Plasma Sterilization: From Research to Production

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Plasma Sterilization: From Research to Production

Plasmasterilisation: Von der Forschung in die Anwendung

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Greek Symbols

$\alpha$  absorption coefficient  
$\Gamma_e$  electron flux  
$\Gamma_{exc}$  norm of excited particles flux  
$\Gamma_{ion}$  ion flux  
$\Gamma_{ion}$  norm of ion flux  
$\Gamma_{neu}$  norm of neutral flux  
$\Gamma_{phot}$  norm of photon flux  
$\epsilon_c$  collisional electron energy loss per electron-ion pair  
$\epsilon_e$  mean electron energy  
$\lambda$  wavelength  
$\lambda_{cut-off}$  cut-off wavelength  
$\mu_e$  electron mobility  
$\nu_{iz}$  electron ionization collision frequency  
$\nu_{IN}$  ion momentum transfer collision frequency  
$\pi$  ratio of a circle’s circumference to its diameter: 3.14159...  
$\Phi$  gas flow

Special Symbols

$\nabla$  nabla operator  
$\varnothing$  diameter
Latin Symbols

\( \ddot{a} \) acceleration
\( A_i \) area
\( A_p \) probe electrode lateral surface area
\( A_{\text{obj}} \) area of an object
\( A_{\text{ref}} \) reference area
\( A_{V_i} \) surface area of volume \( V_i \)
\( b \) initial bioburden
\( c_s \) specific heat capacity
\( C_{\text{el}} \) term for elastic collisions
\( C_{\text{in}} \) term for inelastic collisions
\( CFU \) colony-forming units
\( D_e \) electron diffusion constant
\( e \) elementary charge
\( E \) Energy
\( \vec{E} \) electric field
\( f_E \) electron energy distribution function (EEDF)
\( f_n \) velocity distribution function of species \( n \)
\( f_{\text{pulse}} \) pulse frequency
\( f_v \) electron velocity distribution function (EVDF)
\( I_e \) electron current
\( I_{\text{ion}} \) ion current
\( I_p \) probe current
\( I_\lambda \) spectral intensity
\( I_{\lambda,pk} \) spectral intensity from level \( p \) to level \( k \)
\( I_{\lambda,A} \) spectral irradiation per area \( A \)
\( j_n \) flux of particle \( n \)
\( l_r \) etching depth
\( k \) extinction coefficient
\( k_B \) Boltzmann constant
\( k_{\text{exc}} \) excitation coefficient
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Abstract

Research on plasma sterilization goes back for more than half a century. Plasmas are capable to sterilize through various mechanisms: induced DNA damages by VUV/UV radiation or erosion by radicals, chemical sputtering, or photodesorption. Even though plasma sterilization can be a gentle and rather cold process, there is no process approved by the responsible agencies (FDA or EMA).

Fundamental experimental studies of plasma sterilization are conducted in an inductively coupled low pressure plasma reactor (DICP). This is done by Langmuir probe measurements, optical emission spectroscopy, and microbiological investigations of plasma treated bacterial and fungal spores.

The influence of different spore coats on the plasma sterilization efficacy is studied by using various mutants of Bacillus subtilis endospores with knocked out genes for building up a specific spore coat. The sterilization efficacy is increased independent of if inner, outer, or all coats are missing. Missing pigments (build up by the protein CotA) in the outer coat decrease the resistance against VUV/UV rich plasmas significantly.

The erosion of endospores after a plasma treatment is studied by a developed algorithm, which is capable of detecting and measuring thousands of spores on SEM images.

Plasma simulations of the DICP are conducted with an appropriate hybrid model (HPEM). A good agreement between measurement and simulation is found. Particle and energy fluxes onto an object are calculated.

Based on the fundamental research on plasma sterilization, a prototype of a plasma sterilizer (SKP 101) and afterwards a plasma sterilizer for production (SKP 100) is developed. Syringe packages are sterilized on the outside in these transfer isolators before they can be filled and packaged individually and sterile. The prototype is characterized by means of Langmuir probe measurements and optical emission spectroscopy. The required four-log reduction of B. subtilis and Geobacillus stearothermophilus endospores in the SKP 100 is shown. Furthermore, the SKP 100 has been approved for production by the British health agency MHRA.

B. subtilis endospores are used as a model organism for Paenibacillus larvae (which causes American Foulbrood) to study the plasma sterilization of honeycombs inside the DICP. The plasma sterilization achieves a higher log reduction than the standard physical treatment with flames.
Kurzfassung


Grundlegende experimentelle Untersuchungen der Sterilisationswirkung von Plasmen werden in einem induktiv gekoppelten Niederdruckplasmareaktor (DICP) durchgeführt. Dazu dienen Langmuirsondenmessungen und optische Emissionsspektroskopie als auch mikrobiologische Untersuchungen der plasmabehandelten Bakterien- und Pilzsporen.

Um den Einfluss der verschiedensten Bestandteile von bakteriellen Sporenhüllen auf die Sterilisationswirkung zu untersuchen, werden *Bacillus subtilis* Endosporen, denen Gene für die Bildung spezieller Hüllproteine fehlen, Plasmabehandlungen ausgesetzt. Dabei nimmt die Keimrate der Endosporen, unabhängig davon, ob die innere, die äußere oder beide Sporenhüllen fehlen, nach einer Plasmabehandlung in ähnlichem Maße ab. Fehlende Pigmente in der äußeren Hülle, die durch das Protein CotA gebildet werden, senken signifikant die Resistenz gegenüber VUV/UV-reichen Plasmen.

Mittels eines eigens entwickelten Algorithmus, der anhand von REM-Bildern Tauende Sporen erkennt und ihre Längenverteilungen ermittelt, wird die Erosion von plasmabehandelten Sporen bestimmt.


Um die Sterilisationswirkung von Plasmen auf Bienenwaben experimentell im DICP-Reaktor zu untersuchen, werden *B. subtilis* Endosporen als Modellorganismus für *Paenibacillus larvae*, der die Amerikanische Faulbrut auslöst, verwendet. Im Vergleich zur gängigen Flammensterilisation wird durch eine Plasmabehandlung eine höhere Deaktivierung der Endosporen erreicht.
Introduction

Research on plasma sterilization goes back for more than half a century. Already in 1968 Menoshi filed a patent [1]. However, there is currently no commercial plasma sterilizer available where a plasma is the main sterilizing agent. Although there are two commercial sterilizers available, namely Sterrad (Johnson and Johnson, Irvine, California, USA) and Plazlyte (AbTox Inc., Mundelein, Illinois, USA), which use a plasma, chemicals are the major sterilizing agent [2]. Up to now, plasma sterilization could not keep the promise to be fast and reliable. Moreover, the expectations regarding the treatment of thermolabile materials have not been met yet. At least this applies for the commercial use of plasma sterilization. There are various reasons. One reason is that it is expensive to get a new sterilization technique certified by responsible authorities such as the European Medicines Agency (EMA) or the Food and Drug Administration (FDA). Another challenge for plasma sterilization is that it has to compete with the established industry standards for sterilization. Four examples of common sterilization techniques are autoclaves, electron beams (E-beams), gamma irradiation and ethylene oxide:

**Autoclaves** use water steam at 121 °C (or 134 °C for prions) [3]. These machines are cheap and well established since they have been used for more than one century. But autoclaves have certain drawbacks. Heat-sensitive materials cannot be sterilized. Furthermore, it has been shown that autoclaves have a negative impacts, like a decreased cutting efficiency of rotary nickel-titanium endodontic files [4].

**E-beams** are used in package sterilization. An E-beam sterilizer scans the surface of a package with a beam of electrons with an energy of up to 200 keV [5]. E-beam sterilizers are quite heavy due to their extensive shielding. Their operation requires special qualified personnel with dosimeter control.

**Gamma irradiation** has a deep penetration and commonly used for single use medical devices. Cobalt-60 ($^{60}$Co) serves as an irradiation source. The sterilization plant and especially the radioisotope need extensive shielding for the safety of the operating and maintenance personnel.

**Ethylene oxide** sterilization processes are conducted at temperatures from 20 to 50°C [3]. Thus, thermolabile objects, such as plastics, can be sterilized. Ethylene oxide has good diffusion abilities, so that it is able to sterilize through wrappings. But it is toxic and carcinogenic. Since 1981, it is forbidden to treat food with this gas. It "should only be used when no other method is practicable" in the sterilization of medicinal products [6]. In addition, the FDA generally wants to avoid the usage of it.¹

¹ private communication with two employees from different companies working in the field of sterilization
Nevertheless, the chance to use plasma sterilization as a new and beneficial sterilization technique is still there. It is promising to use a method without toxic chemicals such as ethylene oxide, ionizing radiation such as gamma rays, or to be able to sterilize heat-sensitive materials such as plastics. Plasma sterilization could be established as a niche technique where the classical sterilization methods have drawbacks or will be banned in the future (e.g. ethylene oxide sterilization). Plasma sterilization has recently made some remarkable progress. A utility model of a plasma desktop sterilizer aiming to replace autoclaves in doctor’s surgeries has been developed recently. [7] Another opportunity for the operation of a plasma sterilizer is the treatment of plastic containers for syringes, so-called tubs. E-beams are used in this field of application. Furthermore, the FDA specifies that the "demonstration of a four-log reduction should be sufficient for controlled, very low bioburden materials introduced into a transfer isolator, including wrapped sterile supplies that are briefly exposed to the surrounding cleanroom environment" [8]. This requirement is significantly lower than the usually required six-log reduction. Additionally, plasma sterilizers are significantly lighter than E-beam sterilizers. Thus, plasma sterilizers are less demanding on the pharmaceutical filling facility. All these mentioned requirements are comprehensible reasons for the development of the first plasma based transfer isolator. These considerations led to the development of the prototype Plasma Sterilization Chamber [Sterilisationskammer Plasma] 101 (SKP 101) and the commercial plasma sterilizer SKP 100. The SKP 100 aims to be an alternative in-line transfer isolator for syringe tubs.

Since 2003, research on low pressure plasma sterilization is carried out at the Institute for Electrical Engineering and Plasma Technology [Lehrstuhl für Allgemeine Elektrotechnik und Plasmatechnik] (AEPT) of Ruhr University Bochum (RUB). Halfmann focused on the plasma characterization of a Double Inductively Coupled Plasma (DICP) reactor, performed the first sterilization experiments with this reactor, and investigated the resulting wavelength-dependent sterilization effect [9–11]. At that time the project biological decontamination of surfaces using plasma discharges (BIODECON) was initiated [12]. It was funded as a Sixth Framework Programme by the European Commission (EC). This project had following participants: Center for Plasma Science and Technology (CPST) of the RUB, the Fraunhofer Institute for Process Engineering and Packaging (Fraunhofer-Institut für Verfahrenstechnik und Verpackung) (IVV), the Joint Research Centre (JRC) in Ispra (Italy), the Commissariat à l’énergie atomique (CEA), and AcXys Technologies. Four identical low pressure inductively coupled plasma (ICP) reactors were built and supplied to the project partners at the beginning of the project. These BIODECON reactors are comparable to the DICP reactor, so that many results can be transferred. Within this research project basic plasma sterilization effects and the removal of proteins and prions were successfully investigated [10,11,13–29].

Cooperation

Within this thesis plasma sterilization is investigated by experiments and simulations as an outcome of successful cooperation with the following partners.
Groninger GmbH

The Groninger GmbH (Crailsheim, Germany) builds customized machines for the pharmaceutical and cosmetics industry. Their machines provide sorting, cleaning, sterilizing, filling, closing, assembly, or labelling of containers such as vials, syringes, eyedrop bottles, or nasal sprays. Groninger GmbH designed and manufactured the plasma reactors SKP 101 (prototype) and SKP 100 (transfer isolator) which are investigated in this work. They also provided syringe containers, so that experiments could be done under real conditions.

Space Microbiology Research Group

The Space Microbiology Research Group belongs to the Institute of Aerospace Medicine of the German Aerospace Center [Deutsches Zentrum für Luft- und Raumfahrt] (DLR) (Cologne, Germany). The Space Microbiology Research Group investigates the life and survival of microorganisms under real and simulated extraterrestrial conditions. They are experts in the field of _Bacillus subtilis_ ( _B. subtilis_ ) spore research. Different spore mutants of _B. subtilis_ spores were gratefully provided by the Space Microbiology Research Group.

Institute for Biochemistry and Pathobiochemistry

The Institute for Biochemistry and Pathobiochemistry investigates the multifaceted biochemistry of microorganisms. They study oxidative signalling and redox regulation through reversible modification of proteins. Inoculated wood, wax, and honeycombs were gratefully provided by them.

Fraunhofer Institute for Process Engineering and Packaging

The Fraunhofer Institute for Process Engineering and Packaging [Fraunhofer-Institut für Verfahrenstechnik und Verpackung] (IVV) (Freising, Germany) works in the field of food processes and products, food quality and sensory acceptance, compliance of packaging materials, and processing and packaging machinery. They provided different spores sprayed on surfaces for sterilization tests.
Electronic Version of this Thesis

An electronic version (PDF) of this thesis can be found at:
https://stoodt.org/thesis.html

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1. Fundamentals

Since plasmas offer a broad range of physical and chemical sterilizing agents, they are suitable for sterilization purposes. A characterization of plasmas for sterilization can be carried out on basis of experimental investigations and simulations. Furthermore, investigations of the treated biological samples are necessary in order to assess the efficacy of such plasmas for sterilization.

1.1. Microbiology

To evaluate a sterilization process, microbiological experiments are essential. There are several effects of plasma on biological systems (see section 1.2). This makes it more difficult to understand the sterilization process compared with autoclaves, which use hot water steam for sterilization. Since there is no standard test organism for plasma sterilization processes, there is a lot of work and research required to get the first plasma process certified by medical agencies, such as European Medicines Agency (EMA) or Food and Drug Administration (FDA). It is necessary to choose spores which are resistant to stress factors caused by the specific plasma process and to select test organisms which are accepted by the particular agencies.

Sterilization can be measured by means of the log-reduction $R$. $R$ is the logarithm of the ratio of untreated references to treated samples:

$$R = \log \left( \frac{CFU_{\text{reference}}}{CFU_{\text{sample}}} \right),$$

where $CFU$ is the number of viable spores. A further description of the log-reduction $R$ and sterilization tests can be found in section 2.7.3.

1.1.1. Vegetative Cells and Bacterial Endospores

Bacteria belong to the prokaryotic microorganisms, which means that the chromosome (consisting of deoxyribonucleic acid (DNA)) is not enclosed by a membrane, but is located unprotected in the cytoplasm. If bacteria cells are in an environment which offers enough nutrition and suitable ambient conditions (temperature, pressure, acidity/basicity, ...), they are in a vegetative state. This is the normal state where the cells have metabolism, reproduction, and homeostasis. If these environmental conditions become disadvantageous, some bacteria are able to form a dormant form: the endospore. Endospores can easily be cultivated after several thousand years. Spores are metabolically dormant and
do not undergo cell division, but can survive for a long time in extreme environmental conditions, such as high temperatures, dryness, and vacuum conditions. By way of example, 10% of Geobacillus stearothermophilus (G. stearothermophilus) endospores are augmentable after 62 min in 113°C moist heat [30]. This is the reason that endospores are used as biological indicators for sterilization validation.

The term endospore means from inside the cell, because it is produced inside of a vegetative cell. The spore is morphologically distinct from its vegetative counterpart. Figure 1.1a shows a scheme of a Bacillus subtilis (B. subtilis) endospore. The outer layer, the spore coat, is a complex multilayered structure consisting of the crust as an electron dense layer, followed by the outer and inner coat and the basement layer. The spore coat is impermeable for larger molecules and resistant to toxins. It consists of spore specific proteins and enzymes required for germination. The coat is followed by the basement layer and the outer forespore membrane. Beneath is the cortex consisting of crosslinked peptidoglycan. Inside of the cortex is the inner forespore membrane. In the centre is the core with the nucleoid [31]. The DNA is stabilized by small acid-soluble spore proteins (SASPs). These proteins protect the DNA from irradiation (e.g. ultraviolet (UV) and gamma radiation) and heat, which is one reason for the resistance to external stress.

Besides the structural differences from the vegetative cells, endospores have a lower core water content than the vegetative cells. In contrast to 80 to 90% water content of a vegetative cell, dormant cells have only 10 to 25% water content. This is an important reason for the resistance against heat and chemicals. A unique feature of an endospore is the accumulation of dipicolinic acid (DPA) in the spore core. DPA is particularly important in protecting spore DNA from damaging agents and dry heat as well as in changing the DNA photochemistry [32]. There is also a high amount of calcium ions, which are most bound to DPA. Together it sums up to 10% of the total endospore weight [33].

If the endospore finds itself again in suitable ambient conditions and is surrounded by nutrition, the germination process is triggered. The SASPs serve as an energy source for the germination process. After a few minutes the vegetative cell is soaked up with water and grown out of the spore coat. During this process DNA can be repaired and proteins are formed [34,35].
1.1.2. Mould Spores

Moulds are fungi and belong to the eukaryotes, which means that the DNA is protected by a nucleus. Moulds form spores for reproduction. This dormant state is also called conidia. In contrast to bacterial spores mould spores can be formed exo- or endogenic. For example, Aspergillus niger (A. niger) conidia are formed exogenic. Figure 1.1b shows a scheme of an A. niger spore. Inside of A. niger spores there are nucleus, ribosomes, lipid droplets, and vacuoles, which are surrounded by spore cytoplasm. This is enclosed by the conidium inner and outer wall layer and again enveloped by the intermediate layer. At the outermost of the spore there is the surface coating [36, 37].

1.1.3. Importance of Spores for Plasma Sterilization

Spores are more resistant to stress such as heat or radiation than vegetative cells. Most vegetative bacteria are not suitable for low pressure experiments because they burst due to the rather high amount of water inside the cell. Some bacteria such as Escherichia coli (E. coli) need special drying techniques to be used at low pressure in the vegetative state. Spores can easily be used under low pressure conditions. Due to their resistance to different stress factors, they are important for sterilization tests. Every sterilization process, such as autoclavation or gamma radiation, has a defined test strain. Then, this test strain is used for validation and in-line tests because they are known to be comparably more resistant to a specific stress factor than vegetative cells. Theoretically, viruses or prions could be used for sterilization validation or in-line tests, but to detect functional viruses or prions is by far more time consuming. For instance, prion tests need an animal model. Spores only have to be brought onto a cultural medium and kept under the right environmental conditions. Afterwards, they start to grow and can be counted after a few days. Spore reduction tests also result in quantitative results, see section 2.7.3.

1.2. Plasma Sterilization: Basic Properties

Generally, plasmas are capable to kill bacteria (vegetative cells and dormant spores) and fungi or stop their proliferation [11, 13]. They are also able to inactivate viruses and prions [13, 38–40]. Plasmas can also terminally inactivate ribonuclease (RNase), which is one of the most stable proteins in nature [41]. All of this occurs since plasma is a cocktail of various potential inactivation mechanisms: erosion of biological material by etching with reactive species, photo-desorption, physical or chemical sputtering [13, 26], rupture of the outer membrane by charged particles [42, 43], induced DNA damages by vacuum ultraviolet (VUV) or UV irradiation [11, 44–47], or diffusion of species into cells. Moreover, the flux of energetic particles, radiation, and recombination processes can raise the temperature of microorganisms significantly, which can be lethal alone or in combination with the effects mentioned before. These mechanisms are effective at the same time and have potential synergistic effects [2, 14, 20, 23, 48–53]. However, there are also certain challenges. On the one hand VUV/UV radiation is a fast acting sterilizing
agent, on the other hand only the first few layers of spores can be reached since the deeper layers are shielded. This effect can lead to a biphasic inactivation kinetic. In the first phase the time derivative of the inactivation kinetic is large due to the inactivation based on VUV/UV radiation. In the second phase the time derivative is small because the inactivation is driven by etching and/or heat, depending on the plasma conditions [50].

Which of the inactivation mechanisms plays the major role strongly depends on the plasma conditions (excitation mechanism, pressure, gas composition, ...) and the examined biological object. Every biological object has different resistances. For instance, about 150 kJ m\(^{-2}\) irradiation by a VUV hydrogen lamp is needed to stop 90\% of Deinococcus radiodurans (D. radiodurans) from proliferation [54]. Most research on plasma sterilization is done with bacterial or fungal spores. This has two reasons: there are spores that are resistant to different plasma sterilization mechanisms (such as VUV/UV radiation or reactive oxygen species). Furthermore, they can be handled and evaluated in an easier way than prions or viruses.

**Irradiation with Light**

The first bactericidal effect by sun light was presented in 1877 by Downes and Blunt [55, 56]. Ward presented the first wavelength-dependent sterilization effect in 1893: "the bactericidal action of sun’s rays is due to those in the blue violet half of the spectrum." [57, 58] However, it took until 1929 when the first experiments dependent on UV irradiation were published by Gates [59–61]. UV irradiation has been used for more than a century for the sterilization of air and water [62, 63].

Bacterial endospores are 5 to 50 times more resistant to UV irradiation than their corresponding vegetative cells [32]. This is due to the specific characteristics of endospores [64]:

- An altered conformation of their DNA caused by the presence of a group of SASPs binding to the DNA [32]
- A DNA repair pathway specific for the spore photoproducts [65]
- The accumulation of DPA [66]
- A thick spore protein coating consisting of an electron dense outer coat layer and a lamellar inner coat layer [67]
- Some bacterial endospores have pigments absorbing UV-radiation [64]

DNA is one of the main targets for sterilization based on UV radiation [46]. Inagaki et al. measured the extinction coefficient \(k\) of DNA [68]. With this the absorption coefficient \(\alpha\) can be calculated in dependence on wavelength \(\lambda\):

\[
\alpha = \frac{4\pi k}{\lambda}. \tag{1.1}
\]
Figure 1.2 shows the absorption coefficient $\alpha$ of DNA as a function of wavelength. There is broad absorption with a significant peak at 80 nm. There is also a local absorbance peak at about 260 nm. This is exploited by the commonly used low pressure mercury germicidal lamps emitting at 254 nm. For wavelength greater than 230 nm the endospore and bacterial cell inactivation follow the DNA absorbance [46, 69]. For shorter wavelengths, it diverges. This could be due to absorption in the cell wall. Endospores of B. subtilis in vacuum are approximately ten times more sensitive to UV irradiation than under atmospheric pressure. When B. subtilis endospores are UV irradiated at atmospheric conditions the spore photoproduce 5-thyminyl-5,6-dihydrothymine (TDHT) is produced. In vacuum also two additional isomers of thymine dimer are produced. This is an indication of a partial denaturation of the DNA since it is also found after heat denaturation. Spore photoproduce damage can be repaired during the germination process by nucleotide excision repair or by a specific enzyme (photoproduce lyase) [70, 71].

![Figure 1.2: Absorption coefficient $\alpha$ of DNA measured by Inagaki et al. [68]](image)

To assess the wavelength dependent sterilization a gas mixture with significant and rather homogeneous radiation in the range from 115 to 450 nm is found and sterilization experiments with various cut-off filters are performed. A plasma discharge with a gas mixture of argon/nitrogen/oxygen 100/4/1 sccm at an input power ($P$) of 750 W and a pressure ($p$) of 10 Pa is used. The spectrum of the discharge is shown in Figure 1.3a. It is measured by a VUV monochromator and an Echelle spectrometer. Both are absolutely calibrated and described in section 2.3.2. The samples are treated in the center of the Double Inductively Coupled Plasma (DICP) reactor, as described in section 2.2.1. Following cut-off filters are used: MgF$_2$ ($\lambda_{\text{cut-off}} = 115$ nm), M235 filter ($\lambda_{\text{cut-off}} = 235$ nm), and a glass ($\lambda_{\text{cut-off}} = 300$ nm). In each wavelength range (115 to 235 nm, 235 to 300 nm, and 300 to 450 nm) a similar amount of photons in the range from $2 \cdot 10^{19}$ to $6 \cdot 10^{19}$ photons s$^{-1}$ m$^{-3}$ is emitted. (Endo-)spores of Bacillus atrophaeus (B. atrophaeus) American Type Culture Collection (ATCC) 51189, G. stearothermophilus ATCC 7953, A. niger ATCC 6275, and Aspergillus brasiliensis (A. brasiliensis) ATCC 16404 are sprayed on glass slides. These samples are treated without or with covering of one of the cut-off filters. The filters are separated from the glass slides by a glass ring. A detailed description can be found elsewhere [11]. Figure 1.3b shows the log reduction
dependent on the cut-off wavelength. It can be seen that the log reduction is not much different for all spores except for *A. brasiliensis* in the case without a filter. *A. brasiliensis* spores have a 2.5 to 3 lower log reduction. In the case of the magnesium fluoride filter, it can be seen that the log reduction is reduced by roughly one in comparison without a filter. This means that reactive species such as NO or O combined with the radiation below 115 nm have a smaller impact than the radiation above 115 nm. There are two resonant argon lines at 104.8 and 106.7 nm. The emission of these lines cannot be measured with the VUV set-up since it uses a photomultiplier with magnesium fluoride. Therefore these argon lines have been calculated [72]. The experiment with the M235 filter shows that there is no difference in the log reduction of the two tested bacterial endospores compared to the experiment with the magnesium fluoride filter. This means on the one hand that radiation in the spectral range from 115 to 235 nm has no relevant sterilization effect on *B. atrophaeus* and *G. stearothermophilus* endospores. On the other hand, radiation between 235 and 300 nm is efficacious for sterilizing these two endospore strains. This spectral range fits to the local maximum in the DNA absorbance in Figure 1.2. The sensitivity of the tested mould spores is completely different. Radiation above 235 nm has no impact on *A. niger* and *A. brasiliensis* spores. This could be due to pigments in the cell wall since both moulds are black. The experiment with the cut-off filter at \( \lambda_{\text{cut-off}} = 300 \text{ nm} \) shows that radiation above 300 nm has no impact on any of the tested (endo-)spores, at least at treatment times of 60 s.

