Investigations on the Mechanisms of Sterilization by Non-Thermal Low-Pressure Nitrogen-Oxygen Plasmas

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Preface

Pre-publication

Parts of this dissertation have been published with expressive permission of the supervisor in the following journal articles:

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Authorship Statement

This dissertation is comprised of studies that were carried out at the Institute of Food Science and Biotechnology of the University of Hohenheim, Stuttgart, Germany and at the Facilities of Corporate Research and Development of the Robert Bosch GmbH, Gerlingen-Schillerhöhe, Germany. The co-authors contributed to the publications in the following ways:

Dr. Jochen Feichtinger (Robert Bosch GmbH) supervised the plasma systems and provided helpful discussions on the physical aspects of this work. He assisted in setting up an additional plasma system at the University of Hohenheim.

PD Dr. Christian Hertel (University of Hohenheim, later German Institute of Food Technologies, Quakenbrück, Germany) supervised all studies of this dissertation. He assisted in the interpretation of the results and the discussion of the publication manuscripts.

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Table of Contents

Symbols and Abbreviations

Symbols

A	Adenine (in nucleic acid sequences)
C	Cytosine (in nucleic acid sequences)
D_1	Decimal reduction value in the first phase of an inactivation kinetics
D_2	Decimal reduction value in the second phase of an inactivation kinetics
g	Earth gravitational acceleration 9.81 m s^{-2} (for specification of relative centrifugation forces)
G	Guanine (in nucleic acid sequences)
M	Molarity (mol per liter)
M	Amino base (adenine or cytosine in nucleic acid sequences)
N	Viable count after treatment
N_0	Initial viable count (before treatment)
Q	Ratio of detectable copy numbers in a sample
q	Copy number of a specific nucleic acid fragment as determined by quantitative polymerase chain reaction.
T	Thymine (in nucleic acid sequences)

Symbols of physical units are used according to SI standards.

Abbreviations

A.	*Aspergillus* (genus taxon)
a.u.	Arbitrary units
ATCC	American Type Culture Collection
B.	*Bacillus* (genus taxon)
BGSC	Bacillus Genetic Stock Center at the Dept. of Biochemistry, Ohio State University, Columbus, Ohio, USA.
cfu	Colony forming units
D.	*Deinococcus* (genus taxon)
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid

DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen (German stock collection of microorganisms and cell cultures), Braunschweig, Germany
DTA	Dextrose-tryptone agar
EDTA	Ethylene diamine tetra acetate
EGTA	Glycol ether diamine tetra acetate
EN	Euronorm (European standard)
G.	*Geobacillus* (genus taxon)
LTH	Stock collection of the Institute of Food Science, Stuttgart-Hohenheim, Germany
M.	*Micrococcus* (genus taxon)
MW	Microwave
NER	Nucleotide excision repair
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PTFE	Polytetrafluoroethylene
qPCR	Quantitative polymerase chain reaction
R.	*Rhodotorula* (genus taxon)
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAL	Sterility assurance level
SASP	Small acid-soluble proteins
SP	Spore photoproduct (5-thyminyl-5,6-dihydrothymine)
SPL	Spore photoproduct lyase
STE	Sodium chloride-Tris-ethylene diamine tetra acetate
TE	Tris-ethylene diamine tetra acetate
UV	Ultra-violet

1 Introduction

1.1 Principles of plasma sterilization

A plasma is, in a physical sense, a non-condensed (gaseous) state of matter consisting entirely or partially of charged particles such as electrons and ions. Plasmas are classified as either "thermal" or "non-thermal" by the mean temperatures of their heavy particles like ions and neutral species (atoms, molecules). In the presented work, only non-thermal plasmas are relevant with a temperature of the heavy particles below 400 K (Moisan, *et al.* 2001) and a mean thermal energy of their electrons of typically 1 to 5 eV (with 1 eV corresponding to a temperature of 11,600 K). Such plasmas are also commonly called "non-equilibrium plasmas" or "cold plasmas" because of the temperature difference between heavy and light particles, and respectively, because of the fact that the heavy particles are dominating the transfer of thermal energy to surrounding objects. Another property characterizing plasmas is the *plasma density* that expresses the number of charged particles (usually referring to free electrons) per unit volume. The plasma density can be determined with Langmuir probe measurements. Factors influencing the plasma density include position relative to the plasma source, gas pressure, type of process gases, temperature, type and geometry of the plasma source, and excitation power.

Non-thermal plasmas can be produced by applying electric or electromagnetic fields to gases or vapors or mixtures of both (and hence are also called electric discharges) at atmospheric pressure or at reduced pressure. The electric field can ignite a plasma by accelerating free electrons initially present in gases in

small amounts. The electrons abstract new electrons from atoms or molecules through collisions (impact ionization). Simultaneously, the process of recombination of the charged particles counteracts the ionization process, resulting in an equilibrium sustained by the energy being introduced into the plasma by the externally applied electric field. The collisions between particles inside the plasma also produce radicals and excited states of particles. Spontaneous de-excitation can result in emission of photons in the range from infrared to deep UV below wavelengths of 200 nm. The visible part of the emission spectrum causes the plasma to be perceived as a glowing cloud. As well as the emitted high-energy photons, chemically highly reactive excited particles and radicals can interact with materials in the surroundings of the plasma, such as biological materials on the surface of sterilization goods. For the purpose of decontaminating objects from biological material, the plasma can be used as a voluminous source of potential agents such as UV radiation and chemically reactive particles. The agents produced inside the plasma and their possible effects on biological materials are illustrated in Fig. 1.1.

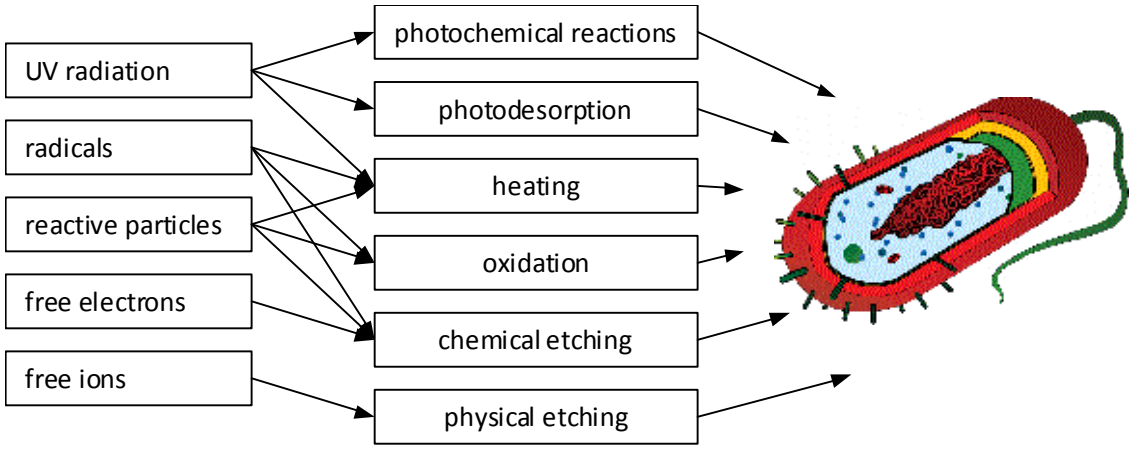


Fig. 1.1. Schematic illustration of putative sterilization active components of plasmas (left) and possible effects of their interaction with biological material (center). (Drawing created by M. Ruiz Villareal, taken from Wikimedia Commons, December 2010.)

Heating may be caused by exothermic reactions and by absorption of radiation. Chemical etching is an erosion mechanism and is mainly caused by reactive

oxygen species and radicals forming volatile compounds from solid by chemical reactions (slow combustion) on surfaces. Photodesorption is another erosion mechanism driven by photons bearing enough energy to break chemical bonds and thus form volatile products, e.g. from biomolecules. Physical etching may be caused by the impact of heavy particles (ion bombardment) and is only likely in situations where ions are accelerated against the sterilization target, e.g. by applying a directed electric field. The concentrations in which the agents occur in a plasma depend greatly on the device setup, the operating conditions (gas pressure – reduced pressure or atmospheric, type and power of plasma excitation) and especially on the gas composition (reviewed in Moisan, *et al.* 2001, Laroussi 2005, Boudam, *et al.* 2006, Moreau, *et al.* 2008). In addition, it is of importance whether the substrate to be sterilized is in direct contact with the plasma or located remote of it. In remote plasmas UV radiation has been established be the dominating agent in sterilization of bacterial spores (Philip, *et al.* 2002). Compared to the presently established chemical methods such as treatment with ethylene oxide or peroxide, or physical methods such as treatment with ionizing radiation, plasma-based sterilization processes have multiple advantages. For example, these processes are fast, easy to control, energy-efficient, and do not require handling of dangerous substances (Yardimci and Setlow 2010).

In most work on sterilization processes, including the one presented here, bacterial spores (endospores) are used as a model for microbial contamination. Spores are physiologically nearly inactive dormant forms produced (among others) by members of the genus *Bacillus* under nutrient limiting conditions. Their properties potentially relevant to their interaction with plasma are briefly presented in the following.

Bacterial spores are characterized by morphological and physiological differences as compared to vegetative bacterial cells, including a modified envelope consisting of several layers (Fig. 1.2) and a greatly reduced water content (reviewed in Setlow 2006).

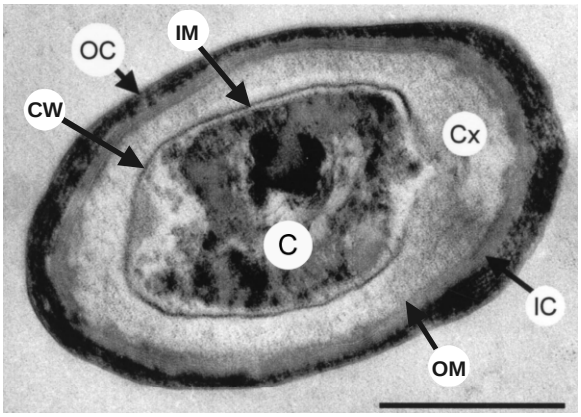


Fig. 1.2. TEM micrograph of a *Bacillus subtilis* spore thin section with its layers indicated from outside to center: outer coat (OC), inner coat (IC), outer membrane (OM), cortex (Cx), germ cell wall (CW), inner membrane (IM), and core (C). Bar is 500 nm. Modified from Driks (1999). Copyright American Society for Microbiology. Reproduced with permission.

In the core area components essential to viability, such as nucleic acids, ribosomes and enzymes are present. The core also contains high concentrations of dipicolinic acid (DPA), which is thought to be related to the low water content in the dormant spore. Another significant specialty of spores is the presence of small acid-soluble proteins (SASP) that bind to the DNA and mediate protection against damages caused by heat (Fairhead, *et al.* 1993) and by UV-radiation (Mason and Setlow 1986, Douki, *et al.* 2005). By binding to the spores' DNA, SASP alter its photochemistry resulting in the formation of a special "spore photoproduct" (5-thyminyl-5,6-dihydrothymine) between adjacent thymidine residues upon UV irradiation. At the same time the formation of the two general DNA photoproducts, cyclobutane type dimers and (6-4)-type photoproducts is suppressed. Spores contain the enzyme spore photoproduct lyase (Spl) that specifically restores the spore photoproduct back into two thymidine monomers during germination. Spl can also be complemented by RecA dependent repair pathways. The combination of SASP and Spl constitutes a highly effective mechanism that contributes significantly to the UV resistance of spores (Setlow 2006).

The spore core is surrounded by the inner membrane. The functional integrity of this membrane is essential to the spores' viability. As long as the membrane is intact, it acts as an effective diffusion barrier even for small molecules and

therefore is likely to be a major factor in the spores' resistance to chemicals (Nicholson, *et al.* 2000).

The spore coat consists of several layers of proteins. It does not directly influence resistance to UV radiation. However, it is involved in resistance to several oxidizing chemicals. Presumably, it acts as a "reactive protective shield", that intercepts oxidants before they can harm essential components in the spore core (Setlow 2006).

Spores must undergo germination and outgrowth, before they can proliferate as vegetative cells. The processes of germination and outgrowth require the inner membrane to be intact, the DNA to be in a reparable or intact state and certain proteins to be functional. During germination enzyme activity and synthesis pathways become reconstituted as the spores take up water. Therefore, it is not before germination, that enzyme mediated repair systems can become effective.

As a second microbial candidate with a potentially high plasma resistance, the bacterium *Deinococcus radiodurans* was included in this work. It is widely known for its high resistance to ionizing radiation and UV radiation and desiccation (reviewed in Cox and Battista 2005). These properties are linked to special structural and metabolic features of this organism. Each of the cells contains four copies of its genome enabling a high potential for recombinational DNA repair mechanisms. Furthermore, the cells can form tetrad units (Fig. 1.3), which also may confer high resistance. *D. radiodurans* has been employed in plasma sterilization research in the past (Mogul, *et al.* 2003, Cooper, *et al.* 2009) and was included in this work for a very close investigation because of its high resistance against ultra violet (UV) radiation, oxidation, and desiccation (Makarova, *et al.* 2001, Wang, *et al.* 2008). Without having to undergo a complete turnover, like germination and outhgrowth like bacterial spores, it enabled the exploration of sublethal injuries.

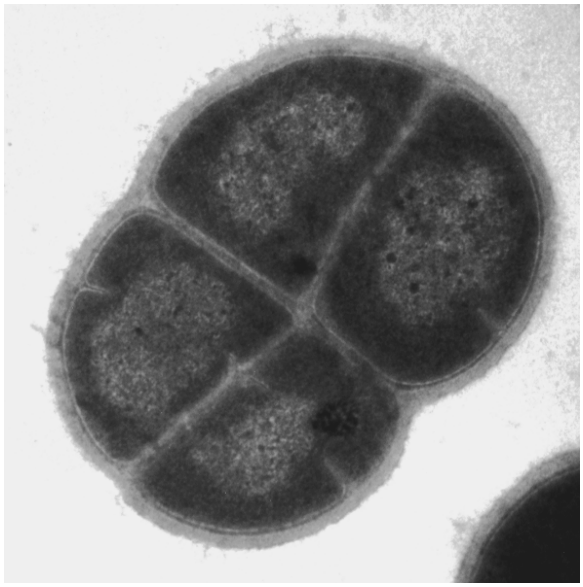


Fig. 1.3. TEM micrograph of a thin section of a tetrad of *Deinococcus radiodurans* cells. Each of the individual cells has an incomplete internal compartmentation allowing exchange of nucleic acids (appearing as lightly stained cloudy material). Each cell has four compartments with each of them containing one copy of the genome. (Image reproduced with kind permission of Michael Daly (Uniformed Services University of the Health Sciences, Bethesda, MD).

