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# BACTERICIDAL EFFECTS AND FORMATION OF ROS/RNS IN WATER SPRAYED THROUGH COLD AIR PLASMA

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Bio-decontamination and chemical effects induced by plasma in water upon electro-spraying through cold atmospheric pressure plasma of DC-driven positive transient spark discharge in air were investigated. Inactivation of *Escherichia coli* in plasma treated water was determined in dependence on the solution pH (controlled by buffers) and correlated with chemical changes induced in water. Productions of H<sub>2</sub>O<sub>2</sub>, nitrites, nitrates, peroxyxynitrites and pH changes were determined. The degree of inactivation and oxidative damage of bacteria increased with increasing acidity of the solution. Acidified nitrites interacting with H<sub>2</sub>O<sub>2</sub> were determined as the most important bactericidal ROS/RNS agents in water treated by air plasma.

## 1. Introduction

Nowadays it is evident that cold atmospheric pressure plasmas can efficiently kill various microbes, even highly resistant forms such as bacterial spores and biofilms, and foster interesting phenomena in the cells of higher organisms leading to various therapeutic effects. The mechanisms of plasma-cell interaction are not fully understood yet, although a great research effort has been dedicated to elucidate the respective roles of various plasma agents in the interaction with live cells, such as charged particles, neutral reactive species, UV radiation, electric field and heat [1]. The most studies agree on that the bactericidal effects of atmospheric pressure cold plasmas are dominantly due to reactive neutral species (mostly radicals) and perhaps some ions, such as superoxide anion O<sub>2</sub><sup>-</sup>.

Plasmas generated in air in contact with water are of the great interest because they produce large quantities of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and seem to be the most efficient in bio-decontamination [2-8]. Even He or Ar plasma jets perform the strongest bactericidal efficacy with O<sub>2</sub> or air admixtures or when the rare gas plasma entrains air components [2]. Air plasma treatment of water and aqueous solutions typically leads to acidification [2-3, 9-15] which can normally be explained by dissolution of NO<sub>x</sub> species formed in the air plasma in water [2, 9-13]. Some authors report acidification by other mechanisms, even in pure O<sub>2</sub> plasma by the action of O<sub>2</sub><sup>-</sup> anions [14]. Acid environment itself, despite being crucial in bactericidal effect of plasma treated water, did not prove to be the main antibacterial agent. Several authors reported that addition of various acids resulting in the same pH as in the plasma treated solutions (usually 2.5-4.8) does not lead to the same bactericidal efficacy. It is acid environment in synergy with plasma agents leads to the bacterial inactivation [2, 9-10]. However, it is not clear enough yet, which plasma agents and which ROS/RNS have dominant roles in water decontamination and what is the associated plasma-induced water chemistry. Hydrogen peroxide, nitrate and nitrite anions, as well as other species such as peroxyxynitrites, have been identified in plasma-activated water [2-3, 7-10, 16].

In this work we investigated the chemical effects induced in water electro-sprayed through DC-driven positive transient spark discharge. We measured formations of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), nitrites (NO<sub>2</sub><sup>-</sup>) and nitrates (NO<sub>3</sub><sup>-</sup>) and peroxyxynitrites (ONOO<sup>-</sup>) in plasma treated water. Production of these chemical species was correlated with the pH changes and with bactericidal effects observed on *Escherichia coli*, suspended in plasma treated solutions. This work follows our previous studies of bio-decontamination of electro-sprayed water in the transient spark [17-18].

## 2. Methodology

The experimental setup for DC-driven transient spark in point-to-plane geometry, with a high voltage (HV) hypodermic hollow needle electrode enabling water flowing through the discharge zone and a mesh electrode is depicted in Fig. 1. The inter-electrode spacing was usually kept at 10 mm. A positive DC high voltage was applied through the ballast resistor  $R$  (5-10 M $\Omega$ ). The discharge voltage was measured by a high voltage probe Tektronix P6015A. The discharge current was measured: on a 1  $\Omega$  resistor or by a current monitor Pearson 2877. The current and voltage signals were processed by a digitizing 200 MHz oscilloscope Tektronix TDS 2024. The contaminated water flowed directly through the high voltage hollow needle electrode. The effect of electrostatic spraying occurred when the high voltage was applied on the needle electrode [17-19]. The typical discharge visual appearance is shown in the photograph in Fig. 2.