![Spectrum and log reduction graphs](image)

(a) Spectrum measured with a VUV monochromator and an Echelle spectrometer, the dashed lines (---) mark the cut-off wavelengths at 115, 235 and 300 nm

(b) Log reduction \( R \) for various cut-off filter: *B. atrophaeus* (○), *G. stearothermophilus* (▽), *A. niger* (□), and *A. brasiliensis* (△), \( t = 60 \text{ s} \)

Figure 1.3.: Filter experiments in an Ar/N\(_2\)/O\(_2\) 100/4/1 sccm discharge in DICP at \( P = 750 \text{ W} \) and \( p = 10 \text{ Pa} \)

Munakata et al. used synchrotron radiation to measure the action spectrum of *B. subtilis* endospores [45]. Figure 1.4 shows the ratio of non augmentable spores dependent on synchrotron radiation. This experiment differs from the filter experiment in the DICP reactor. In contrast to the experiment presented before, the endospores are irradiated with a photon flux, which is at least four magnitudes higher than the the photon flux in the filter experiment. Another difference is that Munakata irradiated the samples
with monochromatic radiation which have a full width at half maximum (FWHM) of less than 5 nm, whereas the samples in the filter experiment are irradiated with the full radiation spectrum just missing lower spectral wavelengths. It can be seen in Figure 1.4 that these endospores are insensitive to radiation of 300 nm. Except at three additional wavelengths, namely 100, 190, and 290 nm, the inactivation is rather constant, maybe a bit decreasing to lower wavelengths and with a small dip at 255 nm. The drop at 290 nm is probably due to the non absorbance of the DNA. The dips at 100 and 190 nm could be due to a proteinous outer layer [45]. Since the result of Munakata diverges from the DNA absorption measurement, it means the DNA is shielded by the spore coat and proteins in the core, so that the broad absorbance in the VUV range is not detectable in the inactivation.

![Graph showing inactivation vs wavelength](image)

**Figure 1.4.** Ratio of non augmentable *B. subtilis* endospores irradiated with $1 \cdot 10^{20}$ photons m$^{-2}$ at defined wavelengths, the value at 300 nm is not depicted and amounts to 7 % [45]

### Reactive Species

Reactive species such as O, O$_2$, O$_3$, OH, and NO have a potential sterilization effect. Such species can cause strong oxidative stress on spores, especially on the outer structures. Figure 1.5 shows images taken with a scanning electron microscope (SEM) of *G. stearothermophilus* endospores untreated and treated in the biological decontamination of surfaces using plasma discharges (BIODECON) reactor for 30 s in an argon/oxygen discharge [15]. The erosion of endospores can clearly be seen. This can have various reasons. Photodesorption by VUV/UV radiation can break chemical bonds and volatile compounds can be formed [48]. Reactive species such as atomic oxygen can directly etch endospores. This process can be enhanced by VUV/UV radiation or ion bombardment. Raballand et al. treated *B. atrophaeus* ATCC 51189 endospores with a particle beam of argon ions, oxygen molecules, and oxygen atoms, separately or combined in a vacuum chamber [24]. The morphology of the endospores is evaluated by an SEM with a lateral resolution of 1.5 nm. Figure 1.6 shows these endospores exposed to different particle beams. An exposure to oxygen atoms alone has no effect, neither in morphology nor
in a sterilization effect. This can be explained by the inability of oxygen atoms to etch hydrocarbon films below 400 K [73, 74]. The exposure with argon ions with an energy of 200 eV only slightly alters the surface texture. This can be explained by the fact that physical sputtering is effective above 200 eV [75]. With the combination of an argon ion and an oxygen molecule beam, the morphology has changed significantly. There are etch channels in the spore coat. The etching of the combined beam can be explained by chemical sputtering. This is the simultaneous impact of ions and oxygen molecules. The ions break the bonds of the material and the oxygen molecules saturate these bonds until volatile species such as CO or CO$_2$ are formed [76, 77]. With the combination of argon ions, oxygen molecules, and oxygen atoms, the endospores are etched significantly more than compared with the result before. This can be explained by deeper penetration of oxygen atoms compared with oxygen molecules due to their smaller size. The physical sputtering cascade of argon ions opens bonds deeper in the material where only oxygen atoms can reach [24].

![SEM images](image)

(a) Untreated  
(b) Treated for $t = 30$ s in Ar/O$_2$ 20/1 sccm at $P = 200$ W and $p = 10$ Pa

Figure 1.5.: SEM images of *G. stearothermophilus* endospores: untreated and etched in the BIODECON reactor [15]
1.3. Plasma Simulations

Plasma simulations are a good complement to measurements because not all plasma properties can be measured. Furthermore, it can be difficult to measure some properties at certain locations. Measurements can also influence the plasma such as Langmuir probes, which are inserted into the plasma. Optical emission spectroscopy (OES), which is performed through optical windows of a plasma reactor can only yield spatially integrated results. When plasma sterilization is examined, it is important to know the amount of high energy photons which are produced all around the sterilization object. The amount of these photons can be measured by OES, but depending on the reactor geometry it is difficult to measure this around all sides of the object. But if it is possible to perform a reliable plasma simulation, even this photon flux to all surfaces can be estimated.

Plasmas can be simulated by various approaches including analytical models, fluid models, Boltzmann models, Monte Carlo models, particle-in-cell–Monte Carlo collisions (PIC-MCC) models, and hybrid models. Every model has advantages and limitations. Depending on parameters such as pressure, energy coupling, or excitation frequency a suitable plasma model is chosen [78, 79].

**Fluid Model**

In a fluid model single particles such as atoms or electrons are not taken into account, but macroscopic quantities such as density, mean velocity, or mean energy are considered. In order to obtain these quantities, the continuity and transport equations for every plasma species have to be solved. These equations are derived by using moments of Boltzmann’s equation. These are coupled with Maxwell’s or Poisson’s (for electrostatic
solutions) equation to obtain a self-consistent electric field distribution. If the collision frequency is larger than the excitation frequency, the drift-diffusion approximation can be used. For this purpose, the inertia of charged particles is neglected and the mean velocities respond immediately to the electric field. These approximations are valid for argon discharges with a pressure above \( \approx 15 \text{ Pa} \) and an excitation frequency of \( 5.56 \text{ MHz} \). If pressure is lower or excitation frequency is higher, the full flux equation has to be used. Equations (1.2) to (1.7) list the typical equations used in fluid simulations of plasmas, where magnetic fields are neglected and single ionization of one species is considered [79]. These equations can be extended for more ion species. Since ions are usually assumed to be at about neutral gas temperature, the energy balance equation is only solved for electrons.

\[
\begin{align*}
\frac{\partial n_e}{\partial t} + \nabla \cdot \vec{\Gamma}_e &= S_e \quad \text{(Continuity eq., elec.) (1.2)} \\
\vec{\Gamma}_e &= -\mu_e \vec{E} - \nabla(n_e D_e) \quad \text{(Drift – diff. flux, elec.) (1.3)} \\
\frac{\partial (n_e \epsilon_e)}{\partial t} + \nabla \cdot \left( \frac{5}{3} \left( \epsilon_e \vec{\Gamma}_e - n_e D_e \nabla \epsilon_e \right) \right) &= -e \vec{\Gamma}_e \cdot \vec{E} - n_e \epsilon_e \nu_{iz} \quad \text{(Energy, elec.) (1.4)} \\
\frac{\partial n_i}{\partial t} + \nabla \cdot \vec{\Gamma}_i &= S_i \quad \text{(Continuity eq., ions) (1.5)} \\
\frac{\partial \vec{\Gamma}_i}{\partial t} + \nabla \cdot (\vec{\Gamma}_i \vec{v}_i) &= \frac{e_n}{m_i} \vec{E} - \frac{\nabla (n_i T_i)}{m_i} - \nu_{iN} \vec{\Gamma}_i \quad \text{(Flux, ions) (1.6)} \\
\nabla^2 \Phi &= -\frac{e}{\epsilon_0} (n_i - n_e) \quad \text{(Poisson’s eq.) (1.7)}
\end{align*}
\]

\( n_e \) and \( n_i \) are the electron and ion density, \( t \) is the time, \( \vec{\Gamma}_e \) and \( \vec{\Gamma}_i \) are the electron and ion flux, \( S_e \) and \( S_i \) are the source terms for electrons and ions (collisions, radiation), \( \mu_e \) is the electron mobility, \( \vec{E} \) is the electric field, \( D_e \) is the electron diffusion constant, \( \epsilon_e \) is the mean electron energy (\( \epsilon_e = \frac{2}{3} T_e \)), \( \epsilon_c \) is the collisional electron energy loss per generated electron–ion pair, \( \nu_{iz} \) is the electron ionization collision frequency, \( \nu_{iN} \) is the ion momentum transfer collision frequency, \( \vec{v}_i \) is the ion mean velocity, \( T_e \) and \( T_i \) are the electron and ion temperature, and \( m_i \) is the ion mass.

One advantage of fluid simulations is that it is possible to handle complex chemistry at fast computation speed. Also two or three dimensional field simulations can be executed at reasonable times. A drawback is that only mean velocities can be handled, so an electron energy distribution has to be assumed, such as a Maxwellian or Druyvesteyn distribution. The assumption that the energy gain of particles by the electric field is balanced by collisional losses is another disadvantage. It is a good assumption for ions or atoms, but not for the light electrons.
### Boltzmann Model

Instead of the rather simple fluid model one could solve the Boltzmann equation for every species:

\[
\frac{\partial f_n}{\partial t} + \vec{v} \cdot \nabla_r f_n + \vec{a} \cdot \nabla_v f_n = C_{el}(f_n) + C_{in}(f_n).
\]

(1.8)

\(f_n(\vec{r}, \vec{v}, t)\) is the velocity distribution function of species \(n\), \(\vec{r}\) is the position, \(\vec{v}\) is the velocity, \(\vec{a}\) is the acceleration in the electric field, and \(C_{el}(f_n)\) and \(C_{in}(f_n)\) are terms for elastic and inelastic collisions.

Solving the Boltzmann equations for all species is difficult and time-consuming. It is only used in simple cases or if suitable approximations can be made [80].

### Kinetic Simulations with Monte Carlo Model

In a kinetic simulation individual particles or super-particles (one super-particle represents \(n\) real particles) are calculated. The explicit trajectory of single particles is computed with Newton’s law under the influence of the electric field. Collisions are calculated with random numbers (whether and what kind of a collision takes place). After each collision, the new energies and scattering angles are calculated. Since single particles are calculated, Monte Carlo simulations yield energy distribution functions of the particles. Especially electron energy distribution functions (EEDFs) are very valuable. In order to achieve reliable results, enough particles have to be calculated and this takes rather long computation time. This model is not self-consistent since the electric field has to be given as input parameter.

### Kinetic Simulation with Particle-in-Cell-Monte Carlo Collisions Model

The Particle-in-cell–Monte Carlo collisions (PIC-MCC) model is an advanced Monte Carlo model. The trajectory and the collisions of particles are calculated as described in the previous section. In a PIC-MCC simulation the electric field is calculated self-consistently. This is done by the projection of charged particles onto a grid to gain the charge density distribution. The electric field distribution is calculated from this by Maxwell’s or Poisson’s equation. Since this calculation is demanding for every real charged particle, super-particles are used. A super-particle can represent about \(10^4\) to \(10^6\) real particles.

The computation of PIC-MCC models takes even more time than the computation of Monte Carlo models explained above. The calculation time of PIC-MCC models is proportional to the number of super-particles. As a thought experiment, a Maxwellian distribution with an average electron energy of 2 eV has four orders of magnitude fewer electrons at 20 eV than at 2 eV. Thus, it is essential that enough super-particles have to be used to get a sufficient statistical representation of all interesting energy regions especially for energetic particles. Otherwise the processes with energetic particles such as ionizations are simulated inadequately and, therefore, lead to wrong results.
**Hybrid Simulation**

A hybrid modelling approach combines various simulation principles mentioned above. Thus, it is possible to govern the different time and length scales in a convenient computation time. Hybrid models need fewer computation time than PIC-MCC models, but the time needed is still rather long. Time scales can vary from picoseconds (integration time for solving Maxwell’s equations) to seconds (time to reach steady state of surface chemistry). Length scales can vary from micrometres (boundary sheath thickness) to metres (dimension of a typical plasma reactor). The whole plasma simulation is divided into different computational modules addressing different physical processes. In this hierarchical approach it is possible to separate the simulation in different modules with specific input and output variables. Thus, it is possible to calculate every physical process with the most appropriate approach, for example by using different particle transport modules depending on the pressure regime. Hence, all modules are transparent to the other. A further advantage of this hierarchical approach is that modules can be switched depending on the outer conditions, for example by replacing the excitation module of capacitively coupled plasma (CCP) to inductively coupled plasma (ICP). Kushner gives a formulaic approach for hybrid modules [81]:

- Compartmentalize physical processes into relatively independent modules.
- Establish hierarchical relationships between the modules (information flow between modules).
- Determine the relative time scales for integration within a module to resolve the physics and the time for exchange of information between modules.

A typical option with this approach is to choose a kinetic model for the fast electrons and a fluid model for ions and neutral atoms.
2. Experimental Set-up and Diagnostics

2.1. Object for Sterilization: Polystyrene Containers for Syringes

In order to produce sterile pre-filled syringes it is necessary to bring sterilized empty syringes into a sterile filling machine. The empty syringes tubes are produced and packaged into a polystyrene container. Typically, 100 syringe tubes are packaged inside one container, a so-called tub. Nets containing empty syringe tubes, are hanging inside a polystyrene container. Figure 2.1 shows images of these containers. The solid polystyrene body has a thickness of 1 mm and is sealed with Tyvek foil made by DuPont (Wilmington, Delaware, USA) on top. Tyvek is a high-density non-woven polyethylene foil, which is a microbial barrier but gas and vapour permeable. The tubs have a height of 95 mm and dimensions of 260 mm × 230 mm and 210 mm × 180 mm, for top and bottom, respectively. The closed tub itself is packaged inside a wrapping made of polystyrene foil and Tyvek. Tyvek serves as a gas window. This allows to sterilize the whole packaged tub by an ethylene oxide (C₂H₄O, oxirane) sterilization process. Thus, it is achieved that the outside and the inside (including the syringe tubes) of the tub are sterile. After this the syringe tubs are transported to the filling facility.

![Tub inside of its wrapping with Tyvek gas window](image1)
![Open tub](image2)

Figure 2.1.: Photographs of syringe tubs

In the filling facility the tubs have to be unwrapped inside the grey area before entering the transfer isolator. The transfer isolator separates the sterile area from the grey area.
2. Experimental Set-up and Diagnostics

The grey side is a clean but unsterile environment (at least by regulations). The transfer isolator sterilizes the syringe containers on the outside, so they can enter the sterile filling machine. Figure 2.2 shows a general scheme of an aseptic filling line. The tubs are unwrapped on the grey side and fed into the transfer isolator. The tubs exit the transfer isolator on the sterile side. After that, the syringes are filled and packaged individually within a sterile environment.

When the tub is unwrapped in the grey area the outside of a tub is not sterile any more, at least by regulations. The syringes inside the tub stay sterile because of the Tyvek foil. This foil poses several challenges for a low pressure plasma process. At first, the pumping power has to be reduced during the evacuation phase, otherwise the heat-sealed joint will burst. A burst Tyvek foil has to be avoided to keep the syringes sterile. The whole tub has to stay intact during the sterilization process. The sensitivity to temperature of the Tyvek foil poses another challenge. Since Tyvek is made of polystyrene like the rest of the tub, it melts at 240°C. In order to avoid damages of the foil and the body, the glass transition temperature must not be reached. The glass transition temperature of polystyrene without additives amounts to 86 to 96°C [83, 84]. ICP modules of a plasma reactor can easily provide enough power to the plasma, so that the Tyvek foils is pierced and even that the tub body can be deformed. The biggest challenge in plasma sterilization of tubs, is to sterilize the object as fast as possible on all sides while keeping the tub intact. It was shown during the design qualification (DQ) procedure that no plasma is formed inside the tub and no surface properties of the syringes are altered if suitable process parameters are used.

Some measurements cannot be conducted with regular tubs, since the time needed for e.g. a phase resolved Langmuir probe measurement is longer than a regular tub can withstand the plasma conditions. Thus, two tub dummies made of borosilicate glass were produced: one cylindrical shaped dummy used in the DICP reactor and one cuboid-shaped dummy used in the Plasma Sterilization Chamber [Sterilisationskammer Plasma] 101 (SKP 101). Since the dummy tub used in the DICP reactor has the shape of a cylinder, results gained with this dummy can be compared with the cylindrically symmetric plasma simulation. Furthermore, since the dummies are made of glass, this inert material is easier to be used in simulations. The cylindrical dummy has a diameter of 230 mm and a height of 75 mm. The cuboid-shaped dummy has the same dimensions as a tub.
2.2. Equipment for Sterilization Experiments

Three different low pressure plasma reactors are used for the experiments: the DICP reactor, the SKP 101, and the Plasma Sterilization Chamber [Sterilisationskammer Plasma] 100 (SKP 100). The DICP reactor is used for basic research whereas the SKP 101 is a prototype sterilization reactor. The SKP 100 is an industrial plasma based transfer isolator. All used plasma reactors are ICPs, since they produce dense and voluminous plasmas.

2.2.1. Double Inductively Coupled Plasma Reactor

The Double Inductively Coupled Plasma (DICP) chamber consists of an electropolished stainless steel cylinder with an inner diameter of 400 mm and a height of 200 mm. This electrically grounded cylinder is enclosed by two quartz panes, resulting in a volume of 251. The power supply can deliver up to 5 kW at 13.56 MHz. The electrical power is fed through a coaxial cable to a custom built matchbox. This matchbox serves as a matching network and a power splitter regarding the two planar coils above and below the two quartz panes. The radio frequency (RF) power supply can be driven by a custom build pulse generator, which is capable of delivering pulsing frequencies from $10^{-2}$ to $10^6$ Hz and duty cycles from 0 to 100%. The chamber can be pumped down either by a roots pump combined with a rotary vane pump or a turbomolecular pump combined with a rotary vane pump. A base pressure of $10^{-4}$ Pa is achieved by the turbomolecular pump. A scheme of the DICP reactor is depicted in Figure 2.3. Four mass flow controller are connected to a gas shower at the top of the stainless steel cylinder. In the frame of this work, argon, hydrogen, nitrogen, and oxygen are used solely or as mixtures. The gas flow can be adjusted from 40 to 1000 sccm for argon and from 0.5 to 20 sccm for molecular gases. With this, a working pressure of 0.5 to 50 Pa is obtained.

![Figure 2.3: Cut-away scheme of the DICP](image)

An engineering drawing and a further detailed description of the DICP reactor can be found in [9,10]. Up to three glass slides with spores or endospores can be placed on sample holders made of glass inside the reactor. Flat samples such as glass slides are placed vertically and horizontally in the middle of the reactor. The flanges for attaching the plasma
2. Experimental Set-up and Diagnostics

Figure 2.4.: Comparison of electron density \( n_e \) and electron temperature \( T_e \) dependent on the distance to the chamber wall in a pure argon discharge (100 sccm) at \( p = 10 \text{ Pa} \) and \( P = 500 \text{ W} \) (——), 750 W (－－－), and 1000 W (— · —): results from the old matchbox [9] are without and from the new matchbox with errorbars.

diagnostics are at the same height as the samples. Polystyrene tubs (see section 2.1) can also be placed inside the DICP reactor. The tub stands on glass supports, so that they are located vertically and horizontally in the middle of the reactor.

**Improved Matching Network**

The plasma characterizations published by Halfmann were carried out with another matching network [9–11]. Within the framework of this thesis, an upgraded matching network, which was built by Aurion Anlagentechnik GmbH (Seligenstadt, Germany), is used. It has been renewed for delivering powers up to 5 kW. The matching network used beforehand was designed for an output power of 3 kW. The new matchbox is also more effective at the same input power.

Figure 2.4 shows Langmuir probe measurements of the electron density \( n_e \) and electron temperature \( T_e \) with the old and the new matchbox in a pure argon discharge 100 sccm at a pressure of 10 Pa for various powers. Measurements with the old matchbox have been performed by Halfmann [9]. The results of the electron density measurement at 500 W with the new matchbox are approximately the same as the 1000 W measurement with the old matchbox. Furthermore, the electron temperature \( T_e \) drops generally from 1.4 eV with the old matchbox to 1.1 eV with the new matchbox. With the old matching network the discharge would switch into E-mode at 500 W and 10 Pa in an argon discharge with an admixture of 5% of a molecular gas such as hydrogen, nitrogen, or especially oxygen. The E-mode is characterized by a low plasma density and a weak light emission. The plasma generation is attributed to the electric field between individual coils or between coils and grounded parts of the chamber. An ICP plasma discharge starts in E-mode at low power. If the power is increased the discharge suddenly transits into H-mode. This
mode is characterized by an increased plasma density and light emission. The plasma generation is usually attributed to an induced azimuthal electric field generated by the oscillating magnetic field. A further description of the E-H-mode transition can be found elsewhere \cite{85}. With the new system, the discharge remains in H-mode for a lower powers and higher pressures at a specific gas mixture. For the typical admixture of 5\% molecular gas (H\textsubscript{2}, N\textsubscript{2}, or O\textsubscript{2}) to argon, as used by Halfmann \cite{9}, the new system switches into E-mode at powers lower than 200 to 220\,W.

### 2.2.2. Prototype Plasma Sterilization Chamber SKP 101

The Plasma Sterilization Chamber [Sterilisationskammer Plasma] 101 (SKP 101) is a prototype of the industrial grade plasma sterilization reactor SKP 100 for syringe containers made of polystyrene. The integration of such a plasma sterilization reactor into an aseptic filling line is described in section 2.1. Knowledge gained in experiments performed at the DICP reactor are incorporated in the development and design of the SKP 101, which was designed and manufactured by groninger GmbH. The prototype consists of a cuboid-shaped stainless steel chamber with a volume of 56\,l. The power is inductively coupled into the reactor by two identical discharge modules (ICP 3000W4 from PAC GmbH, Bochum, Germany) through glass panes at the bottom and at the top of the reactor. Each discharge module consists of an effective planar ICP antenna \cite{86} and a matching network. A cut-away scheme of the chamber is shown in Figure 2.5. The power is delivered by two 3\,kW sources. Both RF power supplies operate at a fixed frequency of 13.56\,MHz and can be pulsed separately to ensure an acceptable surface temperature at all sides of the polystyrene container. If one power source is pulsed, both generators have to be set in pulsing mode at the controlling unit. Because of this most experiments are conducted with a 99\% duty cycle of the bottom coil. The vacuum chamber is pumped down by a roots pump in combination with two rotary vane pumps. One mass flow controller is connected to the gas shower on the bottom of the plasma chamber. One polystyrene container can be placed on four stainless steel supports, see Figures 2.5 and 2.6. A further description can be found elsewhere \cite{82}.

### 2.2.3. Industrial Plasma Reactor SKP 100

The industrial plasma reactor SKP 100 has a similar set-up as the SKP 101 described in section 2.2.2. The chamber volume is nearly twice as big. The reactor is equipped with four ICP modules (ICP 3000W4 from PAC GmbH, Bochum, Germany). With this, two syringe tubs can be sterilized at the same time.