1.2 Applications of plasma sterilization

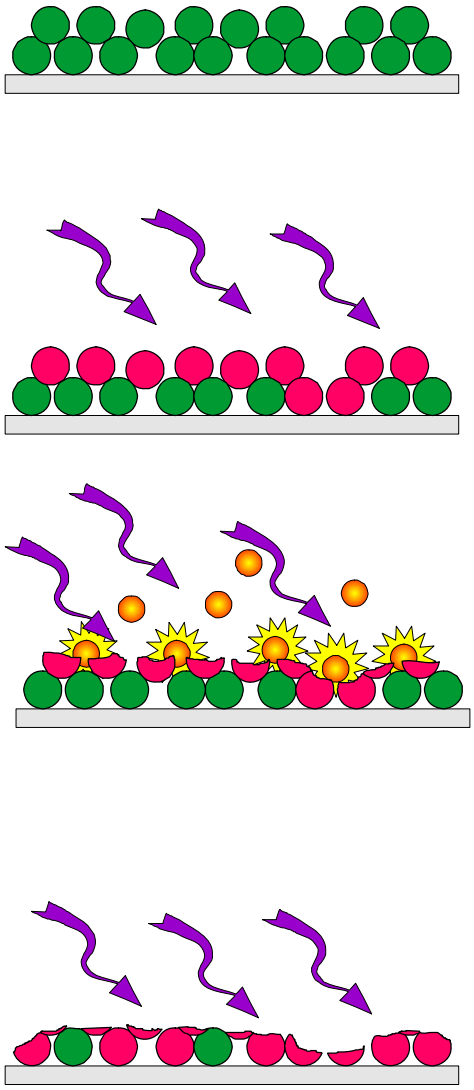
Resulting from the introduction of specialized high-tech polymer materials for medical instruments and novel polymeric packaging materials for liquid pharmaceuticals and foods, there is a need for low-temperature sterilization processes that are safe to operate and that produce reliable sterilization even of complex shaped objects. A novel process with a potential to fulfill these requirements works with low-temperature gas plasmas. Although the temperature of the objects to be sterilized is maintained at material compatible levels, it has been shown that plasma treatment can effectively inactivate a wide range of microorganisms including spores (Kelly-Wintenberg, *et al.* 1999, Feichtinger, *et al.* 2003, Lee, *et al.* 2006). Currently, plasma treatment of packaging (Deilmann, *et al.* 2008, Deilmann, *et al.* 2009, Muranyi, *et al.* 2010) and of foods to improve their microbiological safety is subject of research (Vleugels, *et al.* 2005, Basaran, *et al.* 2008, Selcuk, *et al.* 2008). More than producing sterility, plasma treatment even has the potential of depyrogenation (Hasiwa 2006, Kylián, *et al.* 2006) and inactivation of prions (Rossi, *et al.* 2006). Thus this technique is also widely investigated for its suitability in medical applications like treatment of implants or surgical instruments (Lerouge, *et al.* 2001,

Messerer, *et al.* 2005). In medicine, plasma based techniques are also currently under assessment for applications in dermatology (Heinlin, *et al.* 2010) and dentistry (McCombs and Darby 2010). Other applications for plasma sterilization include surface decontamination after bioterrorism attacks (Herrmann, *et al.* 1999) and planetary protection from carry-over of biological material in space missions (Bol'shakov, *et al.* 2004, Schuerger, *et al.* 2008).

A commercial system marketed for sterilizing medical devices called Sterrad® (ASP Johnson and Johnson, Norderstedt, Germany) is based on the action of hydrogen peroxide vapor and incorporates ignition of a plasma in its sterilization cycle (Krebs, *et al.* 1998). Because in this process the plasma is only used to decompose residues of hydrogen peroxide and its reaction products and it is not substantially directly involved in producing sterility, it has been suggested to designate such processes as "plasma assisted" (Laroussi 2005).

1.3 Current state of investigations on plasma sterilization mechanisms

Systematic investigation of the mechanisms of plasma interaction with biologic materials has started in the mid 1990s (Chau, *et al.* 1996) and was initially focused on analyzing inactivation kinetics of *Bacillus* endospores in correlation with changes of their microscopic appearance. Consequently, for sterilization with low-pressure plasmas, Moisan *et al.* (2001) proposed a general model, which is derived from the observation of at least two sequential phases of rapid and slow inactivation of microorganisms and from properties of the plasmas used (Fig. 1.4). The inactivation rates in the first and second phase are limited by the action of UV radiation (fast inactivation) and reactive particle mediated erosion of the biological material (slow), respectively. Although all of the plasma generated agents act simultaneously, the occurrence of sequential inactivation phases is a consequence of the fact that the germs are present in a stacked form on the bioindicators used as experimental models for worst-case contamination (Fig. 1.5). Actually, microbial contamination normally does not occur as a monolayer of germs, but as aggregates of germ clumps with dust particles or as biofilms.



(a) Before plasma exposure, all individuals are viable (indicated by dark green color). The individuals are partially stacked on the carrier surface.

(b) At the beginning of the plasma exposure, the top layer of individuals is rapidly inactivated (indicated by light red color) by the UV radiation (depicted as arrows) emitted by the plasma. The top layer is shielding the individuals below from the radiation.

(c) In the further course of plasma exposure, the biomass of the already inactivated individuals is eroded by etching and photodesorption. This process is slow compared to the inactivation by UV-radiation. Etching is driven by chemically active particles, e.g. activated oxygen species (illustrated as shaded circles), while photodesorption is caused by high-energy UV-radiation breaking covalent bonds in the biomass and thus forming volatile reaction products.

(d) After the top layer is widely eroded, the individuals can be reached by the UV radiation and can be rapidly inactivated as stated in (a).

Fig. 1.4. Schematic illustration of the inactivation model for highly UV-emitting plasmas proposed by Moisan *et al.* (2001, 2002).

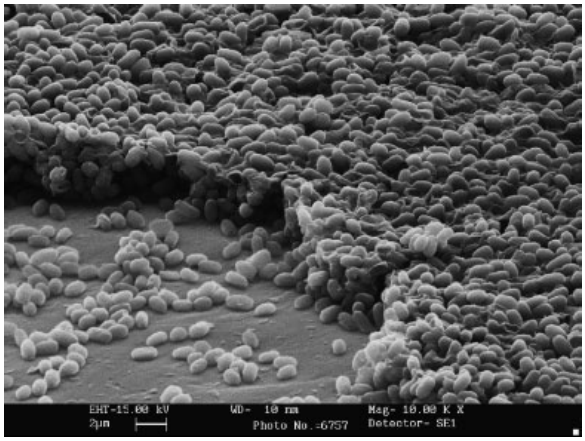


Fig. 1.5. Scanning electron micrograph showing heavily stacked *Geobacillus stearothermophilus* spores on a commercial bioindicator (reproduced from Rossi *et al.* (2006). Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission).

In the further time course, investigations on the effects of plasma on cell components were carried out. Mogul *et al.* (2003) showed that low-pressure oxygen plasma can degrade lipids, proteins and DNA of *Deinococcus radiodurans* cells. Kim and Kim (2006) reported the inactivation of enzyme activity and accumulation of reactive oxygen species inside *Escherichia coli* cells after treatment with atmospheric pressure helium-oxygen plasma. Exposure of *E. coli* cells and spores of *Bacillus globigii* to atmospheric pressure plasmas was shown to cause mutations in surviving cells leading to changes of their metabolic characteristics (Laroussi, *et al.* 2002, Laroussi, *et al.* 2006). However, apart from using standard culturing techniques to determine survival, there is still no consensus about which parameters are useful to characterize plasma sterilization processes and which methods are suitable to analyze them.

1.4 Problem statement and scientific objective

A number of studies have been published about the effectiveness and kinetics of inactivation of spores or killing of bacteria in various plasmas. As a result of these observations, an empiric inactivation model was deduced (Moisan, *et al.* 2001). Still little is known about the details of interaction of the various plasma agents with the diverse components of bacterial cells or spores. Especially, the question remains open, which components of a cell or spore are the primary targets, and which of the agents are most effective in the inactivation process and which molecular mechanisms are involved therein. The acquisition of such knowledge is necessary to identify parameters suitable to control, monitor, and assess the safety of plasma-based sterilization processes.

The aims of the present study are to elucidate which components of a cell or spore are the primary targets in low-pressure plasma sterilization, and which of the putative agents contained in the plasma are most effective in the inactivation process. To accomplish this, in the presented work two strategies were pursued that have hitherto not been applied in published plasma sterilization studies:

- Study the inactivation of spores of suitable well-characterized *Bacillus subtilis* mutants after treatment with various plasmas. Observed differences in the effects of plasma on these mutants are discussed with respect to their phenotypes. This in turn enabled to draw conclusions about the targets of plasma sterilization in the spores. Moreover, a large selection of mutants and a large fund of literature about resistance phenomena and resistance mechanisms are available for *B. subtilis*. This knowledge is used to relate the effects plasma exposure to established mechanisms of other inactivation methods, such as UV irradiation and treatment with oxidizing chemicals.
- Characterize the response of live (injured sublethally) *Deinococcus radiodurans* cells after treatment with various plasmas to investigate which components of the cells are being repaired. *D. radiodurans* is a vegetative bacterium known to be highly resistant to irradiation, oxidation and desiccation. In the presented work, it was used as a model organism because it enabled studying the response to plasma exposure in live cells immediately after plasma treatment without having to undergo a complete turnover process like spore germination. The processes involved in recovery of plasma-induced damages were investigated on a metabolic and on a molecular level.

Suitable methods novel to this area of research have been selected and adapted for these investigations.

1.5 Outline

The presented work consists of three major parts.

In the **first part** (results in section 3.1), a screening of the survival of bacteria, bacterial spores, fungal spores, and yeast after exposure to low-pressure low-temperature nitrogen-oxygen plasma was performed. The aims of this screening were to characterize the sterilization performance of the Bosch pilot

plant plasma equipment and to establish methods and select the microorganisms for more in-depth investigations.

In the **second part** (section 3.2), the effect of plasma treatment on *Bacillus subtilis* spores was investigated with respect to inactivation and changes in proteins, DNA, and membranes. To identify the primary targets of plasma sterilization, spores of *B. subtilis* mutants defective in two independent DNA repair systems (*splB1* and *uvrA42*), synthesis of DNA protecting small acid-soluble proteins (Δ*sspA* Δ*sspB*), and protein coat structure (Δ*cotE*) were included. This part was published in a journal article (Roth, *et al.* 2010a).

In the **third part** (section 3.3), an alternative approach to elucidate the mechanisms of plasma sterilization was used by investigating the response of sublethally injured, but still viable bacterial cells to plasma treatment. The principle of this approach had already been employed to investigate heat injuries in *Staphylococcus aureus* (Iandolo and Ordal 1966), *Salmonella* Typhimurium (Tomlins and Ordal 1971), and *B. subtilis* (Miller and Ordal 1972). In the present study, this approach was adopted and modified to examine the response of bacterial cells to plasma treatment. *D. radiodurans* was used as model organism because of its high resistance against ultra violet (UV) radiation, oxidation, and desiccation (Makarova, *et al.* 2001, Wang, *et al.* 2008). The general pathways necessary for the cells to recover after plasma treatment were investigated up to the level of transcription of genes involved in the stress response. This part was published in a journal article (Roth, *et al.* 2010b)

2 Materials and Methods

2.1 Microorganisms, culturing and preparation of germ suspensions

The strains of the microorganisms used in this work are listed in Table 2.1. For preparation of substrates for the plasma exposure experiments, purified suspensions were produced from all organisms as described in the following.

Table 2.1. Microorganisms used for plasma sterilization experiments.

Strain	Form	Origin/description
Rhodotorula glutinis H48	vegetative cells	LTH; isolate from food
Micrococcus luteus 10BG	vegetative cells	Isolate from ambient air of a pharmaceutical packaging plant
Deinococcus radiodurans R1 (DSM20539)	vegetative cells	DSMZ; type strain, originally isolated from irradiated food
Aspergillus niger DSM 1988	conidia	DSMZ; non-toxigenic test strain
Bacillus licheniformis 90F	endospores	Isolate from ambient air of a pharmaceutical packaging plant
Bacillus atrophaeus DSM 675 (ATCC 9372)	endospores	DSMZ; test organism for dry heat sterilization acc. to EN 866
Bacillus pumilus DSM 492	endospores	DSMZ; test organism for radiation sterilization acc. to EN 866
Geobacillus stearothermophilus DSM 5934 (ATCC 7953)	endospores	DSMZ; test organism for steam sterilization acc. to EN 866.

The imperfect yeast *R. glutinis* H48 as well as the Gram positive bacterium *M. luteus* 10BG were cultured on CASO-Agar (Merck) for 2 d at 30°C. *Deinococcus radiodurans* R1 was routinely cultured by incubating for 2 days at

30°C in dextrose tryptone broth (The composition and preparation of all culture media and buffers is specified in appendix 8.1). Purified suspensions of vegetative cells of these organisms were prepared immediately before use for the plasma exposure experiments (see 2.2). To do so, cells were harvested from agar plates by suspending the colonies in cold sterile water or by centrifugation of broth cultures (15 min at 3,000 × *g*). Cells were washed once with cold sterile water (15 min at 3,000 × *g*), resuspended in cold sterile water. The viable cell densities (colony forming units per microliter) of the suspensions were adjusted as appropriate for the preparation of the plasma exposure substrates with sterile water by their photometric absorbance at 600 nm (optical density). The correlation of the optical density and viable cell counts had been determined beforehand by colony counting.

The mold *A. niger* DSM 1988 was grown on wort agar (see appendix 8.1) for 5 d at 30°C. To harvest the conidia, the agar plates were overlaid with sterile sea sand and shaken gently. The sand containing the conidia was transferred to a flask and flooded with PBS supplemented with 0.05% Tween 80 (Sigma). The conidia were separated from the sand by gently agitating the flask and treatment in an ultrasonic bath for 1 min. The supernatant suspension of conidia was washed twice with PBS by centrifugation (3,850 × *g*, 15 min, 4°C) and finally resuspended in sterile deionized water. The viable number of conidia was determined by plate counting on Rose Bengal agar. The suspension was stored at 4°C until used.

Endospore forming bacteria were routinely cultured on dextrose tryptone agar (DTA; see appendix 8.1) at 30°C, 37°C, or 56°C for *B. pumilus* DSM 492, *B. licheniformis* 90F and *B. atrophaeus* DSM 675, and *G. stearothermophilus* DSM 5934, respectively. For preparation of purified spore suspensions, strains were grown on Schaeffer's sporulation medium agar plates (Harwood and Cutting) by incubating at 37°C until abundant sporulation occurred (up to 5 d) as monitored by phase contrast microscopy. From the agar plates, the biomass was harvested with a sterile spreader using 5 ml of ice-cold deionized water and subjected to centrifugation (10 min, 5,000 × *g*, 4°C). In *B. atrophaeus* and *G. stearothermophilus* strains only about 50% of the cells sporulated. To remove

residual non-sporulated cells, the crude suspension were subjected to sodium bromide density gradient centrifugation as described by Nicholson and Law (1999). The spore suspension was washed five times by resuspension in ice-cold water and centrifugation and finally stored at 4°C in deionized water. Spore counts were determined by plating onto DTA. The spore preparations contained > 99% phase bright spores and were used no longer than two weeks. Sporulation conditions are known to influence resistance properties of the spores (reviewed in Nicholson, *et al.* 2000). To minimize this, spores of all *B. subtilis* strains were produced under the same conditions.

