical and the bacterial inactivation efficiency has been suggested. This finding is highly relevant for future applications of electric discharges to bacterial inactivation. Future experiments and simulations will focus on evaluating all contributors, and thus bringing forth more understanding on the mechanisms of bacteria inactivation in water.

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