Master Thesis

Czech
Technical
University
in Prague

F3

Faculty of Electrical Engineering
Department of Circuit Theory

Bacterial Inactivation by Physiologically Relevant Liquids Activated by Atmospheric Pressure Non-thermal Plasma

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Field of study: Biomedical Engineering and Informatics
Subfield: Biomedical Engineering
May 2018

MASTER'S THESIS ASSIGNMENT

I. Personal and study details

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Branch of study: **Biomedical Engineering**

II. Master's thesis details

Master's thesis title in English:

Bacterial Inactivation by Physiologically Relevant Liquids Activated by Atmospheric Pressure Non-thermal Plasma

Master's thesis title in Czech:

Inaktivace bakterií fyziologicky významnými roztoky ošetřenými netermálním plazmatem za atmosférického tlaku

Guidelines:

1. Do literature research about non-thermal plasma and plasma activated solutions.
2. Learn about effects of plasma activated solutions.
3. To measure chemical and physical properties of plasma activated solutions.
4. Plan experiments to prove antibacterial effects of plasma activated solutions.
5. Discuss results of experiments and compare them to results found in literature research.

Bibliography / sources:

[1] FRIDMAN, Alexander A. Plasma chemistry. New York: Cambridge University Press, 2008. ISBN 0521847354.
[2] FRIDMAN, Alexander A. a Gary FRIEDMAN. Plasma medicine. Chichester, West Sussex, U.K.: John Wiley, 2013. ISBN 0470689706.
[3] LAROUSSI, M. Plasma medicine: applications of low-temperature gas plasmas in medicine and biology. New York: Cambridge University Press, 2012. ISBN 1107006430.
[4] TRAYLOR, M. J, PAVLOVICH, et al. Long-term antibacterial efficacy of air plasma-activated water. Journal of Physics D: Applied Physics. 2011, 44(47), 472001.
[5] YOO, S.R., J.S. PARK, S.M. RYU, et al. A method for generating plasma activated water and its biological assessments. IEEE International Conference on Plasma Science. 2012, p. 151.

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Date of master's thesis assignment: **19.02.2018** Deadline for master's thesis submission: **25.05.2018**

Assignment valid until: **17.02.2019**

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III. Assignment receipt

The student acknowledges that the master's thesis is an individual work. The student must produce his thesis without the assistance of others, with the exception of provided consultations. Within the master's thesis, the author must state the names of consultants and include a list of references.

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Date of assignment receipt	Student's signature

Acknowledgements

My appreciation goes to my supervisor Ing. Vladyslava Čeledová, Ph.D. for all her support, guidance and patience during the work on my Master's Thesis. She helped me with all the experimental work at the Department of Physics, FEE, CTU in Prague. I want to express my deep gratitude to my supervisors at RWTH Aachen Robert Müller, MS.c. and Dr. Hendrik Ballerstedt for the opportunity to work in the laboratory at RWTH Aachen, for their support and guidance. My thanks goes also to my family for all the support I got.

Declaration

This Master's Thesis was done as a part of the double degree studium at CTU in Prague and at RWTH Aachen.

I declare that the presented work was developed independently and that I have listed all sources of information used within it in accordance with the methodical instructions for observing the ethical principles in the preparation of university thesis.

Prague, 25. May 2018

Abstract

This Master Thesis is focused on the examination of antibacterial effects of plasma activated physiologically relevant solutions such as plasma activated water (PAW) and plasma activated saline solution (PASS). For this purpose, one gram-positive and one negative-bacteria are used. Plasma induces formation of various reactive oxygen and reactive nitrogen species in aqueous solutions. And as a result, the final solution becomes bactericidal. This phenomena is called plasma activation and was described by many researchers [BRI16, DOB11, GAU06, KAM09]. Dispite the huge interest to the topic, the mechanism of bacteria inactivation is still not well understood. Hence, the current work was done to examine the bactericidal efficiency of PAW and PASS prepared using positive corona discharge generated by well known point-to-plane geometry.

In this work, non-thermal plasma at the atmospheric pressure is used. In the activated solutions are measured chemical and physical changes of species which are present. Nitrites, nitrates, hydrogen peroxide, ozone and pH are measured and compared to other experiments. Demineralized water or saline solution were activated by the positive corona discharge for several time sequencies in the range from 0 to 12 minutes and then bacteria were exposed to these solutions. The bactericidal effect was then evaluated. Effectivity of PAW and PASS was evaluated by the comparison of the amount of colony forming units cultivated on Lysogeny broth agar surface.

The goal of the thesis is to examine the effects of the different setup conditions on the generated ions occurring in the solution after activation and finding the ideal conditions to generate highest amount of these species.

Keywords: Plasma activated water (PAW), corona discharge, plasma, *E. coli*, *B. subtilis*, bacteria inactivation, non-thermal plasma

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Abstrakt

Cílem této diplomové práce je prozkoumat antibakteriální účinky plazmatem aktivovaných fyziologicky relevantních roztoků, jako například plazmatem aktivované vody (PAW) či plazmatem aktivovaného fyziologického roztoku (PASS). Porovnání cidních účinků těchto roztoků bylo provedeno na jedné gram-pozitivní a jedné gram-negativní bakterii.

Plazma produkuje širokou škálu reaktivních dusíkatých iontů a reaktivních kyslíkatých iontů, které následně reagují s vodními roztoky. Výsledkem je roztok s baktericidními účinky. Tento fenomén je nazýván aktivace plazmatem a byl již popsán v mnoha publikacích [BRI16, DOB11, GAU06, KAM09]. Stále však není dostatečně prozkoumán mechanismus inaktivace bakterií. Tato diplomová práce zkoumá baktericidní účinky PAW a PASS připravené pomocí kladného korónového výboje. Pro aktivaci roztoků je použit stejnosměrný výboj generující netermální plasma. Následně jsou v těchto roztocích změřeny koncentrace některých chemických sloučenin (dusičnanů, dusitanů, peroxidu vodíku a ozónu). Tyto výsledky jsou porovnány s ostatními studiemi.

Demineralizovaná voda nebo fyziologický roztok byly aktivovány kladným korónovým výbojem po definovaný čas v rozsahu 0 až 12 minut a následně byly do těchto roztoků přidány bakterie. Vyhodnocení efektivity použitého procesu a setupu bylo provedeno sčítáním kolonií po kultivovaci na LB agaru. Výsledkem této práce je porovnání účinků PAW a PASS pro různě nastavené parametry setupu na množství iontů generovaných plazmatem a následně difundovaných do roztoku.

Klíčová slova: plazmatem aktivovaná voda (PAW), korónový výboj, plazma, *E. coli*, *B. subtilis*, inaktivace bakterií, netermální plazma

Překlad názvu: Inaktivace bakterií fyziologicky významnými roztoky ošetřenými netermálním plazmatem za atmosférického tlaku

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Chapter 1

Introduction

The plasma medicine is a relatively new field of research which combines the plasma physics and the clinical medicine. The plasma can be used to activate solutions, which gain antibacterial properties as the result of the plasma treatment [ABU18, BAK16, DOB11]. These solution could be used for clinical treatment for their efficiency, low-cost and simplicity of their preparation and no need for the medicals. The relation between generated species and efficiency of the bacterial inactivation are studied to maximaze the efficiency with minimal demands. Some researches already shown, that increasing activation time and bacteria treatment time increases these effects up to 8 orders of magnitude reduction of bacteria. Nevertheless, exact role of all available species in the solution in the process of inactivation is still not well understood. In many researches, nitrites and pH for the experiment and efficiency comparison are measured. The other species such as nitrates, ozone etc. are not investigated for the different experimental setup and conditions. This work focuses on ability of the plasma discharge to generate enough species such as nitrites, nitrates, ozone and hydrogen peroxide to change the chemical properties of the solution. The results of the antibacterial effects of the activated solutions are compared to other available studies. For the purpose of the microbiological part, two model organisms *E. coli* and *Bacillus subtilis* were treat to study induced bactericidal properties of water and physiological saline solution.

1.1 Goals and Structure of the Thesis

In the Chapter 2 the theoretical basics are illustrated, which are relevant in the context of this Master thesis. Several relevant discharges used for the plasma activation of the solutions are described in this chapter.

In the practical part of this Thesis, the relation between the setup settings and the properties of the activated solutions were examined. Therefore, in the Chapter 3, chemical background of ions in the interest of this work are described in details and methods to determine such ions are presented.

In the Chapter 4 (practical part), results of several ions such as nitritas, nitrates etc. measurement and comparisson to the other publications are presented. The dependencies of bactericidal effects and concentrations of chemical compounds on the activation time, the distance between electrodes and the applied voltage is exsperimentally investigated. The antibacterial effects of ativated solution are shown on one gram-negative and one gram-positive bacterium and results are compared to other studies.

Chapter 2

Theoretical Background

In this part, main theoretical basics are briefly described. The physical description of plasma and electrical discharges is mentioned, followed by the introduction to the plasma activated solutions (PAS) with respect to the species produced by the discharge and diffused in the solution. Biological basics are presented at the end of this chapter.

2.1 Plasma

A plasma is known as fourth state of matter. When the solid substance is heated, the kinetic energy of molecules increases. Particles increase distances from the points they are oscillating around. With sufficient molecule velocity, the bond between the par-ticles is disturbed, the crystalline lattice is broken and the liquid is formed. When the liquid is heated, it starts to evaporate. By the evaporation, molecules on the surface of the liquid acquire a kinetic energy greater than potential energy and they overcome the forces that attach them to the other molecules and the molecules escape to the free space over the liquid and gas is formed. When the gas is heated enough firstly partially ionization and later complete ionization occurs. Such ionized gas is called plasma [GOL95].

Plasma can be described as (partially) ionized gas (gas composed of ions, free electrons and possibly neutral atoms and molecules), but there are at least two conditions, which need to be full-filled, that it can be called "plasma":

1. collective behaviour
2. quasi-neutrality

As collective behaviour describes property that plasma (as a whole) is a capable to generate electric and magnetic fields and interact with such fields.

Due to the presence of free charged particles, a space charge and an electrostatic field are generated in the plasma volume, which retroactive-ly interacts with the charged particles. That yields to compensation of fluctuation of charge density and plasma appears to be electrically neutral in macro view [FIT15]. Quasi-neutrality means, that plasma should have almost equal number of positive ions and negative electrons in plasma areas, moreover all three linear dimensions are greater than Debye length.

2.1.1 Plasma parameters

The plasma stretches over wide ranges in kinetic temperature, particle density, ionization degree and other parameters. Plasma can be produced at low-pressure, atmospheric-pressure or high-pressure environment. Some parameters are described in following chapters.

Particle density

Particle density n_s is the number of species s in the volume of one cubic meter. Plasma is from its definition quasi-neutral, that means, that the density of negative charge carries (e.g. electrons, negative ions) is equal to the density of positive ions (equation 2.1) [FIT15]:

$$n_e \cong n_i \equiv n. \tag{2.1}$$

In electronegative gases (e.g. Air) we can assume, that the number of negative ions is much smaller than a number of electrons.

Temperature and thermal velocity of particles

One of the major parameters of plasma is the temperature of particles. An idealized plasma consists of equal number of electrons (with mass m_e and charge -e) and ions (with mass m_i and charge $+e$). In this case e means the magnitude of the particle charge. The kinetic temperature T_s of species s is then defined:

$$T_s = \frac{1}{2} m_s \langle v_s \rangle^2, \tag{2.2}$$

where m_s is the mass of particle s (it represents either electrons or ions), v_s is a particle velocity and angular brackets represent ensemble average (Reif 1965).

Because in the plasmas ions and electrons are normally characterized by the same temperature T, a thermal velocity v_t (typical particle velocity) can be defined [FIT15] as:

$$v_{ts} = \sqrt{\left(\frac{kT_s}{m}\right)}, \tag{2.3}$$

where k is Boltzmann constant and T_s is the thermodynamic temperature of the species. Because the thermal velocity of ion particles is usually far smaller than the electron thermal velocity, we can define the thermal velocity of ions as:

$$v_{ti} = \sqrt{\left(\frac{m_e}{m_i}\right)} \cdot v_{te}, \tag{2.4}$$

Temperature T_s is in general function of particle position in plasma.

By the temperature of ions and electrons we can divide plasmas into two main groups (see Table 2.1):

Low temperature plasma		**High temperature plasma**
Thermal	Non-thermal	
$T_i \approx T \approx 300$ K $T_i \gg T_e \lesssim 10^5$ K	$T_e \approx T_i \approx T \lesssim 2 \cdot 10^4$ K	$T_i \approx T_e \gtrsim 10^7$ K

Table 2.1: Classification of plasma by its temperature

Thermodynamic temperature T_s is measured in electron-volts (1 J is equal to $6,24 \cdot 10^{18}$ eV, 1 degree of Kelvin $= 8,62 \cdot 10^{-5}$ eV). The low temperature plasma can be classified into two groups according to the temperature of its species - thermal and non-thermal plasma. The temperature of electrons in the thermal plasma is approximately equal to the temperature of ions and neutral species and it doesn't exceed 2 x 10^4 K. The ions temperature in the

non-thermal plasma is approximately equal to the temperature of neutral species (approx. 300 K) and the electron temperature is much higher than the temperature of ions. The ion and the electron temperature are in the high temperature plasma higher than 10^7 K.

In this Master Thesis non-thermal plasma is used. There are two main advantages about using non-thermal plasma. FRelatively high electron temperature (approx. 10^4 – 10^5 K), reactive processes that require high activation energy can be realized; And because of low gas, ions and neutrals temperature (approx. 300 K), there is no thermal dissociation of reaction products and the thermal stress of treated interfaces is minimal. These properties make non-thermal plasma suitable for biomedical applications and treatment of heat sensitive materials.

Degree of ionization

This parameter expresses the concentration ratio of charged particles to all particles in plasma (in the simplest case we consider that plasma consists of ions, electrons, and neutrons). In this case the degree of ionization is defined [MEI13]:

$$\chi = \frac{n_e}{n_e + n_n}, \tag{2.5}$$

where n_e and n_n are particle density of electrons and neutrons.

According to this parameter plasmas can be divided into two main groups:

- Weakly or partially ionized plasma, for $\chi \ll 1$
- Strong ionized plasma, for $\chi \sim 1$

Degree of ionization can vary in between 10^{-8} and 1. The concentration of charged particles of the non-thermal plasma is much lower than the concentration of all particles ($n_e \ll n_i$) and therefore it is weakly or partially ionized. The ionization degree of the non-thermal plasma can be then estimated as [MEI13]

$$\chi = \frac{n_e}{n_n}, \tag{2.6}$$

Collisions in plasma

Collisions between charged particles are affected by the long range of the Coulomb forces. For plasmas with a large plasma parameter λ, binary collisions present and a collision frequency $\vartheta_{ss'}$ may be defined, that indicates the rate of the scattering of species s by species s'. A total collision frequency for species s is defined as a sum of partial collision frequencies for all species s' [FIT15], that means:

$$\vartheta_s = \sum \vartheta_{ss'}. \tag{2.7}$$

This parameter measures a frequency with which a particle trajectory undergoes a major angular change due to Coulomb interactions with other particles. In this case, the frequency is not the inverse value of time between collisions because of small angle scattering events. This frequency is inverse of the typical time needed that particle reaches enough collisions to deviate a particle trajectory through 90° and therefore is this frequency also called 90° scattering rate.

Mean-free-path is defined [FIT15]:

$$\lambda_{mfp} \equiv \frac{v_t}{\vartheta_s} \tag{2.8}$$

and it describes the mean path between collisions (90∘ scattering events). ϑ_s is the total collision frequency and v_t is thermal velocity of that particle.

If the mean-free-path λ_{mfp} is much smaller than the length-scale L of the observation $\lambda_{mfp} \ll L$, then the plasma is collisional or collisions dominate. If the observation length-scale L is larger than the mean-free-path, the plasma is called collisionless.