For the systematic investigation of spore inactivation in the plasma, the *B. subtilis* wild-type and mutant strains listed in Table 2.2 were used.

Table 2.2. *Bacillus subtilis* wild-type and mutant strains used in this study.

Strain	Relevant characteristic	Reference
168	*trpC2*	(Burkholder and Giles 1947)
1A757*	Wild-type (Trp^+)	
PS3328†	Derivative of 168, Δ*cotE*	(Paidhungat, *et al.* 2001)
1S111*	Derivative of 1A757, Δ*sspA* Δ*sspB* (referred to as $\alpha^-\beta^-$)	(Mason and Setlow 1986)
1A488*	Derivative of 168, *splB1*	(Munakata and Rupert 1974)
1A345*	Derivative of 168, *uvrA42*	(Munakata and Ikeda 1969)

* Strains were obtained from the BGSC.

† Strain was provided by P. Setlow, University of Connecticut Health Center,Farmington CT, USA.

The *B. subtilis* strains were cultured as described above for the other endospore forming strains, but for strain PS3328, tetracycline (Sigma-Aldrich) was added to a final concentration of 20 $\mu g\ l^{-1}$. Preparation of spore suspension was done without addition of antibiotics as described above. For details on the genotypes of the mutant strains and how they were verified by PCR see appendix 8.3.

2.2 Preparation of microorganism-coated substrates for plasma exposure experiments

Plasma treatment in vacuum required the organisms to be present in an anhydrous state. In the experiments, two different carriers – glass slides and plastic vials – were coated with organisms and subjected to a vacuum drying process. The resulting ready-to-be-plasma-treated organism loaded carriers were designated as "substrates".

2.2.1 Glass slide substrates

Microscopic slides were used as carriers for the spores and microbial cells. The surface of the slides was flamed briefly to sterilize them and make them evenly wettable with 50 µl of an aqueous germ suspension. To delimit the area to be contaminated, a circle of 20 mm diameter was drawn onto the glass surface using a lacquer paint marker. The so prepared glass slides were placed side into sterile microbiological polystyrene petri dishes (Sarstedt, Germany) and handled with sterile forceps in the further process.

For coating slides with *Bacillus* spores or *Aspergillus* conidia, stock suspensions (prepared as stated in section 2.1) were diluted with cold deionized water to yield the desired germ number per substrate in a volume of 50 µl. Aliquots of 50 µl of the diluted suspension were transferred onto the slides with a standard manual pipette. The spores or conidia were allowed to sediment on the slide surface for 1 h at 4°C before freezing for 30 min at –25°C. For coating slides with *D. radiodurans* cells, aliquots of 50 µl of a purified cell suspension (see section 2.1) were used and processed in the same way.

To dry the slides covered with frozen suspensions, the slides (inside the sterile petri dishes) were loaded into the chamber of a SpeedVac vacuum concentrator (Savant, Farmingdale, NY, USA). To keep the suspensions frozen during the drying process, the SpeedVac was equipped with a glass plate pre-cooled to –25°C to support the petri dishes. The SpeedVac was operated without the rotor at full vacuum to allow the slides to be freeze-dried within 30 min. A dense and uniform layer of partially stacked spores or cells was obtained on the glass carriers appearing as an opaque area (Fig. 2.1).

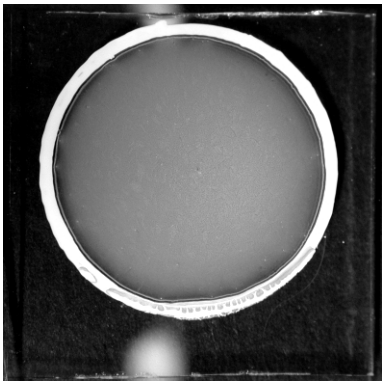


Fig. 2.1. Non plasma treated glass slide substrate coated with 1.5×10^8 *B. subtilis* spores and freeze-dried as used for the final plasma experiments (magnification approximately 2-fold).

2.2.2 Plastic vials

To study the characteristics of plasma sterilization of three-dimensional objects, vials made of cyclic olefin copolymer (Schott, Müllheim, Germany) as used for packaging of liquid pharmaceuticals were taken as carriers to produce substrates for the plasma experiments. These vials had an outer diameter of 20 mm and a total volume of 6 ml. In contrast to glass slides, the plastic vials have a poor water wettability resulting in a non-uniform deposition of the microorganisms from the aqueous suspensions. To overcome this problem, the vials were subjected to nitrogen plasma for 5 s before they were used as carriers for the microorganisms. This process changed the surface energy of the vials and made them wettable allowing them to be evenly contaminated with 120 µl of the microorganism suspensions in a ring shaped area of 140 mm^2 on their inner bottom surface (Fig. 2.2).

(a) **(b)**

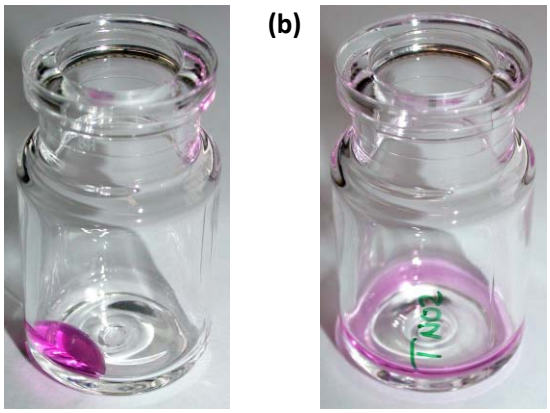


Fig. 2.2. Wettability of cyclic olefin copolymer vials with 120 µl water before (a) and after (b) pre-treatment with nitrogen plasma for 5 s before coating with microorganisms. For the experiments, only pre-treated vials were used. The area contaminated is indicated by the purple dye.

Purified suspensions of spores, conidia or vegetative cells (see section 2.1) were diluted with cold sterile water to obtain the desired viable counts in a volume 120 µl. Aliquots of this volume were transferred to the bottom of the pre-treated vials. The organisms were allowed to sediment for 20 minutes at ambient temperature and then dried in a SpeedVac concentrator under moderate vacuum (so as to avoid boiling) within 30 minutes.

Attempts to produce vial substrates loaded with the Gram negative bacterium *E. coli* or with vegetative cells of *B. subtilis* resulted in a loss of viability of more than 95% after drying and re-hydrating, even though the cryoprotectants trehalose, raffinose and myo-inositol (Potts 1994, Leslie, *et al.* 1995) have been applied. In this case, the small amount of viable cells is embedded into biomass that had already been killed during the preparation process. The dead biomass is shielding the viable cells from direct plasma access and thus, it is to be expected that it greatly influences the results of plasma sterilization experiments. Although such kinds of substrates might reflect realistic contamination situations, they were considered inappropriate for the presented work aiming at analyzing the killing mechanisms of plasmas. Therefore, vegetative cells of *E. coli* and *B. subtilis* were excluded from further investigation.

2.3 Plasma exposure

For the plasma treatments, two vacuum plasma systems designated as DSA701 and M450 were used. Both systems featured the same basic construction and the same type of microwave plasma source and were operated under comparable conditions. The DSA701 system is a proprietary setup of Robert Bosch GmbH and was used for plasma treatment of the wild-type organisms on glass slide substrates and plastic vials. Because of regulatory restrictions, the use of genetically modified organisms in the further experiments required transferring the experiments to the facilities of the Institute of Food Science and Biotechnology of Hohenheim University (Stuttgart, Germany). There, the

commercially available portable M450 plasma system (Muegge Electronic, Reichelsheim, Germany) was used.

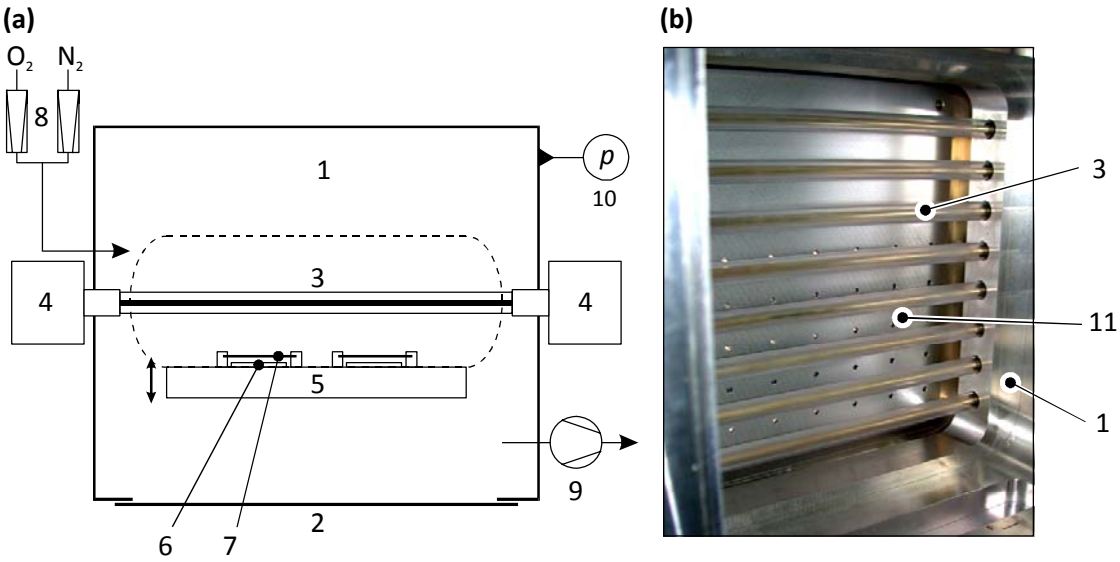


Fig. 2.3. Generic scheme of the vacuum DSA701 and M450 plasma systems (a) and inside view of the vessel of the M450 vacuum plasma system (b). (1) evacuable stainless steel vessel; (2) vessel door; (3) Duo Plasmaline® plasma source (the approximate extension area of the plasma is shown as broken line); (4) magnetron heads; (5) substrate mount allowing the distance between substrates and plasma source to be adjusted; (6) glass slide substrate coated with spores; (7) optical filter plate (optional); (8) gas flow controllers; (9) vacuum pump, (10) pressure gauge, (11) backplate with gas outlet bores.

As depicted in Fig. 2.3a, the vacuum plasma systems consisted of an evacuable vessel, a plasma source, a substrate mount, and means to control the gas composition and gas pressure inside the vessel. A defined atmosphere was maintained by the controlled flow of a mixture of oxygen and nitrogen through the vessel and continuous operation of the vacuum pumping system.

The plasma source consisted of linear microwave antennas (Duo-Plasmaline, Muegge Electronic, Reichelsheim, Germany) driven by two 2.45 GHz magnetron microwave (MW) generators each with a maximum continuous power output of 2 kW. The Duo-Plasmalines consist of a concentric arrangement of a linear metal antenna at the center surrounded by a quartz

tube. A flow through of cooling air fills the space between the antenna and the tube. The antenna emits microwave energy into the evacuable vessel through the quartz tube allowing to ignite a plasma near the outside of the tube. On the DSA701 the power sources also permitted operation in pulsed mode with a maximum power output of 4 kW in the pulse-on phases. In the DSA701, the Duo-Plasmalines were arranged horizontally whereas on the M450 eight shorter Duo-Plasmalines were arranged vertically along the back wall of the vessel (Fig. 2.3b). The Duo-Plasmaline features a homogenous axial plasma density (number of free electrons per unit volume) of typically some 10^{16} m^{-3} as characterized by Langmuir probe measurements if operated correctly. The plasma density decreases greatly with radial distance from the Plasmaline (Kaiser, *et al.* 1999, Schulz, *et al.* 2003). Plasma homogeneity was adjusted using stub tuners located on both sides of the plasma source to yield equal spore inactivation at the two substrate positions.

In both systems, a substrate mount that allowed positioning of the glass slide substrates in variable distances parallel to the plasma source (Fig. 2.4) was installed. Additionally, the substrate mount provided the possibility to cover the glass slide substrates with optical filter plates, thus shielding the plasma-generated particles and parts of the plasma's radiation emission spectrum. The mount was made of the plasma resistant dielectric material PTFE in order to minimize its influence on the plasma properties near the samples. For plasma treatment of the plastic vials, the DSA701 plasma system was additionally equipped with a roller mechanism arranged parallel to the plasma source. This mechanism allowed vials to be rotated during the plasma treatment.

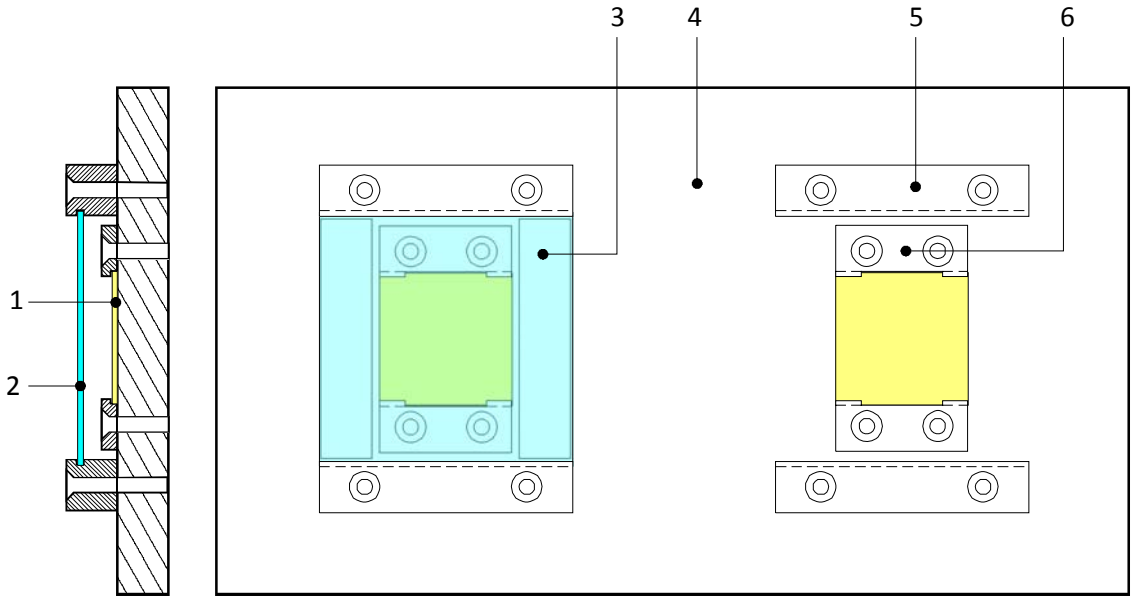


Fig. 2.4. Substrate mount assembly with spore coated glass slide (1), optical filter plate (2), block limiting lateral plasma access (3), PTFE base plate (4), filter plate holder (5), glass slide holder (6). Drawing not to scale.