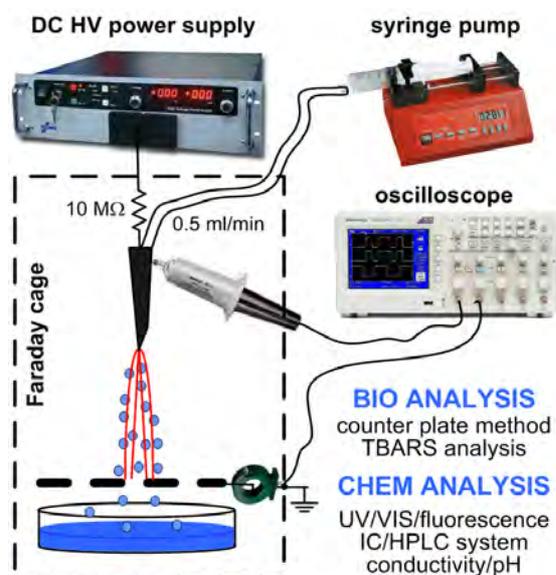


Fig. 1. The experimental setup



Fig. 2. Photograph of the transient spark with electro-spray of water in 1 cm needle-mesh gap, water flow rate 0.5 mL/min, 14 kV.

Measurements of hydrogen peroxide and peroxyxynitrite formed in plasma treated water were performed by colorimetric methods using UV/VIS spectrophotometer Unicam Helios Gamma and UV/VIS/Fluorescence microplate reader Thermo VarioScan Flash, respectively. The concentration of hydrogen peroxide was determined by the reaction of  $\text{H}_2\text{O}_2$  with titanium ions with the absorbance measurements at 407 nm. The concentration of peroxyxynitrite was determined by the reaction with 2,7-dichlorodihydrofluorescein diacetate (DCF-DA) with absorbance measurements at 500 nm and with fluorescence measurements using excitation and emission wavelengths of 502 and 523 nm, respectively (PerkinElmer LS 45, 50 Hz) [20, 21]. The concentrations of nitrites and nitrates were measured by ion chromatography using a HPLC system Shimadzu LC-10Avp with UV (210 nm) and suppressed conductivity detection. Analyses were made by means of a 7- $\mu\text{m}$  Allsep A1 anion exchange column (10 cm  $\times$  4.6 mm) with 0.85 mmol  $\text{L}^{-1}$   $\text{NaHCO}_3$ /0.9 mmol  $\text{L}^{-1}$   $\text{Na}_2\text{CO}_3$  as the eluent (flow rate of 1.2 mL  $\text{min}^{-1}$ ). Changes of pH and electrolytic conductivity in plasma treated water were measured by pH and conductivity probes (WTW, Adwa).

Aqueous solutions of various initial pH and conductivities  $\sigma$  used for plasma treatment experiments were prepared by addition of different salts to deionized water ( $\sigma = 1 \mu\text{S/cm}$ ,  $\text{pH} = 5.5$ ):

- $\text{NaH}_2\text{PO}_4$  solutions ( $\sigma = 0.6 \text{ mS/cm}$ ,  $\text{pH} = 5.5$ ) mimic the natural conductivity of tap water ( $\sim 0.6 \text{ mS/cm}$ ).  $\text{NaH}_2\text{PO}_4$  salt has similar chemical composition with the phosphate buffer described below but no buffering activity. In this work we designated these non-buffered solutions as **'water'**.
- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  phosphate buffered (PB) solutions ( $\sigma = 0.6 \text{ mS/cm}$ ,  $\text{pH} = 6.9$ ), designated as **'PB'**.
- physiological NaCl (saline) solutions (NaCl concentration 0.85% vol.,  $\sigma = 6.35 \text{ mS/cm}$ ,  $\text{pH} = 6.7$ ), non-buffered, designated as **'saline'**. Saline solution is a natural cell environment.
- physiological NaCl (saline) solutions (concentration 0.85% vol.,  $\sigma = 6.0 \text{ mS/cm}$ ,  $\text{pH} = 6.9$ ) with  $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$  phosphate buffer, designated **'PBS'**.

Bio-decontamination effects were tested on Gram-negative *Escherichia coli* (CCM3954) in water with initial populations from  $10^6$  to  $10^8$  colony forming units per mL (CFU/mL). Cultivated bacteria in the liquid nutrient were centrifuged several times and diluted in water/saline solution to obtain desired concentrations. Alternatively, bacteria pre-cultivated on gel discs were directly dissolved in the desired solution under study. The plasma experiments with bacteria suspensions were performed with positive transient spark and repeated 10-15 times. The number of bacteria cells in the solution was assayed by counting colony forming units cultivated on agar plates (MFC, HiMedia, Mumbai, India; Biolab).