A typical magnitude of the collision frequency may be approximated by a relation between plasma parameter Λ and plasma frequency ϑ_{pe} [FIT15]:

$$\vartheta \sim \frac{ln\Lambda}{\Lambda} \cdot \vartheta_{pe}. \tag{2.9}$$

If $\vartheta \ll \vartheta_{pe}$, the plasma is weakly coupled, and collisions do not interfere witch plasma oscillations. On the other hand, if $\vartheta \gg \vartheta_{pe}$, the plasma is strongly coupled, and collisions prevent plasma oscillations.

Using equation for the Debye length, the equation 2.9 can be rewritten [FIT15]:

$$\frac{\lambda_{mfp}}{\lambda_D} \sim \frac{\Lambda}{ln\Lambda} \tag{2.10}$$

and in weakly coupled plasma this ration is large.

Combining equations for coupling and plasma parameter leads to [FIT15]

$$\vartheta = \frac{3 \cdot e^4 \cdot ln\Lambda}{4 \cdot \pi \cdot \epsilon_0^2 \cdot \sqrt{m}} \cdot \frac{n}{\sqrt{T_s^3}}. \tag{2.11}$$

From this equation follows, that high temperature plasmas are collisionless (higher temperature leads to smaller collision frequency) and the low temperature plasmas (non-thermal) are collisional.

Low-pressure, high-pressure and atmospheric-pressure plasma

Low-pressure plasma is produced in closed system, where the pressure achieves 10^2 to 10^{-4} Pa. A large mean free path is typical for this kind of plasma and therefore it fits to generation of directed ion currents to the target surface. Low-pressure systems are, however, expensive.

High-pressure plasma is generated in special gas discharge lamps and the pressure may reach up to 10^8 Pa. This plasma has no special usage for surface or other kind of treatment.

Atmospheric-pressure plasma is produced at approximately atmospheric pressure (approx. 10^5 Pa). The main advantage of atmospheric-pressure plasma is no need for special systems (e.g. air pumps); Plasma generators are widely available and cost-effective. Hence, atmospheric-pressure plasma is used for tissue and bacteria treatment and plasma medicine.

2.1.2 Electrical discharges

Electrical discharges can be classified into two main groups (self-sustained and non-self-sustained discharges) with respect to the need of an external ionization agent for a generation of charged particles. UV radiation, RTG radiation or electrons from a heated cathode can be used as a ionization agent. If the agents are removed, the non-self-sustained discharge disappears. Self-sustained discharges exist further without this agent. Classification of all discharges according to the applied voltage and the type of generated plasma is shown in the Table 2.2.

Power supply	Type of discharge	Ion and Neutral species temperature [K]	Type of plasma
AC	Dielectric barrier discharge	300 - 400	Non-thermal
AC, DC, pulse, HF	Plasma jet	300 - 400	Non-thermal
DC, pulse	Corona discharge	300 - 400	Non-thermal
DC, pulse	Streamer	1.000 - 10.000	Thermal
AC	Sliding discharge	1.000 - 5.000	Thermal
AC, DC	Arc discharge	2.000 - 8.000	Thermal
HF	Microwave discharge	1.000 - 9.000	Thermal

Table 2.2: Types of electrical discharges [VLK16]

Electrical Breakdown

In the case of electrical breakdown, an insulator (gas for discharges in the gas) is changed into a conductor. This depends on the pressure and external electric field strength. The uniform electric field between two parallel electrodes placed in a distance d, in a neutral gas with pressure p and with applied voltage U is defined [MEI15]:

$$|\mathbf{E}| = \frac{U}{d}. \tag{2.12}$$

Free electrons are released from cathode and they are accelerated in the uniform electric field toward to anode. In the case, that their energy is above a threshold for ionization, an ionization of neutral gas appears with typical mean free path λ_i and an electron-ion pair is created. The number of produced electron-ion pairs is described with the equation [PIE10]

$$\mathrm{d}n_e = \alpha \cdot n_{e0} \cdot \mathrm{d}z, \tag{2.13}$$

where α is the first Townsend coefficient, $\mathrm{d}z$ is a distance in the z-direction, n_{e0} is the number of electrones emited by the usage of the ionization agent and $\mathrm{d}ne$ is the number of new created pairs. This leads to exponentially increase of pairs number [PIE10]:

$$n_e = \alpha \cdot n_{e0} \mathrm{e}^{\alpha z}. \tag{2.14}$$

In the Figure 2.1 is depicted how new electron-ion pairs are created. The Townsend coefficient express the number of electron-ion pairs generated per unit length by a negative ion moving from cathode to anode and is defined as inverted value of mean free path [PIE10]:

$$\alpha = \frac{1}{\lambda_i}. \tag{2.15}$$

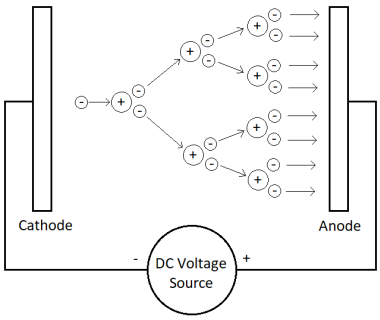

Figure 2.1: Visualization of Townsend avalanche with a constant Townsend coeficient

The total number of produced electron-ion pairs depends on the gas pressure p and distance between electrodes d. Probability of ionization is then estimated [MEI15]:

$$P_i = \frac{n_e(z)}{n_{e0}} = \mathrm{e}^{\frac{-z_i}{\lambda_i}}, \tag{2.16}$$

where z_i is the ionization length (path length of electrons in the electric field to reach kinetic energy necessary for ionization).

DC discharges

DC electrical discharges are generated by the DC power supply. Initiation of several types of DC discharges depends on applied voltage and current and this is depicted by Volt-ampere characteristics (see figure 2.2). This characteristic applies for low-pressure conditions, when the distance between electrodes can be larger. The V-A characteristic consists of 3 main parts, which corresponds to each type of discharge:

- Dark discharge is up to 10^{-5} A. Townsend discharge and corona discharge belongs in here.
- Glow discharge appears in the range of aproximately 10^{-5} and 1 A. There are three types of glow discharge: subnormal (in between 10^{-5} and 10^{-4} A), normal (from 10^{-4} to 10^{-2} A) and abnormal glow (from 10^{-2} to 1 A)
- Arc discharge – arcdischarge can be non-thermal (from 1 to 100 A) or thermal (from 100 to 10 000 A)

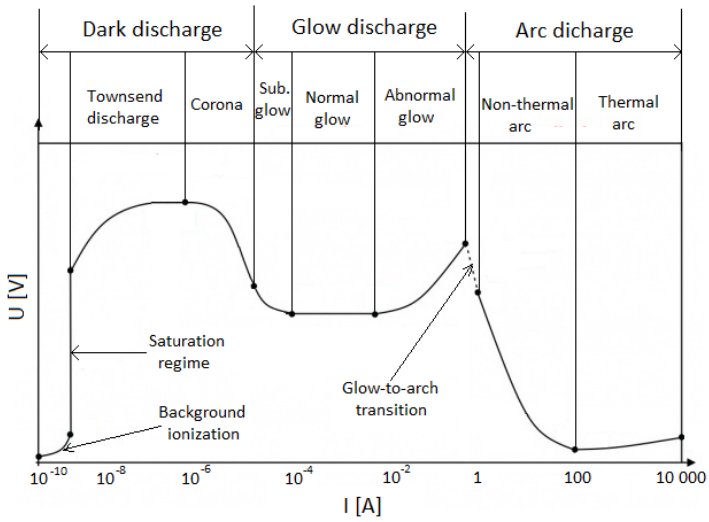

Figure 2.2: Volt-ampere characteristic of the DC discharges [VLK16]

Corona Discharge

Corona discharge is a discharge that occurs around the electrodes with small radius of curvature and with large gradient of electric field. It generates non-thermal highly colisional plasma with the temperature of ions and neutral species in the range of 300 K (room temperature). Depending on the used power supply and electrode arrangement corona can be positive, negative, bipolar, AC or HF type. To generate unipolar positive or negative corona discharge, the point-to-plane electrode arrangement can be used (see Fig. 2.3). Corona discharge is generated around the point electrode and several discharge regions can be described.

Ionization region is around the active electrode, where the electric field reaches high values. Within this region is high energy to support ionization of species. In the drift region, which is further from the active electrode, ions and electrons drift and react with neutrals. The energy in this region is too low for ionization and to react with other ionized particles.

Two different types of DC coronas exist:

1. Unipolar corona discharge, which burns stably around the active electrode. or at least the electrical pulses have high repetition frequency, that the ion flow in the drift region seems to be continuous. For the positive corona, positive ions are produced and for the negative corona dominate negatively charged ions.

2. Bipolar corona occurs at higher currents and especially at positive point polarity. In this case a donductive plasma is produced in the ionization region faster, than it can be absorbed by the point electrode.

However, both types of corona may coexist. The current density of unipolar corona discharge is distributed over the plane electrode according to Warburg's law [GOL85]:

$$j(\theta) = j_0 \cdot \cos^5 \theta, \tag{2.17}$$

where *j(θ)* is the current density for the angle θ between perpendicular axis in between point and pland electrodes. This angle is defined [GOL85]:

$$\tan \theta = \frac{r}{d}, \tag{2.18}$$

where r is the distance between point electrode and a place on the plane electrode and d is the distance between electrodes. Initial current density j_0 can be approximated by [GOL85]:

$$j_0 = \frac{I}{2 \cdot d^2}, \tag{2.19}$$

where I is a total corona current and d is a distance between electrodes.

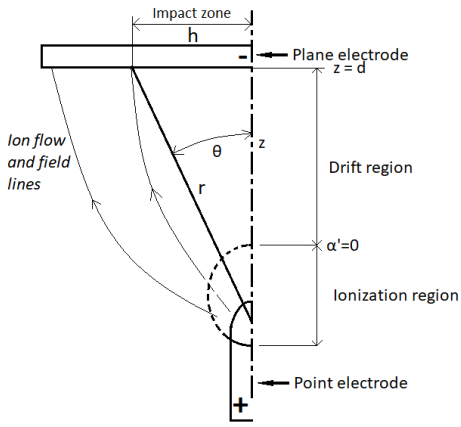

Figure 2.3: Point-to-plane corona geometry [GOL85]

The current of the unipolar corona discharges generated by using point-to-plane electrode arrangment is limited by the saturation currect, that is equal to:

$$I_{sat} = 2 \cdot \mu \cdot \epsilon_0 \cdot \frac{U^2}{d}, \tag{2.20}$$

where μ is the ion mobility and ϵ_0 is the permitivity.

Minimal Voltage

The minimal Voltage required to initiate a visible corona discharge (also called "corona inception voltage" or "corona onset level") can be found by the Peek's law [PEE20]:

$$U_{min} = m_v \cdot E_0 \cdot r_w \cdot ln\left(\frac{d}{r_w}\right), \tag{2.21}$$

where m_v is an irregularity factor of the electrodes (for clean wires is $m_v = 1$), r_w is the radius of the wires in cm, d is the distance between the center of the electrodes in cm, E_0 is the "visual critical" electric field given by [ADA15]:

$$E_0 = g_o \cdot \delta \cdot \frac{1 + c_e}{(\sqrt{\delta \cdot r_w})}, \tag{2.22}$$

where δ is the air density factor with respect to SATP (25 °C and 76 mmHg), g_0 is the "disruptive electric field." (30-32 kV/cm in air [ADA15]), c_e is an empirical dimensional constant (0.301 cm$^{\frac{1}{2}}$ in the air). For the density factor follows:

$$\delta = \frac{0,392 \cdot p}{273 + t}, \tag{2.23}$$

where p is the actual pressure in mmHg and t is the room temperature in °C.

In the Table 2.3 minimum onset voltage for radius of needle electrode with radius 50 µm (used in this work), room temperature 23 °C, pressure 760 mmHg and for vertical distances in between 4 mm to 7 mm is shown.

r_w [mm]	l [mm]	U_{min} [V]
0,05	4	3 580
0,05	5	3 770
0,05	6	3 920
0,05	7	4 040

Table 2.3: Minimal onset voltage for several setup settings

For the initiation of corona discharge higher voltage than the minimal onset voltage has to be used (in this case higher than 4 kV).

Electrical stress distribution

The range of the corona active zone depends on the applied voltage, the distance between the electrodes and the radius of the positive electrode. The

ionization zone is the field zone, where electrical field $E > 21$ kV/cm. The point electrode can be aproximated by a hyperboloid surface and the distribution of electric field between the point and the plane electrodes along the axis between the electrodes is given by [ADA15]:

$$E(x) = \frac{2U}{ln\left(\frac{4d}{r}\right)} \cdot \frac{1}{2 \cdot x + r_e - \left(\frac{x^2}{d}\right)}, \tag{2.24}$$

where *E(x)* is the electric stress in the distance x given in kV/cm, U is the applied voltage in kV, d is distance between electrodes in cm, r_e is the radius of the electrode tip in cm, x is the distance from the tip of the point electrode in cm.

In the Figure 2.4 electrical stress distribution is depicted for different voltages in the range of 7 to 10 kV, distance between electrodes is 4,47 mm and radius of the point electrode is 50 µm.

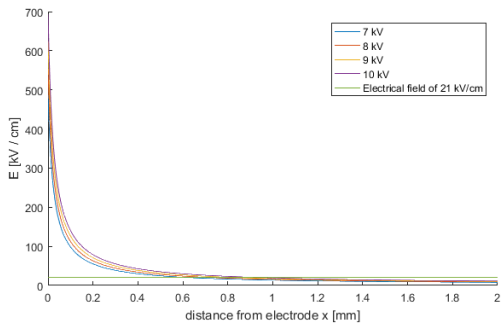

Figure 2.4: Electrical stress distribution for distance between electrodes $d =$ 4,47 mm, the voltage U in the range of 7 kV to 10 kV and the radius of the electrode tip $r_e = 0{,}05$ mm

In the Figure 2.5 the ionization zones ξ (distances from positive electrode along the axis between the electrodes, where the electric field > 21 kV/cm) for the different voltages are shown.

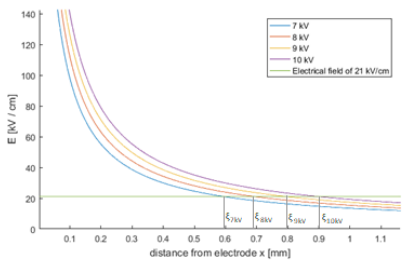

Figure 2.5: Ionization zones for the different voltages

2.2 Plasma activated solution

Plasma induce the prolonged chemical effects into treated liquids. These effects are usually called "plasma activation". Different biologically relevant liquids e.g. water, saline solution, broth etc. can be used to be treated. There are several possible ways to treat the aquaneous solution with plasma:

1. Indirect activation using plasma afterglow and diffusion of radicals into water. In this case, the solution is treated indirectly, it means, the solution is not a part of electrical circuit.
2. Direct plasma treatment means, that the solution is placed inside the electrical circuit and

There are several major species, generated by the plasma over aquaneous solution surfaces:

1. Charged particles
2. Reactive species
3. UV radiation
4. Electric field
5. Heat

2.2.1 UV radiation

UV radiation is electromagnetic radiation with wavelength smaller, than visible light, approx. in the range of 100 – 400 nm. It can be divided into more groups/ranges depending on their wavelength. UV-A (315-400 nm), UV-B (280-315 nm), UV-C (200-280 nm) and VUV (100 – 200 nm) exists, but by the corona discharge has the UV radiation no significant effect. However, for completness the UV radiation is described in this chapter. Antibacterial effects of UV radiation are well known, and UV radiation is also a common way for sterilization. UV-B radiation is destructive for living organism because its energy is capable to decompose or disturb proteins or other vital organic compounds. However, it is not possible to use for medical purposes, because

its longer exposition may have serious consequences for the human metabolism and if the DNA is disturbed, it may cause a cancer disease. This radiation is also capable to destroy one-cell organism by changing a structure of its DNA molecule. Moreover, the UV radiation can cause organel disruption, affect osmotic pressure inside the cell or trigger lysis. UV-C radiation is the hardest UV radiation and it is one of two possibilities to form ozone – when it reaches the two-atom molecule of oxygen, the radiation transmits the energy to produce atomic oxygen and then an ozone may be formed. This radiation is carcinogenic and it starts to ionize. VUV (vacuum UV) has smaller wavelength than 200 nm. This radiation significantly reduces oxygen molecules to form O_3 [ISO 21348].