For the plasma treatments, the glass slides substrates or plastic vials were placed onto the mount or roller mechanism using sterile techniques, respectively. The mount was positioned at a defined distance to the plasma source. Then the vessel was evacuated to a pressure of 10 Pa. The gas flow was started and the plasma was ignited by switching on the MW power when the vessel pressure had equilibrated to 70 Pa. Plasma treatments were done at a pressure of 70 Pa, MW power of 4 kW in continuous mode, gas mixture 80% (v/v) N_2 and 20% (v/v) O_2 unless stated otherwise. The mixture of pure gases was used instead of air to produce a constant initial process environment largely devoid of water vapor and carbon dioxide for the plasma treatments. On the M450 system, for exposure longer than 10 s, pauses of 50 s with the plasma being switched off were introduced after every 10 s to prevent excessive heating of the substrates. In some experiments, the UV/vis emission spectra of the plasma were recorded using an Avantes AVS-SD 2000 spectrometer equipped with an optical fiber. After the plasma treatment, the vessel was

flooded with filtered air to atmospheric pressure and the substrates were removed with sterile practices.

2.4 Determination of surviving cell counts

To resuspend the organisms from the glass slides substrates after plasma exposure, each slide substrate was placed evenly on the bottom of a sterile beaker containing a magnetic stir bar. For experiments with *Bacillus* spores or *Aspergillus* conidia, 2 ml of cold sterile deionized water and for *D. radiodurans* cells, 2 ml of DT broth (autoclaved in two-fold concentration and diluted with sterile deionized water immediately before use) were added. Then, the biomass was resuspended from the slides using a magnetic stirrer at 300 min^{-1} for 2 min.

To resuspend the organisms from plastic vials, 2 ml of resuspension buffer (Table 8.2) was filled into each vial and 10 sterile glass beads (1 mm diameter) were added after plasma treatment. The vials were then sealed with sterile rubber stoppers and agitated on a vortex-mixer for 90 s. This relatively harsh procedure was necessary to suspend the organisms completely because of their strong adherence to the plastic surface of the vials.

Viable counts were determined from the suspensions by plating on DTA or Rose Bengal agar for *Aspergillus* conidia using an automatic spiral plater (Don Whitley Scientific, Shipley, UK). When only a small number of survivors were expected, the pour plating method was employed. The plates were incubated at 30°C for 18 h for *Bacillus* spores or for 48 h for *D. radiodurans*. Based on the arithmetic mean of the colony counts of at least four replicates of non-plasma exposed substrates (N_0) and that of plasma exposed substrates (N), survival was calculated as $N \cdot (N_0)^{-1}$. Corresponding errors were estimated through error propagation and included in the graphs as error bars.

2.5 Modeling of inactivation kinetics

Mean values of the inactivation kinetics data were fitted to a biphasic inactivation model (Cerf 1977) with the GInaFiT macro for Microsoft Excel (Geeraerd, *et al.* 2005), which uses a sum of squared errors based algorithm for parameter estimation. In the biphasic model, the relation between survival and exposure time is given by equation 1.

$$N \cdot (N_0)^{-1} = f \cdot e^{-k_1 \cdot t} + (1 - f) \cdot e^{-k_2 \cdot t} \quad (1)$$

Therein N and N_0 are the numbers of survivors and the initial population, respectively, f is a dimensionless constant designating the transition point from the first phase to the second, k_1 and k_2 are the inactivation rates for the two distinct phases, and t is the exposure time. Survival at the transition from the first to the second inactivation phase was estimated by $N_{Tr} \cdot (N_0)^{-1} = 1 - f$ and the corresponding plasma exposure time was determined by numerical solving.

As an appropriate measure for the quality of the non-linear model fits, the root mean sum of squared error (RMSE) was used. For all fits, the magnitude of RMSE was comparable to that of the precision of the experimental data points (as estimated by the coefficients of variation of the replicates, typically in the range of 10% to 20%). This indicates, that the characteristics and the complexity of the used biphasic model are well suited to represent the experimental data (Ratkowski 2003, Geeraerd, *et al.* 2005).

Since the biphasic model can be interpreted as superimposition of two log-linear kinetics (first order) weighted by f, decimal reduction values (D values) can be estimated for the first and the second phase as follows.

The general log-linear model is given in equation 2.

$$N \cdot (N_0)^{-1} = 10^{-t/D} \quad (2)$$

To estimate D_1 for the first phase, we set $f = 1$ and combine equations 1 and 2:

$$e^{-k_1 \cdot t} = 10^{-t/D_1} \qquad D_1 = \ln 10 \cdot (k_1)^{-1} \quad (3)$$

To estimate D_2 for the second phase, we set $f = 0$ and combine equations 1 and 2:

$$e^{-k_2 \cdot t} = 10^{-t/D_2} \qquad D_2 = \ln 10 \cdot (k_2)^{-1} \tag{4}$$

The *D*-value expresses the exposure time required for a reduction of surviving cell counts by 90% of the initial value. It is of obvious importance to sterilization practice and thus it is a widely used parameter for log-linear kinetics. *D* values are presented in this work to enable comparison with other kinetics data of other studies.

2.6 Screening for auxotrophic mutants

The *Bacillus* spore suspensions from four replicate substrates were pooled, diluted and spread onto DTA plates to yield about 200 colonies per plate upon incubation at 30°C for 24 h. Colonies of auxotrophic mutants were identified by replica plating onto Spizizen's minimal medium (Table 8.1 in appendix) supplemented with 50 µg ml^{-1} L-tryptophan.

2.7 Determination of catalase activity and dipicolinic acid release from spores

An aliquot (4 ml) of an ice-cold aqueous suspension of plasma treated spores was centrifuged (5 min, 10,000 × *g* at 4°C). The supernatant was filter sterilized and stored at –25°C. The pellet was suspended in 200 µl of 10 mmol l^{-1} phosphate buffer (pH 7.0) containing Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany). Zirconia beads (0.3 g, 0.1 mm diameter) were added and the spores were disrupted by shaking in a Mini Bead Beater (Biospec, Bartlesville, OK, USA) for 15 min. After centrifugation (5 min at 10,000 × *g*) the supernatant was used to determine the catalase activity photometrically (Goldblith and Proctor 1950). Catalase activity was expressed as µmol H_2O_2 converted per minute using an external calibration standard of bovine liver catalase (Sigma-Aldrich). To determine the release of dipicolinic acid (DPA) from plasma injured spores, the supernatants were thawed and concentrated four fold under vacuum. The DPA content was assayed according

to Scott and Ellar (1978). Results are reported in nanograms of DPA per substrate. The buffers and reagents required are listed in appendix 8.1.

2.8 Monitoring of the recovery of sublethally injured cells

Five aliquots (each 2 ml) of the suspensions of plasma treated *D. radiodurans* cells were each transferred to a separate baffled Erlenmeyer flask. To four of the flasks, one of the antibiotics cerulenin (25 µg ml^{-1}), chloramphenicol (1 µg ml^{-1}), penicillin G (0.5 µg ml^{-1}), and rifampicin (0.5 µg ml^{-1}) was added, respectively. One flask remained without addition of antibiotics. As control, the biomass of a non plasma treated substrate was resuspended in 2 ml of DT medium and also transferred to a baffled Erlenmeyer flask. All cell suspensions in the flasks were incubated at 30°C for 8 h on a rotary shaker (300 min^{-1}). To differentiate sublethally injured cells from non-injured or recovered cells, samples of 100 µl were taken periodically during the incubation, diluted, and plated on DT agar (standard medium) and on DT agar supplemented with 150 mM NaCl (stress medium) with an automated spiral plater. The plates were incubated aerobically at 30°C for 48 h and viable cell counts were expressed as means of cfu per substrate and standard deviations.

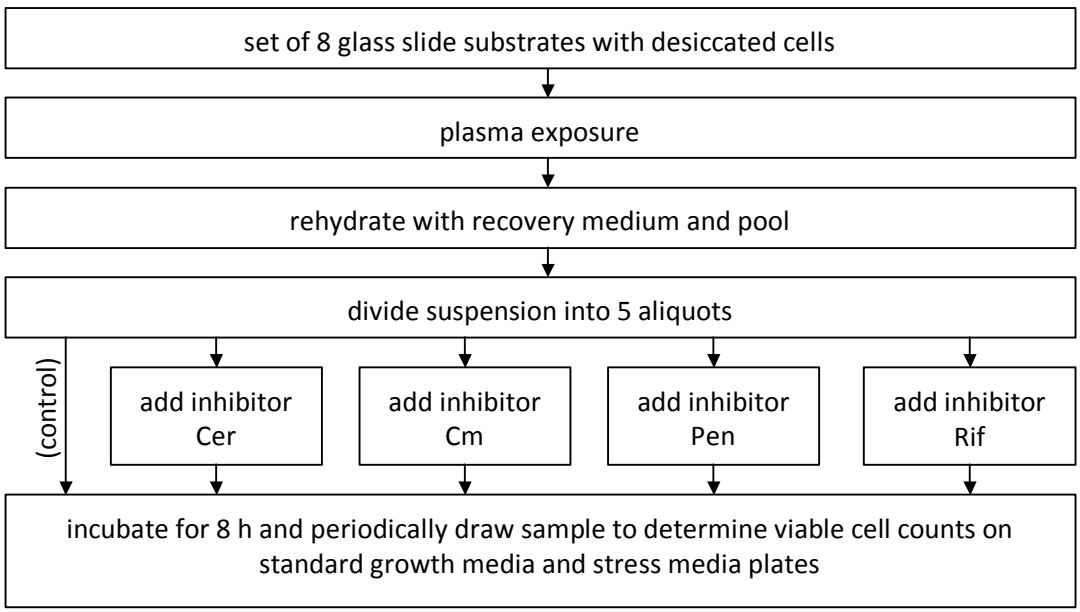


Fig. 2.5 Flow scheme of the assay workflow for monitoring the recovery of sublethally injured cells (Cer: cerulenin; Cm: chloramphenicol.; Pen: penicillin G; Rif: rifampicin).

2.9 Determination of transcription levels in cells recovering from plasma treatment

Suspensions of plasma treated *D. radiodurans* cells were incubated in DT medium at 30°C for 8 h under agitation (300 min^{-1}). At regular intervals, samples of 1 ml were taken (containing around 10^6 to 10^8 cells) and subjected to total RNA isolation and quantitative RT-PCR on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) to monitor the level of transcription of seven selected genes (Table 2.3) involved in stress response. In addition, an aliquot (100 µl) was taken at any one time and subjected to monitor the recovery of sublethally injured cells by plating on standard and stress medium (see section 2.8).

Table 2.3. Target genes selected to monitor repair processes in *D. radiodurans* R1 after plasma exposure.

Target ID/ gene	Function
DR1343 / *gap*	Carbohydrate metabolism, housekeeping gene
DR0261	Nudix family protein, housecleaning and DNA repair
DR0479	Penicillin-binding protein 1, cell wall synthesis
DR1819	UV damage endonuclease, DNA repair
DR1913/ *gyrA*	DNA gyrase subunit A, DNA replication, transcription, DNA repair
DR2220	Tellurium resistance protein, Oxidative stress resistance
DR2275/ *uvrB*	Excinuclease subunit B, DNA repair
DR2340/ *recA*	RecA protein, DNA repair

For preparation of total RNA, samples (1 ml) were immediately mixed with 2 ml of RNAprotect Bacteria reagent (Qiagen, Hilden. Germany). Total RNA was purified from this mixture using RNeasy spin columns (Qiagen) and eluted from the columns with 50 µl water according to the protocol for enzymatic digestion and mechanic disruption provided by the manufacturer. Due to the low number of cells in the samples, it was not possible to measure the concentrations of total RNA in the eluates. Removal of contaminating traces of DNA and first strand cDNA synthesis was carried out with 12 µl of RNA eluate using Quantitect reverse transcription reagents (Qiagen) following the

manufacturer's protocol. PCR Primers and sequence specific fluorescent probes (non-hydrolysable QuantiProbes, Qiagen) were designed (oligo nucleotide sequences see Table 8.8 in appendix 8.2) based on the *D. radiodurans* strain R1 genome sequence (cmr.jvci.org). Relative amounts of target gene transcripts were determined in 25 µl reactions by real time PCR (95 °C/15 min, then 45 cycles of 94 °C/30 s, 56 °C/30 s, 76 °C/30 s) using 1.5 µl cDNA as template and normalized to transcripts of gene DR1343 (*gap*). Target gene transcription levels in plasma treated cells were calculated relative to the transcription in non plasma treated cells (used as calibrator samples). For this calculation, the method by Pfaffl *et al.* (2001) was employed to account for differences in amplification efficiencies which were determined for each primer pair and probe set using template dilution series. Reverse transcriptase negative (RT^-) controls were performed and measurements lower than double the amount of these controls were excluded from analysis.

2.10 Determination of DNA damage

To determine the degree of DNA damage in the plasma-treated organisms semi-quantitatively, real time PCR based ratio detection systems (Bauer, *et al.* 2004) were employed. This method assumes a random distribution of defects along the DNA double strands and therefore the probability of detecting such defects is increasing with the length of the DNA fragment in examination. It enables the detection of damages like thymidine dimers (Sikorsky, *et al.* 2004) and double or single strand breaks. Absolute copy numbers of a longer "reporter fragment" and of a short "standard fragment" contained therein were determined by real time PCR. The degree of DNA damage was expressed as ratio *Q* of the detectable copy numbers between the reporter fragment and the standard fragment.

Real time PCR was carried out on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA) in duplicate 25 µl reactions using Quantitect SYBR Green reagents (Qiagen, Hilden, Germany). Serial dilutions of genomic DNA from non plasma treated organisms were used as an

external standard. Amplification specifities were checked by melting curve analysis.