Figure 2.7 shows two photos of SKP 100. It can be completely integrated in an aseptic filling line and is equipped with vacuum grippers for tub handling at the grey and sterile side as well as with two roller conveyors on each side. The SKP 100 serves as a transfer isolator and separates the sterile and the grey side of a production facility for the filling of pre-filled single packaged syringes.
2.2.4. UV + Heat Set-up

To examine the synergistic effect of VUV/UV radiation and heat, an experiment is developed, where photon dosage and heat can be controlled independently. Figure 2.8 shows a scheme of the set-up. Two deuterium lamps are available one enclosed by a magnesium fluoride window (X2D2 L9841 from Hamamatsu Photonics K.K., Hamamatsu City, Japan) and one enclosed by a quartz window (X2D2 L9518 from Hamamatsu Photonics K.K.). Thus, one lamp has a cut-off wavelength of 115 nm and one of 185 nm. These lamps are placed directly without an air gap above a magnesium fluoride widow, which is fitted to a vacuum chamber. Inside the chamber, a halogen lamp is fixed upon a height adjustable stage. The halogen lamp serves as a heat source for the sample above and does not emit UV radiation [7]. The temperature of a sample is measured by a PT-100 element and the current of the halogen lamp is adjusted so that the desired temperature is reached. The photon dosage can be adjusted by changing the distance between sample and deu-
2.2. Equipment for Sterilization Experiments

(a) Syringe tubs are placed inside SKP 100

(b) Plasma treatment of syringe tubs in SKP 100

Figure 2.7.: Photographs of SKP 100 integrated in an aseptic filling line [82]

---

Figure 2.8.: Scheme of the UV + heat set-up
2. Experimental Set-up and Diagnostics

A deuterium lamp by a height adjustable stage. Generally, the spectrum of a deuterium lamp is similar to the spectrum of a hydrogen plasma discharge. The spectra of these lamps and a comparison to a hydrogen discharge can be found elsewhere [7, 87]. The photon flux of the deuterium lamp is high enough to mimic the photon flux onto a syringe tube inside the SKP 101 in a hydrogen discharge. It is possible to exchange the magnesium fluoride window with different cut-off filters at the top of the vacuum chamber to yield different spectral ranges. Another option is to vent the chamber and thus, only photons above 200 nm reach the sample.

2.3. Plasma Diagnostics

**DICP Reactor**

The plasma diagnostics performed at the DICP reactor are conducted through flanges at the middle height of the reactor (100 mm from the bottom quartz plate). The microbiological experiments with glass slides are conducted at the same height as the plasma diagnostics are performed, see section 2.7.

**SKP 101**

Langmuir probe measurements are performed above and besides the tub in the SKP 101. Quantitative optical emission spectroscopy (OES) is conducted through four quartz windows above, below, and at two sides of the tub. Optical windows and spatial resolved Langmuir probe measurements are depicted in Figure 2.5.

2.3.1. Langmuir Probe

The Langmuir probe was introduced by Irving Langmuir in 1924 [88, 89]. It is part of the group of invasive plasma probes.

**Theory**

Although there are different types of Langmuir probe shapes, the cylindrical type is the most common one and, thus, the one used throughout this work. The principle of cylindrical Langmuir probes is based on the orbital motion limited (OML) theory. Therefore, it is necessary that the lateral surface area of the cylindrical electrode is much greater than the top cap. A further introduction into the OML-theory can be found elsewhere [90]. The basic principle of Langmuir probes is to record a current-voltage curve with a thin wire inside a plasma. Therefore, a wire made of a material with high melting temperature (usually tungsten) is supplied with a voltage ramp and the resulting current is measured. A helpful introduction to Langmuir probe diagnostics is given by Chen [91]. The electrode is surrounded by a boundary sheath. The sheath voltage $V_{sh}$ is given by the difference...
of the probe potential $V_p$ and the plasma potential $V_s$: $V_{sh} = V_p - V_s$. If the potential of the probe is much larger than the plasma potential $V_p \gg V_s$, the probe collects an electron current $I_e$ resulting in a negative probe current. On the contrary, if $V_p \ll V_s$ is valid, an ion current $I_{ion}$ is collected. Usually the negative probe current is plotted against the probe voltage resulting in a positive $I_e$ and a negative $I_{ion}$. Figure 2.9 shows an example of a current-voltage curve measured within the DICP reactor. Generally, the $I - V$ curve is divided into three regions: ion saturation, transition region, and electron saturation.

![Current-Voltage Curve](image)

Figure 2.9.: A measured current-voltage curve of a Langmuir probe measurement (——), its first derivative (−− −) and its second derivative (—· —). The unit on the ordinate is only valid for the $I - V$ curve.

The ion saturation region is at the far left side of the $I - V$ curve where the sheath voltage $V_{sh}$ is negative. Thus, most electrons are repelled and positive ions are accelerated towards the electrode. This region ends at the floating potential $V_f$ where the probe current $I_p$ is zero. It follows that the absolute ion current equals the absolute electron current: $|I_{ion}| = |I_e|$. After that the transition region begins where the sheath voltage $V_{sh}$ is still negative, but electrons are only partly repelled and the ion current $I_{ion}$ is negligible. This part of the $I - V$ curve is exponential if the electrons follow a Maxwellian distribution. The transition region ends when the sheath voltage $V_{sh}$ is zero and, therefore, the probe potential is equal to the plasma potential: $V_p = V_s$. At this point the electron saturation starts. From there on, the whole thermal flux of electrons reaches the probe electrode. Further to the right of the $I - V$ curve the gradient of the probe current $I_p$ is smaller, but it is still growing due to sheath expansion.

The electron velocity distribution function (EVDF) can be calculated from probe current in the transition region ($V_f < V_p < V_s$) according to Druyvesteyn [92]:

$$f_v \left( v = \sqrt{\frac{2e}{m_e} (-V_{sh})} \right) = -\frac{4m_e}{A_p e^2 V_{sh} \frac{d^2 I_e}{dV_{sh}^2}},$$

(2.1)
with \( f_v \) representing the EVDF, \( e \) the elementary charge, \( m_e \) the electron mass, and \( A_p \) the probe electrode lateral surface area. This formula is only valid for an isotropic velocity distribution, but also applies for non-Maxwellian distributions. From this, one can calculate the EEDF \( f_E(E) \):

\[
f_v(v)dv = f_v(v)\frac{dv}{dE}dE = f_v(v)\frac{1}{\sqrt{2m_eE}}dE = f_E(E)dE
\]

\[
\Rightarrow f_E(E) = -\frac{\sqrt{-8m_eeV_{sh}}}{A_pe^3}d^2I_e \text{ d}V_{sh}^2, \text{ with } E = -eV_{sh}.
\]

Thus, the electron density \( n_e \) can be determined by the zero’th moment of the EEDF:

\[
n_e = \int_0^\infty f_E(E)\, dE.
\]

The mean electron energy \( \bar{E} \) is given by the normalized first moment:

\[
\bar{E} = \frac{\int_0^\infty Ef_E(E)\, dE}{\int_0^\infty f_E(E)\, dE}.
\]

If the EEDF is Maxwellian, the electron temperature \( k_B T_e \) is given by:

\[
k_B T_e = \frac{2}{3} \bar{E}.
\]

For a Maxwellian EVDF

\[
f_v(\vec{v}) = n_e \left( \frac{m_e}{2\pi k_B T_e} \right)^{\frac{3}{2}} \exp \left( \frac{m_e \vec{v}^2}{k_B T_e} \right)
\]

the electron current \( I_e \) in the transition region is given by [91]:

\[
I_e(V_{sh}) = e n_e A_p \sqrt{\frac{k_B T_e}{2\pi m_e}} \exp \left( \frac{eV_{sh}}{k_B T_e} \right).
\]

Therewith, the electron density \( n_e \) can be calculated at a sheath potential of zero \( V_{sh} = 0 \) V. Hence, the probe electrode is at plasma potential \( V_p = V_s \) and \( n_e \) results in:

\[
n_e = \frac{1}{eA_p} \sqrt{\frac{2\pi m_e}{k_B T_e}} I_e(V_s).
\]

The plasma potential \( V_s \) can be calculated by the zero-crossing of the second derivative of the \( I - V \) curve:
2.3. Plasma Diagnostics

\[
\left. \frac{d^2 I_e}{dV_{sh}^2} \right|_{V_p=V_s} = 0 \ A \sqrt{V^2}. \tag{2.10}
\]

Since the \( I - V \) curve is strictly increasing, the floating potential \( V_f \) is simply given by the zero-crossing of the probe current:

\[
I_p|_{V_p=V_f} = 0 \ A. \tag{2.11}
\]

There may be more than one zero-crossing in measurements due to noise in the measuring signal. In this case, the floating potential cannot be clearly determined by this method. One improvement is inserting a floating electrode into the plasma to measure the floating potential directly.

Most equations given above contain the electron current \( I_e \), which cannot be measured directly. The probe current is given by the sum of electron current and ion current:

\[ I_p = I_e + I_{\text{ion}}. \]

The ion current can be neglected for the second derivative of the electron current in real measurements \[90\]. Therefore, the probe current can be used. At plasma potential \( V_s \) the ion current is also negligible due to the much higher mean energy of the electrons compared to the ions. Thus, the probe current can be used within Equation (2.9).

**Automatic Probe System 3**

The Automatic Probe System 3 (APS3) is a Langmuir probe system developed by the Chair of Electrotechnology at the Technische Universität München (Germany) and the Institute for Electrical Engineering and Plasma Technology [Lehrstuhl für Allgemeine Elektrotechnik und Plasmatechnik] (AEPT) at the Ruhr University Bochum (RUB) \[82, 90, 93\]. This Langmuir probe system is used throughout this work. A scheme and a block diagram of the APS3 is shown in Figure 2.10. The probe head consists of a cylindrical tungsten electrode with a diameter of 25 or 50 \( \mu \)m and a length of 3 to 5 mm. The probe electrode is held by a ceramic capillary tube (\( \varnothing \) 1 mm, length 8 mm). This tube is fixed in a floating electrode (\( \varnothing \) 8 mm, length 15 mm), which is attached to a ceramic tube (\( \varnothing \) 8 mm). This ceramic tube is moved by a stepping motor. Two different APS3 probes are used: one can be moved up to 200 mm without an offset and the other one can be moved up to 180 mm inside the plasma reactor offering an offset of 150 mm. The first probe is used for measurements at the DICP reactor and the second one for measurements at the SKP 101. Both probes are identically constructed except for the offset and the travel range.

A voltage ramp (\(-80 \text{ V} < V_p < 80 \text{ V}\)) is supplied to the tungsten electrode and the resulting current is measured. A band-stop filter for 13.56 MHz and higher harmonics is interconnected to eliminate disturbances by the plasma excitation frequency. The voltage ramp is centred either around the floating potential measured at the floating electrode or around a predefined floating potential. This voltage offset can vary between \( \pm 320 \text{ V}\). The maximum spatial uncertainty is about 9 mm \[93\]. A measurement of one current
value takes 25 μs. Thus, also pulsed plasmas below $f_{\text{pulse}} = 20$ kHz can be measured [94].

Every $I - V$ curve measured for this work has a voltage step of 0.1 V and each single current value is a mean value of at least 100 measurements. The APS3 yields spatial and time resolved electron density, EEDF, as well as floating and plasma potential. Temporal evolution of electron density over one pulse can be measured since the APS3 can be synchronized with driving frequency.

### 2.3.2. Optical Emission Spectroscopy (OES)

Optical emission spectroscopy (OES) is a non invasive plasma diagnostic method. Measurements of atomic or molecular irradiation enable the determination of VUV/UV radiation as a sterilization agent.

**Spectrometers**

Three different spectrometers are used for quantitative OES an Echelle spectrometer (ESA 3000 from LLA Instruments, Berlin, Germany), a grating spectrometer (QE65000 from Ocean Optics, Dunedin, Florida, USA), and a VUV/UV monochromator (Jobin-Yvon AS50, Longjumeau, France) equipped with a solar-blind photomultiplier.
2.3. Plasma Diagnostics

**Echelle spectrometer** This spectrometer has a spectral range from 200 to 800 nm and a resolving power of $\frac{\lambda}{\Delta \lambda} = 13333$. This corresponds to the FWHM of the apparatus function of 0.015 to 0.06 nm. The Echelle spectrometer is capable of resolving rotational structures of bound-bound transitions of diatomic molecules. Thus, it is possible to obtain rotational temperatures. The spectrometer is relatively and absolutely calibrated by a combination of a pure nitrogen and a nitrogen oxygen plasma plus a tungsten ribbon lamp. The calibration procedure is elaborately described elsewhere [95, 96].

**QE65000** This spectrometer has a resolution of 0.3 nm and a spectral range from 200 to 800 nm. The QE65000 is convenient for measuring broad band emission spectra such as hydrogen continuum irradiation due to its low resolution. This spectrometer is calibrated by the same procedure as the Echelle spectrometer as described above.

**VUV/UV monochromator** The VUV/UV spectrometer consists of a monochromator, a photomultiplier (Hamamatsu PMT R1080 from Hamamatsu Photonics K.K., Hamamatsu City, Japan), and a counting system (Hamamatsu Photon Counting Unit C9744 and Counting Unit C8855-01). A stepping motor turns the mirror grating. It takes approximately 1 h to record a full spectra. Due to this, it was not possible to take a spectrum at the SKP 101 because the maximum plasma-on time for relevant powers amounts to about 5 min. The photomultiplier is enclosed with MgF$_2$, which allows the determination of the lower wavelength boundary of the set-up. The spectrometer has a spectral range from 115 to 300 nm and a resolution of 0.3 nm. The VUV/UV spectrometer set-up is calibrated by taking the known spectra of the branching ratios in the Lyman-Birge-Hopfield (LBH) bands of molecular nitrogen into account. Bibinov et al. described the procedure elsewhere [97].

**Corona Model**

In non-equilibrium plasmas the distribution of the excited states is not determined by the Boltzmann distribution such as in plasmas in thermal equilibrium. An approximation for non-equilibrium plasmas is the corona model. It describes the process in the corona of the sun, but can be applied to technical plasmas where the electron density is rather low, the electron temperature is high, and the radiative excitation is negligible, at least for the considered state. So, de-excitation is only possible by spontaneous emission and excitation only takes place through electron impact collisions. These assumptions simplify the line intensity to:

$$I_{\lambda,pk} = n_1 n_e k_{\text{exc}}(T_e),$$

with $I_{\lambda,pk}$ the line intensity from a spontaneous radiation process from level $p$ to level $k$, $n_1$ the density of the ground level, and $k_{\text{exc}}$ the excitation coefficient dependent on the electron temperature $T_e$. 

(2.12)
2.4. Surface Diagnostics

2.4.1. Surface Temperature and Thermal Flux Measurements

Knowing the surface temperature of sterile goods is important to be able to treat heat-sensitive materials such as plastics and to estimate the temperature impact on spores. For the development of the SKP 101 it was crucial to know the surface temperature since the sterile good is a polystyrene tub possessing a glass transition temperature of 86 to 96 °C [83, 84].

Surface Temperature

Two different types of sensors are used: a resistance thermometer PT-100 and a selection of dry chemical colour changing stripes (40 to 260 °C with $\Delta T = 4$ to 6 °C from Kager GmbH, Dietzenbach, Germany). The PT-100 sensor set-up can measure up to $T \approx 90$ °C.

Thermal Flux

The thermal probe Q-Sensor 103 (INOVAP GmbH, Dresden, Germany) is used to measure the energy flux onto surfaces. The energy flux is calculated from a time dependent temperature curve:

$$Q = m_s c_s \left[ \left( \frac{dT}{dt} \right)_{heating} - \left( \frac{dT}{dt} \right)_{cooling} \right]_{T_f},$$

where $Q$ the thermal flux, $m_s$ the mass of the sensor, $c_s$ the specific heat capacity, $T$ the temperature measured by the sensor, and $T_f$ the temperature at where the derivatives are calculated. Figure 2.11 shows an example of a time dependent temperature curve measured with Q-Sensor 103. To determine the energy flux, it is necessary to measure the heating and the cooling of the probe. Therefore it has to be ensured that the plasma is switched on after starting the measurement and then switched off. In this way the cooling phase is measured for some time. The energy flux is evaluated at the fitting temperature $T_f$. Further information can be found elsewhere [98, 99].

2.4.2. Profilometry

As a surface profiler, a Dektak 6m Stylus Profiler (Veeco Instruments Inc., New York, USA) is used. A surface profile is measured by moving the sample beneath a diamond-tipped stylus. The displacement of the tip is measured electromechanically and a height profile is recorded [100].
2.4. Surface Diagnostics

Figure 2.11.: Time dependent temperature measured with the Q-Sensor 103 in the SKP 101

Determination of BSA Ring Height

In order to measure the removal of the model protein bovine serum albumin (BSA) the profiler mentioned above is used. First a 0.1% solution of dried BSA and distilled water is prepared. This solution is pipetted onto a glass slide and is allowed to dry. This leads to a coffee ring kind of structure of BSA, as described in [17, 20, 101]. This ring is measured at several marked positions before and after plasma treatment. With the measured height difference the etching rate is calculated. Figure 2.12 shows examples of BSA ring profiles: untreated and treated in an argon/oxygen discharge.

Figure 2.12.: BSA ring profiles: untreated (——) and treated in an Ar/O₂ 100/5 sccm discharge at $P = 5 \text{ kW}$ and $p = 10 \text{ Pa}$ for $t = 120 \text{ s}$ (— — —) and 240 s (—— · —)
Determination of Film Thickness

To measure a deposited film a silicon wafer sample is partly covered by tape. After the deposition process the tape is removed and a sharp step remains. This is measured by the surface profiler. The silicon samples usually have a width of 10 mm. Four to five height measurements are performed per sample at different positions. This method is also used for the determination of an etching rate. Four to five positions are marked on the sample and measured before and after a plasma treatment. The marks ensure that the same position is measure before and after treatment. From this, the height difference is calculated and thereby the etching rate is determined by dividing it by the treatment time.

2.4.3. Scanning Electron Microscope

Images of spore samples are obtained by an SEM (JSM-6510 QSEM from JEOL Ltd., Tokyo, Japan), which is equipped with an Everhart-Thornley secondary electron detector. It has a resolution of up to 3.0 nm and 8.0 nm at an electron energy of 30 keV and 3 keV, respectively. The maximum digital image resolution is 5000 pixels × 3840 pixels [102]. Electron energies between 10 to 30 keV are used in this work. The biological samples are sputtered with gold to prevent charging during measurement.

2.5. Algorithm for Detection and Measurement of Spores

When treating biological samples such as bacterial endospores in a plasma, it is important to know how the biologic material is spread out on the surface of a sample such as a glass slide. If spores solution is dropped onto a surface, the spores form a structure that is reminiscent of a coffee ring. This coffee ring structure forms because of a capillary flow inside of the drop. This flow transports dispersed solids to the edge of the ring to maintain the initial contact line [101]. The penetration depth of UV radiation is in the order of the endospore dimension of 1 to 3 µm [48]. Reactive species only react with the surface of spores. Thus, it is important to have monolayer spores, so that every spore is reached by the plasma. Otherwise, there will be a bulk of untreated spores. The best method of bringing a monolayer of spores onto a surface is by spraying [103]. To ensure that only monolayers are present, it is necessary to control the biological samples by SEM images. For controlling larger surface areas it is convenient to automatically capture SEM images and use an algorithm to determine the surface density of spores and detect zones where multilayer occur. An extensive description of the used algorithm is given by Klein [104]. Based on this algorithm a spore length determination algorithm is developed. This is described extensively by Vierling [105]. A scheme of these two algorithms is shown in Figure 2.13.

The algorithms make use of the Image Processing Toolbox from MATLAB (The MathWorks Inc, Natick, Massachusetts, USA). In the beginning, the SEM images are filtered by a low-pass filter to remove noise and single pixel errors. After that, a grey value is
2.5. Algorithm for Detection and Measurement of Spores

Figure 2.13.: Scheme of the algorithm for spore density and length determination

1. Filter SEM image with low-pass filter
2. Define grey threshold
3. Produce binary image
4. Label every object
5. Separate touching spores and relabel
6. Count spores
7. Determine local spore density
8. Reject objects not fitting in $0.75 \ A_{\text{ref}} < A_{\text{obj}} < 1.25 \ A_{\text{ref}}$
9. Reject objects not fitting in $0.75 \ A_{\text{ref}} < A_{\text{obj}} < 1.25 \ A_{\text{ref}}$
10. Reject non-elliptic-like objects
11. Determine length of every object

Legend:
- Density determination
- Length determination
determined. Therefore, the brightest pixel is found. From this coordinate the whole pixel row and column is taken. Figure 2.14 shows a part of a row with the brightest pixel. It can be seen that it does not look exactly like a spore. There are two maxima due to the edge effect of the SEM. The derivative function \( z_s(x) \) of the row and column is calculated and set to zero where the value of the derivative function is 0.9 times the value of the derivative function at the brightest pixel:

\[
z_s(x) = \begin{cases} 
0, & \text{for } -0.9 \frac{dz(x_{mb})}{dx} < \frac{dz(x)}{dx} < 0.9 \frac{dz(x_{mb})}{dx}, \\
\text{elsewhere}
\end{cases}
\]

(2.14)

with \( x_{mb} \) = position of maximum brightness.

Therewith, it is easy to find the edges of the spore. From this, the threshold grey value is determined as 0.4 times the difference between the minimum of the spore brightness and the background, as shown in Figure 2.14. The value of 0.4 is chosen to be above the expected noise level. Hence, the binary image is produced. Pixels with a brightness value below the grey value threshold are set to zero and all other pixels are set to one. After that, every connected domain is labelled. Subsequently, there are two options: determination of the spore density or the spore length. The spore density is the number of spores per area and the spore length is the longest distance inside a spore.

The spore density is an important parameter to know if monolayer samples are required. It is often the case that spores are situated next to other spores and touch each other. For

![Figure 2.14.: Determination of the grey value threshold. Top: brightness value of the spore with the brightest pixel. Bottom: modified derivative function \( z_s(x) \), see Equation (2.14); the background and spore minimum values (---) as well as 40\% (---) of the distance between them [104]]
this case there are different options implemented to separate these spores. One option is to erode every object. This works reliable if the spores are only slightly touching. Another option is to subtract an edge image determined by a Sobel operator. A further option is to perform a distance transformation followed by a watershed transformation. But for this, there should only be little non-spore material such as culture medium on it. Otherwise it will potentially be recognized as a spore. After this separation step, every object fitting into 0.75 to 1.25 times a reference area $A_{\text{ref}}$ is detected as a spore. With this, the spore count per image as well as the spore density per image can be determined. Furthermore, local spore densities are calculated and, therefore, spore clusters are detected and marked. Figure 2.15 shows an example of the determination of spore density.

![SEM image](image1)

![Processed image with coloured spore density](image2)

(a) SEM image  
(b) Processed image with coloured spore density

Figure 2.15.: Spore density of *B. subtilis* PY79 endospores determined by the algorithm

It is also possible to measure the spore length of every spore. To achieve that, a few steps of the algorithm described above have to be changed, as shown in Figure 2.13. Touching spores cannot be separated any more because the spore length is slightly altered during this program step. From this, it can therefore be concluded that only samples with sufficient low spore densities and with a low amount of residuals such as culture medium can be evaluated. To determine the spore length, an ellipse is fitted to every spore object and the major axis of the ellipse, corresponding to the cross-section of the spore, is measured as the spore length. An example is shown in Figure 2.16. Since the spore length varies for every spore, it is necessary to measure many spores. As a reference measurement 1058 untreated *B. subtilis* PY79 endospores have been processed. Figure 2.17 shows a Gaussian fit of a histogram with 50 bins of this measurement as well as a probability plot of this measurement. The probability plot shows the probability that a spore has a specific maximum length. The comparison to the normal distribution shows a good agreement. For all spore length results, 650 to 1600 spores are measured. The mean length of *B. subtilis* endospores is determined as 1.33μm. All measured spore samples shown in this work are pipetted on glass slides. The spore deposition process is described in section 2.7.3. Areas with low enough spore densities inside the coffee ring are recorded by SEM and evaluated by the algorithm. Only spores which are dropped onto glass slides are used since the development of a spore spraying process at the German Aerospace Center
2. Experimental Set-up and Diagnostics

Figure 2.16.: Example of a spore length determination of *B. subtilis* PY79 endospores: fitted ellipse (---) and determined spore length (——)

(a) Gaussian fit of spore length histogram with 50 bins

(b) Probability plot of the maximum spore length, the dashed line (---) represents a normal distribution

Figure 2.17.: Gaussian fit and probability plot the length of untreated *B. subtilis* PY79 endospores

[Deutsches Zentrum für Luft- und Raumfahrt] (DLR) was not finished until recently [103]. However, it looks set to become a more homogeneous spore distribution for plasma experiments, especially for spore length measurements.