2.2.2 Reactive species

If the discharge is generated in the air, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated. ROS, e.g. OH or O_3 (ozone) occurs in the plasma and then react with water, where the H_2O_2 is produced. ROS in liquid in the close vicinity of bacteria cause a disruption of the lipid membrane of the cell. As a result, the cell membrane stops preventing a transport ofundesirable molecules into the cell, that can lead to apoptosis and cell death. ROS are reported as responsible for cell damage caused by PAS [PAR16]. RNS are generated in the plasma and in the liquid. Into this group of radicals belong reactive forms of nitrogen, such like N_xO_x, NO_x, ONOO etc. The antibacterial efficiency of ROS and RNS is related to their half-life and energy. The half-life time is a time required for the species to reduce their amount to the half. In the case of unstable species it is time needed to react and produce a stable species. In the followin table (see Table 2.4) species with their half-life are shown.

Species	Name	Half-life	Energy
$OH^{\bullet}$	Hydroxyl radical	$10^{-}9$ s	
NO_2^-	Nitrogen dioxide	$< 10^{-}6$ s	
O_2^-	Superoxide anion	$> 10^{-}6$ s	
NO	Nitric oxide	0,1 - 6,4 s	
H_2O_2	Hydrogen peroxide	10 - 10^2 s	

Table 2.4: Reactive species and their half-life[DVO17]

Several species, which can occur in the air close to the corona discharge are depicted in the Figure 2.6. See Appendix B for a complete list of species that are produce by the corona discharge. Some of these species react with the water to produce other species and some of them are diffused in the solution. Reactions of species with water and the production of new species are depicted in the Figure 2.7.

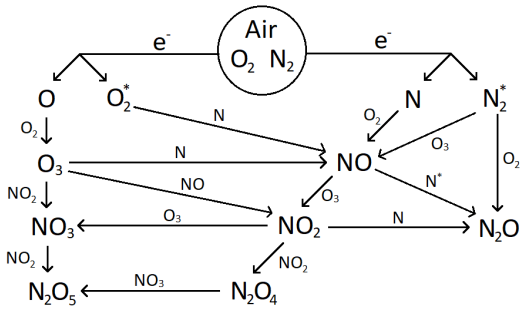

Figure 2.6: Species produced by the corona discharge in the air [MEI15]

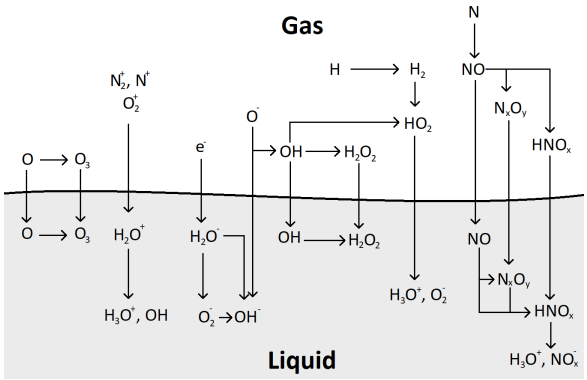

Figure 2.7: Species produced by the corona discharge in the air and water [MEI15]

2.2.3 Heat

Heat, in general, can be used for bacterial inactivation. Various methods using a moist or a dry heat are known. Some bacteria (e.g. *E. coli* are not well heat resistant and they can experience a heat shock already at approx. 42 ∘C, on the other side some bacteria have a well resistance against the heat shock. The temperature about 170 ∘C must be used for a sterilization of most known bacteria. However non-thermal plasmas operate at the room temperature, that is not enough to cause damage to the bacteria. By the indirect activation of solutions without bacteria used in this work, the bacteria are not exposed to the discharge and the heat has no significant role in the treatment performed in this work. [CEL17].

2.2.4 Electrical field

Corona discharge is ignited around electrodes with small radii of curvature with the large gradient of electric field. The electrical field by the discharge reaches high values only at the close vicinity of the electrode (see Figure 2.4). In the further distances, the electrical field is too small to cause any damage to bacteria and its effect can be neglected for the discharge used for the purpose of this work [CEL17].

2.3 Microbiology

Antibacterial effects of plasma activated solutions on prokaryotic organisms were tested using model organisms representing two main groups of bacteria categorized according to the structural differences in their cell walls (described further).

2.3.1 Bacteria

The term "bacteria" covers a large group of prokaryotic microorganisms. The shape of bacteria varies from rod shape, spheres to spirals and its length is mostly only about a few micrometres. [DVO17]

Bacterial structure

On the surface of a bacterial cell is a cytoplasmatic membrane, which is permeable only for molecules smaller than 1 nm. However, this membrane also contains proteins which can transport larger molecules into the cell. Most of the bacteria have the cell wall made of peptidoglycan, that differs and depends on the type of bacteria. The structure of the wall is the criterium for bacterial classification to gram-positive or gram-negative. A bacterial envelope can consist of an another layer called capsule made of lipopolysaccharides and lipoproteins. Its function is to form and protect the bacteria. The bacteria can have flagella, fimbrae or other structures on the membrane. The cell wall of a gram-positive and a gram-negative bacteria is depicted in the Figure

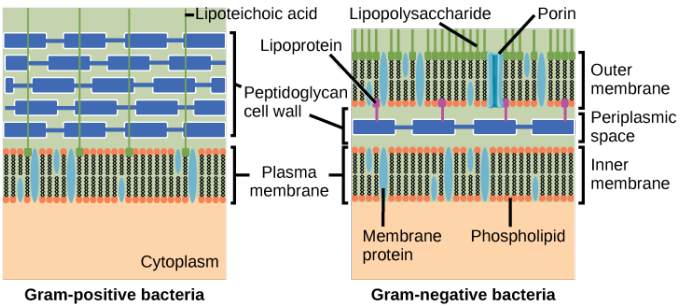

Figure 2.8: A cell wall of gram-positive and gram-negative bacteria. [DON16]

2.8. Within the cell is a cytoplasm containing nutrients, proteins, ribosomes necessary for photosynthesis, single circular bacterial chromosome of DNA (nucleoid) and other es-sential components.

Bacterial growth

Bacteria placed into a medium with suitable conditions (pH, temperature, nutritions) usually grows and replicate. The replication is realized by a cell division – all the content of the cell is duplicated and then the bacterium divides itself to two same cells. If the bacteria are in a medium, the growth curve can be drown (see Figure 2.9). This curve has 4 main phases of bacterial growth:

1. Lag phase – Bacteria need to adapt to conditions in the new high-nutrien medium. Number of the cells doesn't increase and the cells that didn't adapt themselves to a new environment die. Overall the olume, the mass of the cells and a sensitivity to physical and chemical influences increases. Compounds necessary for division are synthesized and the number of en-zymes is increased.

2. Exponential (Log) phase – Cells are adapted to the environment. They start division. Generation time is decreased and growth rate increases. The culture reaches the maximum growth rate. The cells reproduce exponentially until the nutrients are depleted.

3. Stationary phase – This phase is caused by the depleting of nutrients. The growth rate is decreasing and the number of cells undergoing division seems to be equal to that being dead. Metabolites are accumulated in the environment.

4. Dead phase – The bacteria lose the ability to divide and the number of the dead cells exceeds the number of the new reproduced cells. The total

number of the cells decreases.

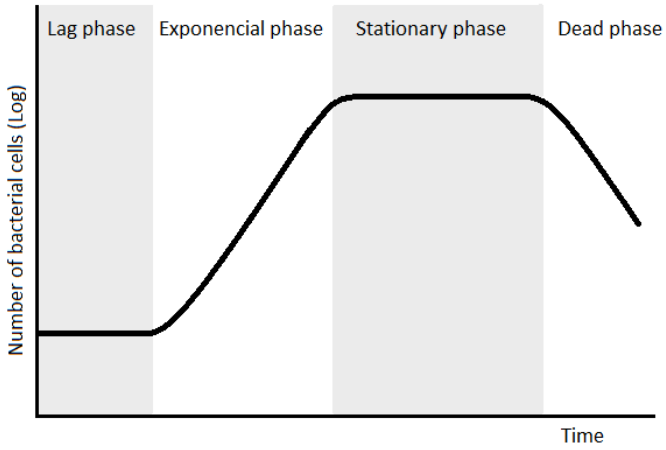

Figure 2.9: Bacterial growth curve

Bacteria die when they are not able to reproduce anymore. The number of the cell colonies may be determined by counting the cells in a given volume of the cultivation media or by measuring of turbidity of the given volume.

2.3.2 Gram stain

The bacteria can be classified into two predominant groups – Gram-positive and Gram-negative. To classify bacteria into one of these broad groups, a staining method devised by H. C. Gram in 1884 has been routinely used in classical microbiology. The classification is based on the different structure of the bacterial wall and accordingly its capacity to retain the crystal violet dye, when subjected to the procedure. The method consists of the following steps:

1. Fixation of the probe on the microscope glass
2. Primary stain – crystalic violet
3. Fixation of colorization by the Lugol iodine solution
4. Decolorization by acetone or ethanol
5. Counter stain

As the result, the gram-positive bacteria are stained red/pink and the gram-negative bacteria are stained blue/purple.

Gram-positive bacteria

The cell wall of Gram-positive bacteria is formed generally of a single membrane made of several layers of peptidoglycan (90 %) and polysaccharides interspersed with teichoic acids (typical for Gram-positives). No outer membrane is present. During the staining procedure the dye crystal violet enters the cell and forms a blue colour complex with a Lugol solution. Alcohol is not able to go through the cell wall and dissolves the complex. Counter stain (with safarin) leads to dark purple colour. Some prominent Gram-positive bacterial species are:

- coccoid-shaped: *Staphylococcus*, *Streptococcus*, *Enterococcus*
- rod-shaped: *Corynebacterium*, *Clostridium*, *Listeria*, *Bacillus*

Bacillus subtilis

Bacillus subtilis is a gram-positive non-pathogen rod-shaped bacterium found in soil, water and the gastrointestinal tract of ruminants and humans. The bacterium can form a protective endospore (morphologically different type of cells), that helps it to tolerate extreme environmental conditions – e.g. high temperature, drought, salinity, high pH, solvents and high doses of ionizing radiation. The estimated lifetime of the endospores ranges from thousands to millions of years. The cell is 2-3 µm long and its width is 0,7 – 0,8 µm. *B. subtilis* is motile using flagella, which gives it the ability to move quickly in liquids. *B. subtilis* is one of the best studied gram-positive bacteria and it is a model organism to study bacterial chromosome replication and cell differentiation. [SRO11]

Gram-negative bacteria

The cell wall of Gram-negative bacteria is formed by a thin layer of peptidoglycan (10 %) between two membranes of lipopolysaccharides. These bacteria contain 17 amino acids including aromatic acids in the cell wall. They don't form spores and they multiply by transverse division. They mostly use flagella to move. These bacteria are well protected from many antibiotics because of their outer membrane. Many representatives of this group are pathogenies.

Generally the gram-negative bacteria are more dangerous, than the gram-positive ones. During the step of decolorization within Gram staining, the crystal violet and iodine complexes are decolorized by the alcohol. Safranin stains bacteria into a red color. Examples of gram-negative bacteria are:

- G- coccus: *Neisseria*
- G- coccobacillus: *Haemophilus influenzae*, *Bordetella pertussis*, *Legionella*, *Brucella*
- G- bacillus: *Klebsiella*, *Escherichia coli*, *Enterobacter*, *Citrobacter*, *Vibrio*, *Pseudomonas*, *Proteus*, *Helicobacter pylori*, *Yersinia*, *Salmonella*

Escherichia coli

Escherichia coli (*E. coli*) is a gram-negative rod-shaped bacteria. It belongs to a family of Enterobacteriaceae. The length of this bacteria is up to 2-3 µm and its width is 0,6 µm. It uses flagella to be motile. Some types of *E. coli* can form slime shells that are composed of polysaccharides. There are two types of fimbriae on its surface. One consists of acidic hydrophobic proteins and this enables bacteria to attach to the epithelium of the host and to colonize that host. The second type of fimbriae is so called sex pili, they are important for conjugation events.

The outer membrane of *E. coli* is covered by a lipopolysaccharide layer and consists of the lipid bilayer, where a lot of membrane proteins are anchored. The space between inner membrane and cell wall is called periplasmatic space. The cell wall of E. coli consists of a thin layer of peptidoglycan, which is responsible for the rigid shape of bacteria. Beneath the peptidoglycan layer there is a cytoplasmic membrane. It consists primarily of proteins, lipopolysaccharides, and phospholipids. Many biochemical processes such as the respiratory chain and the ATP synthesis are located here. The cytoplasm of bacterial cell is a viscous aqueous solution containing dissolved organic and inorganic substances. The ribosomes present in this solution accelerate a protein synthesis and the cell division. Under optimal conditions (37 °C, sufficient amount of nutrients) the time of regeneration is approx. 20 minutes. AThe molecule of the bacterial DNA, where all the bacterial genetic information is stored, is located in the cytoplasm. *E. coli* can grow at the temperature between 8 – 48 °C, but the optimal temperature is 37 °C. The range of the pH for the growth is 6 – 8.

The bacteria can be found in the gastrointestinal tract of warm-blooded

animals, including humans. For humans, *E. coli* as a part of gastrointestinal microflora is very beneficial, because it produces many substances, which prevent the spread of pathogenic bacteria and participate in the creation of some vitamins (vitamin K). *E. coli* cannot exist outside the host for a long period. Therefore its presence (e.g. in the drinking water) points to the presents of contamination by feces. E. Coli is one of the best investigated microorganism, that is why it is a model organism for gens and clinical studies. [GER10, VAL11]

Chapter 3

Used methods

The experimental setup with its parameters is described in this chapter. The experimental process containing bacteria preparation, liquid activation, treatment and evaluation is also mentioned. Methods for determination of nitrites, nitrates, hydrogen peroxide and ozone are described in details.

3.1 Experimental Apparatus

Plasma activated solution can be prepared using many different plasma sources and experimental procedures. In this work, positive corona arrangment was used. The setup was inspired by previous work done at Czech technical university in Prague [BAK16]. For the purpose of the first part of the experiment, setup without airflow was used and thereafter, tubes with an airflow were added to the setup.

3.1.1 Experimental setup without airflow

The experimental setup is depicted in the Figure 3.1. The electrical part of this setup consists of the High Voltage DC power supply with a maximal output voltage U_{max} = 10 kV (1), a point electrode (positive) realized with a sharpened brass rod with diameter d_1 = 1,07 mm (6) and a hollow plane

electrode (grounded) realized with a brass plane penetrated with a hole of diameter $d_2 = 8$ mm (7). Positive electrode is placed in a non-conductive tube (5) guaranteeing defined volume around the electrode. The plane electrode is attached to the one side of non-conductive tube. On the other side the tube is penetrated with two holes (4). A natural airflow is throught these holes in the direction of the discharge allowed (positive to grounded electrode). Petri dish with activated solution (8) is placed on the non-conductive support (3) under the plane electrode. The electrode system is attached to a support (2), which allows to adjust the distance between the plane electrode and the Petri dish.

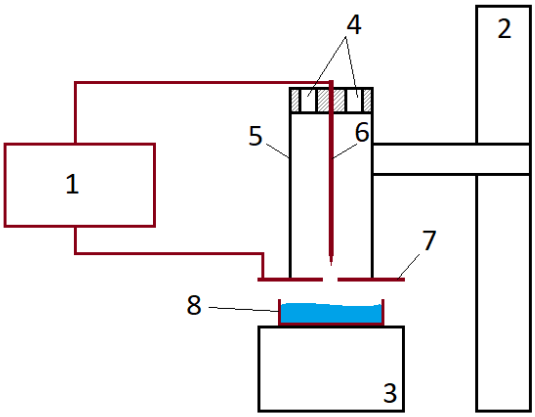

Figure 3.1: Experimental setup without airflow

Important parameters for the experiments are depicted in the Figure 3.2. The vertical distance between electrodes d_3 can be set in the range of $0 - 6$ mm. The real distance between electrodes is then:

$$d_{el} = \sqrt{\left(\frac{d_2}{2}\right)^2 + d_3^2} \tag{3.1}$$

The distance between plane electrode and the Petri dish d_4 can be adjusted in the range of $4 - 7$ mm. The voltage in the range of $7 - 10$ kV is used.