For the experiments with *B. subtilis* spores, 1 ml aliquots of the spore suspensions from each of four replicate substrates were pooled and collected by centrifugation (10 min; 10,000 × *g*; 4°C). For chemical decoating, the sediment was suspended in 200 µl of 50 mmol l^{-1} Tris-HCl (pH 8.0) containing 8 mol l^{-1} urea, 1% sodium dodecyl sulfate, 10 mmol l^{-1} EDTA, and 50 mmol l^{-1} dithiothreitol and incubated for 90 min at 37°C (Fairhead, *et al.* 1993). The decoated spores were washed three times by repeated centrifugation with cold water. Disruption was accomplished by suspending the spores in 200 µl STE buffer (10 mmol l^{-1} Tris-HCl, pH 8.0; 10 mmol l^{-1} EDTA; 150 mmol l^{-1} NaCl) containing 2 mg ml^{-1} lysozyme and incubating 60 min at 37°C. Chromosomal DNA was purified from the disrupted spores using the High Pure PCR Template Preparation Kit (Roche, Penzberg, Germany). Concentrations of DNA were determined by fluorometry using the QuantiT HS DNA Kit (Invitrogen). The DNA samples were analyzed in a ratio detection system with PCR primer pairs Bs_dnaK855f/Bs_dnaK1254r and Bs_dnaK1154f/Bs_dnaK1254r amplifying a 400 bp reporter fragment and a 101 bp standard fragment from the *dnaK* locus, respectively (primer sequences see Table 8.6 in appendix 8.2). Both fragments were amplified in separate reactions using 0.2 µmol l^{-1} of each primer and 0.25 ng template DNA per reaction with the following thermal profile: 95°C/15 min, 45 cycles of 95°C/30 s and 60°C/90 s.

For investigation of *D. radiodurans*, suspensions of plasma treated cells were incubated in DT medium at 30°C for 8 h under agitation (300 min^{-1}) and samples of 1 ml were regularly taken. For isolation of genomic DNA, cells were harvested by centrifugation (5,000 × *g*, 3 min), resuspended in 1 ml of ethanol, and incubated at room temperature for 10 min to remove the surface protein layer (Earl, *et al.* 2002b). After complete evaporation of ethanol, cells were incubated for 60 min at 37 °C in 200 µl PBS (Sambrook and Russell 2001) containing 20 mg ml^{-1} lysozyme (Serva, Heidelberg, Germany). DNA was purified using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's

instructions. For the ratio detection system, the primer pairs DR1343r/DR1342f2 and DR1343r/DR1343f were used to amplify a 510 bp fragment and an internal 144 bp fragment from the DR1343 locus, respectively (primer sequences see Table 8.7 in appendix 8.2). All reactions were performed using the following temperature profile: 95°C for 10 min, 40 cycles of 94°C/30 s, 45°C/30 s and 72°C/60 s.

3 Results

3.1 Characterization of the inactivation of microorganisms by microwave-induced low-pressure plasma

The properties of a plasma depend on multiple conditions related to process parameters and experimental setup. Thus, it is necessary to characterize the performance of the plasma system with respect to a particular application like sterilization. In this section, the performance of the plasma system for sterilization of bacterial spores, bacterial cells, fungal conidia and yeast cells on flat surfaces and inside of plastic vials is presented.

3.1.1 Inactivation kinetics of bacterial and fungal spores, bacteria, and yeast

To characterize the sterilization process on the DSA 701 plasma system, inactivation kinetics of *D. radiodurans* cells, *A. niger* conidia and spores of *B. pumilus* and *B. atrophaeus* were recorded. All biologic specimens were dried on glass slide substrates and subjected to plasma in both continuous and pulsed mode. The plasma exposure parameters were: microwave power 2 kW in continuous mode, 4 kW in pulse on phases; pressure 10 Pa; process gas mixture of 80% oxygen and 20% nitrogen; substrates placed at 1 cm distance to plasma source. Error bars indicate ± one standard error of at least four replicates. The high oxygen content of the plasma employed in these experiments made it highly erosive due to etching but resulted in a relatively low UV emission (Fig. 3.1).

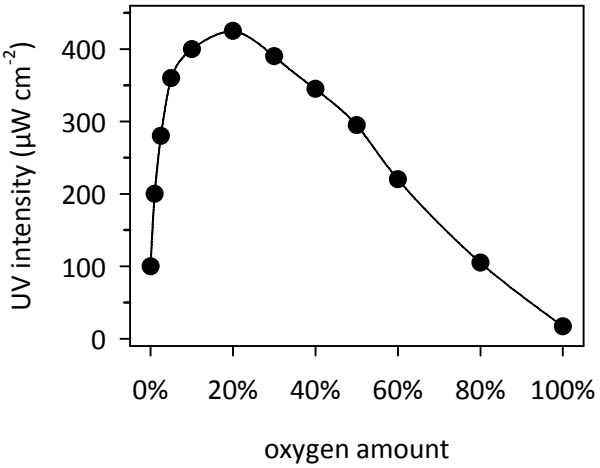


Fig. 3.1. UV emission intensity of microwave plasmas with different oxygen amounts in nitrogen-oxygen mixtures. The intensities were integrated between 200 nm and 280 nm of the emission spectra. (Hägele (2005).)

The resulting survival curves (Fig. 3.2) are plotted against the microwave energy input to make them comparable between both modes. The plasma exposure times in continuous and pulsed mode are proportional to the energies by factor 0.5 s kJ^{-1} and 2.5 s kJ^{-1}, respectively. In the pulsed mode, the plasma was switched on for 10 ms periods at a frequency of 10 Hz, resulting in a substantial reduction of surface temperatures on the substrates (Hägele 2005). In this plasma, *D. radiodurans* exhibited the highest resistance among the organisms investigated (Fig. 3.2a). In continuous mode, the inactivation kinetics can be modeled to a log-linear behavior (first order kinetics) with a decimal reduction value of D = 6.9 kJ (corresponds to D = 3.5 s; coefficient of correlation r^2 = 0.9722). Whereas in pulsed mode, a pronounced shoulder without significant killing at the beginning of the plasma exposure was observed. In the further course of exposure, however, the apparent killing rate was comparable to that in continuous mode. Conidia of *A. niger* showed log-linear inactivation characteristics in continuous and pulsed mode with D = 3.9 kJ (2.0 s; r^2 = 0.9986) and D = 8.0 kJ (20 s; r^2 = 0.9964), respectively (Fig. 3.2b). Of the spores of the two *Bacillus* species, 5 log units were inactivated extremely rapidly in both operation modes apparently following inactivation kinetics with tailing (Fig. 3.2c and d).

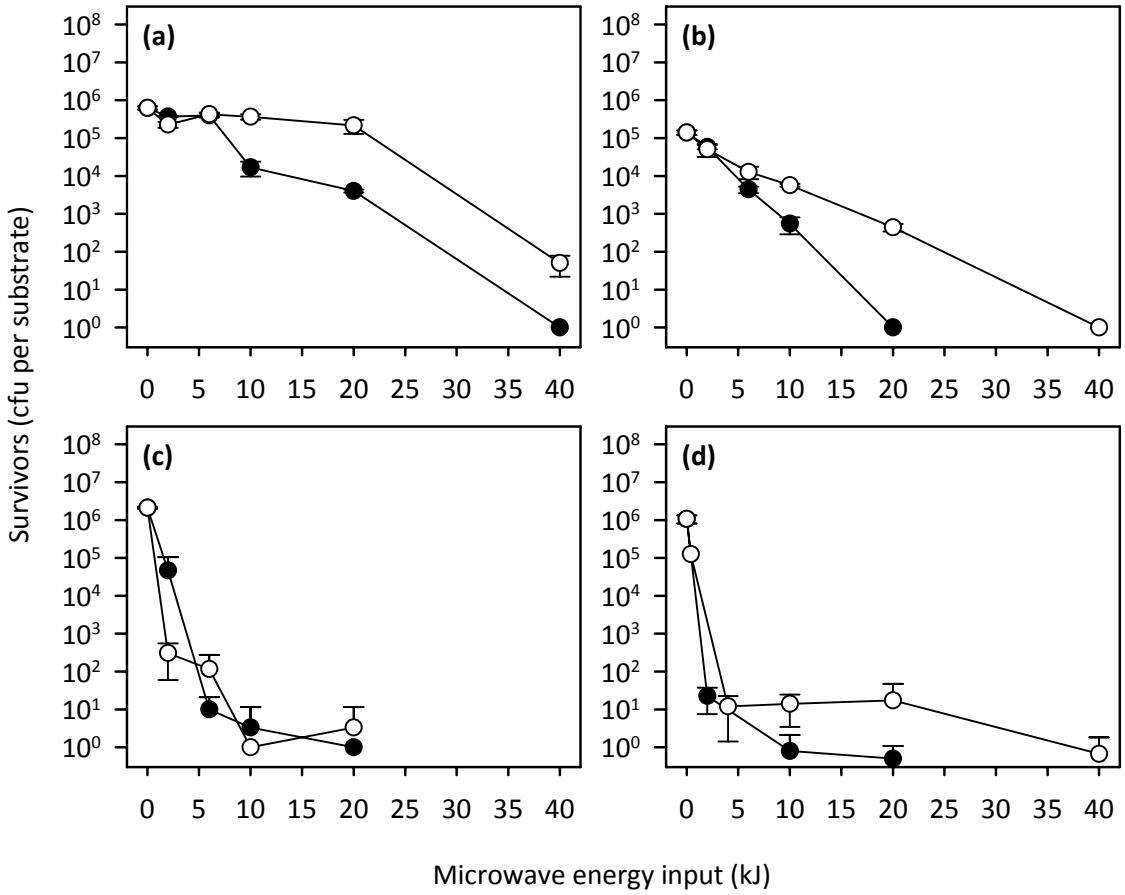


Fig. 3.2. Inactivation kinetics of *D. radiodurans* R1 cells (a), *A. niger* DSM 1988 conidia (b), *B. pumilus* endospores DSM 492 (c), and *B. atrophaeus* DSM 675 endospores (d) treated with plasma in continuous mode (●) or pulsed mode (pulse:pause ratio 10:90 ms; ○). Organisms were freeze-dried on glass slide substrates before plasma treatment. All experiments were performed on the DSA701 plasma system with the following parameters: microwave power 2 kW in continuous mode, 4 kW in pulse on phases; pressure 10 Pa; process gas mixture of 80% oxygen and 20% nitrogen; substrates placed at 1 cm distance to plasma source. Error bars indicate ± one standard error of at least four replicates.

3.1.2 Plasma sterilization of plastic vials

The DSA701 system provided the possibility to perform plasma treatments of three-dimensonal objects. To investigate plasma sterilization under practical conditions, vials made of cyclic olefin copolymer (Topas®) as used for packaging of liquid pharmaceuticals were taken as substrates. These vials had

an outer diameter of 20 mm and a total volume of 6 ml. The plasma system was equipped with a roller mechanism allowing the vials to be rotated during plasma treatment. Thus, like the experiments with glass slide substrates described above, the organisms in the vials are also in direct contact with plasma.

Plastic vials contaminated with cells of *M. luteus*, *D. radiodurans*, and *R. glutinis*, conidia of *A. niger* and spores of *B. licheniformis*, *B. subtilis* or *G. stearothermophilus* were subjected to plasma exposure to record their inactivation kinetics. For these experiments, the plasma's nitrogen-oxygen mixture ratio had been optimized for maximum UV emission (Fig. 3.1) at 80% nitrogen and 20% oxygen which was found to obtain higher sterilization efficiency than the previously used high-oxygen plasma (Hägele 2005). Also, the plasma was operated in pulsed mode to maintain the surface temperature of the vials at a material compatible level. No deformations or visible material damages of the vials were observed after plasma exposure.

For all organisms, the limit of detection (4 cfu per vial) for survivors was reached within 120 s of pulsed plasma exposure (Fig. 3.3). None of the inactivation curves followed a first order kinetics and therefore, no decimal reduction values could be calculated. Notably, each kinetics exhibited a portion of fast inactivation at its beginning followed by a portion of slower inactivation in the further course of plasma exposure. The highest inactivation in this first phase was observed for the spores of the bacilli (Fig. 3.3e) and cells of *M. luteus* (a) while it was lowest for the conidia (c) and deinococcal cells (d). Regarding this characteristic, cells and *R. glutinis* showed an intermediate resistance against plasma. One can speculate a plateau, as observed with *Deinococcus* in the results presented above (see 3.1.1), to be present from the reduced inactivation rate in the phase between 10 s and 60 s of plasma exposure (Fig. 3.3b).

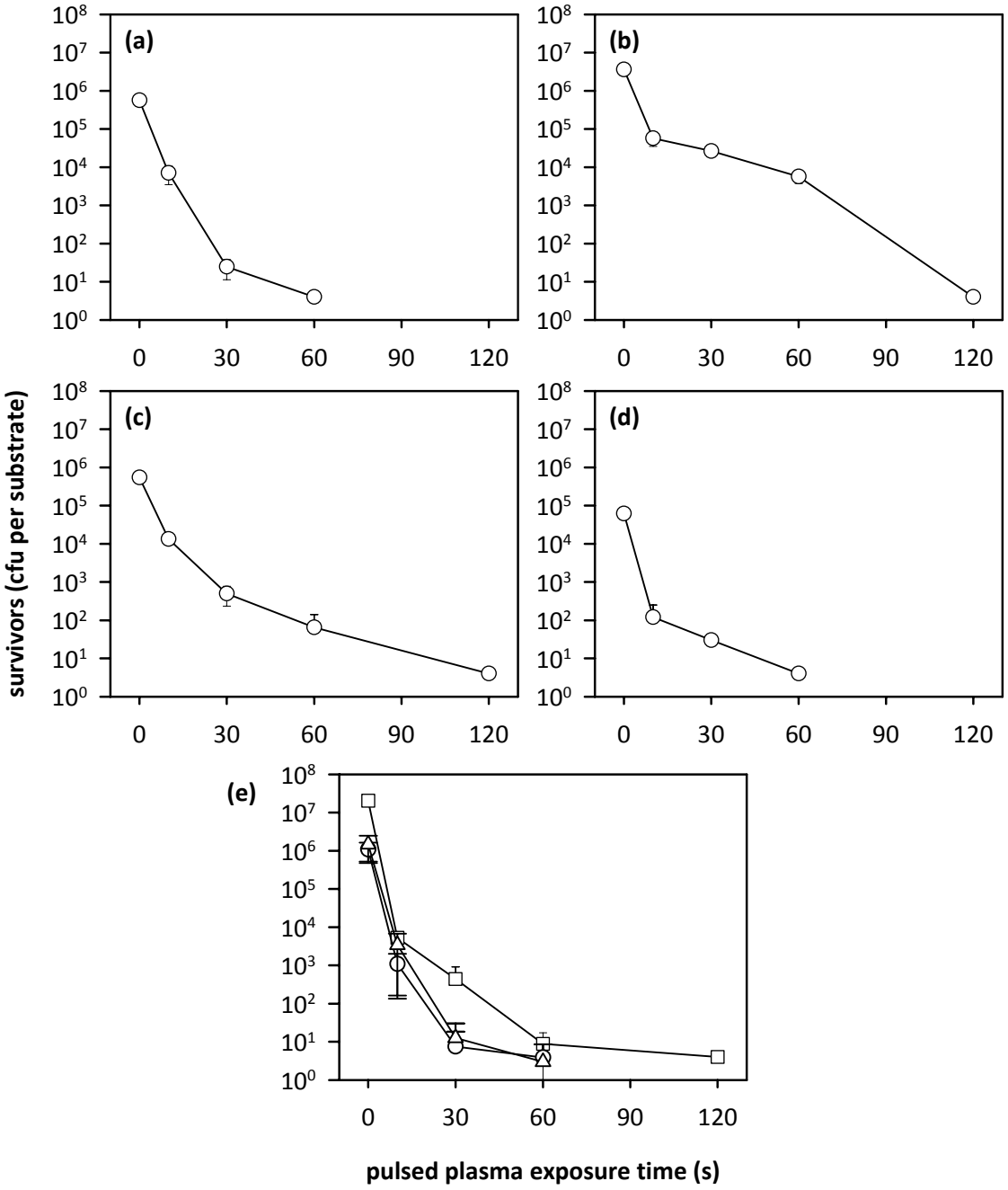


Fig. 3.3. Inactivation kinetics of *M. luteus* 10BG (a), *D. radiodurans* R1 (b), *A. niger* DSM 1988 conidia (c), *R. glutinis* H48 cells (d), and endospores (e) of *B. licheniformis* 90F (□), *G. stearothermophilus* DSM5934 (△), and *B. subtilis* 168 (○) dried on the inner bottom surface of cyclic olefin copolymer vials. The vials were plasma treated under rotation (3 min^{-1}) in a distance of 1 mm to the plasma source using the following parameters: microwave power 8 kW in pulsed mode (10 ms on, 90 ms off); pressure 70 Pa, process gas mixture 80% nitrogen and 20% oxygen. Error bars indicate ± one standard error of five replicates.