2.6. Plasma Simulation with the Hybrid Plasma Equipment Model

Plasma simulations of the DICP reactor are performed to yield space resolved plasma parameters outside of the measurement location at the half height of the reactor. Another reason for plasma simulations is to investigate other reactor geometries for rather large objects for sterilization. The Hybrid Plasma Equipment Model (HPEM) developed by
Kushner is used as simulation tool [81]. HPEM was initially developed by the Computational Optical and Discharge Physics Group (CODPG) of the University of Illinois and is now further developed by the Computational Plasma Science and Engineering Group (CPSEG) of the University of Michigan. It is a comprehensive modelling platform for the low pressure regime (below thousands of pascals) [81]. It makes use of the hybrid plasma simulation approach as described earlier, see section 1.3. A hands-on description of HPEM is given by Engelhardt [106].

2.6. Modules

HPEM is flexible through many available modules to address a wide variety of plasma discharges with different excitation options (e.g. CCPs, ICPs or magnetrons) and different geometries (e.g. cylindrical tubes or cuboids). Figure 2.18 shows the different modules, which are available in HPEM, and the connection between them. Since these modules are transparent, it is only important that all necessary input values are present, but irrelevant which module provides these. Since electrons and ions are calculated by different modules, they are treated separately, namely electrons can be calculated by a Monte Carlo simulation and ions can be calculated by a fluid simulation.

Figure 2.18.: Scheme of different modules available within the Hybrid Plasma Equipment Model, the X’s mark the used models [81, 107]
2.6.2. Time Slicing

In a hybrid modelling approach it is typical that the modules are executed in sequence. That is a disadvantage if parallel computing is available. HPEM can be used with parallel processing so that more than one module can be processed at the same time. This is achieved by the time-slicing technique. For a certain amount of time, which corresponds to the dynamic time scale, the input values are kept constant or only vary in a predetermined way [81]. Thus, the modules can be executed in parallel and the input values are updated on the dynamic time scale or when they change by a certain amount (e.g. a few percent).

2.6.3. Chemical Reactions

Neither all atomic or molecular states, nor all chemical reactions can be taken into account in a simulation. For each reaction, the corresponding probability or collision cross section must be known. This is only the case for a few reactions and a few states. The reaction data used in this thesis is supplied by Kushner and CPSEG of the University of Michigan. Two different gases are used in the HPEM simulations: argon and hydrogen. Argon is used in a first step to test the simulation and to compare it with measurement data. Hydrogen is used to simulate plasmas that are used in sterilization experiments.

Argon Discharge

Table 2.1 shows the five argon states, which are used in the HPEM simulation. The implemented reactions are given in appendix A.1.1.

<table>
<thead>
<tr>
<th>species</th>
<th>threshold / eV</th>
<th>remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar</td>
<td>0</td>
<td>ground state</td>
</tr>
<tr>
<td>Ar^{4s}</td>
<td>11.60</td>
<td>excited state, metastable</td>
</tr>
<tr>
<td>Ar^{4s}</td>
<td>11.60</td>
<td>excited state, transitory</td>
</tr>
<tr>
<td>Ar^{4p}</td>
<td>13.10</td>
<td>excited state</td>
</tr>
<tr>
<td>Ar^{+}</td>
<td>16.00</td>
<td>ion</td>
</tr>
</tbody>
</table>

Table 2.1.: Argon states used in HPEM simulations

Hydrogen Discharge

Hydrogen discharges are more difficult to simulate than argon discharges since there are molecular states and even more relevant atomic states. For this reason, only a small fraction of all possible hydrogen states is used in this simulation. Table 2.2 shows the used molecular and atomic hydrogen states. The H^{2}_{2} state is special because in the original HPEM implementation it is composed of a combination of the states c^{3}Π (11.87 eV) and b^{3}Σ (8.80 eV). c^{3}Π is a resonant state emitting in the wavelength range from 100 to
200 nm upon relaxation. \( b^3\Sigma \) is an unstable state, which dissociates in two H atoms. The radiation process from \( \text{H}_2^* \) is just given by one process (\( \text{H}_2^* \rightarrow \text{H}_2 + h\nu \)) in HPEM. In this case, both states \( c^3\Pi \) and \( b^3\Sigma \) would contribute to this process. However, this is not in agreement with reality. To correct this, the \( b^3\Sigma \) state is replaced by the \( a^3\Pi \) state \( (11.88 \text{ eV}) \), which emits in the wavelength range from 200 to 300 nm and also relaxes to the ground state. So, \( \text{H}_2^* \) is still a combined state, but with two resonant relaxations \([106] \). The amount of photons calculated by an HPEM simulation is the sum of photons emitted from these two states. This enables to compare the simulated photon amount to the measured one. The reactions for the hydrogen discharge are kindly provided by Shoeb from CPSEG \([108, 109] \). Appendix A.1.2 lists the reactions implemented in the HPEM simulation.

<table>
<thead>
<tr>
<th>species</th>
<th>threshold / eV</th>
<th>remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{H} )</td>
<td>0</td>
<td>ground state</td>
</tr>
<tr>
<td>( \text{H}^* )</td>
<td>10.09</td>
<td>excited state</td>
</tr>
<tr>
<td>( \text{H}^+ )</td>
<td>13.56</td>
<td>atomic ion</td>
</tr>
<tr>
<td>( \text{H}_2 )</td>
<td>0</td>
<td>ground state</td>
</tr>
<tr>
<td>( \text{H}_2^* )</td>
<td>11.87</td>
<td>combined excited states: ( a^3\Pi ) and ( c^3\Pi ), see text</td>
</tr>
<tr>
<td>( \text{H}_2^+ )</td>
<td>15.43</td>
<td>diatomic ion</td>
</tr>
<tr>
<td>( \text{H}_3^+ )</td>
<td>4.5</td>
<td>triatomic ion</td>
</tr>
</tbody>
</table>

Table 2.2.: Hydrogen states used in HPEM simulations

2.7. Microbiological Materials and Methods

To study the effect of a plasma discharge on spores, different kinds of *Bacillus* endospores and fungi spores are used. Within all experiments (endo-)spores are used since they are more resistant against induced stress than vegetative cells, see section 1.1.

The plasma laboratory of AEPT has the biosafety level (BSL) 1. Therefore, it is possible to work with genetically modified microorganisms. It is not allowed to work with pathogens such as *Paenibacillus larvae* (*P. larvae*). For this a BSL 2 laboratory is necessary.

2.7.1. Endospores and Spores

In microbiological research it is important to know the exact strain of the microorganism used. Microorganisms are either exchanged between researchers directly or are obtained from a culture collection. Examples for culture collections are: the Bacillus Genetic Stock Center (BGSC), the American Type Culture Collection (ATCC), and the German Collection of Microorganisms and Cell Cultures [Deutsche Sammlung von Mikroorganismen und
Zellkulturen (DSMZ). These non-profit organizations supply biological material to researchers and companies. These culture collections also categorize and enumerate the microorganisms. The identifiers are an assembly of the acronym of the culture collection and a running number. For instance, a specific strain of \textit{E. coli} has the identifiers ATCC 8739 and DSM 1576. The culture collections also provide information and literature about the specific strains. There is also another naming scheme. Biologists produce far more strains than the culture collections want to collect. New strains are produced by mutations. If a microbiologist has produced a new strain it is typically numbered and named after the researcher or the culture collection of the university, like the \textit{B. subtilis} PY79 (Peter Youngman) [110] or the \textit{E. coli} K-12 (Stanford University) [111].

\textit{Bacillus subtilis} W168, PY79, and DSM 4181

\textit{B. subtilis} is widely used in laboratories and industry since it is the model microorganism for gram-positive bacteria and, therefore, the most researched gram-positive bacteria. \textit{B. subtilis} forms rod shaped endospores. Although \textit{B. subtilis} is used heavily in research and industry, the genomic heritage is only poorly understood [112]. In this work, three strains of \textit{B. subtilis} endospores are used: the W168, the PY79, and the DSM 4181. The W168 strain is only used in beehive experiments (see chapter 5), the PY79 strain is used for spore length determination (see section 3.2) and spore mutant experiments (see section 3.3), and the DSM 4181 strain is used for sterilization experiments on syringe tubs (see section 4.3.4).

168 The 168 strain was mutagenized with X-rays by Burkholder and Giles from a domesticated version of the wild type Marburg strain [112, 113]. A sublethal X-ray dose induced auxotrophy (the cell is not able to produce a single needed nutrition) amongst the survivors. Thus, the "one gene-one enzyme" model could be enlarged to \textit{B. subtilis}. The wild type Marburg strain was lost when the Yale institute ended its \textit{B. subtilis} studies [112].

W168 The W168 was produced in the Noboru Sueoka laboratory at Princeton [112]. The 168 strain was transformed with W23 [114] DNA to the W168 strain [115].

PY79 The PY79 strain was derived by Youngman [110] from the CU1769 strain (Zahler, unpublished), which traces its genomic heritage to W168. PY79 also shares genetic regions with the strains 23 [113], W23 [114], W168 [116,117], and Mu8u5u1 [116,117] at least. Strain 23 is derived also from the Marburg strain. The strains W23, W168, and Mu8u5u1 are derived from the 168 strain. The whole genome of the PY79 strain is available [118]. A comparative DNA study about the heritage of the 168 and PY79 strains can be found elsewhere [112].

DSM 4181 (SA 22) The endospores of the \textit{B. subtilis} DSM 4181 (SA 22) strain are the standard test spores for H$_2$O$_2$ sterilization processes [119,120].
2.7. Microbiological Materials and Methods

**Geobacillus stearothermophilus ATCC 7953 and ATCC 12980**

*G. stearothermophilus* is a thermophile bacterium, which habitats are hot springs or ocean sediments. The vegetative cell can grow at a temperature range of 30 to 75°C. The microorganism is formerly known as *Bacillus stearothermophilus*, but was renamed in 2001 [121]. Its endospore is heat resistant. Therefore, it is the standard test spore for hot steam sterilization methods such as autoclaves [122–124]. An SEM image of an endospore of *G. stearothermophilus* is shown in Figure 2.19a. Two different strains are used: ATCC 7953 and ATCC 12980.

**Aspergillus brasiliensis ATCC 16404**

*A. brasiliensis* is a black fungus, which can be found in soil or on grapes. It can also be present in indoor environments, such as beverage filling lines. *A. brasiliensis* was reclassified from *A. niger* in 2007 [125]. *A. brasiliensis* is resistant against UV irradiation. An SEM image of an *A. brasiliensis* spore is shown in Figure 2.19b.

### 2.7.2. *Bacillus subtilis* Endospore Mutants

The endospore coat is the first barrier to environmental stress. In order to investigate the role of various coat layers in spore resistance against low pressure plasma several mutants of *B. subtilis* PY79 are used. *B. subtilis* endospores are chosen since their spore coat structure and assembly have been closely studied. About 70 spore coat proteins have been identified [31]. 50 to 80% of total spore proteins are in the spore coat [126]. However, the function of only a few of these proteins have been identified. Some of these spore coat proteins are essential for coat morphogenesis [31, 127]. Some of these morphogenetic proteins are investigated: CotXYZ, CotE, CotH, CotO, SafA, SpoIVA, SpoVW, and SpoVID. An absence of any of these proteins causes severe structural defects in the spore coat [31, 128, 129]. Figure 2.20 shows the spore coat structure with the corresponding

- (a) *G. stearothermophilus* endospore
- (b) *A. brasiliensis* spore [9]

Figure 2.19.: SEM images of a *G. stearothermophilus* and an *A. brasiliensis* spore
proteins. *B. subtilis* PY79 strains with the knocked out genes *spoVID*, *safA*, *cotE*, *cotA*, *cotX*, *cotYZ*, *cotVW*, or both *safA* and *cotE* are used to investigate the contribution of each spore coat layer, and CotA-dependent pigmentation to the resistance in low pressure plasma sterilization. All spores are gratefully prepared, and analysed by Raguse and Möller from DLR. Table 2.3 shows the used genotypes of *B. subtilis* and which spore coat component these mutants are missing. The notation $\Delta$gene means that a genotype has the specific gene knocked out, e.g. $\Delta$cotE is the genotype missing the cotE gene. Therefore the protein CotE is not synthesized and thus the outer coat is not formed. The sample preparation is described in section 2.7.3. A transmission electron microscopic analysis of the spore mutants can be found elsewhere [130].

### 2.7.3. Sample Preparation and Examination

#### Glass Slides

The *B. subtilis* PY79 endospores are prepared by Raguse and Möller from the Institute of Aerospace Medicine, Radiation Biology Department, Research Group Astrobiology at the DLR in Cologne, Germany. The endospore solution is prepared and purified to remove residuals of culture medium and salts. This is necessary to be able to perform the spore length measurements described in section 2.5. The spore solution is pipetted

---

Figure 2.20.: The assembly of the spore coat of *B. subtilis* is directed by the morphogenetic proteins SpoIVA, SafA, CotE, CotX, CotYZ, and CotVW. SpoVID is responsible for encasement, CotA conducts the spore pigmentation located in the outer coat [31,130]
## 2.7. Microbiological Materials and Methods

<table>
<thead>
<tr>
<th>strain</th>
<th>genotype</th>
<th>missing spore coat component</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE594 (PY79)</td>
<td>wild type (prototroph)</td>
<td>none</td>
<td>[128]</td>
</tr>
<tr>
<td>PE697</td>
<td>$\Delta$spoVID</td>
<td>all coat layers (spore coat morphogenetic protein, promotes encasement of the spore)</td>
<td>[128]</td>
</tr>
<tr>
<td>PE277</td>
<td>$\Delta$safA</td>
<td>inner coat (morphogenetic protein associated with SpoVID, major organizer of inner spore coat)</td>
<td>[128]</td>
</tr>
<tr>
<td>PE618</td>
<td>$\Delta$cotE</td>
<td>outer coat (outer spore coat morphogenetic protein, controls the assembly of the outer spore coat layer)</td>
<td>[128]</td>
</tr>
<tr>
<td>PE1720</td>
<td>$\Delta$safA, $\Delta$cotE</td>
<td>inner and outer coat</td>
<td>[130]</td>
</tr>
<tr>
<td>MR06</td>
<td>$\Delta$cotA</td>
<td>pigmentation (located in the outer coat)</td>
<td>[130]</td>
</tr>
<tr>
<td>PE620</td>
<td>$\Delta$cotX, $\Delta$cotYZ</td>
<td>crust (outermost layer of the spore coat)</td>
<td>[128]</td>
</tr>
<tr>
<td>PE566</td>
<td>$\Delta$cotVW</td>
<td>crust (outer spore coat proteins (insoluble fraction), formation of the outer section of the spore crust)</td>
<td>[131]</td>
</tr>
</tbody>
</table>

Table 2.3.: Used *Bacillus subtilis* strains, the notation $\Delta$gene means that a gene is knocked out in the spore [130]
onto an autoclaved glass slide and dried. The endospores form a coffee ring with a large amount of spores within the ring. Inside of such a ring there are large areas with a monolayer of endospores. An example of the middle area can be found in figure 2.15a [103,130].

Endospores of *B. subtilis* DSM 4181 and all other non-*B. subtilis* (endo-)spores are prepared by the Fraunhofer Institute for Process Engineering and Packaging [Fraunhofer-Institut für Verfahrenstechnik und Verpackung] (IVV) in Freising, Germany. The spores are harvested, separated from the vegetative cells, and cleaned. In contrast to the spores from the DLR, these spores are diluted in aqueous solution and sprayed with a two-component jet onto glass slides in a 1 cm × 1 cm square. Figure 2.21a shows an example of a monolayer of *B. atrophaeus* endospores sprayed on a glass slide. The whole process used by the IVV is described elsewhere in more detail [132–134].

**Stainless Steel Coupons**

Stainless steel coupon samples are prepared and evaluated by Raguse and Möller from the Space Microbiology Research Group from the DLR. The V4A stainless steel coupons (Wilms Metallmarkt Lochbleche GmbH & Co. KG, Cologne, Germany) have a diameter of 7 mm and a thickness of 1.5 mm. They are autoclaved and spore suspension is pipetted onto them. These samples are dried at ambient conditions. Every coupon carries about $1 \cdot 10^6$ endospores [130].

**Syringe Tubs**

The inoculated syringe tubs are prepared by the IVV. The spore preparation is the same as described above. These spores are sprayed on the whole area of one tub side (e.g. Tyvek foil). Spraying monolayers of spores on tubs is more challenging. Tubs are made of polystyrene, on which water has a greater contact angle than on glass. If the spore solution is too dense, the spores can form clusters. Figure 2.21b shows an example of a cluster of *G. stearothermophilus* endospores, which were sprayed on a tub side. These clusters can be avoided by using a not too dense spore solution and by frequent spraying.

**Beehive Material**

The biological sample preparation and evaluation of beehive material is carried out by Priehn and Leichert from the Institute for Biochemistry and Pathobiochemistry, RUB, and Aumeier and Kirchner from the Faculty of Biology and Biotechnology, RUB. Since tests with honey combs are time consuming, first tests are made with wood and wax, which are the major constituents of frames used in beehives. The whole process is extensively described elsewhere [135].
2.7. Microbiological Materials and Methods

(a) *B. atrophaeus* endospores sprayed on a glass slide [9]

(b) *G. stearothermophilus* endospores sprayed on a tub side

Figure 2.21.: SEM images of spores sprayed by the IVV

**Wax** For sample preparation beeswax is autoclaved and dried for five days at 80°C to remove the water of the sterilization process. 6 ml of this beeswax is poured under sterile conditions into petri dishes (60 mm diameter). After solidification these plates are cut in half under sterile conditions.

**Wood** Rectangular (40 mm × 20 mm) pieces are cut out of wooden tongue depressors (VWR, Darmstadt, Germany). These rectangles are autoclaved and dried over night at 60°C.

**Frames and Honeycombs** The so-called *Zander* hive frames used at the beekeeping in the botanical garden of the RUB do not fit into the DICP chamber. Therefore, custom miniature hive frames in the size of 9.5 cm × 9.8 cm were made. These frames are made of wood, glue, and stainless steel wire crimps. A beeswax foundation with a cell diameter of 5.4 mm is fixed into these frames. The beeswax foundation serves as a starting base and wax reservoir so that the bees only build small chambers for worker bees and have enough wax for a cell. Only worker bees chambers are favoured since they have the best cell size for honey production. Eight of these small hive frames are fixed into a Zander hive frame, see Figure 2.22a. These Zander frames are inserted into the super of a beehive at the botanical garden during honey flow. This super is segregated from the brood nest by a queen excluder. The bees build the cells and fill them with honey. The frames are taken out of the beehive and the honey is extracted. The empty combs are placed in the beehive again so that the bees remove the rest of the honey and repair the cell wall damages. Figure 2.22b shows an image of a frame with an empty honeycomb after the process mentioned before. A further description can be found elsewhere [135].

Afterwards, the beeswax, wood, and honeycomb samples are sprayed with *B. subtilis* W168 endospores. Sprayed samples are treated and, subsequently, the endospores are
Figure 2.22.: Image of a Zander hive frame and a miniature hive frame with an empty honeycomb [135]

recovered. The recovery process is described in the next subsection

**Sterilization Tests**

The procedure of a sterilization test differs for each surface material:

**Glass Slides and Tubs from IVV** Glass slides or tubes are prepared at the IVV as described above and sent to the Ruhr University Bochum. Samples are treated inside a plasma reactor. If the spores are sprayed on tubes, only that part of the tub with spores on it is cut out with a hot knife. Afterwards, all samples are washed with Ringer-Tween solution and packaged in vials or plastic bags. These bags are welded up. All samples are sent back to the IVV for further evaluation [132–134].

**Glass Slides or Stainless Steel Coupons from DLR** Both sample types for sterilization experiments are treated in the DICP and taken back to the DLR. The treated samples are covered with 10% aqueous polyvinyl alcohol (PVA) solution. After drying the spore-PVA layer is stripped off and dissolved in sterile distilled water. 95% of spores can be recovered by this method [35, 103, 130].

**Wax from Beehive Experiments** Three samples from one sprayed wax piece are extracted and heated to 70°C in distilled water. These samples are mixed with Tween 80 and are incubated in a thermomixer for 30 min. After that the samples are incubated for 2 to 4 h at room temperature [135, 136].
Wood from Beehive Experiments  The wood samples are given into a Falcon tube with distilled water and Tween 80. Afterwards, they are rotated for one hour in a centrifuge at 37 °C \[135\].

Honeycombs from Beehive Experiments  100 mg of beeswax are extracted from the whole honeycomb and treated like the wax samples above. Afterwards the hive frame is dissembled and the wood samples are treated as mentioned above \[135, 136\].

After the procedures described above dilution series are made. The dilutions are pipetted on culture medium and kept at spore specific surrounding conditions inside an incubator. Colony-forming units (CFUs) are enumerated. It is assumed that every viable spore forms a colony. More details of the procedure can be found elsewhere \[35, 130, 132–135\].

Results of the sterilization tests are given as log reduction $R$. A ratio of the CFU of the treated samples and the reference (initial bioburden $b$) is calculated. $R$ is given as:

$$R = \log \left( \frac{CFU_{\text{reference}}}{CFU_{\text{sample}}} \right). \quad (2.15)$$

$10^5 \text{ to } 10^6 \text{ CFU}$ can be recovered from untreated glass slide or tub samples, which leads to a density of $10^9 \text{ to } 10^{10} \text{ CFU m}^{-2}$ on glass slides and $2.1 \cdot 10^6 \text{ to } 1.2 \cdot 10^8 \text{ CFU m}^{-2}$ on tubs. The spore density of wood samples amounts to $4.2 \cdot 10^8 \text{ to } 7.9 \cdot 10^8 \text{ CFU m}^{-2}$ and $5.4 \cdot 10^8 \text{ to } 1.6 \cdot 10^9 \text{ CFU m}^{-2}$ on wax samples.

In a process validation for the sterilization of equipment, containers, and closures for drug fillings a log reduction of six or higher "under the most difficult to sterilize conditions" should be demonstrated according to the FDA \[8, 137\]. For aseptic processing isolators the FDA demands: "Normally, a four- to six-log reduction can be justified depending on the application" \[8\].

Another characteristic for sterilization processes is the D-value. The D-value is given by the time needed to achieve a one log reduction. For \textit{G. stearothermophilus} ATCC 12980 endospores the D-value for moist heat sterilization measured in a biological indicator evaluator resistometer (BIER) at 121.1 °C amounts to $D = 3.3 \text{ min}$ \[30\].

2.7.4. Model Protein Bovine Serum Albumin

Bovine serum albumin (BSA) is chosen as a model protein since it is a cheap, innocuous, and water soluble protein. Furthermore, it was extensively used in the BIODECON project \[13, 15, 20, 26, 28\]. The protein is derived from cattle. It is the most abundant blood plasma protein. BSA consists of 583 amino acids and has a molecular weight of 66.4 kDa \[138, 139\]. Figure 2.23 shows the crystal structure of BSA measured by X-ray diffraction. It is used for the study of protein removal in the DICP. For this, BSA in aqueous solution is dropped on glass slides and measured by profilometry, as described in section 2.4.2.
Figure 2.23.: Crystal structure of bovine serum albumin from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (PDB ID: 3V03) [140]
3. Investigation of Basic Sterilization Mechanisms

3.1. Etching of a Model Protein

Etching of biological material can be one important effect when objects, which come into contact with blood circulation, are sterilized. Prions which cause Creutzfeldt-Jakob disease (CJD) have to be removed or deactivated from reusable medical tools. Also pyrogens such as lipoteichoic or peptidoglycan from gram-positive or lipopolysaccharide (LPS) from gram-negative bacteria have to be removed from all materials which are introduced into the bloodstream. Otherwise this could lead to a septic shock. These pyrogens are heat resistant and challenging to remove by dry or moist heat [141–143]. It was shown that peptidoglycan and lipid A (the major pyrogenic component of LPS) can be removed in the BIODECON reactor [15].

BSA is selected as a model for proteins since it was used before in the BIODECON project, as described in section 2.7.4. The samples are prepared as described before (see section 2.4.2) and placed on the sample holder, so that the samples are kept in the centre of the discharge.

The first experiments are conducted to test the etching of the standard gas mixtures used before in the BIODECON project [9,13]. Figure 3.1a shows the etched ring height after a treatment time ($t$) of 2 and 4 min in argon 100 sccm, argon/hydrogen 100/5 sccm, and argon/oxygen 100/5 sccm discharges at an input power of 5 kW and a pressure of 10 Pa. The ring height profiles of the argon/oxygen experiment are shown in Figure 2.12. The etching rates for the tested gas mixtures are 0.5, 0.9, and 3.5 nm s$^{-1}$ for Ar, Ar/H$_2$, and Ar/O$_2$, respectively. The argon/oxygen mixture shows the highest etching rate, as expected. The etching rate is less than one third compared to results obtained in the BIODECON reactor [26]. The samples in the BIODECON reactor are placed close to the ICP torus. In the DICP the samples are positioned in the centre of the reactor. Thus, they are further away from the main heating and dissociation zone.