3.1.2 Experimental setup with airflow

For the experiments performed in Aachen, the airflow is introduced to the plasma source through the penetration in the upper part of the non-conductive tube using an airflow source. The setup is depicted in the Figure 3.3. The air flowrate is measured by the Flow meter 0-15 L/min G 3/8-" Swivel nut x G 3/8" male out and it is set in the range of 0 to 15 L/min.

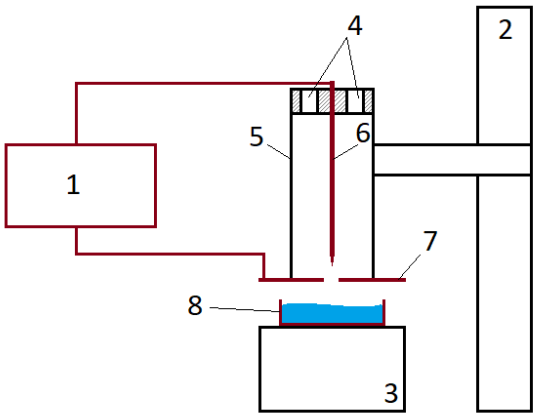

Figure 3.2: Important parameters of the experimental setup

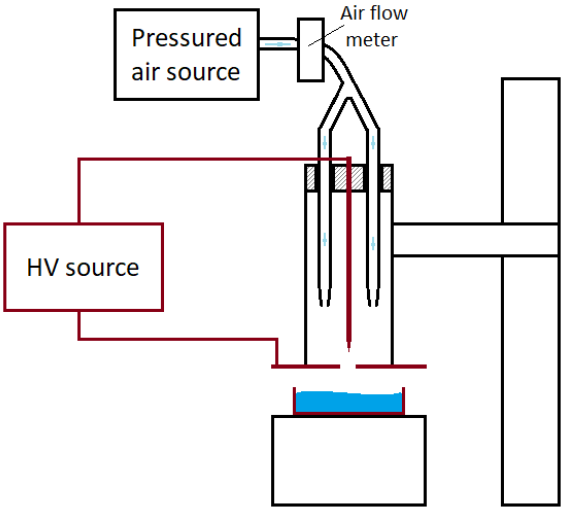

Figure 3.3: Experimental setup with airflow

3.2 Experimental procedure

The whole experimental procedure consists of five separate steps: inoculation, solution activation, exposition (treatment), cultivation and evaluation. These steps are detailed described in following chapters.

3.2.1 Inoculation

First, a concentrated bacterial solution is prepared. 100 µl of frozen bacteria in glycerine with concentration 10^8 is resuspended in a LB broth medium and stirred on a Petri dish with an LB agar. The agar plate is left in an incubator for at least 20 hours to grow colonies. Then one forming unit is taken and diluted in 25 ml of LB liquid medium in a 250 ml flask. Inoculated medium is placed into shaker with constant temperature of 37 °C and humidity 70 % and left for 20 hours for a cultivation. After the cultivation, 20 ml of this suspension is centrifuged in a plastic test tube for 10 minutes at 10 000 rpm. The supernatant is poured out and concentrated bacteria are resuspended in the sterile physiological saline solution. The solution is again centrifuged, the supernatant is poured out and the bacteria are resuspended in 10 ml of the sterile physiological saline solution. The solution absorbance is measured with McFarland densitometer to check approximate bacteria concentration (1 – 2 MFU relies to 10^7 CFU/ml). The concentration is approximately 10^8 CFU/ml for *E. coli* and 10^9 CFU/ml for *B. subtills*.

3.2.2 Solution activation

4 ml of physiological saline solution or demineralized water (both sterile) was placed in a Ø4 cm Petri dish and the solution was indirectly activated with plasma generated using electrical discharge. The distance d_3, the applied voltage U and the period of the treatmentare varried to estimate the dependencies if the concentration of the chemical compounds and the bacteria inactivation.

3.2.3 Bacteria treatment

100 µl of the bacterial suspension (10^8 CFU/ml for *E. coli* and 10^9 CFU/ml for *B. subtilis* are added to 3 ml of the activated solution and left for 2 hours at the room temperature. The bacterial concentration is measured every 30 minutes and the final concentration is measured after two hours of the treatment.

3.2.4 Cultivation and Evaluation

Cultivation is done using the classic disc method. 100 µl of the liquid sample is spread onto an agar surface (LB agar) using plastic fisher brand. The sample is diluted by the factor of 10 in the saline solution for at least 5 times to obtain the concentration of 500 CFU/ml, so about 100 colonies appears on an agar plate, because with this amount, colonies are large enough and the computation is simple. The Petri dishes are then placed into an incubator tempered to 37 °C for about 20 hours, where the colonies grew and became visible. Number of colonies is counted and recalculated to obtain initial concentration in the treated solution.

3.2.5 Effects of radicals

To compare results of plasma activated water treatment, also treatment with separate radicals soluted in water are used. For this part of the test, the solutions of nitrites, nitrates and hydrogen peroxide with two defined concentration for each of them are prepared. 100 µl of bacterial suspension is added into 3 ml of this solution and left for 2 hours. The bacterial concentration is measured every 1 hour and the final concentration is measured after two hours of the treatment. Cultivation is prepared in the same way as described above. After cultivation, the number of CFU/ml on the plates is evaluated and the concentration of bacteria after treatment is determined.

3.2.6 Effects of solution

Because placing of bacteria into the demineralized water may cause osmotic shock, effect of demineralized water and physiological solution onto bacterial inactivation is measured. 100 µl of bacterial suspension is added into 3 ml of the physiological solution or the demineralized water and left for 2 hours at the room temperature. The bacterial concentration is measured every 1 hour and the final concentration is measured after two hours of the treatment. Cultivation and evaluation is prepared as in the previous chapters.

3.3 Measurement of chemical species

As mentioned in Chapter 2.2.2, the plasma activated solution contains many various ions and reactive species e.g. NO_2, O_3, N_2O_5 and other N_xO_x molecules, H_2O_2 etc. This work focuses on a determination of nitrates (NO_2), nitrates (NO_3), hydrogen peroxide (H_2O_2) and ozone (O_3). For all these species spectrophotometric methods are used.

3.3.1 Determination of nitrites

Nitrite ion (NO2-) is a symmetric anion of N and O with equal length bonds between the elements and the bond angle about 135° (see Figure 3.4). Molar mass of nitrite ion is 46,01 g/mol. The simplest way to determine nitrites

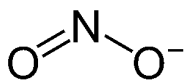

Figure 3.4: Molecule of nitrite ion

in the water is to use Griess assay, first described in 1858 by Peter Griess. The Griess assay is performed following these steps (used acids can vary):

1. Reagent A (usually called *NED*) was prepared dissolving 0,05 g of N-(1-naphthyl)-ethylenediamine dihydrochloride in twice distilled water and the volume is adjusted to 50 ml.
2. Reagent B (usually called SA) was prepared by dissolving 0,1 g of sulphanilamide in 5% solution of orthophosphoric acid (H_3PO_4). The volume of resulting solution is 50 ml.
3. The reagent A and the reagent B are mixed together in the ration 1:1. Final solution should be stored in a dark bottle in a fridge and it is stable for at least one month.

This method can be used for concentration of nitrites in between 0,0 – 4,5 mg/l, because in this range, the calibration curve is linear. For higher concentration the curve is not linear, therefore the concentration cannot be estimated correctly. 0,5 ml of the sample and 0,5 ml of the Griess reagent are mixed together and in a presence of nitrites, the solution changes its colour to pink. If the colour is too dark, the concentration of nitrites is over the

limit and the concentration needs to be reduced (e.g. by dissolving 50 µl of the sample in 450 µl of the demineralized water to get the final concentration ten times lower than before). An absorbance of final solution is measured by Spectrophotometer Synergy Mx biotek at wavelength 540 nm (the peak maximum; see 3.6, where the spectrum is shown for wavelengths in the range of 400 and 600 cm).

Chemical reaction

When the sulphanilamide is added into the solution with NO_2^- it reacts with the nitrites and a diazonium salt is formed. The azo dye agent is formed after the reaction of the diazonium salt with the N-(1-naphthyl)-ethylenediamine (see Figure 3.5) and the solution changes its colour to pink.

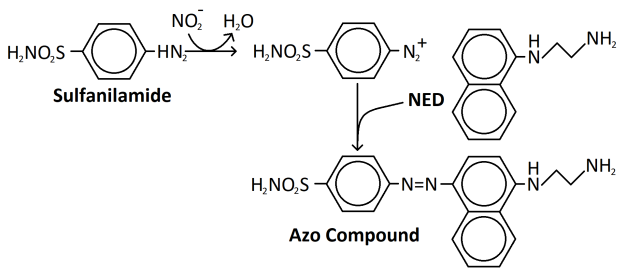

Figure 3.5: Chemical reaction of the Griess reagent with NO_2^-.

To determine the real concentration of the nitrites in the solution, the calibration curve should be prepared. For this step, the nitrite reference solution should be used and it can be prepared by dissolving of 0,068 g of Natrium nitrite ($NaNO_2$) in the demineralized water and adjusting the volume to 100 ml. Concentration of nitrites in te reference solution is 500 mg/l. The calibration curve is prepared by diluting the reference solution to get concentrations of the nitrites in the range of 0,0 and 4,5 mg/l. This measurement should be done against demineralized water to exclude an error caused by absorbance of the reagent.

3.3.2 Determination of nitrates

A nitrate anion (NO_3^-) is a polyatomic ion (charged ion composed of two or more atoms covalently bonded that acts as a single unit). The nitrate ion contains three atoms of Oxygen and one atom of Nitrogen. The nitrogen atom in the centre is surrounded by identically bounded the Oxygen atoms

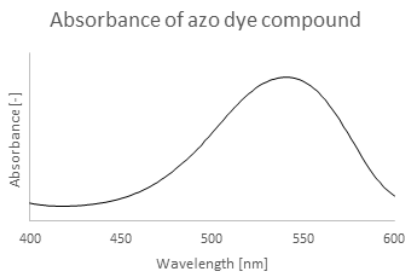

Figure 3.6: Absorbance of the azo dye compound in the range of 400 and 600 nm. The maximum of the absorbance is at 540 nm.

in a trigonal planar arrangement (see Figure 3.7). Molar mass of NO_3^- is 62,00 g/mol. There are many methods to determine concentration of nitrates

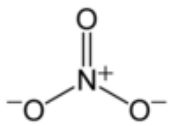

Figure 3.7: Molecule of nitrate anion

in the water. The simplest method is to use ultraviolet spectrophotometry, but the absorbance of UV light is affected by the ions such as nitrites, that is why this method cannot be used to determine the exact concentration of nitrates.

Another group of methods includes two steps determination. In the first step the nitrates are reduce to the nitrites and in the second step the nitrites are measured. Because of presence of nitrite in the plasma activated water, the final absorbance by these methods is affected by the initial concentration of the nitrites in the solution and the nitrites that are reduced from nitrates. Therefore the exact concentration of initial nitrite concentration needs to be known to determine concentration of reduced nitrate.

One of the possible method to determine the nitrate concentration is for example the method based on using Zinc or Cadminum-Copper powder. In this work method with Vanadium trichloride (VCl_3) [SCH14] is presented. NO_x reagent is prepared by following these steps:

1. Reagent A (saturated Vanadium trichloride reduction solution) is prepared by dissolving 400 mg of VCl_3 in a small amount of twice demineralized water, 4,2 ml of HCl (37 wt.%) is added into this solution and the volume is adjusted to 50 ml by adding demineralized water. This

procedure leads to a particle-free solution and this solution is stable for at least one month.

2. Reagent B: Griess reagent. Preparation of this reagent was described in previous chapter.

3. NO_x reagent is prepared by mixing the Reagent A and the Reagent B in the ration 5:2. This reagent is stable for at least one month stored at the room temperature.

The range of the nitrates concentrations that can be measured by this method is 0,00 – 1,00 mg/l (for this range the calibration curve is linear), the calibration curve for nitrites is linear in the range 0 – 0,5 mg/l.

0,5 ml of sample is added to 100 µl of NO_x reagent and the solution is well mixed. The reaction time is related to the temperature. At the room temperature, the solution needs at least 10 hours to complete the reaction. Using higher temperature, the reac-tion time decreases: at 40 °C the reaction time is approx. 120 min, at °C only 5 minutes are necessary to complete the reaction (see Figure 3.8.

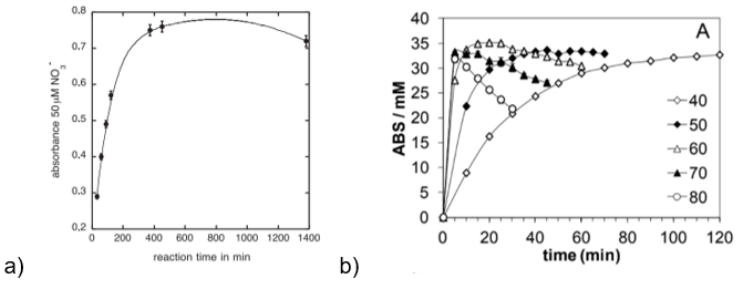

Figure 3.8: Absorbance of reagent (a) for different reaction times [SCH14], (b) with dependence on the temperature, in which the reaction takes place [GAR14]

In the presence of nitrites, the solution after a certain time is coloured to pink as in the method using Griess assay. As was already mentioned this method measures sum of initial nitrites and nitrates reduced to nitrites, so the final maximum limit can be reached faster than with Griess assay. Because this takes more time than reaction with Griess assay and it is not possible to estimate, if the final concentration is over the limit, it is necessary to reduce the solution concentration and prepare at least 10x and 100x reduced concentration series (10x reduced: dissolving 50 µl of plasma activated solution into 450 µl of double demineralized water, 100x reduced: dissolving 5 µl of the plasma activated solution into 495 µl of the demineralized water). This solution with the reduced concentration is added to 100 µl of NO_x reagent. All these measurements are done against the demineralized water. If the concentration is over the limit, the final solution

is colourless. Absorbance is measured after the reaction is completed by the spectrophotometer Synergy Mx biotek at 540 nm.

For the determination of the nitrates, the nitrate and the nitrite calibration curves are prepared. The nitrite reference solution from previous measurement of the nitrite concentration is used to prepare the nitrite calibration curve. The nitrate reference solution is prepared by dissolving of 0,0816 g of the Potassium nitrate (KNO_3) in double demineralized water and adjusting the volume to 100 ml. Concentration of nitrates in this stack solution is 500 mg/l. These reference solutions are dissolved in the demineralized water to prepare the concentration series in between the range of the measurement limits.

The final absorbance is a combination of absorbance of the nitrites, the nitrates and the reagent:

$$ABS_{NO_x} = ABS_{NO_3} + ABS_{NO_2} + ABS_{reagent} \tag{3.2}$$

Using reference solutions of nitrites and nitrates leads to:

$$ABS_{NO_2} = S_{NO_2} \cdot [NO_2] + ABS_{reagent}, \tag{3.3}$$

$$ABS_{NO_3} = S_{NO_3} \cdot [NO_3] + ABS_{reagent}, \tag{3.4}$$

where ABS_{NO_2} and ABS_{NO_3} is the absorbance of the nitrite (NO_2^-) and the nitrate (NO_3^-) calibration standard, respectively; S_{NO_2} and S_{NO_3} are the slopes of the calibration curves for the NO_2^- and the NO_3^-, respectively; $[NO_2]$ and $[NO_3]$ are the concentration of the NO_2^- and the NO_3^-, respectively; $ABS_{reagent}$ is the absorbance of the reagent measured against the demineralized water. Combination of these two equations leads to:

$$ABS_{NO_3} = S_{NO_2} \cdot [NO_2] + S_{NO_3} \cdot [NO_3] + ABS_{reagent}. \tag{3.5}$$

The concentration of the NO_3^- is then expressed:

$$[NO_3] = \frac{ABS_{NO_x} - ABS_{reagent} - S_{NO_3} \cdot [NO_2]}{S_{NO_3}}. \tag{3.6}$$

Concentration of the nitrite $[NO_2]$ is for every measured sample known from the previous measurement with the Griess reagent. As is shown in the Figure 3.9, the calibration curve for nitrite and nitrate when using this reagent is linear in the range of 0 and 0,7 mg/l and 0 and 1 mg/l, respectively.