3.1.3 Comparison of the inactivation performance of two low-pressure microwave plasma systems

The results presented above provide information about the sterilization characteristics of the plasma system that enable comparison to data obtained by other workgroups with other plasma equipment. To provide a more detailed insight into the mechanisms of plasma sterilization, the approach to study the response of mutants of *B. subtilis* was chosen. Because the Bosch pilot plant lab was not authorized to work with these genetically modified organisms, it was necessary to transfer the experiments to the Food Microbiology lab of the Hohenheim University. There, the commercially available mobile plasma system M450 (Muegge, Reichelsheim, Germany) was used to perform the plasma exposures. The M450 was operated with the same parameters (gas composition, pressure, gas flow rates scaled down according to vessel volume ratios) and featured the same type of Duo-Plasmaline® microwave plasma source as the Bosch pilot plant plasma system DSA701.

To compare the sterilization performances of the DSA701 and M450 plasma systems, inactivation kinetics of *B. subtilis* wild-type spores were recorded (Fig. 3.4). To minimize the effect of heating, the plasma was manually switched off for 45 s after each 5 s of plasma exposure. In the plasma of the M450, the spores were inactivated more rapidly than in the pulsed plasma of the DSA701. However, considering that in the 30 s process duration, the pulse on phases sum up to only 3 s, the inactivation on the DSA701 was extremely efficient. Both inactivation curves exhibit two distinguishable phases with respect to the inactivation rate. Therefore, biphasic model fits were applied to the data sets with the GinaFiT tool (see 2.5). The parameters of the model fits are listed in Table 3.1 along with the calculated decimal reduction values *D* for the two phases.

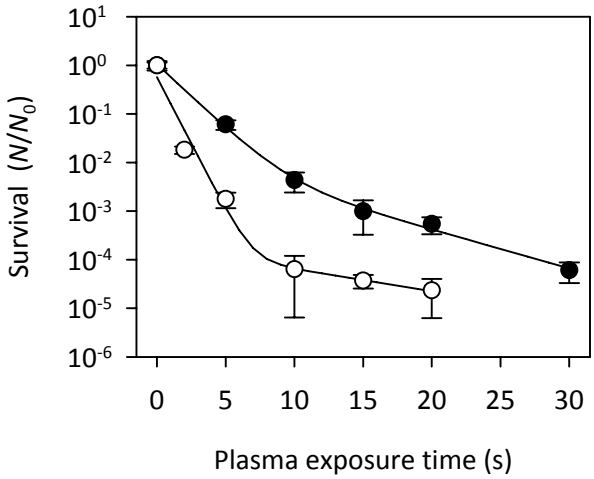


Fig. 3.4. Comparison of the inactivation kinetics of *B. subtilis* 1A757 endospores on glass slide substrates on the DSA701 (●) or on the M450 microwave plasma system (○). The DSA701 system was operated in pulsed mode (10 ms on, 90 ms off) and the M450 in continuous mode (but pausing the plasma for 45 s after each 5 s of exposure to minimize heating). Both systems were set to 4 kW microwave power and 80% nitrogen and 20% oxygen as process gas mixture at a pressure of 70 Pa. The lines represent biphasic model fits. Error bars indicate ± one standard error of at least four replicates.

Table 3.1. Parameter values f, k_1, and k_2 obtained by applying a biphasic model to the inactivation kinetics data of spores of *Bacillus subtilis* 1A757 shown in Fig. 3.4.

System	f	t_{Tr} [*] (s)	$N_{Tr} \cdot (N_0)^{-1}$ [†]	k_1 (s^{-1})	D_1 (s)	k_2 (s^{-1})	D_2 (s)
DSA701	0.984600	7.2	1.6×10^{-2}	0.6	3.7	0.2	12.6
M450	0.999674	6.9	1.9×10^{-4}	1.3	1.8	0.1	21.8

[*] Plasma exposure time at the transition from the first to the second inactivation phase as calculated from the model.

[†] Spore survival at the exposure time t_{Tr} as calculated from the model parameters.

The difference of the inactivation efficiencies of the two plasma systems is likely caused by an optimized design of the plasma source of the DSA701 concentrating the plasma at the location of the substrates whereas the plasma of the M450 is generated in a non-directed manner around the source. However,

since the setup and operation of both systems are basically identical and the inactivation kinetics both follow the same biphasic characteristic, the mechanisms participating in the inactivation process are expected to be the same.

3.2 Investigation of *Bacillus subtilis* spore inactivation

3.2.1 Effects of plasma treatment on DNA, membranes and proteins of spores

To investigate the effect of plasma treatment on membranes, DNA and proteins of spores, the leakage of DPA, generation of auxotrophic mutants, and spore specific catalase (KatX) activity, respectively, were determined in spores of *B. subtilis* 168, which have been subjected to plasma treatment under moderate conditions (5 cm distance from the plasma source, exposure up to 30 s). The results are compiled in Fig. 3.5.

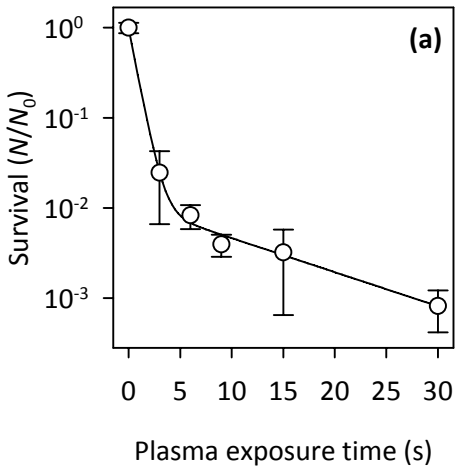


Fig. 3.5a. Effect of plasma treatment on viability of *B. subtilis* 168 endospores. Purified spores were lyophilized on glass slides and exposed to plasma at a distance of 5 cm to the plasma source. Error bars indicate ± one standard error. Initial spore count (N_0) was 1.2×10^8 cfu per substrate. The line represents the biphasic model fitted to the data. The experiments were performed on the DSA701 plasma system using the following parameters: microwave power 4 kW; pressure 70 Pa, process gas mixture 80% nitrogen and 20% oxygen.

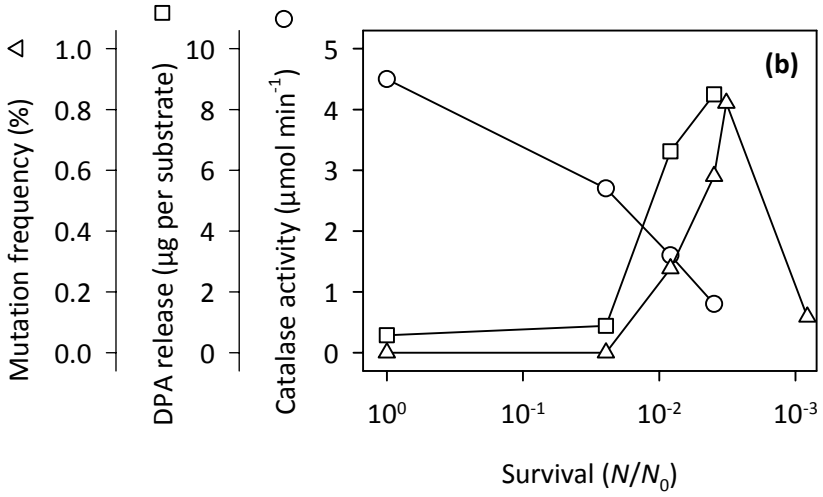


Fig. 3.5b. Effects of plasma treatment on dipicolinic acid (DPA) release, catalase activity, and mutation of *B. subtilis* 168 endospores. Survival data corresponds to Fig. 3.5a.

Viable spore counts decreased over plasma exposure time and after 30 s of treatment a reduction of 3 orders of magnitude was obtained. A biphasic model was applied to the inactivation data as described in 2.5 and resulted in a good fit (parameters $f = 0.988846$, $k_1 = 1.4\ s^{-1}$, $k_2 = 0.1\ s^{-1}$, $N_0 = 1.2\times10^8$; RMSE = 0,0977). This suggests an inactivation kinetics consisting of two distinguishable log-linear phases: a rapid inactivation phase ($D_1 = 1.7$ s) at the first 4.2 s of plasma exposure followed by a slow one ($D_2 = 26$ s). When spores were subjected to one evacuation and gas flow cycle without ignition of the plasma, no inactivation was observed.

Plasma treatment of the spores caused release of DPA, generation of auxotrophic mutants and reduction of KatX catalase activity. Monitoring of these effects was possible for the first 15 s plasma exposure only. Thereafter, the continued plasma exposure resulted in a degradation of organic material into volatile products. Although 97% of the spores were inactivated during the first 3 s of plasma exposure, only a minor release of DPA to the suspension medium was observed (Fig. 3.5b). In the subsequent phase up to 9 s of exposure, an enhanced DPA release was observed, while the inactivation rate of the spores was decreased. After 3 s, first auxotrophic mutants occurred and the mutation

frequency increased to a maximum of 0.82% at 15 s exposure. Further exposure revealed a remarkable drop in the mutation frequency, likely caused by the low surviving fraction of the spores and the resulting lack of sublethally injured spores. In contrast, the activity of KatX, a catalase present in the core of *B. subtilis* spores (Casillas-Martinez and Setlow 1997), decreased almost linearly with exposure time and fell under the limit of detection after 15 s of plasma exposure.

3.2.2 Inactivation kinetics of *B. subtilis* mutant spores

To further investigate the mechanism of action of plasma treatment, spores of the *B. subtilis* mutants PS3328, 1S111, 1A488 and 1A345 as well as of the wild-type strain 1A757 were generated and used for preparation of substrates. These substrates were treated at a distance of 2 cm to the plasma source, leading to faster spore inactivation compared to the experiment described above. As the substrates were placed well inside the plasma, the spore containing area normally appearing opaque became progressively clear during exposure for longer than 5 s, indicating a proceeding erosion of the biologic material. As shown in Fig. 3.6, the response of survival to plasma exposure time suggested a biphasic inactivation for all strains with a first phase (first 2.5 s to 5.9 s of plasma exposure) of rapid inactivation followed by a second phase of slow inactivation. Various models (Weibull-type, log-linear with tail, and biphasic) were applied using GInaFiT and the most appropriate fits were actually produced by the biphasic model of Cerf (1977). Biphasic model fits were applied to the inactivation kinetics data of the spores of the five *B. subtilis* strains and the parameters obtained are shown in Table 3.2.

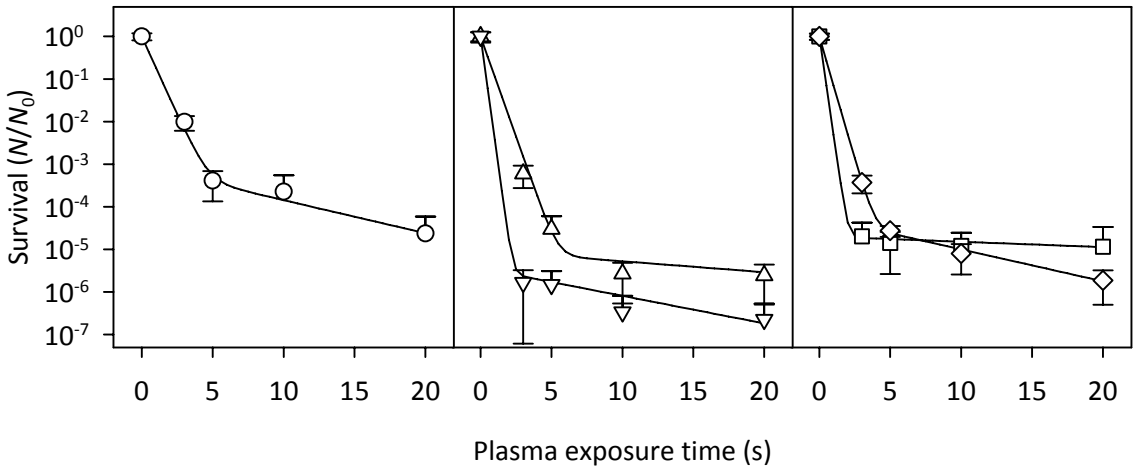


Fig. 3.6. Inactivation kinetics of spores of *B. subtilis* strains 1A757 (○), PS3328 (△), 1S111 (▽), 1A488 (◇), and 1A345 (□) on glass slide substrates obtained by direct plasma exposure on the M450 system with microwave power 4 kW; pressure 70 Pa, process gas mixture 80% nitrogen and 20% oxygen. The distance from plasma source to the substrates was 2 cm. Initial viable spore counts (N_0) before exposure were 1.5 ± 0.1 × 10^8 cfu per substrate for strains 1A757, PS3328, 1S111, 1A488 and 1.4 ± 0.2 × 10^7 cfu per substrate for strain 1A345. The line represents the biphasic model of inactivation fitted to the data points. Error bars indicate ± one standard error of at least four replicates.

Table 3.2. Parameter values f, k_1, and k_2 obtained by applying a biphasic model to the inactivation kinetics data of spores of *Bacillus subtilis* wild-type and mutant strains (shown in Fig. 3.6).