To examine the etching of proteins on larger objects, BSA samples are fixed to the cylindrical glass dummy described in section 2.1. Figure 3.1b shows the etching rate at different positions on the dummy. The samples on top are placed at the edge of the dummy (position 1), at half radius (position 2) and the centre (position 3). The samples at the side are evenly distributed around the dummy. It can be seen that the etching rate on top of the dummy is about two to three times higher than on the side. A higher etching rate compared to the experiment performed before can be seen. Now the samples on top are
closer to the heating zone. The highest etching rate on top amounts to 10 nm s\(^{-1}\), which is closer to the 12.5 nm s\(^{-1}\) obtained in the BIODECON reactor [26].

### 3.2. Spore Etching

Etching of spores can be a plasma sterilization mechanism if spores are etched to the core and dry out. Etching may not only aid in the inactivation of the spore, but also removes a multilayer of spores, which shield each other from irradiation and incoming particles. To assess the etching of endospores, an atomic-force microscope (AFM) would be the first choice since the samples would not have to be coated with gold and the etching of a single spore could be examined [144]. However, spores vary in size and therefore determining the etching of only a few spores is not representative. Figure 2.17 shows the length distribution of *B. subtilis* spores. In order to measure hundreds of spores, SEM images are taken and evaluated by the specially developed algorithm described in section 2.5. Two different gas mixtures are used: an argon/oxygen plasma to test the algorithm and different ratios of hydrogen/oxygen plasmas to assess the etching. All investigated microorganisms in this section are *B. subtilis* PY79 endospores. The samples are prepared and the sterilization results are evaluated by Raguse and Möller from the DLR.

#### 3.2.1. Argon/Oxygen Plasma

The first etching experiments are performed in an argon/oxygen 100/5 sccm plasma at a pressure of 5 Pa and an input power of 1 kW in the DICP. This mixture is chosen to obtain
3.2. Spore Etching

a rather fast etching and to test the algorithm in real conditions. Stapelmann et al. have shown in the comparable BIODECON reactor that the highest etching rate of BSA in an argon/oxygen plasma is at 5\% oxygen admixture to argon [26]. The maximum etching rate of BSA amounts to 12.5 nms\(^{-1}\). It was also shown that the maximum etching rate of amorphous hydrogenated carbon (a-C:H) films is between 5 to 10\% oxygen admixture to argon in the BIODECON reactor [13]. The maximum etching rate of a-C:H films amounts to 4.5 nms\(^{-1}\).

The spore etching depth \(l\) as a function of different times is shown in Figure 3.2a. Furthermore, the etched distance in every time interval is given in this figure. The mean etching rate is given by \(r_e = l / t\). The mean etching rate for every time interval (\(\cdots\)) is calculated and depicted in Figure 3.2b. The etching rates in the last three time intervals vary from 0.13 to 0.67 nms\(^{-1}\). In the first 10 s the etching rate amounts to 1.75 nms\(^{-1}\). The mean etching rate for the entire 120 s treatment (\(\bigcirc\)) amounts to 0.58 nms\(^{-1}\). Thus, the etching rate of \(B. subtilis\) PY79 endospores is 20 times lower than the etching rate of BSA and eight times lower than the etching of a-C:H films. It can be seen that the

![Figure 3.2a](image1)

![Figure 3.2b](image2)

![Figure 3.2c](image3)

Figure 3.2.: Etching of \(B. subtilis\) PY79 endospores in an Ar/O\(_2\) 100/5 sccm discharge at \(p = 5\) Pa and \(P = 1\) kW
endospore length is reduced drastically in the first 10 s of the plasma discharge which then slows down and increases after 30 and 60 s again. The high etching rate in the first seconds cannot be an effect of the vacuum since the spore length is determined in SEM under vacuum conditions. It could be residual nutrition material or similar compounds attached to the spore which could have a high etching rate. It could also be a plasma effect since it takes a few seconds until the plasma is fully established. The increasing etching rate starting at 30 s could also be an effect of the different layers of endospores as described in section 1.1.1. Or it could be a temperature effect. The temperature after 60 s and 120 s are at about 62 °C and 93 °C, respectively. Therefore, the etching could be enhanced by it. Assuming the endospore is clean, it can be estimated which layer is etched away. After 60 s the spore is etched 30 nm deep. From a spore cross section by McKenney et al. [127] it follows that the crust is etched away. After 120 s, 70 nm are etched and also a part of the outer coat layers is removed. Figure 3.2c shows a single etched endospore after 120 s. This should be the outer layer. Right of the spore some residuals can be seen, most likely culture medium.

### 3.2.2. Oxygen/Hydrogen Plasma

#### Substrate Temperatures

During the spore etching experiments the surface temperature should stay low in order to have a similar temperature throughout the treatment. One reason is to prevent thermal assisted hydrogen etching. Since it starts at a few hundred degrees Celsius [145], the other reason is more important: The endospore should not be thermally damaged. Ninety percent of wild-type *B. subtilis* endospores are augmentable after 60 min of dry heat at $T = 90$ °C [146]. Since it is not completely investigated which temperature an endospore has during a plasma process, the conditions are chosen so that the surface temperature stays below 40 °C.

To achieve temperatures below 40 °C a pulsed plasma is used. Figure 3.3 shows the temperature measurement with a PT-100 sensor for different oxygen/hydrogen mixtures with a total flux of 20 sccm, a pressure of 10 Pa, an input power of 1 kW, a pulse frequency ($f_{\text{pulse}}$) of 1 kHz, and 10% duty cycle. Not all measurements are shown since temperatures of the other mixtures are between the temperatures of the pure oxygen discharge and the O$_2$/H$_2$ 12/8 sccm discharge. The pure hydrogen discharge has a different slope than all other O$_2$/H$_2$ mixtures. This is likely because of oxygen impurities, which are present in the reactor at the beginning of the discharge and influence the plasma more drastically compared to a defined admixture of oxygen. In the etching experiments spores are treated for 1200 s. This treatment is split into four treatments of 300 s each. Between each 300 s treatment the reactor is vented for 60 s to cool down the spores. At the end of one 300 s treatment the surface temperature reaches to 31.5 to 34.9 °C. The sterilization experiments in oxygen/hydrogen mixtures are performed with a treatment time of 200 s. At the end of this treatment the temperature is 29.0 to 30.4 °C for all mixtures.
3.2. Spore Etching

Endospore Etching

Plasmas containing oxygen have the ability to etch all kinds of carbon hydride and, therefore, biological material. Even though oxygen atoms alone cannot etch at temperatures below 400 K, in the presence of ions (chemical sputtering) or VUV radiation they can etch biological material at low temperatures as described in section 1.2. Hydrogen plasmas emit a broad range of VUV/UV radiation and can, therefore, sterilize. The effects of oxygen and hydrogen plasmas can be combined by using a mixture, which both emits VUV/UV radiation and has a high etching rate. Six glass slides with *B. subtilis* PY79 endospores are treated in different oxygen/hydrogen mixtures with a total flux of 20 sccm, a pressure of 10 Pa, an input power of 1 kW, a pulse frequency of 1 kHz, and 10% duty cycle in the DICP. The total treatment time is 1200 s. This treatment is divided into four 300 s sets. Between two sets there is a 1 min cooling phase. The total treatment time is chosen since the endospores are treated in a pulsed plasma discharge with a duty cycle of 10%. Thus, the plasma is only switched on for one tenth of 1200 s. So, it has a comparable treatment time regarding the previous experiment in an argon/oxygen discharge. Three probability plots of an untreated and two plasma treated endospore samples are shown in Figure 3.4b. The corresponding Gaussian distributions can be found in Figure 3.4a. It can be seen that the probability plots of the treated samples shift to the left side and the gradient gets larger. Thus, the mean endospore length gets smaller and has a narrower distribution. Figure 3.4c shows the endospore etching depth $l_r$ for each sample. The corresponding etching rates $r_e$ can be found in Figure 3.4d. The etching rate varies from 0.018 to 0.067 nms$^{-1}$. The lowest etching rate occurs in pure hydrogen and the maximum etching rate occurs in the O$_2$/H$_2$ 12/8 sccm plasma. If the etching rates are calculated for the time the plasma source is switched on, these etching rates are ten times higher and
3. Investigation of Basic Sterilization Mechanisms

(a) Gaussian fit of endospore length distributions of untreated (---) and treated in a 100% O$_2$ (---) and in a 60% O$_2$ (---) discharge

(b) Probability plot of untreated (---, ○) and treated in a 100% O$_2$ (---, ▽) and a 60% O$_2$ (---, □) discharge

(c) Etching depth $l_r$ of the mean spore length as a function of oxygen concentration

(d) Mean etching rate $r_e$ as a function of oxygen concentration

Figure 3.4.: Etching depth of *B. subtilis* PY79 endospores treated in O$_2$/H$_2$ mixtures, $\Phi_{\text{total}} = 20$ sccm, $p = 10$ Pa, $P = 1$ kW, $f_{\text{pulse}} = 1$ kHz, 10% duty cycle, and $t = 1200$ s

in the same order of magnitude as the etching rates in an argon/oxygen discharge shown above.

**Sterilization**

Sterilization experiments are also conducted at the same conditions as the etching experiments. The only difference is a shorter treatment time of 200 s. This treatment time is chosen so that neither complete deactivation nor no deactivation is achieved. The sterilization experiments are also conducted with *B. subtilis* PY79 endospores treated in the DICP. The spores are evaluated by the DLR. Figure 3.5 shows the sterilization results. The initial bioburden amounts to $b = 5.5$. The highest log reduction $R = 4.7$ is in the pure hydrogen discharge. The lowest log reduction amounts to $R = 2.0$ in the pure oxygen discharge. The log reduction $R$ is monotonically increasing from the pure oxygen discharge to the pure hydrogen discharge. The spore etching depth is only determined
3.2. Spore Etching

![Graph showing log reduction R of B. subtilis PY79 endospores as a function of oxygen concentration in O₂/H₂ mixtures: Φtotal = 20 sccm, p = 10 Pa, P = 1 kW, f_{pulse} = 1 kHz, and 10 % duty cycle; t = 200 s (○) and t = 600 s (▼); initial bioburden b = 5.5 (—— < —— )]

Figure 3.5.: Log reduction $R$ of $B. subtilis$ PY79 endospores as a function of oxygen concentration in $O₂/H₂$ mixtures; $ϕ_{total} = 20$ sccm, $p = 10$ Pa, $P = 1$ kW, $f_{pulse} = 1$ kHz, and 10% duty cycle; $t = 200$ s (○) and $t = 600$ s (▼); initial bioburden $b = 5.5$ (—— < —— )

after 1200 s, but the etching after 200 s can be estimated. The endospores are etched by 3.5 to 13.3 nm after 200 s if a constant etching rate is assumed. With this small etching the crust is most likely only partly etched or if so the outer coat is at least still intact, which should has no direct impact on the log reduction. But on the other hand the etching rate could change as a function of time or spore layer as seen in Figure 3.2b in the argon/oxygen etching experiment. But even though the spore would be largely intact. Thus, it is still a radiation based sterilization and the more VUV/UV radiation is emitted, the higher the log reduction. Additionally, two sterilization experiments are performed in pure oxygen and in pure hydrogen with a treatment time of 600 s, see Figure 3.5. To keep the temperature low the treatment is split into three sets of 200 s. In pure hydrogen complete deactivation is achieved, even though there are multilayer of spores present in the ring of dropped spores. In pure oxygen the log reduction increases from $R_{200s} = 2.0$ to $R_{600s} = 3.2$, while the assumed etch depth rises from 8.3 nm to 25 nm. Even after 600 s the outer coat of the endospore should still be intact.

3.2.3. Morphology of Etched Spores

Since the endospores are etched, their morphology changes. Figure 3.6 shows SEM images of an untreated and an etched endospore of $G. stearothermophilus$. The endospores are treated in the SKP 100 in a standard sterilization plasma cycle. In contrast to all other biological samples in this section, these samples are biological indicators (BIs) from Mesa Labs Inc (Lakewood, Colorado, USA). These BIs are made of $0.0762 \text{mm} \times 6.35 \text{mm} \times 69.85 \text{mm}$ stainless steel ribbons. A droplet of $G. stearothermophilus$ ATCC 12980 endospores is located on one end of these ribbons. Furthermore, the images are not taken by the SEM described in section 2.4.3 but by the central SEM of the RUB. It has a better resolution than the one at the AEPT. This central SEM is a LEO Model 1530 (ZEISS SMT, Oberkochen, Germany) and has a resolution of 1 nm at
3. Investigation of Basic Sterilization Mechanisms

Figure 3.6.: SEM images of *G. stearothermophilus* ATCC 7953 endospores, size bars have the same length.

an acceleration voltage of 20 kV. With this it is possible to resolve the endospore structure. Figure 3.6a shows an untreated spore. The knobby structure is the usual shape of *G. stearothermophilus* in vacuum. It can clearly be seen that the etched endospore in Figure 3.6b is smoother in contrast to the untreated spore. The difference in size of these endospores should not be overestimated since only five images of single spores are recorded, which is far too little to estimate the mean spore size. A length distribution of *B. subtilis* endospores can be found in Figure 2.17. AFM images of *B. subtilis* PY79 endospores etched in the DICP have been presented by Raguse et al. [130].

### 3.3. *Bacillus subtilis* Endospore Mutants

The experiments with *B. subtilis* spore mutant strains are performed in cooperation with Raguse and Möller from DLR and Fieberstadt from AEPT. The mutants lack different parts of the coat layer, as seen in Table 2.3. The different endospore mutants are described in section 2.7.2. The UV fluence is measured for all conditions and is given in Table 3.1. Sterilization experiments with different treatment times up to 3 min are performed in the DICP. An inactivation kinetic for every mutant is plotted. Figure 3.7 shows the

<table>
<thead>
<tr>
<th>gas composition</th>
<th>VUV 130-200 nm</th>
<th>UV-C 200-280 nm</th>
<th>UV-B 280-320 nm</th>
<th>UV-A 320-400 nm</th>
<th>total 130-400 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar 100 sccm</td>
<td>0.25</td>
<td>0.18</td>
<td>0.06</td>
<td>0.25</td>
<td>0.74</td>
</tr>
<tr>
<td>H₂ 20 sccm</td>
<td>5.04</td>
<td>3.11</td>
<td>0.54</td>
<td>0.33</td>
<td>9.02</td>
</tr>
<tr>
<td>O₂ 20 sccm</td>
<td>0.70</td>
<td>0.09</td>
<td>0.06</td>
<td>0.01</td>
<td>0.86</td>
</tr>
<tr>
<td>H₂/O₂ 10/10 sccm</td>
<td>1.03</td>
<td>0.32</td>
<td>2.65</td>
<td>0.12</td>
<td>4.12</td>
</tr>
</tbody>
</table>

Table 3.1.: UV fluence in J m⁻² s⁻¹ of plasmas (*P* = 500 W and *p* = 10 Pa) used in the spore mutant experiments [130]
3.3. *Bacillus subtilis* Endospore Mutants

resulting D-value of the different spore mutants treated in pure argon, hydrogen, oxygen, and a hydrogen/oxygen mixture at an input power of 500 W and a pressure of 10 Pa. As expected, wild-type *B. subtilis* PY79 spores have the highest resistance to all tested gases, whereas the hydrogen containing plasmas are significantly more effective. The D-values for all spore mutants follow the trend: $D_{H_2} < D_{H_2/O_2} < D_{O_2} < D_{Ar}$. A high sensitivity to all plasmas is given, when the inner, outer, or all coat layers are missing (ΔspoVID, ΔsafA, ΔcotE, and ΔsafA and ΔcotE). Spores with deficits in crust assembly (ΔcotVW and ΔcotX and ΔcotYZ) are about as resistant as wild type spores. The less pigmented genotype (ΔcotA) is only sensitive to the hydrogen containing plasmas, but in argon or oxygen it exhibits the same resistance as the wild type spores.

The result of the hydrogen containing plasmas is as expected since these plasmas emit the most VUV/UV radiation, as seen in Table 3.1. The argon plasma has the highest D-value. This is in line with earlier experiments with *B. atrophaeus* endospores [10,11]. However, argon plasmas have two resonance lines at 104.8 and 106.7 nm, which seem to have only a weak influence on the sterilization efficacy [72]. This is in accordance with the results by Munakata et al. described in section 1.2 [45]. They found an insensitivity of irradiation at 100 nm and suggested that radiation in this spectral region is absorbed by proteins in the coat. The spore crust seems to play only a minor role in the resistance.

![Figure 3.7.](image-url)

Figure 3.7.: D-value of *B. subtilis* endospore mutants treated in Ar 100 sccm (○), H₂ 20 sccm (▽), O₂ 20 sccm (□), and H₂/O₂ 10/10 sccm (△) at $P = 500$ W and $p = 10$ Pa; used mutants (missing coat layers or defects are denoted in parentheses): wild-type, ΔspoVID (spore encasement, all coat layers), ΔsafA (inner coat), ΔcotE (outer coat), ΔcotA (outer coat protein responsible for pigmentation), ΔcotX and ΔcotYZ (crust), ΔcotVW (crust), and ΔsafA and ΔcotE (inner and outer coat) [130]
to plasma sterilization. The CotA pigmentation is an important resistance mechanism in plasmas which are rich in VUV/UV radiation. This is in agreement with the findings of Hullo et al., who found that *B. subtilis* spores lacking CotA are more sensitive to UV-A and UV-B [147]. UV-C was not investigated in that study.

### 3.4. VUV/UV Lamp Experiments

Different experiments are conducted with the UV + heat set-up described in section 2.2.4. The UV irradiation dosage is adjusted as such that it is the same as in the SKP 101 in the spectral range from 200 to 280 nm at the same treatment time. All samples treated in this section are sprayed and evaluated by the IVV using endospores of *G. stearothermophilus* ATCC 7953.

**UV Irradiation**

The first experiments are conducted in a vented chamber. Thus only UV radiation ($\lambda \geq 200$ nm) reaches the sample. Figure 3.8a shows the log reduction as a function of the substrate temperature. One sample set is irradiated for 14 s with the UV lamp in air at atmospheric pressure and kept at defined temperature for that time. The other sample set is only kept at certain temperatures for the same amount of time. The experiment shows that the irradiation alone results in a log reduction of 1.8. A risen substrate temperature of 70 °C does neither alone nor in combination with the UV irradiation result in a higher log reduction compared to room temperature. At 120 °C the difference between the log reductions with and without UV irradiation almost vanishes. A temperature of 121 °C alone would be enough for the complete deactivation of *G. stearothermophilus* endospores, but the treatment time is not long enough to deactivate all spores. The D-value for moist heat sterilization at 121 °C amounts to 3.3 min. At 150 °C a log reduction of $R \approx 4$ is reached in both cases, though there are still augmentable endospores present due to short treatment time. Figure 3.8b shows the log reduction $R$ of endospores sprayed on polystyrene samples cut out of syringe tubs dependent on the treatment time. The treatment was performed in air at atmospheric pressure. Since polystyrene does not withstand elevated temperatures, the samples are only irradiated for different treatment times. It can be seen that the log reduction rises only slightly from $R = 1.5$ to 2.0 while the treatment time is four times longer from 14 to 56 s. The log reduction on glass slides and polystyrene is the same within the measurement precision, so the substrate material has no influence.

**VUV Irradiation**

To assess the wavelength dependent sterilization effect, different filter experiments are performed with the VUV lamp and vacuum chamber described in section 2.2.4. The filters can be directly mounted on the vacuum chamber by replacing the magnesium fluoride window used in the aforementioned experiment. A MgF$_2$ ($\lambda_{\text{cut-off}} = 115$ nm), a
3.4. VUV/UV Lamp Experiments

(a) Log reduction $R$ as a function of various substrate temperatures of glass slides: without irradiation (⌀) and with UV-irradiation for 14 s (▼)

(b) Log reduction $R$ as a function of treatment time $t$ of polystyrene samples irradiated with UV radiation

Figure 3.8.: Log reduction $R$ of various sterilization experiments with the UV lamp and *G. stearothermophilus* ATCC 7953 endospores sprayed on samples and treated in air at atmospheric pressure, the initial bioburden is marked (——)

CaF$_2$ ($\lambda_{\text{cut-off}} = 130$ nm), a Suprasil ($\lambda_{\text{cut-off}} = 165$ nm), and a M235 ($\lambda_{\text{cut-off}} = 235$ nm) filter are used. Figure 3.9 shows the log reduction for different treatment times performed in vacuum. It can be seen that there is no significant difference of the log reduction for the filters in the VUV region. Only the log reduction of the M235 filter with a cut-off wavelength of 235 nm is different. With this filter only approximately one third of the log reduction of the other filter experiments is achieved. When the result of this experiment is compared to the experiment presented in Figure 3.8a, is seems that the most effective wavelength range for the sterilization of *G. stearothermophilus* endospores is from 200 to 235 nm. The log reduction in air ($\lambda_{\text{cut-off}} \approx 200$ nm) is 1.8 after 14 s which is comparable to the experiments with the VUV filters.
Figure 3.9.: Log reduction $R$ as a function of treatment time $t$ with the VUV lamp covered with different cut-off filters: MgF$_2$ (115 nm) (○), CaF$_2$ (130 nm) (▽), Suprasil (165 nm) (□), and M235 (235 nm) (△), the small variation in time is for a better reading.
4. Development and Investigation of an Industrial Plasma Sterilization Process

In order to develop a plasma sterilization process, first experiments are carried out in the well characterized laboratory set-up DICP reactor, which has already been used for basic sterilization experiments [9–11,47]. It has several flanges for diagnostics and is even large enough to treat whole syringe containers, which makes it a good starting point for the development and a proof of principal of a plasma sterilization process for syringe tubs. Plasma simulations with HPEM are performed to yield plasma parameters all around a tub. With the knowledge gained at the DICP it is possible to design and develop the industrial prototype SKP 101. The prototype is characterized by means of Langmuir probe and OES. Finally, the plasma based transfer isolator SKP 100 is built that is capable to achieve the required sterilization efficacy.

4.1. The Laboratory Set-up: Double Inductively Coupled Plasma Reactor

4.1.1. Irradiation of a Hydrogen Plasma

Since the VUV/UV irradiation plays a major role in low pressure plasma sterilization, it is important to find plasma discharges which emit a significant amount of it [11,46]. Low pressure hydrogen discharges are known to emit a lot of radiation in the VUV/UV range. The intensity at a fixed input power is strongly dependent on the pressure. Figure 4.1 shows the normalized integrated intensity in the UVC wavelength range from 200 to 280 nm dependent on pressure in a 20 sccm hydrogen discharge at an input power of 750 W. It is measured with the dummy tub inside the reactor, as described in section 2.1. Merely the plasma between the chamber wall and the dummy tub is measured since the dummy itself is made of borosilicate, which is non-transparent for radiation in the evaluated wavelength range. The maximum intensity is between 1 and 3 Pa. It is declining at lower and higher pressures.
4. Development and Investigation of an Industrial Plasma Sterilization Process

4.1.2. Sterilization of Syringe Tubs: Proof of Principal

To assess the plasma sterilization of syringe tubs and before building a special purpose sterilizer, first sterilization experiments are performed in the DICP reactor. As a starting point the gas mixture proposed in [9] is tested. It was shown that a log reduction of \( R = 5 \) of \( B. \) atrophaeus ATCC 9372 endospores sprayed on glass slides is reached after 20 s at an input power of 1 kW. A gas mixture of argon/hydrogen/nitrogen/oxygen 100/1.2/1/0.8 sccm was chosen because it emits over a broad wavelength range in VUV and UV. Figure 4.2 shows log reduction \( R \) of \( G. \) stearothermophilus ATCC 12980, \( A. \) brasiliensis ATCC 16404, and \( B. \) subtilis DSM 4181 endospores sprayed on different positions of a syringe tub (Tyvek, bottom and sides) by the IVV. The experiments are performed at a pressure of 10 Pa and input powers from 1.35 to 5 kW for a continuous wave (CW) plasma and 2.5 kW for a pulsed plasma at a pulsing frequency of 1 kHz with 50% duty cycle. These experiments show that it is possible to achieve a log reduction \( R \) of at least larger than four, in some cases even larger than six, dependent on the type of (endo-)spore and applied power, after 30 s at Tyvek or at bottom of a tub. Even with \( A. \) brasiliensis spores a four-log reduction is achieved. This is the most difficult microorganism for plasma sterilization among the tested (endo-)spores [9]. Another experiment is conducted with a contamination of the syringe tub side with \( G. \) stearothermophilus ATCC 12980 endospores which is not depicted in the aforementioned figure. The tubs are treated in the same gas mixture as used before at an input power of 5 kW. A log reduction of 3.1 and 3.9 is achieved after 15 and 45 s respectively. The initial bioburden was 7.4. These experiments show that a four-log reduction on tubs is feasible in a plasma sterilization process.
4.1. The Laboratory Set-up: Double Inductively Coupled Plasma Reactor

(a) *G. stearothermophilus* ATCC 12980: \( P = 2.5 \text{ kW} \) (glass slides, ○, ---), 1.65 kW (Tyvek, ▽, − − −), 2 kW (bottom, □, — · —), and 5 kW (bottom, △, · · · · · ·)

(b) *G. stearothermophilus* ATCC 12980, CW: \( P = 5 \text{ kW} \) (glass slides, ○, ---) and bottom, □, --- · ---) and 1.8 kW (Tyvek, ▽, − − −), and pulsed: \( P = 2.5 \text{ kW} \), \( f_{\text{pulse}} = 1 \text{ kHz} \), 50% duty cycle, and \( t = 30 \text{s} \)

(c) *A. brasiliensis* ATCC 16404: \( P = 2.5 \text{ kW} \) (glass slides, ○, ---), 1.35 kW (Tyvek, ▽, − − −), and 2 kW (bottom, □, --- · ---), the initial bioburden for Tyvek and bottom are the same

(d) *B. subtilis* DSM 4181, CW: \( P = 1.8 \text{ kW} \) (Tyvek, ▽, − − −) and 5 kW (bottom, □, --- · ---), and pulsed: \( P = 2.5 \text{ kW} \), \( f_{\text{pulse}} = 1 \text{ kHz} \), 50% duty cycle, and \( t = 30 \text{s} \)

Figure 4.2.: Sterilization of syringe tubs and glass slides in an Ar/H\(_2\)/N\(_2\)/O\(_2\) 100/1.2/1/0.8 sccm discharge at \( p = 10 \text{ Pa} \): glass slides (○), Tyvek (▽), and bottom (□, △); initial bioburden: glass slides (---), Tyvek (− − −), and bottom (--- · ---, · · · · · ·)
4. Development and Investigation of an Industrial Plasma Sterilization Process

4.2. Hybrid Plasma Equipment Model Simulations

As a starting point for the HPEM simulations, an argon discharge is chosen because it is less complex than a hydrogen discharge. The argon simulation has a 25\% less reactions than the hydrogen simulation. Also there are no dissociation. The hydrogen discharge is simulated to assess a plasma which is used in sterilization experiments.