3.3.3 Determination of hydrogen peroxide

Hydrogen peroxide (H_2O_2) is composed of two atoms of the Oxygen and two atoms of the Hydrogen (see Figure 3.10). H_2O_2 is in the normal state

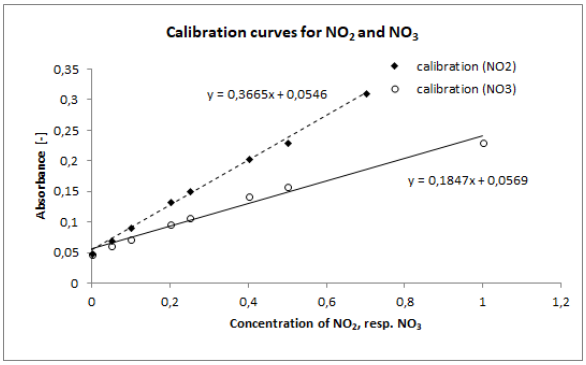

Figure 3.9: Calibration curves for nitrites and nitrates

a clear liquid with a higher viscosity than the water. It is the simplest peroxide (a compound with an oxygen–oxygen single bond) and it is a strong oxidant and a reduction agent. Hydrogen peroxide is thermodynamically highly unstable, reactive and it decomposes to the water and the oxygen:

$$2H_2O_2 \rightarrow 2H_2O + O_2. \tag{3.7}$$

The rate of the decomposition is increased with the pH, concentration and the temperature. Molar mass of hydrogen peroxide is 34,01 g/mol. For

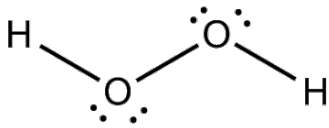

Figure 3.10: Molecule of the hydrogen peroxide

determination of hydrogen peroxide many various methods can be used. In this work spectrophotometric method using reaction of hydrogen peroxide with 4-nitrophenyl boronic acid is used [SU11]. First, several reagents should to be prepared:

1. Stock solution of 4-nitrophenyl boronic acid (1 mM) is prepared by dissolving 0,0167 g of 4-nitrophenyl boronic acid in the methanol and adjusting the volume to 100 ml by the methanol.
2. Tris-HCl buffer (20 mM) is prepared by dissolving 0,2428 g of the TRIS in the demineralized water and adjusting the volume to 100 ml by adding the demineralized water. The pH of the solution should be adapted ideally to ca 10 by adding the Na0 (to increase the pH) or the HCl (to decrease the pH).
3. Stock solution of the 4-nitrophenyl boronic acid is dissolved in the Tris-HCl buffer to get 100 µM solution of the 4-nitrophenyl boronic acid. This is used as the reagent.

The range of the hydrogen peroxide concentrations that can be measured by this method is from 0,16 mg/l to 2,48 mg/l (in this range the calibration curve is linear). For higher concentration, the calibration curve is not linear, and the concentration cannot be determined correctly.

Chemical reaction

When the reagent (consisting of the 4-nitrophenyl boronic acid (NBA) and the TRIS buffer) is added to the solution containing hydrogen peroxide, H_2O_2 reacts with the NBA and the 4-nitrophenol is produced (see Figure 3.11). The solution changes its color to yellow (higher concentration of the hydrogen peroxide produces darker yellow colour). The speed of this method

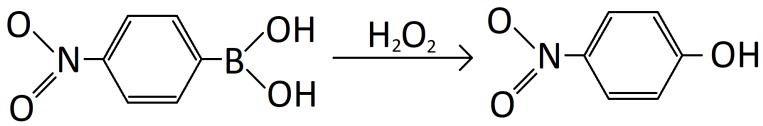

Figure 3.11: Chemical reaction of the hydrogen peroxide and the reagent [SU11]

highly depends on many variables such as the room temperature or the pH of the TRIS buffer. Increasing the temperature and the pH decreases the time needed for complete the chemical reaction (see Figure 3.12). To

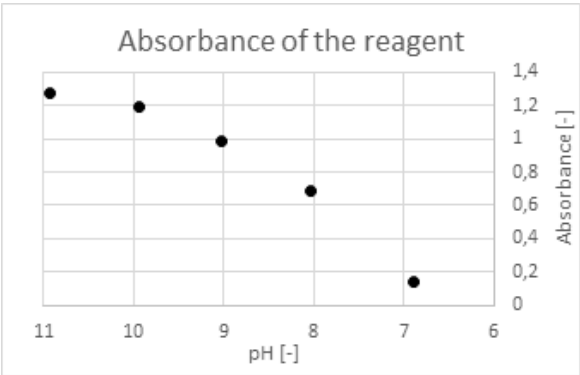

Figure 3.12: Dependence of the absorbance at 400 nm on the pH of the reagent [SU11]

determinate the concentration of the hydrogen peroxide in the solution, the calibration curve should be prepared. First, the reference solution of the H_2O_2 is prepared by dissolving 0,286 ml of the H_2O_2 solution (35 wt.%, stabilized) in the demineralized water and the volume is then adjusted to 100 ml. Concentration of the H_2O_2 in reference stack solution is 1 g/l.

The calibration curve is prepared by dissolving this reference solution in the

demineralized water to get the concentration series in between the range of 0,16 mg/l and 2,48 mg/l. The measurement is done against the demineralized water.

0,5 ml of the sample and 0,5 ml of the reagent are mixed together, and the solution is left for a certain time at the room temperature until the chemical reaction is completed. At the room temperature and pH 11 the reaction is completed in 60 minutes, at the room temperature (25 °C) and pH 8 the reaction is completed in 120 minutes. With the increasing temperature to 80 °C the reaction time can be decreased to 2 minutes. Absorbance is measured by the spectrophotometer Synergy Mx biotek at 400 nm.

Concentration of hydrogen peroxide can be calculated using equation:

$$ABS_{H_2O_2} = S_{H_2O_2} \cdot [H_2O_2] + ABS_{reagent}, \tag{3.8}$$

where $ABS_{H_2O_2}$ is the absorbance of the solution, $S_{H_2O_2}$ is the slope of the calibration curve, $[H_2O_2]$ is the concentration of the hydrogen peroxide and $ABS_{reagent}$ is the absorbance of the reagent (measured against demineralized water). The calibration curve is depicted in the Figure 3.13.

The oncentration of H_2O_2 can be expressed:

$$[H_2O_2] = \frac{ABS_{H_2O_2} - ABS_{reagent}}{S_{H_2O_2}}. \tag{3.9}$$

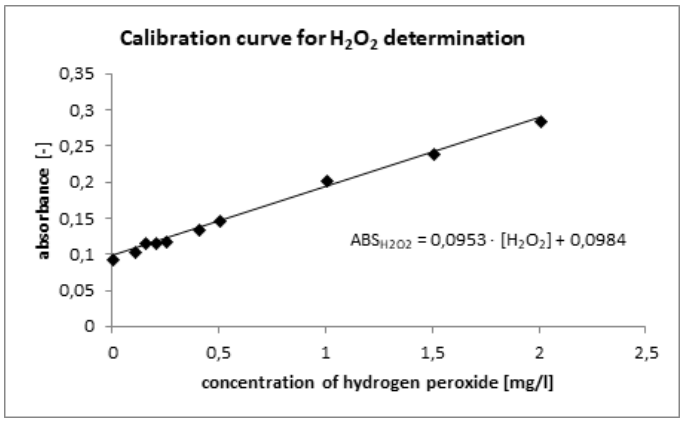

Figure 3.13: Calibration curve for the Boromic acid reagent used for the determination of the hydrogen peroxide

3.3.4 Determination of ozone

Ozone (O_3), also called trioxygen, is allotrope modification of oxygen. Under normal conditions (e.g. room temperature, standard pressure) it is a highly reactive blue coloured gas with a characteristic smell. Naturally ozone is presented in a stratosphere (low concen-trations) and in an ozone layer (higher concentration), where it absorbs most of the UV radiation coming from the Sun.

Molecule consists of three atoms of oxygen bounded together. Angle between bounds of atoms is 116,8°. The ozone molecule has a dipole moment, because it consists of the positive charge in the middle of the molecule and two 1/2 negative charged sides atoms (see Figure 3.14). The ozone can

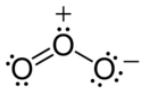

Figure 3.14: Molecule of the ozone

be produced by the exposure ordinary oxygen molecules to the electrical discharges or the shortwave ultraviolet radiation through the two step process. In the first step, the supplied energy splits the two-atom molecule into two separate atoms. These atoms are highly reactive one-atom radicals and they immediately bind to another oxygen molecule to form an ozone:

$$O_2 + \hbar\vartheta \rightarrow 2O, \tag{3.10}$$

$$O_2 + O \rightarrow O_3, \tag{3.11}$$

The ozone is unstable under normal condition. It decomposes to the molecules of the oxygen:

$$2O_3 \rightarrow 3O_2. \tag{3.12}$$

This reaction is accelerated with the increasing temperature and pressure.

There is a variety of the methods to determine the ozone concentration, e.g. chemical methods (Byers and Saltzman, 1959; Hofmann and Stern, 1969; Layton and Kinman, 1970), electrochemical methods (Ehmert, 1959; Hersch and Deuringer, 1963) and optical methods (Alway and Slomp, 1959; Hanst et al., 1961; Bersis and Vassiliou, 1966; Amos, 1970; Sachdev, Lodge and West, 1972). In this work a spectrophotometric method [SHE73] with neutral potassium iodide solution as the reagent is used.

For this method several solutions must be prepared:

1. The neutral potassium iodide reagent is prepared by dissolving 1,361 g of the potassium dihydrogen-phosphate, 1,420 g of the anhydrous disodium hydrogen phosphate and 5,0 g of the potassium iodide in the demineralized water and adjusting the volume to 100 ml. The solution should be stored in the dark bottle in the refrigerator. This reagent is used for lower concentration of ozone. For higher concentration of ozone, only 2,0 g of the potassium iodide are used.
2. The standard iodine solution (10 mM) is prepared by dissolving 0,64 g of the potassium iodide and 0,127 g of the iodine in demineralized water and the volume is adjusted to 100 ml. This solution should be prepared one day before it is used. One mililitre of the solution is equivalent to 240 μg of the ozone (it is equivalent to concentration of the ozone 240 mg/l).

This method can be used for the measurement of the ozone concentration in the range 0,01 – 0,30 mg/l (or for higher concentration between 0,30 – 2,0 mg/l).

Chemical reaction

The ozone molecule reacts with the potassium iodide and the water by forming the diiodide molecule I_2, oxygen molecule O_2 and potassium hydroxide KOH:

$$O_3 + 2KI + H_2O \rightleftharpoons I_2 + O_2 + 2KOH. \tag{3.13}$$

THe diiodide molecule reacts with the iodide anion and the triiodide ion is produced:

$$I_2 + I^- \rightarrow I_3^-. \tag{3.14}$$

The concentration of triiodide ions is spectrophotometrically measured at the wavelength 352 nm.

Similary to other method, the calibration curve should be prepared. This is done by dissolving the reference iodine solution (10 mM) with the neutral potassium iodide reagent to get a solution of the iodine with the concentration 40 µM (1 ml of this solution is than equivalent to 0,96 μg of ozone; it represents ozone concentration of 0,96 mg/l). The final solution is dissolved in the neutral potassium iodide reagent to obtain the concentration series within the range limits. The solutions are mixed and the absorbance is immediately measured at 352 nm against the demineralized water as the reference.

0,5 ml of the sample and 0,5 ml of the neutral potassium iodide reagent are well mixed in the glass tube (the ozone reacts with the plastic tubes and

that can cause an error of the concentration determination). The solution is left for 15 minutes in the cool and dark place and the absorbance of the solution is measured at the wavelength 352 nm.

The absorbance of the solution should not be measured later than 15 minutes after adding the potassium iodide reagent, because the color intensity is not stable and for lower concentrations of the ozone the intensity decreases very fast (see Figure 3.15).

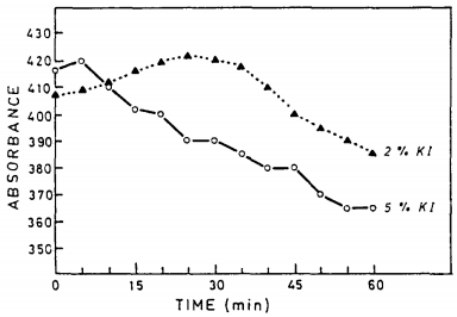

Figure 3.15: Dependence of the absorption of the reagent on the reaction time for 2% solution of KI and 5% solution of KI [SHE73]

Following equation describes the absorbance of the ozone solution:

$$ABS_{ozone} = S_{ozone} \cdot [O_3] + ABS_{reagent}, \tag{3.15}$$

where ABS_{ozone} is the absorbance of the sample containing the ozone, S_{ozone} is the slope of the calibration curve, $[O_3]$ is the concentration of the ozone and $ABS_{reagent}$ is the absorbance of the reagent against the demineralized water. This equation is used for measurement of the triiodide concentration that represents the certain ozone concentration.

The concentration of the ozone can be calculated:

$$[O_3] = \frac{ABS_{ozone} - ABS_{reagent}}{S_{ozone}}. \tag{3.16}$$

Chapter 4

Results

The first part of this chapter is focused on effects of setup changes on properties of produced plasma activated solutions. Results for the different time of activation, the distance between electrodes and the applied voltage are shown. In the second part, effects on bacteria are presented. Thereafter the following results, some variables are used: U - the applied voltage, d_3 - the vertical distance between electrodes, d_{el} - the real distance between electrodes (calculated using the equation 3.1), d_4 - the distance between grounded electrode and the Petri dish, V - the volume of the solution used fot activation, t_A - the activation time and v_{flow} - the airflow rate. The setup with airflow described in Chapter 3.1.2 is used for all measurements.

4.1 Effects of the activation time

Following settings of the experimental setup were used:

U = 10 kV
d_3 = 2 mm; d_{el} = 4,47 mm
t_A = 0 - 12 mm
d_4 = 18,4 mm
V = 4 ml

The solution (demineralized water or saline solution) was activated for the defined period of time in the range of 0 to 12 minutes and right after the

activation pH of the solution was measured using pH meter. Results of the pH measurement are depicted in the Figure 4.1.

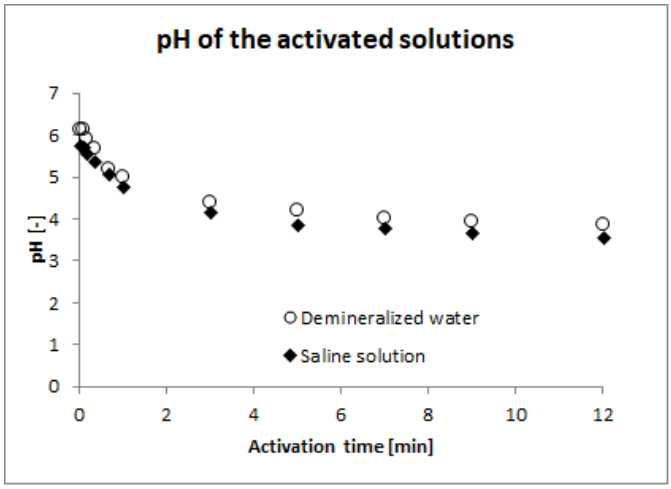

Figure 4.1: Dependence of pH on time of activation for demineralized water and saline solution. Standard devisation od pH value did not exceed a 0,3

The pH value of the demineralized water decreases exponentially from approx. 6,3 to less than 4 with increasing time of plasma activation. This fact is in a good agreement with the data reported by the other researches [BAK16, TIA15, KAM09].