Strain	f	t_{Tr} [*] (s)	$N_{Tr} \cdot (N_0)^{-1}$ [†]	k_1 (s^{-1})	D_1 (s)	k_2 (s^{-1})	D_2 (s)
1A757	0.999143	4.5	8.6×10^{-4}	1.7	1.4	0.2	12.9
PS3328	0.999991	5.9	9.5×10^{-6}	2.2	1.1	0.1	[‡]
1S111	0.999996	2.5	3.6×10^{-6}	5.6	0.4	0.1	[‡]
1A488	0.999944	4.0	5.6×10^{-5}	2.6	0.9	0.2	[‡]
1A345	0.999980	2.5	2.0×10^{-5}	5.3	0.4	< 0.1	[‡]

[*] Plasma exposure time at the transition from the first to the second inactivation phase as calculated from the model.

[†] Spore survival at the exposure time t_{Tr} as calculated from the model parameters.

[‡] D_2 values were not calculated due to the higher error of spore counts near the limit of detection.

The use of the biphasic model allowed a more consistent estimation of D_1-values for the first inactivation phase because the transition between the two phases is mathematically defined and all available experimental data points are included in its calculation.

Based on the characteristics of the inactivation kinetics (Fig. 3.6, Table 3.2), differences in the resistance to plasma between the spores of the wild-type and mutant strain can be observed. Spores of the wild-type strain 1A757 showed the highest resistance resulting in $D_1 = 1.4$ s and $D_2 = 12.9$ s as determined from the biphasic model fit for the first and second inactivation phase, respectively. Spores of the *cotE* (PS3328) and *splB* (1A488) mutants exhibited an intermediate resistance in the first phase with D_1 values of 1.1 s and 0.9 s, respectively, while spores of the *uvrA* mutant (1A345) and the $\alpha^-\beta^-$ mutant (1S111) were highly sensitive. Due to their extremely fast inactivation, there is no data point in the middle of the first phase. Therefore, the estimation of the D_1 values to 0.4 s by the model is accompanied with a greater uncertainty. Due to low spore counts at the end of the second inactivation phase, determination of useful D_2 values was impeded by the limit of detection.

3.2.3 Spore inactivation by fractions of the plasma UV emission spectrum

To investigate the role of radiation involved into spore inactivation in more detail, various optical long-pass filter plates were introduced 2 mm in front of the substrates (Fig. 2.3). This prevented access of short-wavelength-photons and plasma-generated reactive particles. In this setup, spores of the wild-type strain 1A757 and mutant strains PS3328, 1S111, 1A488, and 1A345 were treated in equal plasma processes (10 s exposure at 2 cm distance to the plasma source) but filters with different 50% transmission cut-off wavelengths ranging from 125 nm to 305 nm were used. The plasma used in our experiments emitted UV radiation in the spectral ranges of UV-C (200 nm to 290 nm) as well as of UV-A+B (290 nm to 320 nm + 320 nm to 400 nm) (Fig. 3.7).

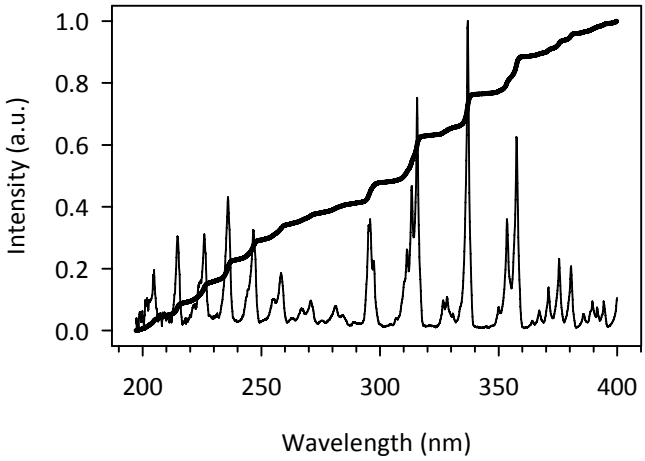


Fig. 3.7. UV-A/B/C Emission spectrum (thin line) of the plasma obtained on the DSA701 system with microwave power 4 kW; pressure 70 Pa, process gas mixture 80% nitrogen and 20% oxygen. The spectrum is normalized to the highest peak at 337 nm and integrated intensity (thick line) is normalized to its maximum. (a.u.: arbitrary units)

As depicted in Fig. 3.8a, the wild-type spores were inactivated less than one log when radiation of wavelengths smaller than 280 nm was excluded. Radiation between 215 nm and 280 nm caused inactivation of more than 3 logs. No difference in spore inactivation was observed when filters with cut-off wavelengths below 215 nm were used as well as when direct plasma access (without filter) was allowed. Spores of the *cotE* mutant strain PS3328 exhibited a spectral inactivation response comparable to that of the wild-type spores. However, direct (unfiltered) plasma exposure resulted in a one log higher inactivation of PS3328 spores than of wild-type spores. On the contrary, PS3328 spores tended to slightly higher survival than wild-type spores when filters with cut-off wavelengths lower than 280 nm were used. This observation has also been reported for UV-inactivation of *cotE* mutant spores and it may be caused by the effect CotE may have on the synthesis or transport of UV resistance factors during the formation of the spores (Riesenman and Nicholson 2000).

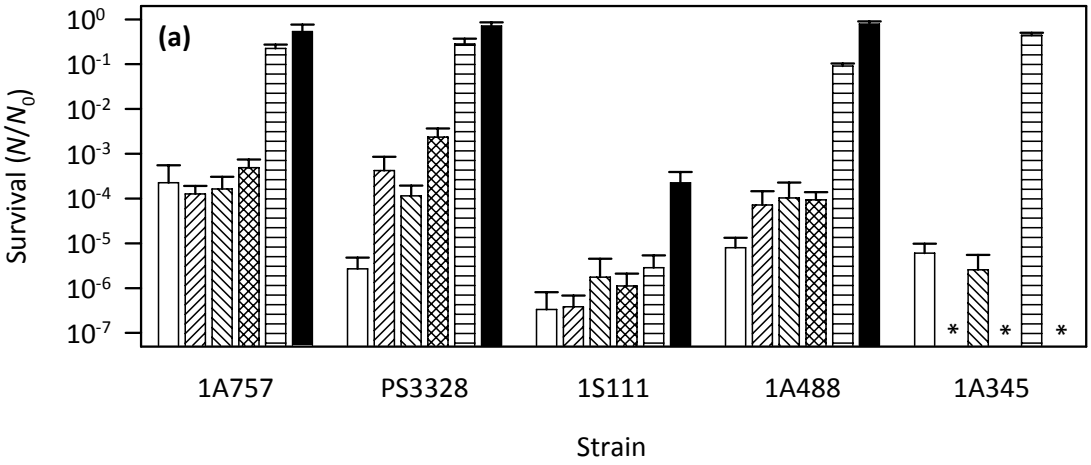


Fig. 3.8a. Inactivation of spores of *B. subtilis* wild-type (1A757) and mutant strains by plasma exposure without shielding (□) and with shielding of the substrates from the plasma-generated activated particles and parts of the emission spectrum by optical long pass filters MgF_2 (▨), SiO_2 (▧), BG24A (▩), WG280 (▤), and WG305 (■). The 50% transmission cut-off wavelengths of these filters were 125 nm, 164 nm, 215 nm, 280 nm, and 305 nm, respectively. All substrates were exposed for 10 s in a distance from the plasma source of 2 cm. (*: not determined for filters MgF_2, BG24A, and WG305).

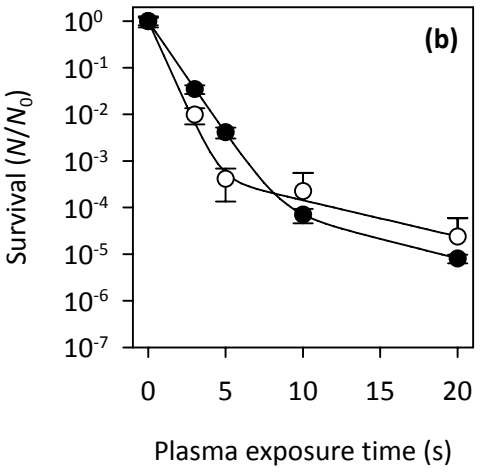


Fig. 3.8b. Comparison of inactivation kinetics of *B. subtilis* 1A757 spores without shielding (○) and with shielding (●) of the substrates by the SiO_2 optical filter during plasma exposure at 2 cm distance from the plasma source. The line represents the biphasic model of inactivation fitted to the data points. Initial viable spore counts (N_0) before exposure were $1.2 \pm 0.6 \times 10^8$ cfu per substrate. In (a) and (b), error bars indicate ± one standard error of at least four replicates. Experiments were performed on the M450 system with microwave power 4 kW; pressure 70 Pa, process gas mixture 80% nitrogen and 20% oxygen

The strongest inactivation was observed with spores of the $\alpha^-\beta^-$ mutant strain 1S111 when filters with cut-off wavelengths of 280 nm and lower were used. In contrast to the observations with spores of the other strains, 1S111 spores still

showed a 3.7 log inactivation when covered with 305 nm long pass filters. Survival of spores of the *splB* mutant strain 1A488 carrying a defect in the spore photoproduct repair pathway was comparable to that of the wild-type and *cotE* mutant spores, when filters were applied. However, the spores of strain 1A488 were still more susceptible to direct plasma exposure than the wild-type spores. Spores of the nucleotide excision DNA repair defective mutant 1A345 exhibited the most pronounced difference in inactivation between plasma treatments using the 280 nm and 164 nm long-pass filters. To investigate the effect of filters on spore inactivation in the different phases of inactivation, an inactivation curve was determined using the SiO_2 optical filter and spores of the wild-type strain *B. subtilis* 1A757. The biphasic model was also applicable to these data with good fitting (Fig. 3.8b, parameters Table 3.3). Use of the filter caused a prolongation of the initial inactivation phase (from 4.5 s to 7.3 s) resulting in an increase of D_1 to 2.1 s. In the second inactivation phase, spore survival as well as the D_2 value was not affected significantly.

Table 3.3. Biphasic model fit parameters related to the inactivation kinetics data of spores of *B. subtilis* wild type strain 1A757 with and without optical filters (see Fig. 3.8b).

Filter	f	t_{Tr} * (s)	$N_{Tr} \cdot (N_0)^{-1}$ †	k_1 (s^{-1})	D_1 (s)	k_2 (s^{-1})	D_2 (s)
(none)	0.999143	4.5	8.6×10^{-4}	1.7	1.4	0.2	12.9
SiO_2	0.999616	7.3	3.8×10^{-4}	1.1	2.1	0.2	11.9

* Plasma exposure time at the transition from the first to the second inactivation phase as calculated from the model.

† Spore survival at the exposure time t_{Tr} as calculated from the model parameters.

3.2.4 Impact of plasma exposure on spore DNA

Since DNA is likely to be a target for spore inactivation, the effect of plasma treatment on the damage of DNA was investigated monitoring the destruction of a particular chromosomal DNA fragment. After exposure of spores of *B. subtilis* 1A757 to plasma (10 s) and extraction of DNA, the concentration of the 400 bp target DNA fragment was determined using quantitative real-time PCR.

Plasma exposure may affect the quality and efficiency of DNA extraction from spores (e.g. by generating cross-links between DNA and other spore core components), which may in turn unpredictably influence the detection of target DNA fragments by PCR. To account for this, a ratio detection system (Bauer, *et al.* 2004) was established, wherein the target fragment copy numbers q_{400} were normalized to those of an internal sub-fragment of 101 bp (q_{101}). The resulting ratio is equal to 1 for non-degraded DNA and attains lower values for increasing degrees of degradation. Since the correlation between the actual degree of DNA damage (in lesions per base pair) and the ratios is unknown, this method was used for qualitative evaluation only. As shown in Fig. 3.9, when the substrates were shielded with the WG280 long-pass optical filters (50% cut-off wavelength 280 nm), the DNA damage was moderate corresponding to the weak spore inactivation.

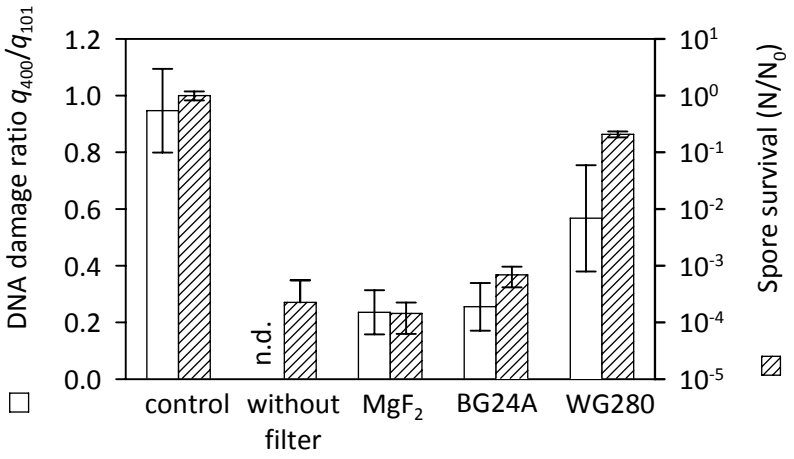


Fig. 3.9. Damage in DNA extracted from *B. subtilis* 1A757 spores after plasma exposure expressed as ratio of the copy numbers of a 400 bp target fragment (q_{400}) and an internal control fragment of 101 bp length (q_{101}) detected by quantitative real-time PCR (□). For comparison, spore survival of the plasma treatments is shown (▨). The spores were plasma treated on glass-slide substrates with and without shielding of the substrates using the optical filters indicated (for details on the filters and exposure conditions see legend of Fig. 3.8a). In the treatment without filters, the spore biomass was eroded so heavily that no detectable traces of DNA could be purified from the samples and therefore the q_{400}/q_{101} ratio could not be determined (n.d.). Error bars indicate ± one standard error of four replicates.

Plasma treatment using filters BG24A and MgF_2 (cut-off below 215 nm and 164 nm, respectively) resulted in a high degree of DNA degradation. Comparing the effects of these two filters, although spore survival decreased when using the lower cut-off wavelength filters, the DNA degradation remained constant (q_{400}/q_{101} = 0.25 and 0.24 for the treatment using filters BG24A and MgF_2, respectively).

To differentiate the effects of UV radiation and interaction of plasma particles on the biologic material, wild-type spores were plasma treated with varying distance to the plasma source either unshielded or shielded with UV transparent filters. When plasma-exposed without shielding, the survival of the spores increased if the distance to the plasma source was increased (Fig. 3.10a).