Nearly all HPEM simulations are conducted in the DICP geometry with a glass block in the middle of the discharge. This glass block shall mimic a syringe tub. Figure 4.3 shows the investigated geometry. There is also a geometry study to test the potential of a larger chamber.

4.2.1. Argon Plasma

The argon chemistry model is described in section 2.6.3. All simulations shown in this subsection are calculated in the DICP geometry.

Simulation with Different Models

Two different models that handle the electrons are compared: the fluid model and the kinetic Monte Carlo model. Figure 4.3 shows the electron density of a simulation with a fluid and a kinetic Monte Carlo model of an argon discharge at a power of 750 W and a pressure of 5 Pa. Figure 4.4 shows the related electron temperature $T_e$. First of all, a plasma torus can be seen, which is typical for ICPs. In that torus the main plasma heating takes place. There is almost no difference in the electron density whether the fluid or the Monte Carlo model is used. Both models lead to the same result. This is different for the electron temperature though. In case of the fluid model the electron temperature $T_e$ is about 1 eV higher than in the kinetic Monte Carlo model. The structure of the electron temperature is finer resolved in case of the kinetic Monte Carlo model. This is due to a rather low pressure. For higher pressures the fluid model gives credible results. Since the DICP is usually operated at a few pascals, all further simulations are performed with the Monte Carlo model for electrons.

Comparison of HPEM Simulations with Langmuir Probe Measurements

To compare the simulation results with measurements, Langmuir probe measurements with APS3 are performed to yield electron densities $n_e$ and electron temperatures $T_e$. In contrast to the Langmuir probe measurements presented before, the probe is attached to a skew flange, which enables the measurement at the side and above the object. Figure 4.5 shows a scheme of the Langmuir probe movement. The x-axis of the plotted electron densities and temperatures represents the radius and starts in the reactor centre axis and points to the reactor wall, so it is easier to compare with the 2D-plots of the HPEM simulations. To mimic a sterilization object a glass cylinder is placed in the DICP in the
Figure 4.3.: Electron densities $n_e$ dependent on space in an argon simulation at $P = 750$ W and $p = 5$ Pa, the electron density is given in m$^{-3}$

Figure 4.4.: Electron temperatures $T_e$ dependent on space in an argon simulation at $P = 750$ W and $p = 5$ Pa, the electron temperature is given in eV
simulations as well as in the measurements. For comparison, values along the same path are extracted from the two-dimensional HPEM results.

Figure 4.6 shows a simulation and a measurement of the electron density $n_e$ in argon 100 sccm at a pressure of 5 Pa and an input power of 500 to 1000 W as well as at 10 Pa and an input power of 750 W. The values are in the same order of magnitude, but differ by a factor of four. The profile is similar. In the HPEM simulation the electron density decreases a bit faster towards the chamber wall than in the Langmuir probe measurement. The corresponding electron temperatures are shown in Figure 4.7. There is a difference between simulation and measurement. The values in the simulation are about twice as high as in the measurement. Also the profile is flat in the measurement and decreasing towards the chamber wall in the simulation. Only the trend is roughly comparable. Lower power results in a higher electron temperature, as well as lower pressure yields a higher electron temperature.

The first tests in argon show that the HPEM simulation is useful with certain limitations. There is no complete agreement, but the trends are similar. The main goal of the first tests with HPEM was to verify the functionality and the applicability on the DICP. The results in argon are not good enough yet, to use HPEM for the simulation in an argon discharge in the DICP. There is some work left to achieve better results such as an optimization of some of the HPEM input parameters to yield results which are more consistent with the measurements. Further simulation effort is put into the simulation of hydrogen, which is used for sterilization.

![Figure 4.5. Scheme of the DICP with the attached APS3 to the skew flange, the arrow shows the measurement path](image-url)
4.2.2. Hydrogen Plasma

Hydrogen plasmas emit in a broad wavelength range from 160 to 350 nm. With a broad amount of VUV/UV radiation it is a candidate for a high efficient plasma for sterilization. Due to that, a pure hydrogen discharge is simulated. The hydrogen chemistry model is described in section 2.6.3. Electrons are simulated by the kinetic Monte Carlo model since only pressures below or equal 10 Pa are simulated by HPEM.

Figure 4.6.: Electron densities \( n_e \) dependent on distance to the reactor centre axis in an Ar 100 sccm discharge at \( p = 5 \text{ Pa} \) and \( P = 500 \text{ W} \) (——), \( 750 \text{ W} \) (— — —), and \( 1000 \text{ W} \) (— · —) and at \( p = 10 \text{ Pa} \) and \( P = 750 \text{ W} \) (· · · · · ·)

Figure 4.7.: Comparison of electron temperatures \( T_e \) dependent on distance to the reactor centre axis in an Ar 100 sccm discharge at \( p = 5 \text{ Pa} \) and \( P = 500 \text{ W} \) (——), 750 W (— — —), and 1000 W (— · —) and at \( p = 10 \text{ Pa} \) and \( P = 750 \text{ W} \) (· · · · · ·)
Overview

Figures 4.8 and 4.9 show the two dimensional electron density $n_e$ and electron temperature $T_e$ of an HPEM simulation in DICP geometry. It is performed in a pure hydrogen 20 sccm discharge at an input power of 750 W and pressures of 5 and 10 Pa. The plasma torus is again evident as in the argon discharge in section 4.2.1. This is an inhomogeneity which leads to different amount of VUV/UV radiation emitted to the surface of the sterilization object in the middle of the discharge. But this inhomogeneity gets smaller when the pressure is lowered as seen in Figure 4.8. To address this inhomogeneity the irradiation of the sterilization object will later be simulated at different pressures.

Comparison of HPEM Simulations with Langmuir Probe Measurements

The measurements in hydrogen are also performed through the skew flange as depicted in Figure 4.5 and described before. Figure 4.10 shows the comparison of electron density $n_e$ of HPEM simulation and APS3 measurement in a hydrogen 20 sccm discharge at input powers of 500 to 1000 W and pressures of 5 and 10 Pa in the DICP. The maximum electron density is in good agreement between simulation and measurement. The trends are the same, for instance higher power leads to a higher electron density. The profile of the simulation shows a steeper decline towards the chamber wall. The parameters for diffusion processes in HPEM are investigated and changed, but no correction is achieved.
4.2. Hybrid Plasma Equipment Model Simulations

Figure 4.9.: Electron temperature $T_e$ in a hydrogen 20 sccm discharge at $P = 750$ W, the electron temperature is given in eV

Figure 4.10.: Comparison of electron densities $n_e$ dependent on distance to the reactor centre axis in an H$_2$ 20 sccm discharge at $p = 5$ Pa and $P = 500$ W (---), 750 W (--), and 1000 W (---) and at $p = 10$ Pa and $P = 750$ W (-----)
The results for the comparison of the electron temperature is depicted in Figure 4.11. The findings are in better agreement compared with the results obtained in argon. The maximum values only differ slightly and the trend between different pressures is correct. The difference in electron temperature between separate input powers is not apparent. The decline towards the chamber wall is more pronounced in the simulation.

Spectroscopic Validation of Electron Temperature and Density with Calculated Photon Intensity

To obtain another validation of the simulation, a plasma inside the SKP 101 is simulated with HPEM and from this the produced photons are calculated. The corona model is used (see Equation (2.12)) for the transition $H\_2$(a-b). The ground state density of hydrogen molecules $n_{H\_2}$, electron density $n_e$, and electron temperature $T_e$ are taken from the simulation. $k_{\text{exc}}$ is taken from an external electric field (EEF) simulation algorithm from St. Petersburg University. This algorithm calculates the rate constants with the electron temperature $T_e$ as an input parameter. OES measurements are performed at the optical windows above and below of a dummy tub at the SKP 101. This reactor is used because it is possible to measure at different spatial points of the reactor whereas the DICP has only one optical window. The drawback using the SKP 101 is that the reactor is not a cylinder as the DICP, but a cuboid, so there is a systematic error. The OES measurements are performed with the QE65000 spectrometer. Therefore, only wavelengths above 200 nm can be measured. Particularly, the wavelength range from 200 to 280 nm is integrated. This wavelength range represents not the total spectral intensity of the hydrogen photons calculated in the corona model. Therefore the calculated photons are corrected accordingly.

Figure 4.12 shows a comparison of OES measurements and HPEM simulations of a hydrogen 20 sccm discharge where $P_{\text{top}} = 3 \text{ kW}$ (25% duty cycle) is applied to the top coil.
and $P_{\text{bottom}} = 3\, \text{kW}$ (99\% duty cycle) is applied to the bottom coil at pressures from 3 to 10 Pa. Simulation and measurement show a good agreement in both measurement positions. One difference is that the maximum intensity occurs at a pressure of 6 Pa in the simulation and at 3 Pa in the OES measurements. So, this comparison shows that the electron density and temperature are in good agreement between measurements and simulation, at least above and below the sterilization object. The position at the side of the object differs between simulation and measurements. This is not surprising since the simulation results presented above show a faster decline of electron densities and electron temperature towards the chamber walls. This results in far less photons in the calculation of the corona model.

**Photon Flux onto Surfaces Calculated by HPEM**

HPEM can also directly export a photon flux of two radiative transitions in hydrogen as described in section 2.6.3. With this, the photon flux of these transitions onto chamber walls or an object inside the reactor can be simulated. For this, it is necessary to define a path on the desired surfaces. Figure 4.13a shows this path definition. The path starts at the bottom left corner and rotates positively around the object.

Figure 4.13b shows the simulated surface irradiation in the DICP by a hydrogen 20 sccm discharge at powers from 500 to 1000 W and pressures of 5 and 10 Pa. The steep decline and increase represent the edges of the object. The highest irradiation is on top and bottom of the object. The reason are the tori at the coils where the electron density and temperature are significantly higher than in the rest of the discharge. The irradiation of the side of the object is probably underestimated since it was shown before that the electron density and electron temperature are also underestimated aside of the object. A decrease of pressure not only leads to an overall rise in irradiation, but the irradiation

![Graph](image_url)
onto the side increases significantly more than at top and bottom. This is crucial for irradiation based sterilization of objects since the less irradiated parts of an object are the ones which define the treatment time and sterilization efficacy. Another option would be raising the power, but with this the irradiation rise is the same on all sides and the energy flux is also increased, which can be problematic for thermolabile sterilization goods.

**Energy Flux onto Surfaces Calculated by HPEM**

Since the thermal stress on sterilization objects inside the plasma is an important aspect, the energy flux is estimated. The energy flux is composed of different fluxes: flux of neutrals, ions, excited species, and photons. The total energy flux $E_{\text{tot}}$ is calculated as follows:

$$E_{\text{tot}} = E_{\text{neu}} + E_{\text{ion}} + E_{\text{rec}} + E_{\text{exc}} + E_{\text{phot}}$$

in which:

$$E_{\text{neu}} = \Gamma_{\text{neu}} \frac{3k_B}{2}(T_{\text{gas}} - T_{\text{surface}})$$

$$E_{\text{ion}} = \Gamma_{\text{ion}} (E_{\text{ionization}} + eV_{\text{sheath}})$$

$$E_{\text{rec}} = \Gamma_{\text{rec}} E_{\text{recombination}}$$

$$E_{\text{exc}} = \Gamma_{\text{exc}} E_{\text{excitation}}$$

$$E_{\text{phot}} = \Gamma_{\text{phot}} \frac{hc}{\lambda}$$
Figure 4.14.: Simulated Energy flux onto a surface in an H\textsubscript{2} 20 sccm discharge at $p = 5$ Pa and $P = 500$ W (——), 750 W (— — —), and 1000 W (—— · —) and at $p = 10$ Pa and $P = 750$ W (····).

with $\Gamma$ the flux of the respective particles and photons, $E_{\text{ionization}}$ the ionization energy, $V_{\text{sheath}}$ the sheath voltage, $E_{\text{recombination}}$ the neutral recombination energy, and $E_{\text{excitation}}$ the excitation energy. Fluxes $\Gamma$, gas temperature $T_{\text{gas}}$, and sheath voltage $V_{\text{sheath}}$ are simulated with HPEM. The ionization and excitation energies are shown in Table 2.2. It is assumed (and simulated) that all ions and excited species leave the surface as neutrals in ground state and leave their intrinsic energy at the surface. Neutrals transfer the difference between their gas and surface temperature (assumed to be 300 K).

Figure 4.14 shows the calculated energy flux onto an object in the middle of the reactor. The path along the surface is the same as before and shown in Figure 4.13a. The result shows that there is a slight increase in the energy flux when the power is raised, but a huge increase when raising the pressure. The main contribution to the energy flux is from the neutral species. This flux is dependent on the density of neutrals and therefore, strongly dependent on the pressure. The simulations are verified by measurements with the thermocouple Q-Sensor 103 described in section 2.4.1. The thermocouple yields energy fluxes in the same order of magnitude as the calculated energy fluxes. The trends for different pressures and powers are the same. It is a good agreement for the rather simple energy transfer model given in Equations (4.1) to (4.6). This means that the energy fluxes can be roughly estimated by the simulation.

4.2.3. Geometry Study

To finalize the results of the HPEM simulations, an enhanced reactor design is simulated. The critical point of the sterilization of an object is to treat it evenly from all sides. In a reactor such as the DICP the critical locations are the side walls of the object. Photon fluxes presented before, show that the intensities at the sides are an order of magnitude
lower compared to the surfaces facing the antennas. The aim of this geometry study is to enhance the photon flux to the side, but not the energy flux. For this, the chamber radius is enlarged by 20 mm resulting in a new radius of 220 mm. The coils are also moved outwards by 20 mm. The input power is raised to compensate the volume gain. Thus, the input power per plasma volume is kept constant. In this geometry only simulations at a pressure of 5 Pa are performed since lower pressure results in an increased photon flux and a decreased energy flux.

Figure 4.15 shows the two-dimensional electron density $n_e$ and electron temperature $T_e$ in a hydrogen 20 sccm discharge at a power of 927 W and a pressure of 5 Pa. It can be seen that the torus shifts outwards and, therefore, an increased electron density at the side occurs. The photon and energy flux towards the surface is shown in Figure 4.16. It can be seen that irradiation on the side of the object is enhanced significantly while the energy flux rises only marginally. The irradiation is increased by 48% in the case of 927 W and 84% in the case of 1237 W. At the same time the energy flux rises only by 0.4%. The enhanced geometry achieves an irradiation gain of an equivalent to an increase of input power of about 250 W. The irradiation of top and bottom of the object is decreased, due to the fact that the torus is moved outwards. But since the side is the critical location, this should be no problem. Another advantage is the slight lowering of the energy flux to the top and bottom side. So, the thermal stress to the most thermally challenged position is lowered.
4.3. The Industrial Prototype SKP 101

The prototype SKP 101 is developed to show that the plasma sterilization of syringe tubs is possible on an industrial scale. It is designed so that it can be integrated into an aseptic filling line. The main challenge is the time constrain for the sterilization of a tub. The production chamber SKP 100 aims to sterilize one syringe tub per minute. Since the SKP 100 can treat two tubs at the same time, one process may last two minutes. A whole process consists of subsequent steps starting with a vented chamber:

1. open the door on the grey side
2. feed in syringe tubs
3. close the door on the grey side
4. evacuate the chamber
5. flush the chamber with the process gas and regulate the process pressure
6. plasma treatment
7. stop the process gas
8. vent the chamber
9. open the door on the sterile side
10. feed out the tubs
11. close the door on the sterile side.

Figure 4.16.: Simulated photon and energy fluxes in an H2 20 sccm discharge at $p = 5\,\text{Pa}$ and in DICP geometry at $P = 750\,\text{W}$ (---) and 1000 W (——) and in improved geometry at $P = 927\,\text{W}$ (---) and 1237 W (· · · · · ·)
4. Development and Investigation of an Industrial Plasma Sterilization Process

The step taking the longest time of this process chain is the evacuation of the chamber. As described in section 2.1 the polystyrene tubs are sealed with a gas permeable Tyvek foil. Due to a limited permeability of the foil the pumping power has to be reduced in the beginning of the evacuation phase, otherwise the heat-sealed joint will burst. In the end, the maximum plasma treatment time cannot be longer than $\approx 15$ to $20\text{s}$. Another tough challenge is the material of the tubs. The glass transition temperature of polystyrene is 86 to $96^\circ\text{C}$ which must not be reached. Otherwise the Tyvek foil will start to get fusings or holes. This means even during the short plasma treatment time the mean input power has to be constrained so that the polystyrene tub withstands the plasma treatment. An advantage of the SKP 101 is, that both coils can be powered and pulsed independently, in contrast to the DICP. With this degree of freedom it is possible to set the power of the top coil as low as the Tyvek foil can withstand the plasma treatment. The bottom coil is set to a higher input power to generate a plasma beside the tub. Usually $3\text{kW}$ (25\% duty cycle) is applied to the top coil and $3\text{kW}$ (99\% duty cycle) to the bottom coil, both at a pulse frequency of $1\text{kHz}$. With these parameters, syringe tubs withstand the plasma treatment without damage.

Hydrogen is chosen as a process gas, because it emits a broad range of intensive continuum radiation from VUV to visible radiation. Especially, it emits strong emission in the VUV/UV region, which has shown to be effective for sterilizing spores, see section 1.2 [11, 46]. VUV/UV radiation is the most important mechanism in the first seconds of a plasma sterilization process [49]. ICP discharges generate dense and voluminous plasmas, so they yield high photon and radical fluxes around objects. Thus, there is no shadowing effect. Usually, the discharge is operated at a few pascals. Diffusion is good at lower pressures and therefore the discharge is more homogeneous around the tub. Another argument for a pressure of a few pascals is the increased VUV/UV intensity as shown in section 4.1.1.

4.3.1. Temperature of Chamber and Tub

Control of surface temperatures is necessary to achieve gentle, but fast sterilization. When the SKP 101 runs continuously the whole chamber heats up, especially close to the bottom, since more energy is applied to the bottom coil. Figure 4.17 shows the maximum surface temperatures measured with temperature stripes (see section 2.4.1) at different locations. Indicated temperatures are reached at the end of one plasma treatment cycle after 20 process cycles ran continuously in advance. The camber walls only heat up slightly, but the glass plates at the coils heat up to a higher temperature, especially at the bottom. There, the measured temperature exceeds $204^\circ\text{C}$, which was the stripe with the highest temperature available. The temperature at the side wall and the Tyvek foil stay below the glass transition temperature of polystyrene. The bottom side of the tub is considerably above the glass transition temperature, but the syringe tub stays intact and no deformation is detected. This is because not the whole bulk polystyrene is heated up, but only the surface layer.
4.3.2. Optical Emission Spectroscopy

Optical emission is an important sterilization mechanism in the examined plasma process. The whole VUV-UV spectrum could not be measured. The VUV spectrometer cannot be used at the SKP 101, since it has only a slow grating monochromator, which takes one hour to record a whole spectrum and the SKP 101 can only be run for less than 5 min at typical operating parameters. Nevertheless, it can be shown than the VUV radiation is established as expected. This is done by measuring the discharge with a solar blind photo multiplier (Russian Standard Type PMT-142), which is sensitive from 115 to 400 nm. The photo multiplier is covered with different cut-off filters: MgF₂ (λcut-off = 115 nm), quartz (λcut-off = 160 nm), and a M235 filter (λcut-off = 235 nm). The spectral integrated relative intensities (λcut-off – 350 nm) are compared with the spectrum of hydrogen published by Zaidel [148], which is convoluted with the transmission functions of the cut-off filters. Good agreement between the measured and the calculated values is found, as shown in Figure 4.18a. The spectrum (in photons s⁻¹ nm⁻¹ m⁻³) published by Zaidel is fitted to a spectrum measured with the QE65000 in the UV-C wavelength range from 200 to 280 nm and depicted in Figure 4.18b. This wavelength range is chosen since it was the lowest range which could be measured with the OES set-up. It shows the Lyman-alpha (Ly-α, H(2-1)) line at 121.6 nm and the broad emission by the Werner H₂(C-X) and the Lyman Band H₂(B-X), as well as the dissociative transition H₂(a-b) in the VUV/UV range.

In hydrogen discharges, radiation from 200 to 280 nm only contributes to 13 % of total photons emitted in the VUV/UV range. A time dependent integral (in photons s⁻¹ m⁻³) of this wavelength range is measured by the QE65000 spectrometer through different optical windows (see Figure 2.5) and pressures as shown in Figure 4.18c. With increasing pressure from 3 to 10 Pa, irradiation dosage decreases by about 35 % at the bottom position. At the top position the radiance is lowered by a factor of 2.2. Whereas at side positions the decrease of the irradiation dosage is about factor of 4.4.

![Figure 4.17.: Surface temperatures measured with temperature stripes in SKP 101 in an H₂ 20 sccm discharge at P_top = 3 kW (25 % duty cycle), P_bottom = 3 kW (99 % duty cycle), p = 6 Pa (99 % duty cycle), f_pulse = 1 kHz and t = 14 s](image-url)
(a) Spectral integrated spectrum \((\lambda_{\text{cut-off}} - 350\,\text{nm})\) published by Zaidel [148] (○) and the measured spectral integrated relative intensities \((\lambda_{\text{cut-off}} - 350\,\text{nm})\) (▼) at \(p = 3\,\text{Pa}\)

(b) Spectrum published by Zaidel [148] fitted to a QE65000 spectrometer measurement at \(p = 3\,\text{Pa}\) (from 200 to 280 nm)

(c) Time dependent integral of the spectral range 200 to 280 nm for \(p = 3\) to 10 Pa measured through following windows (Figure 2.5): top (——), bottom (— —), and sides (— · —)

Figure 4.18.: Spectrum of a hydrogen 20 sccm discharge at \(P_{\text{top}} = 3\,\text{kW}\) (25\% duty cycle), \(P_{\text{bottom}} = 3\,\text{kW}\) (99\% duty cycle), and \(f_{\text{pulse}} = 1\,\text{kHz}\) [82]
4.3. The Industrial Prototype SKP 101

Figure 4.19.: Surface irradiation of each side of a syringe tub in a hydrogen 20 sccm discharge at $P_{\text{top}} = 3 \text{ kW}$ (25\% duty cycle), $P_{\text{bottom}} = 3 \text{ kW}$ (99\% duty cycle), and $f_{\text{pulse}} = 1 \text{ kHz}$ [82]

Figure 4.19a shows the irradiation per area (in photons s$^{-1}$ m$^{-2}$) onto the different tub surfaces. This is calculated by assuming homogeneous photon production on each side of the tub and multiplying the total number of produced photons ($I_{\lambda,i} \times V_i$) with the considered tub surface ($A_i$) divided by the total surface area of that volume ($A_{V_i}$):

$$I_{\lambda,A} = I_{\lambda,i}V_i \frac{A_i}{A_{V_i}},$$

(4.7)

with $I_{\lambda,i}$ the intensity, $V_i$ the volume, and $i$ the tub surface (top, bottom, side). A sketch of $V_{\text{top}}, A_{\text{top}},$ and $A_{V_{\text{top}}}$ is shown in Figure 4.19b. $I_{\lambda,i}$ is taken from the measurement shown Figure 4.18c. The irradiation at the side of a tub is 20\% of the irradiation of the Tyvek foil and 14\% of the irradiation of the bottom of a tub.