The concentration of the produced nitrites depends on the activation time. In the range of 0 and 12 minuts, the concentration increases from 0,0 mg/l to 0,9 mg/l for the physiological solution and from 0 mg/l to 0,6 mg/l for the demineralized water. Curves of nitrite concentrations for both solutions are depicted in the Figure 4.2. Small concentration of nitrites is present after very short time of activation (nearly few seconds). The concentration after 7 minutes of the activation is out of the linear dependency of the concentration curve, that can be caused by the measurement error. Additional measurement should be done to prove its accurancy.

The nitrate concentration in plasma activated solution increases with increasing time of activation from 0,0 mg/l to approx. 0,09 mg/l and 0,06 mg/l for PASS and PAW, respectively. It is shown, that the nitrate concentration increases faster in the saline solution, than in the demineralized water. The curve of nitrate concentration for different activation times is depicted in the Figure 4.3. The concentration after 3 minutes of the activation is out of the linear dependency of the concentration curve, that can be caused by the measurement error. Additional measurement should be done to prove its accurancy.

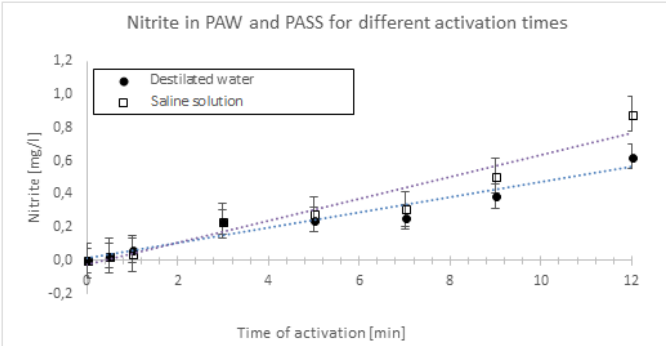

Figure 4.2: Dependence of the nitrite concentration on the time of activation for demineralized water and saline solution

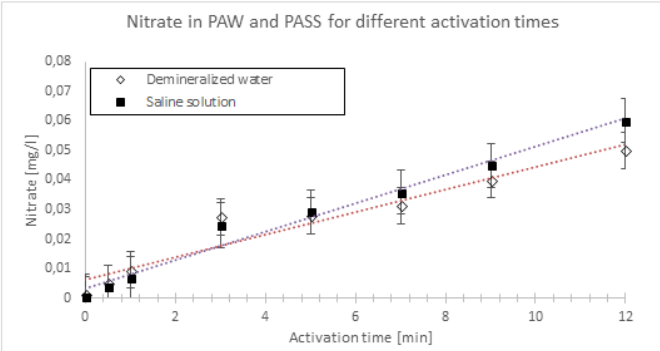

Figure 4.3: Dependence of the nitrate concentration on the time of activation for demineralized water and saline solution

The concentration of the hydrogen peroxide increases linearly with the activation time. Up to 30 seconds, the concentration of H_2O_2 was under the detection limit of this method. After 12 minutes, the hydrogen peroxide concentration was 0,35 mg/l in the water and 0,3 mg/l in the saline solution. The concentration is shown in the 4.4.

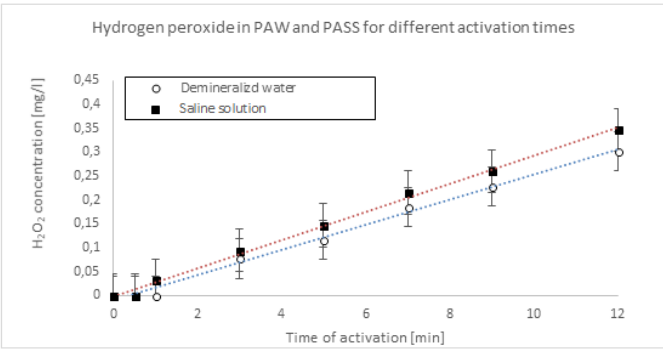

Figure 4.4: Dependence of the H_2O_2 concentration on the time of activation for demineralized water and saline solution

The ozone concentration is depicted in the Figure 4.5. The concentration is lower than 0,1 mg/l even after 12 minutes of activation. This low concentration has no significant impact on bacterial inactivation. The final concentration in the water and in the saline solution doesn't differ, the difference between final concentrations is approx. 0,01 mg/l, that is smaller then a standar error of the measurement. It can be assumed according to the concentration curve, that the saturation of the ozone appears for higher times of activation, because the curve is ascending fot these activation times slower, than for shorter times of activation.

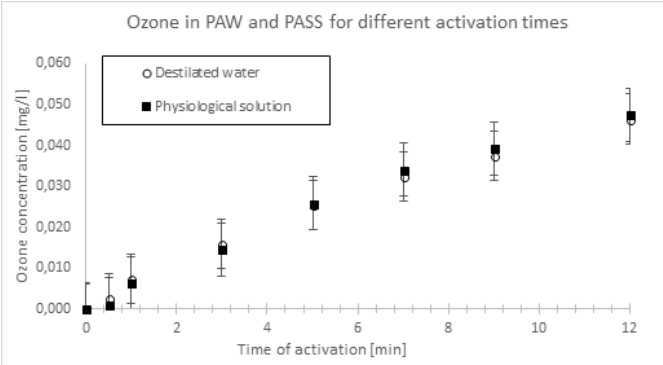

Figure 4.5: Dependence of the ozone concentration on the time of activation for demineralized water and saline solution

4.2 Effects of the distance between electrodes

To examine the dependency of the distance between the electrodes on the produced concentrations of the species, following settings were used:

U = 10 kV
d_3 = 0 - 3 mm; d_{el} = 4 - 5 mm
t_A = 5 and 9 min
d_4 = 18,4 mm
V = 4 ml
v_{flow} = 5 l/min

The vertical distance between electrodes was set in the range of 0 and 3 mm, that corresponds to real distance between electrodes 4 - 5 mm. The airflow 5 l/min was used for this measurement.

pH was measured right after 5 and 9 minutes of the activation and the value was read out once it was not changing anymore. pH was measured in the 2 ml of the sample. The result is depicted in the Figure 4.6. As it is shown, the pH decreases with increasing distance between the electrodes from

approx. 7 to 3,5 and 6 to 3,5 for demineralized water and saline solution, respectively.

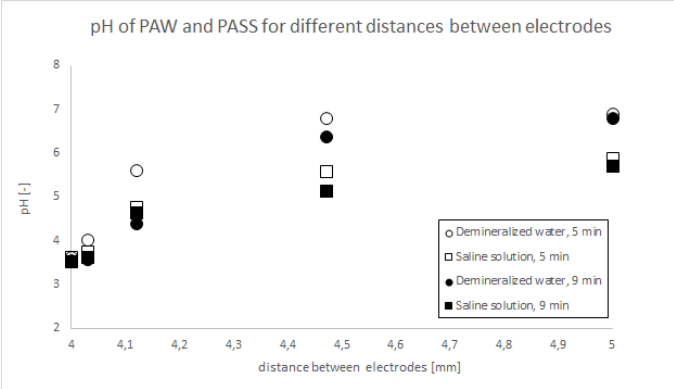

Figure 4.6: Dependence of the pH on the distance between electrodes for demineralized water and saline solution activated for 5 and 9 minutes.

The concentration of nitrites was measured for the same distances between the electrodes. The concentration curve is shown in the Figure 4.7. As can be seen in the figure, the nitrite concentration decreases with the increasing distance between electrodes. In the distance of 4,12 mm, the concentration is decreasing with higher slope (starting at 16 mg/l for the demineralized water and distance 4,0 mm and 12,0 mg/l by the saline solution) to ca 2,0mg/l or 3,0 mg/l when using demineralized water and saline solution, respectively. Up to 5 mm the decreasing is slower - in this distance it decreases from 2,0 mg/l to 0,0 mg/l.

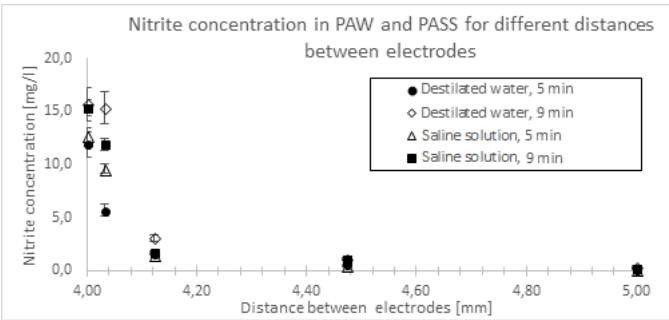

Figure 4.7: Dependence of the nitrite concentration on the distance between electrodes for demineralized water and saline solution activated for 5 and 9 minutes.

Such as for nitrite concentration, the nitrate concentration also decreases with higher slope in the range of 4,00 and 4,16 mm, where the concentration is decreased from 0,42 mg/l and 0,40 mg/l for the demineralized water and the saline solution, respectively, to 0,1 mg/l and 0,8 mg/l for the PAW and PASS, respectively. In the distances between 4,16 mm and 5,00 mm the concentration is the decreasing slowly. All curves are depicted in the figure 4.8.

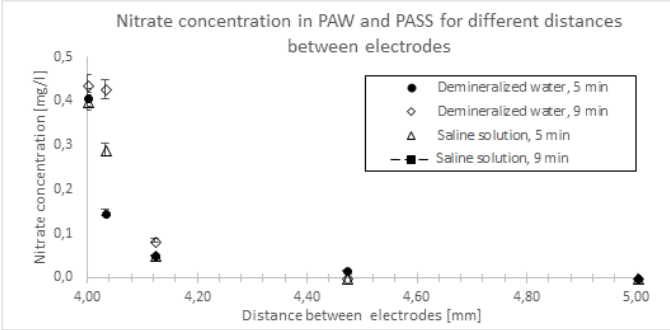

Figure 4.8: Dependence of the nitrate concentration on the distance between electrodes for demineralized water and saline solution activated for 5 and 9 minutes.

The ozone concetration is the highest for the smallest distance between the electrode for both solutions. The concentration reaches 0,30 mg/l and 0,22 mg/l for the demineralized water and the saline solution, respectively. There is very small difference between the 5 and 9 minuts activation, that can be caused y the measurement error. The concentration curve is shown in the Figure 4.9.

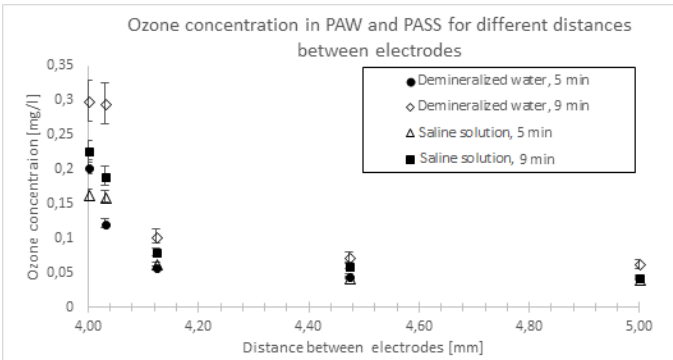

Figure 4.9: Dependence of the ozone concentration on the distance between electrodes for demineralized water and saline solution activated for 5 and 9 minutes.

4.3 Effects of the applied voltage

To examine the dependency of the applied voltage between the electrodes on the produced concentrations of the species, the vertical distance between electrodes was set on 2 mm (that corresponds to 4,47 mm of the real distance between electrodes). As was mentioned, smaller distance increases the amount of species concentrations in the solution. Nevertheless, this distance was taken, because it can be well compared with other measurements done in the previous chapters.

For this part, following settings were used:

$$\boxed{\begin{array}{l} U = 7 \text{ - } 10 \text{ kV} \\ d_3 = 2 \text{ mm; } d_{el} = 4{,}47 \text{ mm} \\ t_A = 5 \text{ and } 9 \text{ min} \\ d_4 = 18{,}4 \text{ mm} \\ V = 4 \text{ ml} \\ v_{flow} = 5 \text{ l/min} \end{array}}$$

The measurement of pH was measured in the 2 ml of the sample right after 5 and 9 minutes of the activation and the value was read out once it was not changing anymore. The pH curve is shown in the Figure 4.10. As it is shown, the pH decreases with increasing applied voltage for both solutions. There is no significant difference between the solutions or activation times.

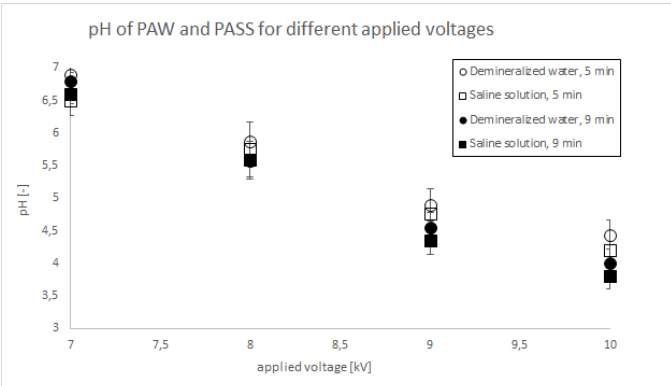

Figure 4.10: Dependence of the pH on the applied voltage for demineralized water and saline solution activated for 5 and 9 minutes.

The measurement of nitrite concentrations was done for the same values of the applied voltage. The result curve is depicted in the Figure 4.11. As can be seen, with increasing applied voltage (and constant distance between electrodes), the nitrite concentration in the activated solution increases for both times of activation. In the range from 7 kV to 9 kV, the slope of the increasing is small and in the range from 9 kV to 10 kV, the concentration increases with higher slope. Concentrations at 10 kV corresponds approximately with the results from previous measurement with the distance between electrode. The small difference can be caused by the measurement error.

The nitrate concentration is depicted in the Figure 4.12. It is shown, that concentration of nitrates increases with the applied voltage. The nitrate concentration increases from approx. 0,3 mg/l to 0,5 mg/l. At 10 kV the concentration approximatly corresponds to the concentration measured in the previous chapters. The small deviation can be caused by the measurement error.

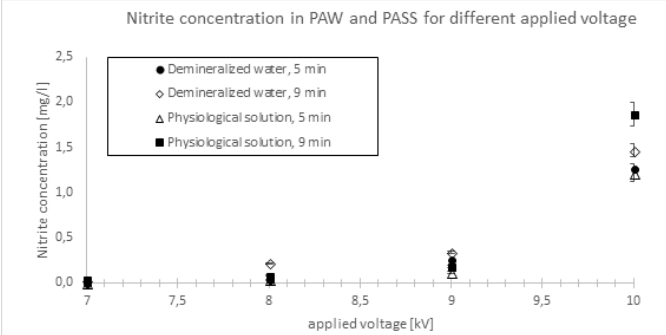

Figure 4.11: Dependence of the nitrite concentration on the applied voltage for demineralized water and saline solution activated for 5 and 9 minutes.

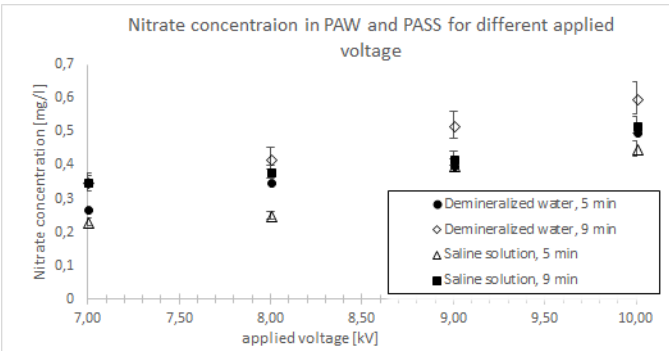

Figure 4.12: Dependence of the nitrate concentration on the applied voltage for demineralized water and saline solution activated for 5 and 9 minutes.