### 3. Results and discussion

#### 3.1. Transient spark discharge (TS)

Electrical parameters and emission spectra of transient spark discharge (TS) operating in atmospheric air with water spray were documented in detail in our previous works [22-23]. The typical voltage and current waveforms of positive TS discharges in 10 mm gap (with electro-spray of water) are shown in Fig. 3. The TS plasma cannot reach equilibrium conditions and remains relatively cold. With water electro-spray through TS, a great care was taken to keep the constant electrical parameters throughout each experiment since the water spray substantially perturbs the discharge regularity (especially jitters the pulse frequency). In order to keep the constant power dissipated into the discharge, the pulse frequency was controlled at  $\sim 1$  kHz. The typical applied power used in this work was 1-2 W, with energy 1-2 mJ per pulse. The water was sprayed through the high voltage hollow needle electrode under a constant flow rate of 0.5 mL/min.

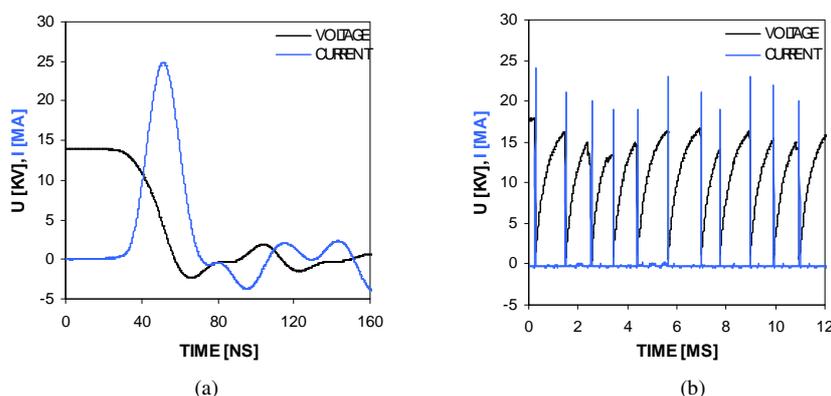


Fig. 3. Typical voltage and current waveforms of TS discharge with electro-spray of water in (a) ns time scale – one pulse, and (b) ms time scale – several pulses, 10 mm gap.

#### 3.2. Bactericidal effects of plasma treated water

Bacterial suspensions of *E. coli* in aqueous solutions were electro-sprayed through the discharge. The bactericidal efficiency expressed as log reduction reached up to 7 logs for both non-buffered water and saline, directly after plasma treatment. A slight enhancement of the bactericidal effect was observed when bacteria were left in the plasma treated water or saline for 4-5 h longer. This indicates ongoing biochemical processes in the plasma activated water post treatment, also reported in [3-4, 10, 14, 15].

The bactericidal effect of plasma treated water and saline was always accompanied with the decrease of pH (down to pH  $\sim 3$ ) and the increase in the solution conductivity (water  $0.6 \rightarrow \sim 1.04$  mS/cm, saline solution  $\sim 6.35 \rightarrow \sim 6.85$  mS/cm). However, additional tests (not shown) performed to evaluate whether low pH is the main bactericidal agent showed that the nitric or hydrochloric acid solution of the same pH did not lead to the same biocidal effects. In agreement with [2, 9, 10, 15], we confirm that rather acid environment in synergy with plasma agents leads to the bacterial inactivation.

To elucidate the role of pH in the plasma induced bactericidal effects, we performed experiments with phosphate buffered solutions (PB and PBS) of sufficient buffer capacity (pH=6.9 remained fairly constant upon treatment or decreased very little). As a consequence, the bactericidal effect was strongly reduced in PB and PBS with respect to non-buffered solutions, but not completely stopped (about 1-log). Fig. 4 compares the bactericidal effect of water vs. PB, and saline vs. PBS obtained immediately after plasma treatment and 5 h later. Interestingly, the decontamination in plasma treated PB and PBS increased substantially after 5 h, without further pH change.