4.3.3. Electron Density and Electron Temperature

All Langmuir probe measurements at the SKP 101 are performed with a dummy tub made of borosilicate glass. This is necessary since a spatial or time resolved Langmuir probe measurement takes up to 5 min and the tub would not stay intact during the whole measurement. The dummy tub has the same dimensions and is placed at the same position as the real tubs.
4. Development and Investigation of an Industrial Plasma Sterilization Process

Langmuir Probe Measurements above the Dummy Tub

Spatial resolved Langmuir probe measurements above the dummy tub are taken from the chamber wall ($x = 0 \text{ m}$) to the center of the discharge ($x = 0.26 \text{ m}$). The temporal progression of the electron density and electron temperature is measured in two positions: at the middle of the tub ($x = 0.26 \text{ m}$) and at the tub edge ($x = 0.13 \text{ m}$). The positions are shown in Figure 2.5.

Figure 4.20 shows the time dependent evolution of the electron density $n_e$ at the two mentioned positions at a pressure of 3 and 10 Pa. The profile of the electron density is dependent upon pressure. At 3 Pa the slope after the end of the top pulse is steeper than at 10 Pa. The decrease of electron density during the on-time of the top coil at 3 Pa at the centre of the tub was not expected. This could be due to an ignition in E-mode [86]. The maximum values in electron density are $n_e = 5 \cdot 10^{16} \text{ m}^{-3}$ and $n_e = 1 \cdot 10^{17} \text{ m}^{-3}$ for 3 and 10 Pa, respectively. At $p = 3 \text{ Pa}$ at tub edge ($x = 0.13 \text{ m}$) the steady state is reached after 150µs when the top coil is powered and then again after 400µs (of the total pulse period) when no power is applied to the top coil. At $p = 10 \text{ Pa}$ and tub edge ($x = 0.13 \text{ m}$) no steady state is reached. In contrast to this, steady state is reached for a pressure of 10 Pa in the centre after 150µs. The profile clearly shows a higher diffusion at a pressure of 3 Pa. At 3 Pa electron density $n_e$ does not fall below $n_e = 4 \cdot 10^{16} \text{ m}^{-3}$ when solely the bottom coil is powered. Whereas at $p = 10 \text{ Pa}$ it drops below $n_e = 1 \cdot 10^{15} \text{ m}^{-3}$.

The result can be interpreted as a superposition of the characteristics of the two ICP sources. Since the tip of the Langmuir probe is closer to the antenna on top, the influence of this is far more pronounced as it can be seen at the drop in electron density after 250µs.

The spatial and temporal electron density evolution over one pulse at a pressure of 3 Pa is shown in Figure 4.21. The temporal profile over the whole chamber has the same trend

![Figure 4.20](image-url)

Figure 4.20.: Temporal profile of the electron density $n_e$ in an H$_2$ 20 sccm discharge at $P_{\text{top}} = 3 \text{ kW} \ (25 \% \text{ duty cycle}), \ P_{\text{bottom}} = 3 \text{ kW} \ (99 \% \text{ duty cycle}), \ f_{\text{pulse}} = 1 \text{ kHz}$ at centre of the tub ($\bigcirc$) and tub edge ($\triangledown$), the vertical dashed line marks the end of the top coil pulse [82]
as shown in Figure 4.20a. Electron density rises from the chamber wall to the centre of the discharge when the tub coil is switched on. When the top coil is switched off, the profile is nearly flat. A slight increase of the density at the tub edge \((x = 0.13 \text{ m})\) can be seen over the whole pulse.

Figure 4.22 shows the spatial distribution of the electron density. A comparison of the spatial electron density distribution from the chamber wall \((x = 0 \text{ m})\) to the centre of the tub \((x = 0.26 \text{ m})\) is shown for two parts of a pulse period: both coils are powered \((0 \text{ to } 250 \mu \text{s})\) and only the bottom coil is powered \((250 \text{ to } 1000 \mu \text{s})\). At a pressure of \(10 \text{ Pa}\) the spatial profile of the electron density has a significant increase from the chamber wall to the centre when both coils are powered, which leads to an electron density of \(n_e = 9 \cdot 10^{16} \text{m}^{-3}\) at the centre of a tub. At a pressure of \(3 \text{ Pa}\) the discharge is more homogeneous with a higher electron density next to the tub. The electron density is \(n_e = 5 \cdot 10^{16} \text{m}^{-3}\) at the centre of the tub. When only the bottom coil is powered, the electron density profile is homogeneous for both pressures. The electron density at the centre of a tub is greater at \(3 \text{ Pa}\) than at \(10 \text{ Pa}\). The electron density difference of both pressures is greater when only the bottom coil is powered. The values are \(n_e = 7 \cdot 10^{15} \text{m}^{-3}\) and \(2 \cdot 10^{15} \text{m}^{-3}\) for \(3 \text{ Pa}\) and \(10 \text{ Pa}\), respectively.

Figure 4.23 shows the spatial profile of the mean electron temperature \(T_e\) over one pulse period for pressures of \(3\) and \(10 \text{ Pa}\). The electron temperature is \(1.5\) to \(1.8 \text{ eV}\) for both pressures up to a distance of \(x = 0.07 \text{ m}\) from the chamber wall. Further to the centre of the tub there is a wide difference of the electron temperatures. The electron temperature rises to \(2.8 \text{ eV}\) at \(3 \text{ Pa}\) towards the tub centre. Whereas at \(10 \text{ Pa}\) the electron temperature decreases to \(0.6 \text{ eV}\). This difference in electron temperature can be used to explain why the optical emission above the tub is higher at \(3 \text{ Pa}\) than at \(10 \text{ Pa}\), as shown in Figure 4.18c. Even though the electron density is higher at \(10 \text{ Pa}\), while the top coil is switched on,
Figure 4.22.: Spatial profiles of the electron density $n_e$ in an H$_2$ 20 sccm discharge at $p = 3$ Pa (○) and 10 Pa (▲), $P_{\text{top}} = 3$ kW (25\% duty cycle), $P_{\text{bottom}} = 3$ kW (99\% duty cycle), and $f_{\text{pulse}} = 1$ kHz, the dashed lines mark the position of the tub edge and tub centre.

Figure 4.23.: Spatial profile of the mean electron temperature $T_e$ (over one pulse period) in an H$_2$ 20 sccm discharge at $P_{\text{top}} = 3$ kW (25\% duty cycle), $P_{\text{bottom}} = 3$ kW (99\% duty cycle), $f_{\text{pulse}} = 1$ kHz while both coils are powered at $p = 3$ Pa (○) and 10 Pa (▲), the dashed lines mark the position of the tub edge and tub centre.
which is the phase where most photons are emitted. But due to the large difference in electron temperature, this is compensated.

**Langmuir Probe Measurements besides the Dummy Tub**

The time dependent evolution of electron density and electron temperature measured besides the dummy tub is comparable to the measurements shown from above the tub. Only results from two instants of time are presented: both coils are powered and solely the bottom coil is powered. To distinguish between both sides of the tub, one is called *long side* and the other one *short side*. The long side of the tub is aligned parallel to the reactor doors. The additional flange on the left side of Figure 2.5 is used to measure next to the long side. One side window, also depicted in the aforementioned figure, is used to measure beside the short side. Figure 4.24 shows electron density and electron temperature dependent on pressure. Measurements for a pressure of 10 Pa are not presented since the signal was too low for a proper evaluation. The electron density stays constant for all pressures at the short side. At this position the electron density is independent of whether the top coil is powered or not. At the long side the electron density rises with higher pressures for both cases. For 6 and 8 Pa, there is also a growing gap between when both coils are switched on and when only the bottom coil is powered. The densities are higher at the long side compared to the short side. This is due to the shorter distance to the center of the coil from the long side. The electron temperature is independent of the status of the top coil. It drops from 1.9 to 0.8 eV while the pressure rises from 3 to 8 Pa. These measurements show that the plasma beside the tub is only generated by the bottom coil at a pressure of 3 and 6 Pa. The top coil has only a small influence at a pressure of 8 Pa. Another result is that the plasma is established homogeneously beside the tub.

### 4.3.4. Sterilization of Syringe Tubs

To test the sterilization efficacy, first experiments are conducted. The experiments are performed in a pure hydrogen discharge since it emits a large amount of VUV/UV radiation. Figure 4.25a shows log reduction of *G. stearothermophilus* ATCC 7953 endospores dependent on the inoculation position of the endospores treated in a hydrogen 20 sccm discharge at a power of $P_{\text{top}} = 3$ kW (25% duty cycle) and $P_{\text{bottom}} = 3$ kW (99% duty cycle), a pulse frequency of 1 kHz, a treatment time of 10 s, and pressures of 3 and 6 Pa. The experiment shows that the log reduction of 4.8 is reached at the bottom and Tyvek inoculation position, which is well above four. But at the side of the tub it is around four for both tested pressures. The sides of a tub are the crucial position for sterilization in such a concept. That is expected since the OES shows that irradiation at the side is only 20% of the irradiation of the Tyvek foil. The sterilization experiments conducted with the DICP showed also the same trend.

To assess the sterilization on the side of a tub, different parameters are varied, namely treatment time and power. Since both coils can be powered, different powers can be applied to each coil for different times. The aim was to go to the limit of a tub without
A power of 3 kW is used for both coils in this experiment, only the duty cycle is altered. Table 4.1 shows the used treatment times and duty cycles for each coil. Figure 4.25b shows the log reduction at the side of the tub. It can be seen that different treatment time and duty cycle variations do not alter the log reduction at the side position, as long as the treatment is pushed to the limit of the tub integrity. To overcome this limit the SKP 101 is improved by a shielding and a new power supply as described in section 4.3.6.

4.3.5. Contamination of Chamber Walls

Deposition

First results from long term operation of the SKP 101 as a sterilizer show that a film is deposited onto the reactor walls. Figure 4.26a shows an image of the bottom of the

![Graph of Electron Density and Temperature](image)

Figure 4.24.: Langmuir probe measurements besides the dummy tub in an H₂ 20 sccm discharge at \( P_{\text{top}} = 3 \text{ kW} \) (25\% duty cycle), \( P_{\text{bottom}} = 3 \text{ kW} \) (99\% duty cycle), and \( f_{\text{pulse}} = 1 \text{ kHz} \) discharge

<table>
<thead>
<tr>
<th>Parameter set</th>
<th>top coil ( t ) duty cycle</th>
<th>bottom coil ( t ) duty cycle</th>
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<tr>
<td>a</td>
<td>10 s 25%</td>
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<td>b</td>
<td>10 s 25%</td>
<td>14 s 95%</td>
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<tr>
<td>c</td>
<td>14 s 15%</td>
<td>14 s 95%</td>
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</table>

Table 4.1.: Parameter sets for the tub side sterilization experiments
4.3. The Industrial Prototype SKP 101

Figure 4.25.: Log reduction of *G. stearothermophilus* endospores ATCC 7953 in an H\textsubscript{2} 20 sccm discharge, the initial bioburden varies between 5.4 and 5.6

chamber. The stainless steel as well as the glass above the bottom coil is coated. Without syringe tubes inside the plasma, there is no deposition of the chamber walls. It is not completely clear what is the exact source of the deposition. A possible cause for this is the tub material. The outer surfaces of syringe tubs consist exclusively of polystyrene. The polystyrene can be etched by hydrogen or oxygen residuals. It could also be a degassing of chemicals from the polystyrene production such as plasticiser or residuals from the ethylene oxide sterilization process. OES measurements show that the hydrogen plasma is contaminated by carbon. In order to investigate the film, one silicon wafer sample is attached to the chamber wall during sterilization cycles until it has a film thickness of 100 nm. This sample is measured by X-ray photoelectron spectroscopy (XPS). An XPS yields in binding energy of atoms inside the film. The binding energies can be looked up in a database and assigned to a type of atom. The depth of penetration is 10 nm. A further description can be found elsewhere [96]. Figure 4.26b shows a film composition of about 79% carbon, 18% oxygen, and 2% nitrogen. Besides the expectable carbon there is an amount of oxygen and nitrogen incorporated into the film. A possible source for that are degassing chemicals or air residuals permeating through the Tyvek foil. Hydrogen will also be part of the film but cannot be detected by XPS due to the missing core electrons [149].

To assess the chamber coating, several silicon wafer samples with a width of 10 mm are fixed inside the reactor. Then syringe tubes are treated inside the plasma reactor. The deposition rate on the wafer samples varies with the position inside the reactor. After 40 process cycles 10 nm are deposited on the side wall of the chamber, which leads to a deposition rate of 0.25 nm process\textsuperscript{-1}. Similar experiments are also performed in the SKP 100. The deposition rate varies from 0.26 to 2.3 nm process\textsuperscript{-1} measured after 40 to 100 processes.
(a) Coated SKP 101 reactor wall

(b) Chemical composition of coated and bare silicon wafer samples determined by XPS: carbon C1S (○), nitrogen N1S (▽), oxygen O1S (□), and silicon Si2p (△)

(c) Etching rates of a-C:H (○) films and films deposited by a tub sterilization process in the SKP 101 (▽) both film types are etched in N2/O2 20/5scm and O2 20scm at p = 3 Pa, in DICP

(d) Etching rates of a-C:H films dependent on the pressure of an O2 20scm (○) and a N2/O2 20/5scm (▽) discharge at P = 5kW, treated in DICP

Figure 4.26: XPS analysis and removal of films deposited by syringe tub sterilization processes
Removal

In order to clean the chamber a plasma etching process is assessed. Silicone samples are fixed at different positions at the bottom of the SKP 101 and tubs are processed, as before, but this time for an undefined amount of plasma time, during other experiments. The total film thickness varies between 50 and 450 nm. There is a strong gradient on each sample. Due to this strong gradient, it is difficult to measure at the same position before and after an etching treatment. These samples are also time consuming and expensive to produce, since whole tubs have to be treated. To simplify the sample production and the determination of the etched samples, the deposited film is compared to an a-C:H film deposited by an acetylene/argon plasma discharge inside the Biodecon reactor. Figure 4.26c shows etching rates of films deposited by plasma sterilization cycles and a-C:H films in different plasmas. The film thickness is measured by a stylus profiler (see section 2.4.2), then fixed to the chamber wall of the DICP and treated by various plasmas. After that, the samples are measured again by a stylus profiler and the etching rate is calculated. Since a cleaning process for a production reactor should be investigated, only available gas mixtures are tested, namely pure oxygen and air. Air is reproduced by a mixture of nitrogen/oxygen 20/5 sccm in the DICP. Both films have comparable etching rates. So it is possible to use a-C:H films to assess the etching of the chamber contamination, at least in the investigated oxygen based etching processes. These films have two advantages: they can be produced in high quantities and the film thickness is homogeneous, and therefore, can be measured reproducible. Since the whole filling line is cleaned and maintained usually once per day, a cleaning cycle of the transfer isolator could be done without an additional time delay during that time. To compare the two gas mixtures in more detail, a pressure variation is performed. For this, a-C:H films are placed on the sample holder and treated inside the DICP at a power of 5 kW. Resulting etching rates can be seen in Figure 4.26d, with a maximum of about 30 nm s\(^{-1}\) and 20 nm s\(^{-1}\) for a pure oxygen and an artificial air discharge, respectively. The pressure of the maximum etching rate for the nitrogen/oxygen discharge is at 10 Pa, whereas it is at 5 Pa in the case of pure oxygen.

To test a cleaning cycle, an etching experiment is performed in the SKP 100 with a-C:H films deposited on silicon wafer samples. Air is chosen as a process gas mixture since it would be possible to run the cleaning cycle without the need of additional gas bottles. The lower etching rate can be balanced by a longer treatment time. The silicon samples are fixed at the bottom of the reactor. The mean etching rate is 6.5 nm s\(^{-1}\). So it is estimated that after one hour of plasma sterilization cycles, a contamination would be etched after 11 s.

4.3.6. Improved SKP 101

To enhance the sterilization efficacy on the sides of a tub, a plasma with higher density and, therefore, higher irradiation should be generated around the tub. At the same time the tub integrity must stay intact. Adding more plasma sources at the side position is not possible since the plasma reactor has to be accessible from two sides. The treatment time is limited by the integrity of the tub. At bottom and at Tyvek position the sterilization
efficacy is very good. To enhance the plasma density on the side without altering the tub a grid is placed between the bottom coil and the tub. The bottom side is chosen because the Langmuir probe measurements showed that the bottom coil determines the plasma density at the side, see section 4.3.3. Since the bottom coil is powered with a maximum of 3 kW and a longer treatment with reduced duty cycles does not improve the sterilization efficacy, the generator is replaced by one which can deliver up to 5 kW. The mass flow controller and pumping system is also exchanged. It is now possible to set the gas flow up to 100 sccm at a pressure below 2 Pa through the chamber to reduce impurities in the discharge.

Grid Experiments

Tests are made with two types of grids: a coarse grid and a fine grid. The coarse grid is a 1 mm thick perforated sheet with holes which have a diameter of 8 mm and a hole distance of 12 mm. The fine grid is a mesh with an aperture size of 1 mm. These stainless steel grids are mounted at a height of $y = 38$ and 58 mm above the bottom of the stainless steel chamber wall. Figure 4.27 shows a scheme of a grid inside the SKP 101.

Figure 4.28 shows temperatures measured with the Q-Sensor 103 at tub bottom position for the two grids at different heights and various plasma parameters. The temperatures measured with the Q-Sensor 103 are higher compared to measurements with temperature strips since the Q-Sensor 103 is enclosed by a copper electrode. The recombination coefficient of metals is significantly higher than of plastics, and therefore, measured temperatures are higher. Nevertheless it is an indicator for the heating of the tub surface. It can be seen that the temperature can be reduced by 50 to 100 °C at the same conditions by the different grids. The lowest temperature is achieved with the fine grid at a height of 58 mm in all conditions. When the power is raised to 5 kW and treatment time is prolonged to 16 s, the temperature with grids stays below the temperature at 3 kW and 14 s without a grid. Thus, a grid can be a promising chance for reducing the thermal

![Diagram of grid](image)

Figure 4.27.: Scheme of a grid mounted inside SKP 101, $y$ is height above the stainless chamber wall where the grid is mounted
4.3. The Industrial Prototype SKP 101

load on the bottom of the tub. Besides this, a challenge for a continuous process is the
heating up of the grid after several consecutive cycles and, therefore, the vanishing of
the cooling effect on the bottom of the tub. To overcome this, a water cooled grid is
installed.

With a water cooled grid, the temperature of different parts of the syringe tub is measured
by a miniature temperature probe incorporated into the tub material by Semmler et
al. [150]. Figure 4.29 shows the temperatures of a syringe tub after one sterilization cycle.
It can be seen that the temperatures are well below the glass transition temperature of
polystyrene of 86 to 96°C.

Figure 4.28.: Temperatures measured with Q-sensor 103 in SKP 101 in an H2 20 sccm
discharge at $P_{\text{top}} = 3$ kW (25\% duty cycle), $P_{\text{bottom}} = 3$ and 5 kW (99\%
duty cycle), $f_{\text{pulse}} = 1$ kHz, $p = 6$ Pa, and $t = 14$ to 16 s: without a grid (○),
a coarse grid at 38 mm (▽), a fine grid at 38 mm (□), a coarse grid at 58 mm
(△), and a fine grid at 58 mm (x).

Figure 4.29.: Maximum temperatures of a syringe tub in the improved SKP 101 measured
with a miniature temperature probe incorporated into the tub material in
an H2 100 sccm discharge at $P_{\text{bottom}} = 5$ kW (CW) and $p < 2$ Pa [150].
Sterilization

With the actively cooled grid it is possible to power the bottom coil with 5 kW (CW) while the syringe tub stays intact for the entire sterilization cycle of 10 to 20 s. The new pumping system allows pressures below 2 Pa, improving the diffusion. It is even possible to omit the top coil and solely use the bottom coil at a power of 5 kW. With the improved SKP 101 sterilization experiments with \textit{G. stearothermophilus ATCC 7953} are performed. Figure 4.30 shows the log reduction dependent on treatment time at Tyvek and side position by Semmler et al. [150]. At side of the tub only treatment times above 10 s are tested. A log reduction of 4 is reached after 13 s and the maximum \( R = 4.5 \) is reached after 16 s at Tyvek foil position. At the side position \( R = 4 \) is also achieved after 13 s and a maximum log reduction of 5 is accomplished after 16 s. Therefore, a D-value of 3.5 and 3.2 s is achieved for Tyvek and at side, respectively.

![Figure 4.30: Log reduction \( R \) of \textit{G. stearothermophilus} endospores dependent on treatment time of Tyvek (----, ○) and side of a tub (−−−−, △) in an \( \text{H}_2 \) 100 sccm discharge at \( P_{\text{bottom}} = 5 \text{kW (CW)} \) and \( p < 2 \text{ Pa} \) in the improved SKP 101 [150]](image)

4.4. The Plasma Based Transfer Isolator: SKP 100

The final plasma sterilizer SKP 100 is built taking into account the results obtained with the DICP and SKP 101. The results of the improved SKP 101 came too late, so they are not incorporated. The SKP 100 has approximately double the size of the prototype SKP 101 and can treat two syringe containers at the same time. Following endospores are tested: \textit{G. stearothermophilus ATCC 7953} and \textit{B. subtilis DSM 4181}. \textit{G. stearothermophilus} and \textit{B. subtilis} are accepted test spores in the pharmaceutical industry for heat and oxidation based sterilization processes, respectively. Figure 4.31 shows a result of the log reduction \( R \) dependent on the inoculation position on the tub.
It can be seen that a log reduction of four is achieved for all endospores. As expected the bottom of the tub is the easiest to sterilize part of the tub followed by the Tyvek foil. Sides of the tub are still the crucial position.

4.5. Remarks

The project started with experiments in the laboratory set-up DICP. It could be shown that plasma can be an alternative method for sterilizing polystyrene containers. HPEM simulations were conducted to yield two-dimensional plasma parameters around an object. The prototype SKP 101 was developed to have a sterilizer with a geometry fitting to the sterilization object. It also has the flexibility to set different input powers for the two ICP antennas. The SKP 101 was characterised by means of Langmuir probe measurements and OES. With these results, input parameters are found for an optimized plasma treatment. Further improvements were made to the SKP 101 to enhance the sterilization on the side walls of the tub. With these improvements it was even possible to omit the ICP module on top of the chamber. Finally, it could be shown that the plasma based transfer isolator SKP 100 is capable to sterilize polystyrene containers for an aseptic filling line.

After two years of product development and one year of parameter studies, the SKP 100 got the approval for ’in production’-status in 2010 by the Medicines & Healthcare products Regulatory Agency (MHRA). The MHRA is the national EMA branch of the United Kingdom of Great Britain and Northern Ireland (UK). In course of the project approximately 2500 syringe tubs were used. Hundreds of sterilization tests had to be performed to show applicability as a transfer isolator. Before approval, design, installation, operational, and process qualification (DQ, IQ, OQ, and PQ) had to be prepared, which is about 10 folders each. The SKP 100 is in production since the approval.
A process monitoring is essential to detect failures in the sterilization process. Since VUV/UV radiation is the main sterilization effect, an optical emission spectrometer is used to observe the process. A simpler spectrometer than the ones used in this thesis is attached to the process chamber for this purpose. The spectral range from 200 to 280 nm is used as a monitoring parameter for the plasma sterilization process. With this, it is possible to decide if a plasma sterilization process has been successful. If the plasma is not established as expected, the tub is not fed out to the sterile area but is rejected to the grey area.