In the figure 4.13 the results of hydrogen peroxide measurement are presented. The concentration increases with increasing applied voltage. At 7 kV, the concentration is approx. 0,1 mg/l for saline solution activated 5 and 9 minutes and for the water activated 5 minutes. The concentration of the water activated 9 minutes at 7 kV is approx. 0,3 mg/l. The hydrogen peroxide concentration increases up to 0,5 mg/l for both solutions activated 5 minutes, to 0,7 mg/l for the water activated 9 minutes and to 0,9 mg/l using saline solution, that was activated 9 minutes.

All mentioned measurement were done at least three times to minimalize the measurement error. In the figures, the calculated average is used with error bars using calculated deviation of the data.

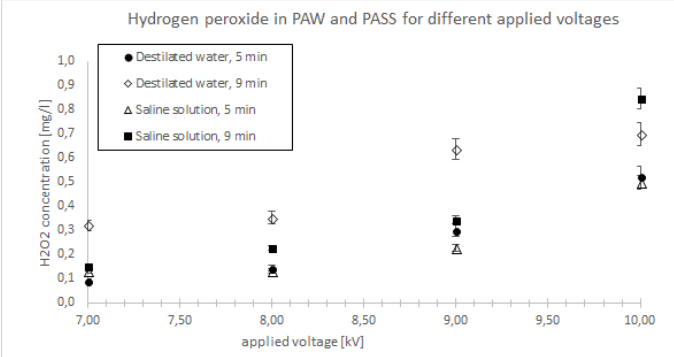

Figure 4.13: Dependence of the hydrogen peroxide concentration on the applied voltage for demineralized water and saline solution activated for 5 and 9 minutes.

4.4 Antibactericial effects of activated solutions

The antibacterial effects were examined on one gram-positive (*B. subtilis*) and one gram-negative (*E. coli*) bacteria stain. The demineralized water and the physiological solution were activated for 5 and 12 minutes to obtain activated solutions. Then 100 µl of bacteria suspension (10^8 CFU/ml for *E. coli* and 10^9 CFU/ml for *B. subtilis*) were added into the solution and left for 2 hours at the room temperature. At the beginning of the measurement and every two hours, CFU of bacteria was measured. In the Figure 4.14 is shown the reduction of the *E. coli* for PAW and in the Figure 4.15 are results for *E. coli* reduction in PASS.

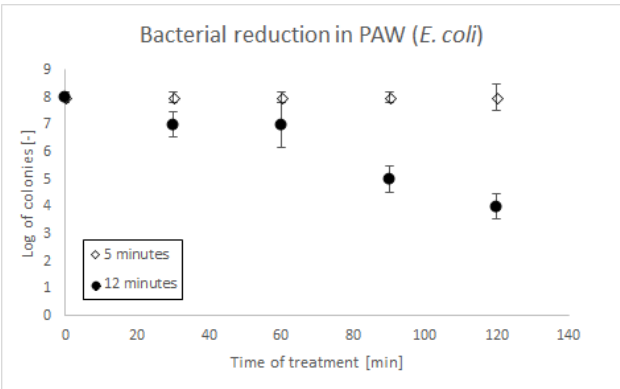

Figure 4.14: Reduction of the *E. coli* number of colonies with the PAW activated for 5 and 12 minutes.

As it is shown in the Figure 4.14, plasma activated water activated for 12 minutes is more efficient. It can be caused by the higher concentration of all species (hydrogen peroxide, ozone etc.) for this activation time. After 120 minutes of the treatment, there is 4 orders of the magnitude reduction.

For activation time 5 minutes, no significant effects of inactivation were examined.

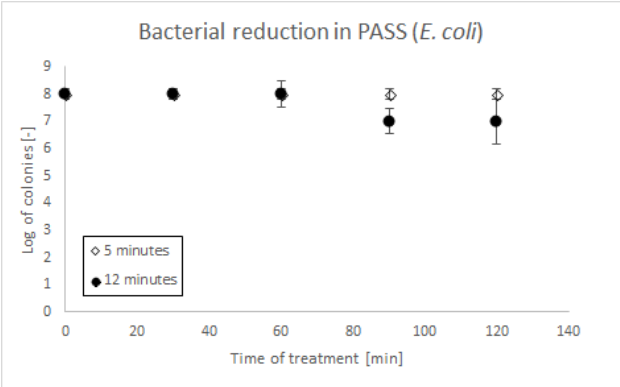

Figure 4.15: Reduction of the *E. coli* with the PASS activated for 5 and 12 minutes.

In the Figure 4.15 effects of PASS on *E. coli* bacteria are depicted. As for the activated demineralized water, there is negligible effect of the 5 minutes activated solution on this bacteria. Using PASS activated for 12 minutes leads to one orger of magnitude bacteria reduction of the *E. coli*.

The same procedure such as for *E. coli* was used also for *B. subtilis*. Bacteria were treat by the solution (PAW or PASS) activated for 5 or 12 minutes. The results of the inactivation are shown in the Figure 4.16 for PAW and in the Figure 4.17 for PASS.

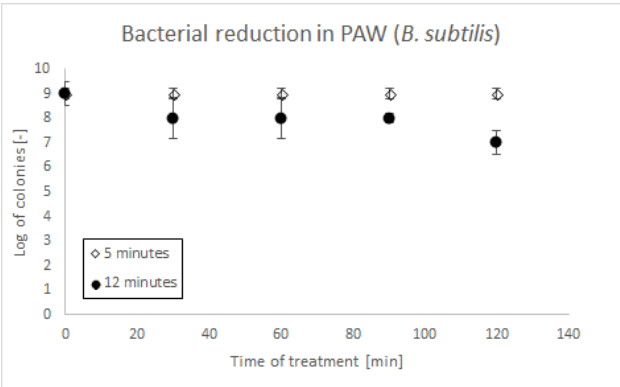

Figure 4.16: Reduction of the *B. subtilis* with the PAW activated for 5 and 12 minutes.

Water activated with plasma for 12 minutes leads to one order of magnitude reduction after 30 minutes of treatment, no other inactivation was present. PAW activated only 5 minutes has no effects on the bacteria (see Figure 4.16). This can be causes by the better resistance of the gram-positive bacteria against unsuitable environment.

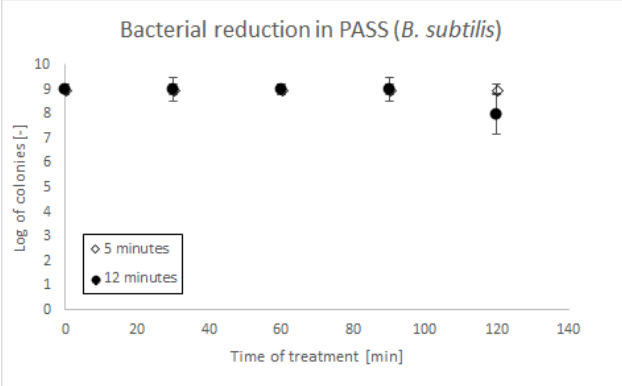

Figure 4.17: Reduction of the *B. subtilis* colonies with the PASS activated for 5 and 12 minutes.

Effects of the PASS can be neglected, because no bacteria were inactivated within 120 minutes of the treatment by this solution. In the PAW activated for 12 minutes, low reduction of the bacteria appeared (after 120 minutes of the treatment the bacteria colonies were reduced by one order of the magnitude). This is depicted in the Figure 4.17.

4.5 Antibactericial effects of chemical compounds

The effects of separate chemical coumpounds (nitrites, nitrates and hydrogen peroxide) were examined. The procedure was the same as for the treatment using activated solutions, the bacteria were, however, placed into solutions made of demineralized water or saline solution and one of the chemical substance. To examine effects of nitrate, solution of demineralized water or saline solution and potassium nitrate was used. The concentration of nitrate was 10 mg/l and 20 mg/l and this measurement was done agains demineralized water and saline solution. Nitrites were examined by using solution with the nitrite concentration of 10 mg/l and 20 mg/l. Solution of hydrogen peroxide with concentration of 5 and 10 mg/l was used to show the effects of this compound.

It was examined, that no of these solutions had a significant effect on the bacteria inactivation within 2 hours. After 120 minutes, all the bacteria presented and no reduction was observed. The used concentration was the hightest concentration measured in the previous measurements, nevertheless it was probably too low to cause any damage to the bacteria cell.

Conclussion

In this work was shown, that the point to penetrated plane electrode experimental setup may be used for producing of plasma activated solu-tions. Such liquids become acidic, that can cause a bacterial death.

According to the experiment results, the plasma activated solutions have anti-bacterial effects and they cause reduction of bacteria colonies. This was confirmed by the measuring of the number of CFU after 0, 30, 60, 90 and 120 minutes of the treatment with the plasma activated water and the plasma activated saline solution. The efficiency of this setup is however smaller, than is mentioned in other studies [LIU17]. In comparison to previous work done at Czech technical university in Prague [BAK16], the results of these experiments were with their efficiency similar. Results from Prague are described in details in the Appendix A. The difference in the results can be caused by the using of the different strains of the used bacteria and their initial concentration. The deactivation of the bacteria *E. coli* and *B. subtills* has better results, when the solution was activated.

The concentration of the all ions and the species increases with the activation time, the applied voltage and with the decreasing distance between the electrodes. The ion concentration can have significant role in the bacteria inactivation. The concentration of the produced hydrogen peroxide was comparable to the study [LIU17], but the concentration of nitrites and nitrates are smaller than in the mentioned study. One of the reason may be the fact, that in such studies the higher voltage was applied (typically more than 12 or 15 kV) or different experimental setup was performed. In this work the source with the voltage only up to 10 kV was used. As was shown

in the results, the applied voltage has sifnificant impact on the produced amount of the ions.

In these experiments it was examined, that to maximize the concentration of the species, distance between the electrodes should be small and the applied voltage should be high.

In general, the activated demineralized water is more effective compared to the activated saline solution The concentrations of all the species are higher in demineralized the water. Also, in the activated water more bacteria were killed and after 2 hours of the treatment the concentration of the bacteria was about 4 logs reduced. On the other hand, for the medical purposes saline solution is more convenient, because it doesn't cause osmotic shock of the cells as well as the demineralized water.

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List of abbreviations

AC	Alternative current
atm	Atmosphere
B. subtilis	*Bacillus subtilis*
CFU	Colony Forming Unit
DBD	Dielectric-Barrier Discharge
DC	Direct current
E. coli	*Escherichia coli*
H_2O_2	Hydrogen peroxide
kV	Kilovolt
LB	Lysogeny broth – type of medium for biological purposes
MFU	McFarland Units
p.a.	For analytical purposes
PAS	Plasma activated solution
PASS	Plasma activated saline solution
PAW	Plasma activated water
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TTP	Tissue tolerable plasma
UV	Ultra violet

Appendix A

Results from the CTU in Prague

Bacterial Inactivation by Physiologically Relevant Liquids Activated by Atmospheric Pressure Non-thermal Plasma

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Abstract. *Non-thermal plasma as a source of reactive species is a topic of great interest. Liquids after plasma activation become acidic and concentrations of nitrite, nitrates and peroxides rapidly increases. Bactericidal properties of such liquids are not well understood yet.*

The purpose of this work is to investigate bactericidal effects of plasma activated liquids. Non-thermal plasma generated by a DC discharge was applied indirectly on distilled water and physiological saline solution. An indirect plasma treatment means that liquids were activated mainly by reactive oxygen and reactive nitrogen species. pH and conductivity of plasma activated liquids were measured and compared with results obtained by other authors. Concentration of nitrite ions was measured using a Griess reagent. The bactericidal properties of plasma activated water was got through a drop test and a disc method for four plasma exposition times and five bacteria treatment times.

Keywords

Non-thermal plasma, plasma effects, microorganism inactivation, plasma activated water, PAW.

1. Introduction

Antibacterial effects of plasma activated liquids (PAL) are well known and documented. Declared decontamination effects depend on the discharge type, on the used liquid and on time of activation. A time of treatment of bacteria by PAW plays a significant role. [1, 2].

Liquids activated with plasma become acidic (~2–3.5 pH) and more conductive. However, conductivity of liquids that were conductive before treatment doesn't change significantly. Nitrite and nitrate anions and hydrogen peroxide are usually created in liquid after activation. These chemicals are claimed to be biocidal [2, 3]. Concentration of these chemicals, as well as pH and solution conductivity are usually measured in term to investigate possible biocidal effects. It is still not known, which of the chemicals of the plasma activated liquid has the major effect on bacteria.

The biocidal effects of PAL on bacteria are in investigation as well. Authors [3, 4] consider that reactive oxygen (ROS) and reactive nitrogen species (RNS) can cause the oxidative stress on bacteria and as the result the lipid peroxidation. The majority of inactivation process occurs on the cell wall and membrane, as the first obstacle on the way of ROS and RNS, but some processes may effect metabolism of prokaryotic cell which can lead to decreasing of cell viability [5, 6].

The efficiency of PAL inactivation strongly depends on different factors, mainly on amount of mentioned species. In this study a simple electrode point-to-hollow plane configuration was used to generate a DC positive streamer. Liquids (DI water and physiological saline solution) were inactivated indirectly.

2. Material

2.1 Bacteria

Gram-negative bacteria *Escherichia coli* (Czech Collection of Microorganisms 4332) was used for all measurements. The bacterium as well as all cultivation mediums were bought in Oxoid s.r.o., Brno, CZ.

Escherichia coli belongs to the family *Enterobacteriaceae*. It is commonly found in the digestive tract of animals, including humans. For humans it is a part of the natural microflora beneficial, since it produces a number of substances that prevent the spreading of pathogenic bacteria. The bacteria reaches a length of 2-3 microns and a width of 0.6 microns [7]. *E. coli* is one of the most explored bacteria and is usually used as a modeling organism of prokaryotic gram-negative cells. Not all *E. coli* strains are pathogenic.

Mueller-Hinton agar was used to cultivate *E. coli*. This agar consists of 30.0% beef infusion, 1.75% casein hydrolysate, 0.15% starch and 1.7% agar.

2.2 Used chemicals

Physiological saline solution (PSS) was used for bacteria dilution and was prepared as a solution of sodium chloride. 0.9% saline of NaCl was used. That means there was 9 g of NaCl in 1 liter of distilled water. This saline was sterilized in autoclave before usage.

Griess assay is used to detect nitrites and relies on diazotization reaction described by Griess in 1879. Griess reagent system is based on a chemical reaction, shown in Fig. 1, which uses sulfanilamide and N-1-napthylethylenediamine dihydro-chloride (NED) under acidic (phosphoric acid) conditions. This system detects nitrites NO^{2-} in variety of liquids. The limits of detection are 1 µmol up to 250 µmol nitrite in a pure DI water using the protocol described in section 3.2. [8].

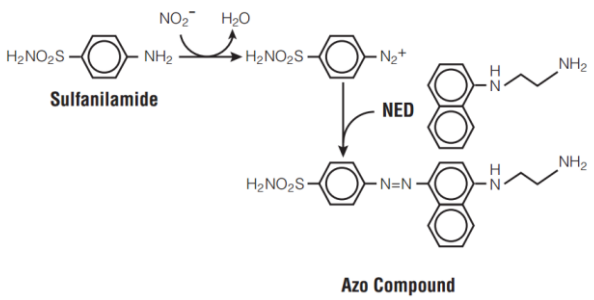

Fig. 1. Chemical reaction involved in the measurement of NO^{2-} using the Griess reagent system [8].

3. Methods

3.1 pH and conductivity

pH and conductivity of 4 ml of PAL or the control sample were measured with the multifunctional water meter Voltcraft. The measuring part of the device was always cleaned with DI water properly before each single measurement.

3.2 Nitrite essay

The Griess reagent was taken out of the refrigerator to allow it to equilibrate to room temperature. Then 1 ml of the sample (PAL or control) was placed to an ependorf microtube. 0.5 ml of reagent was added to the sample and the mixture was left for at least 15 minutes to finish the reaction. 330 µl of each sample was placed into the 96 wells microtiter plate and an absorbance at 530 nm was measured with varioSCAN™ microplate reader. Recalculating value of absorbance to a nitrite concentration was done with the calibration curve of 13 points (see Fig. 2). Griess reagent changes its parameters during the time. The calibration should be done for each measurement. In the case the nitrite concentration was out of the limits the sample was diluted in pure DI water before adding the Griess reagent.