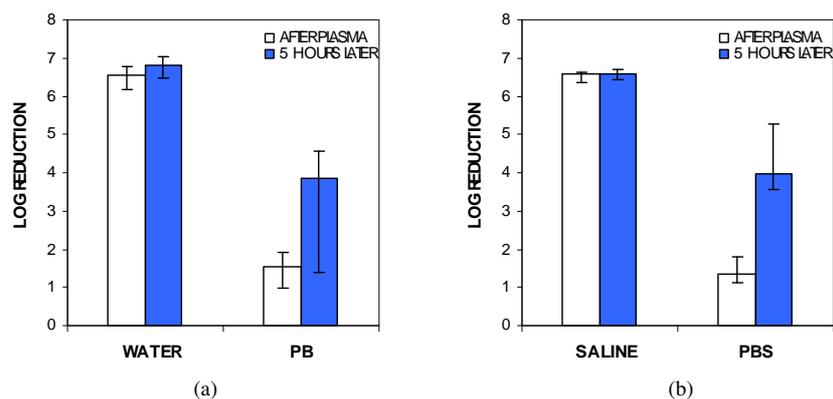


Fig. 4. *E. coli* inactivation of log reduction after plasma treatment and 5 h later obtained in: (a) water (pH~3.3 after plasma and ~3.4 5h later, respectively) and PB solutions (pH~6.2 and ~5.8, respectively); (b) saline solution (pH~2.8 after plasma and ~2.9 5h later, respectively) and PBS (pH~5.6). Medians with error bars showing 25 and 75 percentiles.

### 3.3. Chemical effects induced in water by plasma

In order to evaluate the mechanisms participating in bacterial inactivation, the chemical effects induced in water sprayed through the transient spark discharge were studied in more detail. Since the discharge operates in atmospheric humid air, we focused mainly on the formation of ROS and RNS: hydrogen peroxide ( $H_2O_2$ ), nitrites ( $NO_2^-$ ) and nitrates ( $NO_3^-$ ) and peroxynitrites ( $ONOO^-$ ).

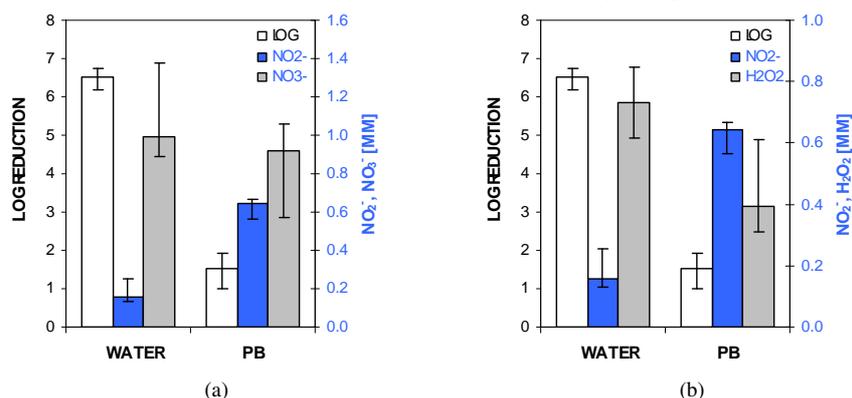


Fig. 6. Bactericidal effect (log reduction) of water and PB solutions related to (a)  $NO_2^-$  and  $NO_3^-$  and (b)  $NO_2^-$  and  $H_2O_2$  concentrations. Medians with error bars showing 25 and 75 percentiles.

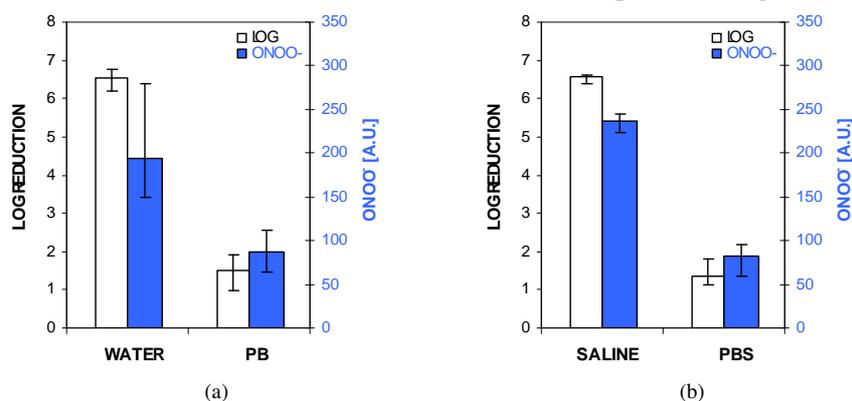


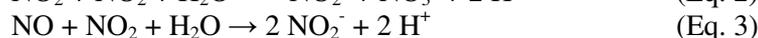
Fig. 7. Bactericidal effect (log reduction) of (a) water and PB solutions, and (b) saline and PBS, after plasma treatment correlated with the relative  $ONOO^-$  concentrations (fluorescence emission at 523 nm with reference subtracted). Medians with error bars showing 25 and 75 percentiles.