The throughput of the SKP 100 is one tub per minute, which is the same as a small electron beam (E-beam) transfer isolator. Fast E-beam sterilizer have a throughput of six tubs per minute [5]. To compete with these machines, the chamber has either to treat more tubs at the same time or the pumping and venting phase have to be reduced. Both comes at the prize of a larger footprint. In the first case the chamber has to be larger and in the second case there has to be some kind of a pre evacuation chamber since the permeability of the Tyvek foil limits the pumping speed. Other possibilities for further development would be to broaden the range of potential products to be sterilized or enhance the sterilization so that it achieves a six-log reduction.
5. Sterilization of Beehive Material

Honeybees are important for the pollination of crops. The worldwide economic value of insect pollination is estimated to $153 \cdot 10^9 \text{€}$ [151]. Bee colonies are threatened by many factors including diseases, parasites, pesticides, and socio-economic factors [152]. The American Foulbrood (AFB) is a serious threat to bees and a notifiable disease in many countries [152]. AFB is caused by the spore-forming bacterium *Paenibacillus larvae* (*P. larvae*). Bee larvae can be infected by 10 spores during the first 24 to 36 h of their lives. In contrast to this, million spores are needed to infect bee larvae older than two days [153]. Adult bees are not infected by *P. larvae*. The physical or chemical inactivation of *P. larvae* spores in a beehive is complicated due to the resistance of spores. In New Zealand the whole infected colony and associated appliances have to be destroyed by burning [154]. In Germany the colony can survive by artificial swarming [155]. With this method the adult bees have to starve for two days, so that they clean their bodies and eat the residual *P. larvae*. The adult bees are then transferred to a clean beehive. Infectious beehive material and brood are treated chemically by boiling in 3% sodium hydroxide or physically by scorching by flames, or by ignition. The physical treatment inactivates only 84% of *P. larvae* spores [156]. The results of the disinfection is often hard to control in the field. Beekeepers have to fight against economic losses due to losing in productivity of a colony when it is rehabilitated by artificial swarming, the waste of beehive material, and the effort for the disinfection of beehives. Thus, it would be desirable to have an efficient, fast, and non-destructive method for the sterilization of beehive material. A gentle but effective plasma treatment may be an option to sterilize the different beehive materials.

Beehives consist of different materials. To show the sterilization efficacy, experiments with wax, wood, and with whole honeycombs are performed. The biological sample preparation and evaluation is described in section 2.7.3. All biological experiments in this section are conducted with *B. subtilis* W168 endospores, which is a close relative to *P. larvae* and has generally regarded as safe (GRAS) status. *P. larvae* is infectious and could not be used in the plasma laboratory of the AEPT. All endospore samples for experiments in this section are prepared and evaluated by Priehn and Leichert from the Institute for Biochemistry and Pathobiochemistry. The study of the sterilization of beehive material is conducted in the DICP reactor.

Substrate Temperatures

Since honeycombs consist of beeswax, the surface temperature has to stay below 62°C. In order to keep the temperature below the melting temperature, the plasma has to be interrupted. Cycles of 5 s continuous plasma treatment are followed by an off-time.
Figure 5.1 shows the surface temperature measured with a PT-100 of nine cycles with different plasma-off-times. The plasma-on-time is kept constant at 5 s. It was not possible to measure while the RF generator was switched on. Instead, the temperature is shown which is present at a few hundred milliseconds after the plasma discharge is switched off. It can be seen that with a cooling time of 20 s the surface temperature stays below the melting temperature of wax. With this cooling time the surface temperature stays below 61 °C after a prolonged treatment of 12 cycles (60 s on, 240 s off). Additionally, the relevant surfaces, namely wood and beeswax, are examined by eye to control that no macroscopic alterations have occurred.

Sterilization of Wax

As a starting point for the sterilization of beehive materials, beeswax samples are treated in plasma discharges. An argon/nitrogen/oxygen 125/6/2.5 sccm discharge at a pressure of 10 Pa and an input power of 1 kW is used, which is similar to a plasma used beforehand [9]. With this plasma, a log reduction $R = 1.2$ at $b = 3.1$ after 45 s plasma (9 cycles) is reached. After this preliminary experiment, a pure hydrogen 20 sccm plasma at a pressure of 5 Pa and an input power of 1 kW is used. This discharge is chosen since it produces more VUV/UV radiation than the other tested gas mixture. With this plasma a log reduction $R = 2.95$ at $b = 3.03$ after 45 s (9 cycles) is achieved. Figure 5.2 shows the log reduction of these two plasma discharges. Subsequently, only the pure hydrogen discharge is used for beehive experiments since it is almost two orders of magnitude more efficient.

![Figure 5.1.: Surface temperature of a glass slide measured with a PT-100 sensor in an H₂ (20 sccm, $p = 5$ Pa, $P = 1000$ W) discharge for different numbers of cycles. Nine cycles with 5 s plasma-on-time and a variation of plasma-off-times: 5 s (◯), 10 s (▽), 20 s (□), and 40 s (△). The dashed line marks the melting temperature of beeswax (62 °C) [135]](image-url)
Sterilization experiments with different cycles are performed. Figure 5.3a shows the log reduction $R$ dependent on the treatment time $t$. After 5 s the log reduction amounts to $R = 1.9$ and rises until $R = 3.0$ after 12 cycles ($t = 60$ s). The initial bioburden is $b = 3.0$ in this experiment. It can be seen that the most effective cycle is the first one. After that the log reduction rises over time until it reaches total sterilization at 10 to 12 cycles ($t = 50$ to 60 s).

**Sterilization of Wood**

Figure 5.3b shows the time dependent log reduction $R$ in a hydrogen 20 sccm plasma at a pressure of $5 \text{ Pa}$ and an input power of $1 \text{ kW}$. The initial bioburden is 2.8. In this experimental series there are two additional subcycle treatment times: 0.1 and 0.5 s. At 0.1 s there is no detectable sterilization and at 0.5 s $R$ amounts to 0.4. The other result is similar to the result of beeswax. There is hardly a sterilization effect below one second, but the first cycle is also the most effective one. From there, the log reduction rises until it reaches $R = 2.7$ after 60 s.

**Sterilization of Honeycombs**

The honeycombs are sprayed with endospores in total, but after plasma treatment they are dissembled into wood and wax samples. During plasma treatment a slight deformation of some honeycomb cells is observed after more than 4 cycles (20 s). To prevent this from happening, the reactor chamber is vented after four cycles to let the honeycombs
Figure 5.3.: Log reduction $R$ dependent on treatment time $t$ in an H$_2$ 20 sccm discharge at $p = 5$ Pa and $P = 1$ kW [135]
cool down. The deformation could have different reasons. Due to the low thermal conductivity of wax and a low heat capacity or due to the thin honeycomb cell walls, the local temperature inside the cell walls could be higher than the measured substrate temperature. There is also a study that concludes that the first thermal phase change in wax from honey bees starts at 35 to 40°C [157].

Figure 5.3c shows the time dependent log reduction of beeswax and wood samples extracted from honeycombs. Since experiments with honeycombs are laborious, only three different treatment times are investigated: 8, 12, and 16 cycles (40, 60 and 80 s). Again, wax samples have a slightly higher log reduction than wood samples. The log reduction rises only minimally over time, as if there are spores which can not be reached by the plasma. The sterilization efficacy is one decade lower compared with the experiments with flat beeswax and wood. The maximum log reductions of wax and wood from honeycombs are $R = 2.2$ and $1.9$, respectively.

Remarks

The log reduction is rather low compared with the sterilization of materials such as glass, metals, or plastics. Beeswax, wood, and complete honeycombs are challenging materials. They are rough and the wax gets soft so that maybe spores are moved inside the wax and out of the reach of plasma. Nevertheless, the log reductions have to be compared with the standard procedures for the treatment of AFB. The physical treatment with flames only reaches $R = 0.8$ and leaves scorch marks on the wood [156]. The chemical treatment of boiling in a 3% sodium hydroxide solution reaches $R = 4.5$ [156]. The Food and Environment Research Agency from the National Bee Unit (NBU) (UK) describes the chemical treatment as "not very practical for the average beekeeper" due to boiling of 40 l caustic soda solution [158]. Suitable protective equipment is necessary. Also the disposal of the used solution can be problematic.

First experiments with the plasma treatment of honeycombs achieved a log reduction of $R = 1.9$ to 2.2. The beeswax is even sterilized, which is not possible with the aforementioned standard procedures. These results are promising for future research and development for a special built plasma reactor for honeycomb sterilization which could fit into a van and can be driven from one infected beehive to another.
6. Conclusion & Outlook

The DICP reactor is used to investigate the general plasma sterilization effects in a well characterized and accessible plasma chamber. The etching of a model protein and *B. subtilis* spores is studied. Different *B. subtilis* endospore mutants are treated to investigate the resistance of different spore coats. The DICP reactor is simulated with HPEM to yield two-dimensional plasma parameters. First proof of concept studies are conducted in the DICP reactor. Knowledge gained in researching the DICP is incorporated into the development of the prototype SKP 101 and the plasma based transfer isolator SKP 100. A plasma characterization of the SKP 101 is performed by means of Langmuir probe measurements and OES. As a further application, first sterilization experiments with honeycombs are conducted.

Investigation of Basic Sterilization Mechanisms

The low pressure Double Inductively Coupled Plasma reactor is used as a well characterized set-up to study basic sterilization effects, namely the etching of spores and the effect of separate spore coats on the sterilization efficacy.

**Etching of Bacillus subtilis Spores**: an algorithm for spore detection and measurement is developed. It can process SEM images and calculate the spore density and the length distribution of thousands of spores. With this algorithm it is possible to directly study the etching effect of plasmas on spores. An etching rate from 0.13 to 1.75 nm s$^{-1}$ was found in an argon/oxygen discharge. Only low etching rates were found in the studied plasma conditions. In these conditions it would take several minutes to etch a single spore layer at moderate temperatures. This means that in typical sterilization conditions in the DICP etching is only a minor sterilization effect. The developed algorithm opens the possibility to study the etching rates of various spores or spore mutants with different spore coat configurations.

**Bacillus subtilis Spore Mutants**: the experiments with *B. subtilis* spore mutants lacking some part of coat showed that the resistance is decreased - no matter if inner, outer, or both coats are missing. Furthermore, it was shown that pigmentation of the outer coat (protein CotA) increases the resistance against VUV/UV rich plasmas.
Development and Investigation of an Industrial Plasma Sterilization Process

A transfer isolator for syringe packages (tubs) made of polystyrene shall be designed as a plasma reactor. A first proof of principal of plasma sterilization of tubs is conducted in the DICP. It is shown that it is possible to reach the required four-log reduction on all sides of the tub. Another outcome of these experiments is the necessity to have a larger plasma chamber and to be able to adjust the power feed to both coils separately.

Hybrid Plasma Equipment Model: the hybrid simulation HPEM is used to gain inside in two-dimensional plasma parameters of the DICP reactor. The simulations are validated by Langmuir probe measurements and OES. The results of the HPEM simulations in hydrogen are in good agreement with corresponding measurements. The photon and energy flux onto surfaces are calculated. With this, it was possible to perform a geometry study and to enhance the irradiation of the crucial syringe tub position, while keeping the energy flux low.

Industrial Plasma Sterilization (SKP 101 & SKP 100): the possibility of a plasma based sterilization process of syringe containers was demonstrated in the DICP reactor. From this starting point, the prototype SKP 101 and the plasma based transfer isolator SKP 100 are developed. The SKPs are cuboid shaped low pressure plasma reactors operated with two or four individual powered ICP modules.

The prototype SKP 101 was characterized by means of Langmuir probe measurements and OES. The spatial and time resolved Langmuir probe measurements showed that the discharge is significantly more homogeneous, when it is operated at 3 Pa. This is the lowest possible pressure with this set-up. The photon production is as well higher at lower pressures, especially at the side of a tub. This is the crucial location of tub sterilization in double ICP set-ups, like the SKPs.

The coating of the chamber wall by tub sterilization processes has been addressed. It was shown that these films consist of carbon, oxygen, and nitrogen. This film can be removed by an air plasma. The chamber wall coating of eight hours of plasma sterilization cycles can be etched in about 90 s.

First improvements have been introduced to the SKP 101 to enhance the plasma sterilization process. Through a shielding on the bottom of the reactor it is possible to raise the input power of the bottom coil and to omit the plasma source on top, while keeping the temperature load on the tub low enough. It was shown that the improved SKP 101 reaches a four-log reduction after 13 s. This prototype accomplishes a D-value of 3.2 to 3.5 s depending on the inoculation location.

The SKP 100 achieves a four-log reduction of B. subtilis and G. stearothermophilus endospores after 10 s. It was approved in 2010 for ‘in production’-status by the MHRA. This is a good starting point for further developments. The sterilization efficacy is good enough so that the SKP 100 can serve as a transfer isolator, but a six-log reduction would broaden the application possibilities. Another improvement would be to broaden the product range which can be sterilized. There are other products, such as bags of eyedrop
bottles, which could be interesting since their shape and requirements are not too different from syringe tubs.

**Sterilization of Bee Hive Material**

To demonstrate the versatility of plasma sterilization, sensitive bee hive materials are decontaminated. It was shown that it is possible to sterilize different materials such as wood and wax. It was even possible to sterilize whole honeycombs with wooden frames, which is not possible with conventional sterilization techniques. A log reduction of about two was determined. This is twice as good as the standard physical treatment with flames.

**Remarks**

In this work, different aspects of plasma sterilization have been addressed. The sterilization effects of VUV/UV radiation and reactive species have been studied. The major sterilization effect in the ICP set-ups used in this thesis are based on VUV/UV radiation. Especially in the industrial set-ups which only allow short plasma treatment times, etching is irrelevant for the sterilization. A homogeneous and dense plasma around the sterilization object is most important to reach all crucial locations.

After years of research and development, it is finally achieved that a pure plasma sterilization process is approved for commercial use and sold to a pharmaceutical company. The SKP 100 has been sterilizing syringe tubs since then. Effort is recently made to facilitate the approval process, [150] so that the acceptance of plasma sterilization is further raised. The SKP 100 has a throughput of one tub per minute, so it is fast enough to compete with a small E-beam sterilizer.
A. Appendix

A.1. Hybrid Plasma Equipment Model

A.1.1. Chemical Reactions of Argon Discharge

Plasma Reactions

Excitation:

\[ e^- + Ar \rightarrow Ar^{m4s} + e^- \]
\[ e^- + Ar \rightarrow Ar^{4p} + e^- \]
\[ e^- + Ar^{m4s} \rightarrow Ar^{t4s} + e^- \]
\[ e^- + Ar^{m4s} \rightarrow Ar^{4p} + e^- \]
\[ e^- + Ar^{t4s} \rightarrow Ar^{4p} + e^- \]

De-excitation:

\[ e^- + Ar^{m4s} \rightarrow Ar + e^- \]
\[ e^- + Ar^{t4s} \rightarrow Ar + e^- \]
\[ e^- + Ar^{t4s} \rightarrow Ar^{m4s} + e^- \]
\[ e^- + Ar^{4p} \rightarrow Ar + e^- \]
\[ e^- + Ar^{4p} \rightarrow Ar^{m4s} + e^- \]

Ionization:

\[ e^- + Ar \rightarrow Ar^+ + e^- + e^- \]
\[ e^- + Ar^{m4s} \rightarrow Ar^+ + e^- + e^- \]
\[ e^- + Ar^{4p} \rightarrow Ar^+ + e^- + e^- \]
\[ e^- + Ar^{t4s} \rightarrow Ar^+ + e^- + e^- \]
\[ Ar^{m4s} + Ar^{m4s} \rightarrow Ar^+ + Ar + e^- \]
\[ Ar^{4p} + Ar^{4p} \rightarrow Ar^+ + Ar + e^- \]
\[ Ar^{t4s} + Ar^{t4s} \rightarrow Ar^+ + Ar + e^- \]
\[ Ar^{4p} + Ar^{m4s} \rightarrow Ar^+ + Ar + e^- \]
\[ Ar^{t4s} + Ar^{m4s} \rightarrow Ar^+ + Ar + e^- \]
\[ Ar^{4p} + Ar^{t4s} \rightarrow Ar^+ + Ar + e^- \]
Charge exchange:

\[ \text{Ar}^+ + \text{Ar} \rightarrow \text{Ar} + \text{Ar}^+ \]

Further inelastic collisions:

\[ \text{Ar}_m^{4s} + \text{Ar} \rightarrow \text{Ar}_t^{4s} + \text{Ar} \]
\[ \text{Ar}_t^{4s} + \text{Ar} \rightarrow \text{Ar}_m^{4s} + \text{Ar} \]

Spontaneous emission:

\[ \text{Ar}_t^{4s} \rightarrow \text{Ar} + h\nu \]

Surface Reactions

\[ \text{Ar}_m^{4s} \rightarrow \text{Ar} \]
\[ \text{Ar}_t^{4s} \rightarrow \text{Ar} \]
\[ \text{Ar}^{4p} \rightarrow \text{Ar} \]
\[ \text{Ar}^+ \rightarrow \text{Ar} \]

A.1.2. Chemical Reactions of Hydrogen Discharge

Plasma Reactions

Elastic collisions:

\[ e^- + \text{H}_2 \rightarrow \text{H}_2^+ + e^- \]
\[ e^- + \text{H}_2^+ \rightarrow \text{H}_2^+ + e^- \]
\[ e^- + \text{H}_3^+ \rightarrow \text{H}_3^+ + e^- \]
\[ e^- + \text{H}_2^* \rightarrow \text{H}_2^* + e^- \]
\[ e^- + \text{H} \rightarrow \text{H} + e^- \]
\[ e^- + \text{H}^+ \rightarrow \text{H}^+ + e^- \]
\[ e^- + \text{H}^* \rightarrow \text{H}^* + e^- \]

Excitation:

\[ e^- + \text{H}_2 \rightarrow \text{H}_2^* + e^- \]
\[ e^- + \text{H} \rightarrow \text{H}^* + e^- \]

De-excitation:

\[ e^- + \text{H}_2^* \rightarrow \text{H}_2 + e^- \]
\[ e^- + \text{H}^* \rightarrow \text{H} + e^- \]
Dissociation:

\[ e^- + H_2 \rightarrow H + H + e^- \]
\[ e^- + H_2 \rightarrow H^* + H + e^- \]
\[ e^- + H^+_2 \rightarrow H + H^* + e^- \]
\[ e^- + H^+_2 \rightarrow H + H \]
\[ e^- + H^*_2 \rightarrow H + H' \]
\[ e^- + H^*_3 \rightarrow H + H_2 \]
\[ e^- + H^*_3 \rightarrow H^* + H_2 + e^- \]
\[ e^- + H^*_3 \rightarrow H + H + H \]

Ionisation:

\[ e^- + H_2 \rightarrow H^+_3 + e^- + e^- \]
\[ e^- + H^*_2 \rightarrow H^+_2 + e^- + e^- \]
\[ e^- + H^*_3 \rightarrow H^* + e^- + e^- \]
\[ e^- + H^* \rightarrow H^* + e^- + e^- \]

Recombination:

\[ e^- + H^+ \rightarrow H \]

Charge exchange:

\[ H + H^+ \rightarrow H^+ + H \]
\[ H_2 + H^+_2 \rightarrow H + H^*_3 \]
\[ H + H^+_2 \rightarrow H^+ + H_2 \]
\[ H_2 + H^+_2 \rightarrow H^+_2 + H_2 \]
\[ H_2 + H^+ \rightarrow H^+_2 + H \]

Spontaneous emission:

\[ H^*_3 \rightarrow H_2 + h\nu \]
\[ H^* \rightarrow H + h\nu \]

Surface Reactions

\[ H + H \rightarrow H_2 \]
\[ H^* \rightarrow H \]
\[ H^*_2 \rightarrow H_2 \]
\[ H^+_2 \rightarrow H_2 \]
\[ H^*_3 \rightarrow H + H_2 \]
Acronyms

*A. brasiliensis* *Aspergillus brasiliensis*. 13, 14, 45, 66, 67

*A. niger* *Aspergillus niger*. 10, 11, 13, 14, 45

a-C:H amorphous hydrogenated carbon. 55, 90, 91

AEPT Institute for Electrical Engineering and Plasma Technology [Lehrstuhl für Allgemeine Elektrotechnik und Plasmatechnik]. 6, 31, 43, 59, 60, 97

AFB American Foulbrood. 1, 97, 101

AFM atomic-force microscope. 54, 60

APS3 Automatic Probe System 3. 31, 32, 68, 70–74

ATCC American Type Culture Collection. 13, 15, 43–45, 51, 59, 62, 63, 66, 67, 87, 89, 94

*B. atrophaeus* *Bacillus atrophaeus*. 13–15, 17, 48, 49, 61, 66

*B. subtilis* *Bacillus subtilis*. 1, 3, 4, 7, 10, 13–15, 39, 40, 44–49, 54–62, 66, 67, 94, 95, 97, 99, 103, 104

BGSC Bacillus Genetic Stock Center. 43

BI biological indicator. 59

BIER biological indicator evaluator resistometer. 51

BIODECON biological decontamination of surfaces using plasma discharges. 6, 15, 16, 51, 53–55, 91

BSA bovine serum albumin. 35, 51–55

BSL biosafety level. 43

CCP capacitively coupled plasma. 20, 41

CEA Commissariat à l’énergie atomique. 6

CFU colony-forming unit. 9, 51

CJD Creutzfeldt-Jakob disease. 53

CODPG Computational Optical and Discharge Physics Group. 41

CPSEG Computational Plasma Science and Engineering Group. 41–43

CPST Center for Plasma Science and Technology. 6
CW  continuous wave. 66, 67, 93, 94

*D. radiodurans*  *Deinococcus radiodurans*. 12

DICP  Double Inductively Coupled Plasma. 1, 3, 4, 6, 13, 14, 22–25, 28, 29, 31, 40, 49–51, 53, 54, 57, 58, 60, 65, 66, 68, 70, 72, 74, 75, 77, 79, 80, 87, 90, 91, 94, 95, 97, 103, 104

DLR  German Aerospace Center [Deutsches Zentrum für Luft- und Raumfahrt]. 7, 39, 46, 48, 50, 54, 58, 60

DNA  deoxyribonucleic acid. 1, 3, 9–15, 44

DPA  dipicolinic acid. 10, 12

DQ  design qualification. 22, 95

DSM  former abbreviation for DSMZ, but it is kept in microorganism strain numbering. 44, 48, 66, 67, 94

DSMZ  German Collection of Microorganisms and Cell Cultures [Deutsche Sammlung von Mikroorganismen und Zellkulturen]. 43, 112

*E. coli*  *Escherichia coli*. 11, 44

E-beam  electron beam. 5, 6, 96, 105

EC  European Commission. 6

EEDF  electron energy distribution function. 19, 30, 32

EEF  external electric field. 74

EMA  European Medicines Agency. 1, 3, 5, 9, 95

EVDF  electron velocity distribution function. 29, 30

FDA  Food and Drug Administration. 1, 3, 5, 6, 9, 51

FWHM  full width at half maximum. 15, 33

*G. stearothermophilus*  *Geobacillus stearothermophilus*. 1, 3, 10, 13–16, 45, 48, 49, 51, 59, 60, 62, 63, 66, 67, 87, 89, 94, 95, 104

GRAS  generally regarded as safe. 97

HPEM  Hybrid Plasma Equipment Model. 1, 3, 40–43, 65, 68, 70–75, 77, 95, 103, 104

ICP  inductively coupled plasma. 6, 20, 22–25, 41, 53, 68, 80, 84, 95, 104, 105

IQ  installation qualification. 95

IVV  Fraunhofer Institute for Process Engineering and Packaging [Fraunhofer-Institut für Verfahrenstechnik und Verpackung]. 6, 7, 48–50, 62, 66
Acronyms

**JRC** Joint Research Centre. 6

**LBH** Lyman-Birge-Hopfield. 33

**LPS** lipopolysaccharide. 53

**MHRA** Medicines & Healthcare products Regulatory Agency. 1, 3, 95, 104

**NBU** National Bee Unit. 101

**OES** optical emission spectroscopy. 1, 17, 28, 32, 65, 74, 75, 81, 87, 89, 95, 103, 104

**OML** orbital motion limited. 28

**OQ** operational qualification. 95

**P. larvae** *Paenibacillus larvae*. 1, 4, 43, 97

**PDB** Protein Data Bank. 52

**PIC-MCC** particle-in-cell–Monte Carlo collisions. 17, 19, 20

**PQ** process qualification. 95

**PVA** polyvinyl alcohol. 50

**RCSB** Research Collaboratory for Structural Bioinformatics. 52

**RF** radio frequency. 23, 25, 98

**RNase** ribonuclease. 11

**RUB** Ruhr University Bochum. 6, 31, 48–50, 59

**SASP** small acid-soluble spore protein. 10, 12

**SEM** scanning electron microscope. 1, 3, 15–17, 36–39, 45, 49, 54, 56, 59, 60, 103

**SKP 100** Plasma Sterilization Chamber [Sterilisationskammer Plasma] 100. 1, 3, 6, 7, 22, 23, 25, 27, 59, 65, 79, 89, 91, 94–96, 103–105


**TDHT** 5-thyminyl-5,6dihydrothymine. 13

**UK** United Kingdom of Great Britain and Northern Ireland. 95, 101

**UV** ultraviolet. 1, 3, 10–13, 15, 26, 32, 36, 45, 57, 59, 61–63, 65, 66, 71, 72, 80, 81, 87, 96, 98, 103, 105
**VUV** vacuum ultraviolet. 1, 3, 11–15, 26, 32, 57, 59, 61–66, 71, 72, 80, 81, 87, 96, 98, 103, 105

**XPS** X-ray photoelectron spectroscopy. 89, 90
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Résumé

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