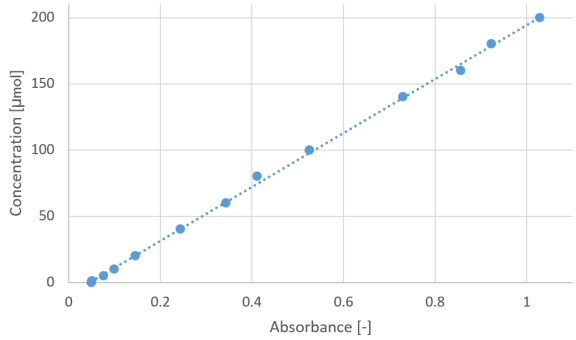

Fig. 2. Griess assay calibration curve.

3.3 Bacterial drop test

The drop test is an easy, fast and economical method to test bacterial concentration. It is widely used for so called blind tests, when one doesn't know exact concentration of bacteria after treatment.

To perform a drop test a control concentration is needed for each plate in order to prevent wrong positive results. Bacterial suspension was diluted 6 times 1:10 (sterile microtubes were used for this purpose) and small drops of 3 µl of 4 different dilutions (6th, 5th, 4th and 3th) were spread on an agar surface (see Fig. 3).

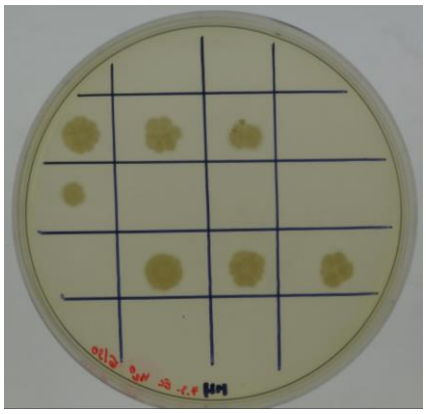

Fig. 3. Drop test for water activated for 6 minutes and 15 minutes of bacterial treatment. In the top raw is the control sample dilution. In the second raw are the maximal dilutions of the sample, but there are no bacteria grew due to inactivation. In the third raw are less diluted bacterial samples.

4. Experiment

4.1 Experimental process

Whole process consists of five steps: inoculation, water activation, exposition, cultivation and evaluation.

4.1.1 Inoculation

It was necessary to prepare a concentrated bacterial solution. 2 ml of frozen bacterial solution in glycerin was

re-suspended in 8 ml of physiological saline solution. The solution absorbance was measured with McFarland densitometer to check an approximate concentration (1 – 2 MFU relies to 10^7 bacteria). 10 ml of the suspension was then centrifuged for 10 minutes in 5000 rpm. A supernatant was though out and a bacterial pellet was re-suspended in 0.8 ml of sterile PSS. The concentration that was obtained by centrifuging was about 10^8 cfu/ml and was used as an initial concentration for all measurements.

4.1.2 Liquid activation

6.5 ml of PPS or DI water was placed in a Ø6 cm Petri dish and was activated indirectly by plasma. To mix liquid properly the orbital shaker was used. Activation time was set to 1, 3, 6 and 9 minutes.

4.1.3 Bacteria treatment

100 µl of bacterial suspension (10^8 cfu/ml) was added to 3 ml of activated liquid and left for 2 h. Bacterial concentration was measured each 15 minutes during the first hour of treatment. The final concentration measurement was made after 2 h.

4.1.4 Cultivation

Bacterial cultivation was done with a drop test and a classic disc method. In the case if classic cultivation method 100 µl of diluted sample was spilled on an agar surface with sterile glass balls. All Petri dishes were placed into the thermostat set for 37 °C for about 20 hours. The colony forming units became visible and their amount was evaluated.

4.2 Apparatus

Plasma was generated by a point-to-hollow plane electrode system (Figs. 4). A high power supply (U_{max} = 15 kV) was used. The positive point electrode was realized by a sharped brass rod of a diameter d_1 = 7 mm. Negative electrode, hollow plane, was realized by a copper plane with a hole with a diameter d_2 = 8 mm. The distance between the electrodes was set to h = 6 mm. The electrode system was closed in a plastic tube to make the stable surrounding atmosphere.

A Petri dish (Ø6 cm) with a treated liquid was placed on orbital shaker under the plane electrode as close to the electrode as possible.

5. Results and discussion

Parameters of plasma activated DI water and plasma activated PSS were measured. pH, conductivity and nitrite were measured at least 3 times. The drop test was done for one time for each solution and the disc volatile test was done for PAW only.

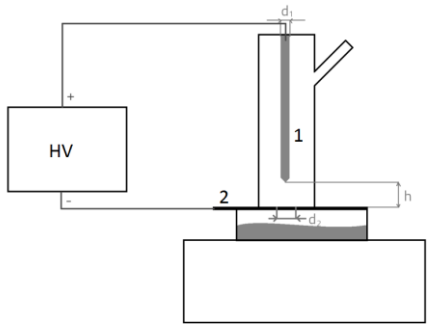

Fig. 4. Experimental setup: power supply HV, a point electrode (1) realized with a sharpen rod (positive electrode) with a diameter d_1=7 mm and hollow plane electrode (2) realized with a plane with hole with diameter d_2 = 8 mm. Distance between electrodes is *h* = 6 mm.

pH of PAW and PAPSS had a similar trend for all measurements. pH decreased for rapidly during the first minute of an exposition. It decreased slightly for longer explosion and it had never overcame the value of 3 (Fig. 5). Even though, plasma activation created an acidic solution, that didn't give optimal terms for bacteria.

A solution conductivity changes depended on the solution itself. In case of PAW conductivity increased rapidly, that could be explained by nitrite and nitrate, and other ions that got into DI water by plasma treatment (Fig. 6).

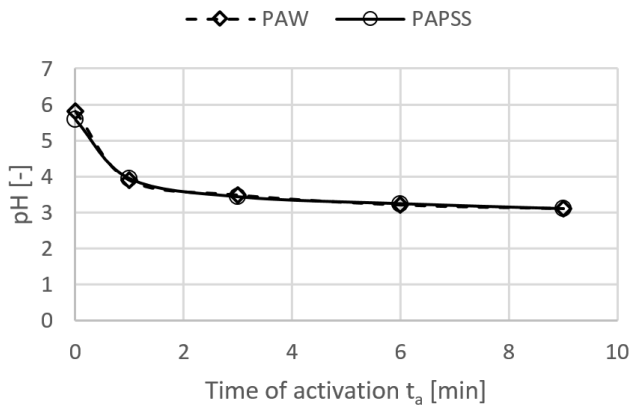

Fig. 5. Dependence of pH on the time of activation for distilled water and saline solution.

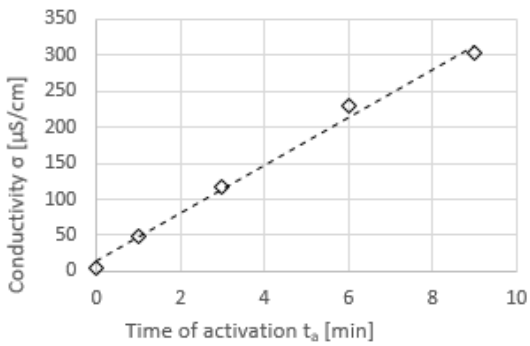

Fig. 6. A dependence of a conductivity of the plasma activated water on an exposition time.

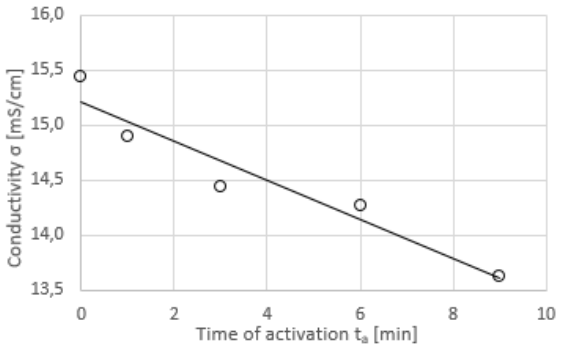

Fig. 7. A dependence of a conductivity of the plasma activated PSS on an exposition time.

A PSS conductivity decreased linearly with an increasing exposition time (Fig. 7). The reason could be in chemical reactions that made ions react with plasma products, so molecules and metastable particles were produced and ions were consumed.

Nitrite concentration increased linearly in both solutions (PAW and PAPSS). A nitrite concentration in PAPSS increased faster, than in PAW, and due to some experimental results the bactericidal effects of PAPSS were better (Fig. 8).

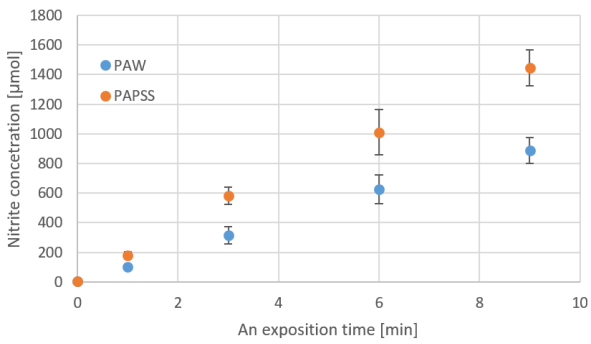

Fig. 8. A nitrite concentration in the plasma activated solutions.

Results of the drop test and the disc volatile method for PAW are shown in Fig. 9 and Fig. 10. The experiments of these methods for PSS need to be repeated because of contamination of initial sample.

The results obtained by the drop test showed inactivation of bacteria up to 6 order of magnitude. Inactivation properties of PAW depend on exposition time and on the time of bacteria treatment in the activated water. The drop test showed inactivation of about 2 orders of magnitude after the first 15 minutes of treatment for all plasma exposition times. For 30 minutes of treatment PAW exposed for only 3 minutes became ineffective, but PAW exposed for 6 and 9 minutes were able to inactivate one more order of magnitude of cells (see Fig. 9).

The maximal inactivation was obtained after 2 hours of bacteria treatment in the PAW exposed to plasma for 9 minutes.

The disc volatile method that is supposed to be more precise showed slightly different results. The decrease of bacteria amount was evident after 15 minutes of treatment for all exposition times. A number of inactivated bacteria was not the same as for the drop test. An observed difference could be caused by plasma setup that could be not so stable during the Drop test, agar contamination or cell counting error.

The maximal inactivation was observed after 2 hours of treatment and 9 minutes of exposition.

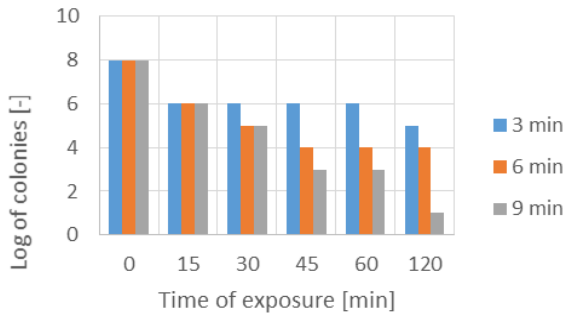

Fig. 9. A bacteria reduction measured with the drop test for a PAW.

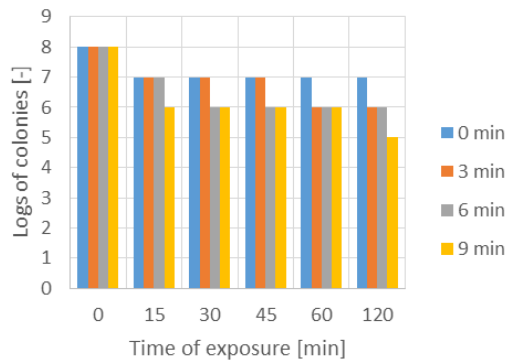

Fig. 10. A bacteria reduction measured with a disc volatile method for the PAW.

6. Conclusion

It is possible to activate liquids by indirect non-thermal plasma treatment. Such liquids become acidic, that can cause a bacterial death. The concentration of nitrite ions increases with time of plasma treatment. Ion concentration can have significant role in bacteria inactivation.

Bacterial inactivation depends not only on the plasma treatment time, but on the time bacteria are treated with the activated solution. The maximal bacterial inactivation was observed for 9 minutes of a plasma exposition and 2 hours of bacterial treatment.

The PAW generated for 3 minutes of plasma treatment caused the significant bacteria concentration decrease after 15 minutes of treatment bacteria with PAW.

The drop test and a disc volatile method showed different results, that could be caused by a pipette error that

Appendix B

List of species produced by plasma discharge

Plasma region		
	Cations	N^+, N_2^+, N_3^+, N_4^+, NO^+, N_2O^+, NO_2^+, H^+, H_2^+, H_3^+, O^+, O_2^+, O_4^+, OH^+, H_2O^+, H_3O^+
	Anions	e, O^-, O_3^-, O_4^-, NO^-, NO_3^-, H^-, OH^-, N_2O^-, NO_2^-
	Neutrals	N, N_2, H, H_2, N_2, H_2O, O, O_2, O_3, OH, HO_2, H_2O_2, NO, NO_2, NO_3, N_2O_3, N_2O_4, N_2O_5, HNO_2, HNO_3, N_2O, HNO
Air gap region		
		NO, N_2O, NO_2, NO_3, N_2O_3, N_2O_4, N_2O_5, HNO, HNO_2, HNO_3, N, N_2, O_2, O, O_3, OH, HO_2, H_2O_2, H_2, H_2O
Liquid region		
	Deionized water	O, O_3, OH, HO_2, HO_3, H_2O_2, N_2, O_2, H_2O, H, H_2, N_2O_3, NO, NO_2, NO_3, N2O_4, N_2O_5, HNO_2, H^+, HO_2^-, OH^-, O^-, O_2^-, O_3^-, NO_2^-, NO_3^-, O_2NOOH, O_2NOO^-, $ONOO^-$, ONOOH, HNO_3, N_2O
	Saline solution	O, O_3, OH, HO_2, HO_3, H_2O_2, N_2, O_2, H_2O, H, H_2, N_2O_3, NO, NO_2, NO_3, N_2O_4, N_2O_5, HNO_2, H^+, HO_2^-, OH^-, O^-, O_2^-, O_3^-, NO_2^-, NO_3^-, O_2NOOH, O_2NOO^-, $ONOO^-$, ONOOH, HNO_3, N_2O, HCl, HClO, HOClH, $HOCl^-$, ClO_2, Cl, Cl^-, Cl_2, Cl_2^-, Cl_3^-,. ClO^-, ClO, ClO_2^-, ClO_2, ClO_3^-, ClO_3, ClO_4^-, Cl_2O, Cl_2O_2, Cl_2O_3, Cl_2O_4, Cl_2O_5, Cl_2O_6, $ClNO_2$, Na^+

Appendix C

List of used chemicals

The list below contains all chemicals used in this work. All chemicals are mentioned with a distributor and a purity if capable.

Chemical substance	**Purity**	**Distributor**
4-Nitrophenylboronic acid	$\geq$ 95,0 %	Merck
Demineralized (deionized) water		
Di-Natriumhydrogenphosphat	$\geq$ 98,0 %, Ph.Eur.	Roth
Hydrochloric acid fuming (37 %)	37 %, p.a.	Roth
Hydrogen peroxide (30 %)	25,0 – 35,0 %	Merck
Iodine potassium iodide solution		Roth
Methanol	$\geq$ 99,95 %	T.H. Geyer
N-(1-Naphthyl)ethylenediamine dihydrochloride	$\geq$ 99,0 %	Merck
Natriumnitrit	$\geq$ 98,0 %, p.a.	Roth
ortho-Phosphoric acid (85 %)	$\geq$ 85,0 %, p.a.	Roth
Physiological saline solution (0,9 %)		
Potassium dihydrogen phosphate	$\geq$ 99,0 %, p.a.	Roth
Potassium iodide	$\geq$ 99,5 %, Ph.Eur.	Merck
Potassium nitrate	$\geq$ 99,0 %	Merck
Sulphanilamide	$\geq$ 99,0 %	Merck
TRIS	$\geq$ 99,0 %, p.a.	Roth
Vanadium(III) chloride	97 %	Merck