Fig. 5 shows the concentrations of nitrites, nitrates and hydrogen peroxide obtained in water and PB solutions after spraying through the discharge. Fig. 5 shows that non-acidic environment of PB buffers resulted in much higher concentrations of nitrites (~0.6 mmol/L) and slightly lower concentrations of

H<sub>2</sub>O<sub>2</sub> (~0.4 mmol/L) and nitrates (~0.9 mmol/L) compared to that for non-buffered solutions, in which the concentrations of H<sub>2</sub>O<sub>2</sub> were ~ 0.7 mmol/L, nitrites ~ 0.2 mmol/L and nitrates ~ 1 mmol/L. Such a significant pH effect on the concentrations of nitrites is result of disproportionation of nitrites into nitrates occurring under acidic conditions:

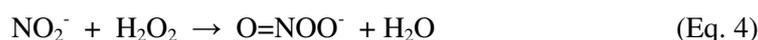


This route is greatly accelerated at pH below ~ 3.5 which correlates with acid-base properties of nitrites ( $\text{p}K_a = 3.3$ ). Since the only difference between buffered and non-buffered solutions was pH, it is reasonable to assume that the initial production rates of nitrites and nitrates by the discharge were the same under both conditions. The mechanism of their formation in water is the result of dissolution of NO<sub>x</sub> formed in air plasma by gas-phase reactions of dissociated N<sub>2</sub> and O<sub>2</sub>. Along with formation of NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> in the plasma treated water, dissolution of NO<sub>x</sub> in water leads to the decrease of pH:



The difference in the concentrations of NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> measured in both solutions is then a result of subsequent post-discharge reactions leading to disproportionation of nitrites into nitrates (Eq. 1).

The formation of nitrates may also proceed via the liquid-phase reaction of NO<sub>2</sub> with OH radical to form peroxynitrous acid (ONOOH) or its conjugate base peroxynitrite (O=NOO<sup>-</sup>), which subsequently decay into NO<sub>3</sub><sup>-</sup>. Or in the presence of hydrogen peroxide, peroxynitrite can be also formed by the reaction of nitrite anion with H<sub>2</sub>O<sub>2</sub>.



This route occurs under acidic conditions and might be responsible for lower concentrations of hydrogen peroxide determined in non-buffered water. Peroxynitrites are relatively strong oxidants with a large bactericidal effect and they may significantly contribute to inactivation process induced by air plasma in water. Under acidic conditions their bactericidal effect is determined mainly by OH radicals formed by the decomposition reaction of peroxynitrous acid [8]. It is likely that with the decreasing pH, the contribution of ONOO<sup>-</sup> to bactericidal effects of plasma increased. The attempt was made to correlate the concentrations of nitrites and H<sub>2</sub>O<sub>2</sub> with the concentrations of ONOO<sup>-</sup> formed in plasma treated water. Since ONOO<sup>-</sup> are highly reactive under acidic conditions, their lifetime is very short and their detection in plasma treated water was possible only qualitative with concentrations estimated in the order of tens of μmol/L. Nevertheless, Fig. 7 shows that the relative amounts of ONOO<sup>-</sup> measured by fluorescence spectroscopy correlate directly with the bactericidal effect. These qualitative measurements indicate an important role of ONOO<sup>-</sup> in bio-decontamination.

At the same time, the antimicrobial properties of nitrites under acidic conditions have to be considered. These so-called “acidified nitrites” are capable of great effect on bacteria [3, 16]. In fact, it seems more reasonable to assume that acidified nitrites are more important route of bactericidal effects than through their reaction with H<sub>2</sub>O<sub>2</sub> upon formation of peroxynitrites (Eq. 4).

#### 4. Conclusions

We investigated the treatment of water or saline non-buffered and buffered solutions contaminated with *E. coli* bacteria electro-sprayed through the cold air plasma of transient spark. The treatment lead to acidification and the production of nitrites, nitrates, peroxides and peroxynitrites. At lowered pH, nitrites are quickly oxidized to nitrates with H<sub>2</sub>O<sub>2</sub> and this is associated with the strong bactericidal effect. At neutral pH in buffered solutions, nitrites are less oxidized and the biocidal effect is weaker. The bactericidal effect correlates with the relative amount of the formed peroxynitrites. The estimated concentrations of peroxynitrites are, however, too low compared to the measured nitrites and H<sub>2</sub>O<sub>2</sub> to consider the reaction (Eq. 4) as the main mechanism of their formation and consider peroxynitrites as the key bactericidal agent. It rather seems that synergistic effects of nitrites and peroxides in acidic conditions are the most responsible for bactericidal properties of water sprayed through air plasma.

## 5. Acknowledgements

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