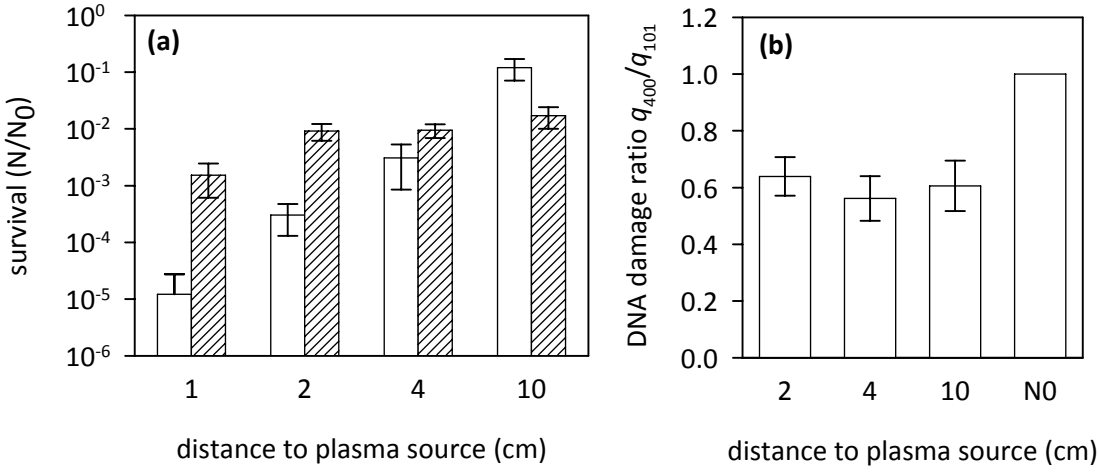


Fig. 3.10. (a) Survival of *B. subtilis* 1A757 spores after 5 s of plasma exposure without shielding (□) and shielded with filter MgF_2 (▨) at various distances to the plasma source. (b) Damage in DNA extracted from *B. subtilis* 1A757 spores after plasma exposure without shielding at the indicated distances to the plasma source. For details on the expression of DNA damage as ratio see Fig. 3.9. (N_0: untreated control). Plasma treatments were done on the M450 system with microwave power 4 kW; pressure 70 Pa, process gas mixture 80% nitrogen and 20% oxygen

Since the volume density of activated particles decreases with increasing distance due to recombination of particles (Kaiser, *et al.* 1999), this indicates that these reactive particles take a distance-dependent part in the inactivation of the

spores. In contrast, survival was nearly constant at all distances greater than 2 cm when the substrates were shielded under UV-transparent filters. This observation indicates that – in contrast to the plasma density – the UV flow is nearly constant in the M450 plasma system with increasing distance to the plasma source. This is likely because of the great area of the plasma source related to the size of the substrates and the reflective inner walls of the evacuable vessel. The effect that at 1 cm distance the spore survival was slightly lower than at the greater distances is likely due to the high plasma density near the plasma source. This seems to cause a significant flow of reactive particles through cracks in the substrate mount and these particles can interact with the substrates below the filter plates.

Interestingly, while at the survival of the spores was greatly influenced by the distance, the amount of DNA damage in the spores was not influenced when they were plasma exposed without shielding (Fig. 3.10b). In combination with the observation that the amount of inactivation caused by UV radiation alone is also constant, this supports the finding that DNA damage in the plasma is mostly effected by UV radiation and not by plasma-generated reactive particles.

Under the conditions chosen for these experiments, about 99% of the spores were inactivated due to DNA damages caused by plasma-generated UV radiation. The additional inactivation that is seen with direct plasma access at distances of 2 cm and below is likely to be due to injuries other than DNA damage and it seems to be caused by agents other than UV radiation.

3.3 Response of *Deinococcus radiodurans* to plasma exposure

3.3.1 Inactivation kinetics

To determine the kinetics of inactivation, glass slide substrates with dried *D. radiodurans* cells were subjected to nitrogen-oxygen plasma treatment on the M450 plasma system with varying exposure times. The plasma process conditions (see section 2.3) used for the experiments were found to be highly

effective in the inactivation of *B. subtilis* endospores (see 3.2.2). As shown in Fig. 3.8, the viable cell count was reduced by six orders of magnitude within 60 s of plasma exposure.

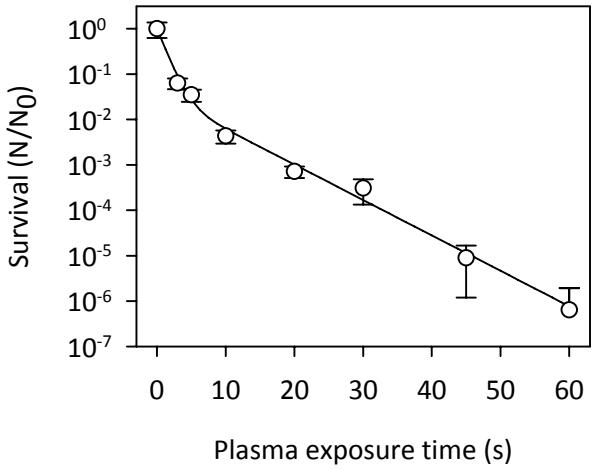


Fig. 3.11. Inactivation kinetics of *D. radiodurans* R1 (○) on glass slide substrates obtained by plasma treatment. Initial viable cell count (N_0) was $8 \pm 2 \times 10^6$ cfu per substrate. The line represents the biphasic inactivation model fitted to the data points. Error bars indicate ± one standard error. Plasma treatments were performed on the M450 system with microwave power 4 kW; pressure 70 Pa, process gas mixture 80% nitrogen and 20% oxygen.

The data suggested an inactivation kinetics consisting of two distinguishable log-linear phases: a rapid inactivation phase at the beginning of plasma exposure followed by a slow one. Thus, using the GinaFiT tool, the biphasic log-linear model (see section 2.5) was applied to the data and produced a good fit. *D* values of 2.6 s and 12.8 s for the first and the second inactivation phase, respectively, as well as the phase transition point at 4.4 s of plasma exposure were calculated from the model parameters $f = 0.963104$, $k_1 = 0.9\ s^{-1}$, $k_2 = 0.2\ s^{-1}$, and $N_0 = 7.2 \times 10^6$.

3.3.2 Recovery from sublethal injuries caused by plasma treatment

When culturing plasma-exposed *D. radiodurans* cells on DT agar, great variations in colony size were observed (Fig. 3.12), compared to non plasma treated cells forming regular sized colonies.

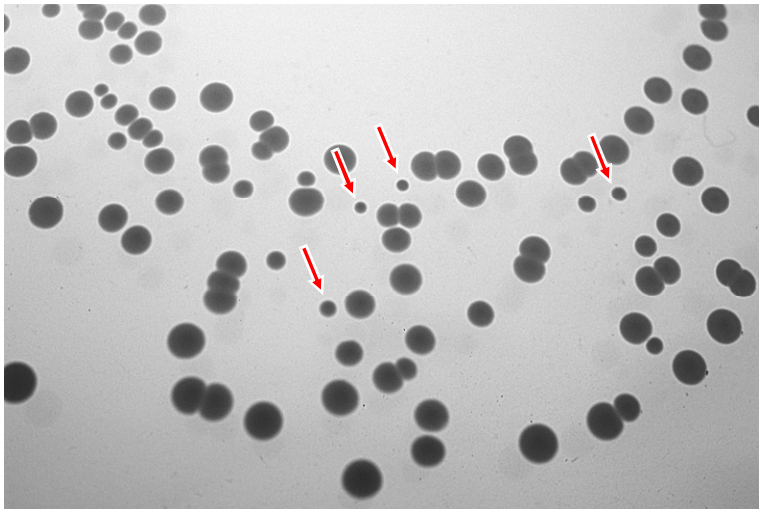


Fig. 3.12. Photograph of *D. radiodurans* R1 colonies on a DT agar plate after 18 h incubation at 32°C. The cells were plated directly after plasma treatment. Arrows mark examples of colonies with aberrant sizes indicating reduced growth rates and/or prolonged lag phases.

This indicates that a part of the survivors exhibited reduced growth rates and/or prolonged lag phases due to injuries caused by the plasma treatment. To investigate these sublethal injuries in more detail, *D. radiodurans* cells were plasma treated on glass substrates for only 5 s on the M450 plasma system, to yield enough survivors. The biomass from the substrates was then resuspended in DT medium and incubated. To monitor the cells' recovery, samples from the DT medium were taken periodically and cell counts were determined on stress DT-agar and compared to that obtained on standard DT-agar (see section 2.8). For the stress DT-agar, a stressor supplement was added to standard DT-agar, which is able to selectively prevent the growth of plasma-injured cells. As stressor supplements bile salts, acetate, nalidixic acid, and mitomycin C were tested , but 150 mmol l^{-1} NaCl was found to be most suitable (data not shown). In addition, to be able to differentiate between recovery processes requiring

fatty acid synthesis, synthesis of proteins (translation), construction of cell walls, and synthesis of RNA (transcription), plasma treated cells were incubated in DT medium containing a specific inhibitor. As inhibitors the antibiotics cerulenin (final concentration 25 μg ml^{-1}), chloramphenicol (1 μg ml^{-1}), penicillin G (0.5 μg ml^{-1}), and rifampicin (0.5 μg ml^{-1}), respectively, were used. The concentrations of the antibiotics were determined to be bacteriostatic for non plasma treated cells (data not shown). The cell counts obtained in the incubations are summarized in Fig. 3.13. When no plasma treatment was performed, the cell counts on stress and standard medium were similar (Fig. 3.13a). By extrapolating the exponential data points, the lag phase was estimated to last for approximately 90 min. This delay might be caused by the rehydration of the cells and repair of injuries caused by desiccation. The plasma treatment was survived by approximately 6% of *D. radiodurans* cells (Fig. 3.13b). Of these survivors, 83% were unable to form colonies on stress medium at the beginning of the recovery. However, in the course of recovery the ability to grow on stress medium was restored, as indicated by similar counts obtained on stress and standard agar. In the presence of antibiotics, the cell counts determined by plating on standard agar generally remained unchanged or decreased during recovery (Fig. 3.13c-f). This indicates that a part of the plasma-treated cells, presumably the sublethally injured ones, were susceptible to the antibiotics to a greater or lesser extent. In the presence of the fatty acid synthesis inhibitor cerulenin (Moche, *et al.* 1999), the cell counts determined on stress agar after recovery attained approximately the same level as on standard agar (Fig. 3.13c). When penicillin G, an inhibitor of bacterial cell wall formation, was used cell counts slightly increased during the initial phase of recovery but then decreased in the further course (Fig. 3.13e). In the presence of the translational and transcriptional inhibitors chloramphenicol (Fig. 3.13d) and rifampicin (Fig. 3.13f), respectively, no recovery of the plasma treated cells could be observed when plating on stress agar.

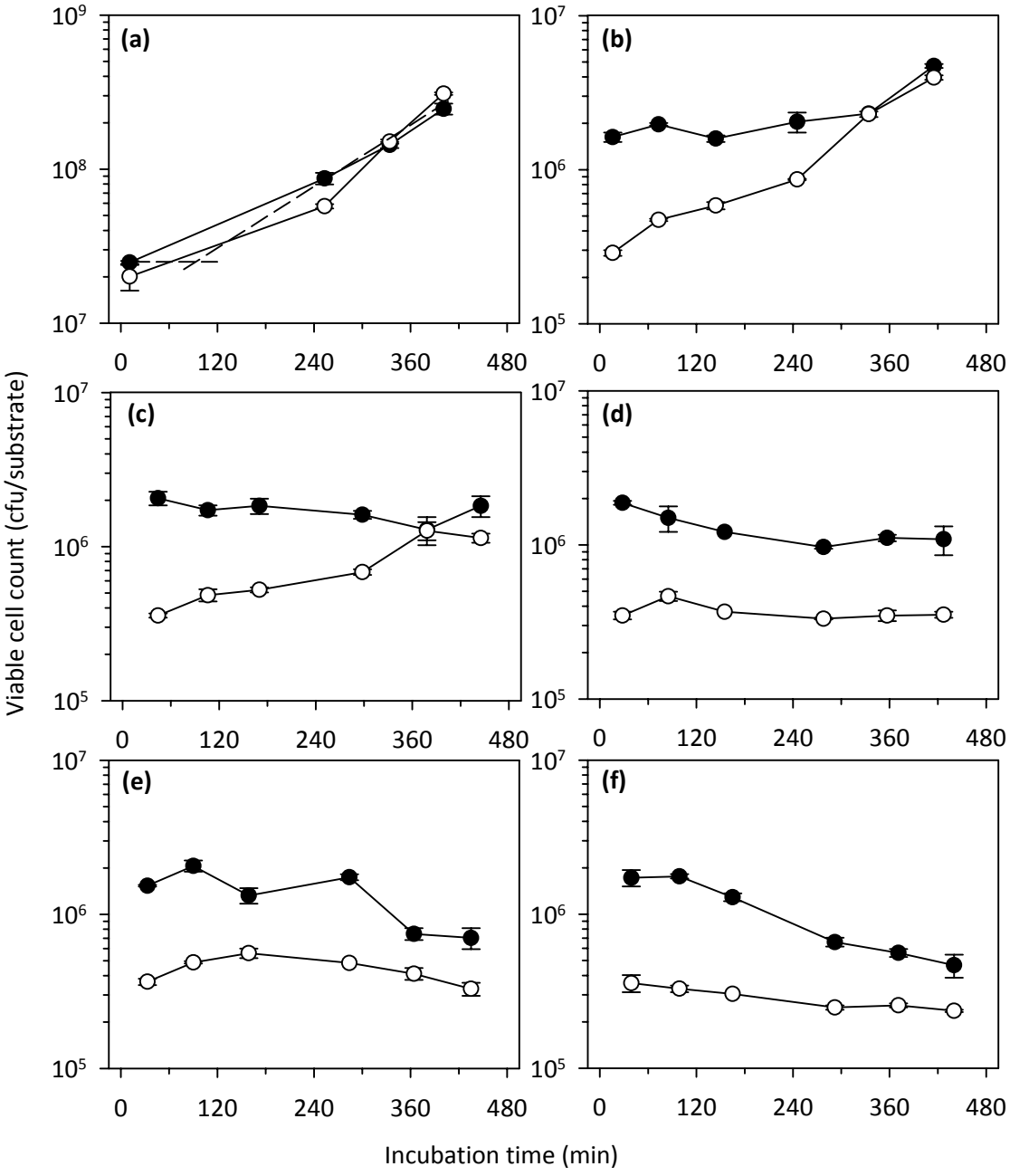


Fig. 3.13. Recovery of untreated (a) and plasma treated (b to f) *D. radiodurans* R1 cells in DT medium or DT medium supplemented with bacteriostatic concentrations of cerulenin (c), chloramphenicol (d), penicillin G (e), and rifampicin (f), as monitored by determination of the viable cell counts on stress DT-agar (○) and standard DT-agar (●). Dashed lines: Extrapolation of exponential data points to estimate the lag phase duration for untreated cells. Error bars indicate ± one standard error. Substrates were plasma treated for 5 s on the M450 system with microwave power 4 kW; pressure 70 Pa, process gas mixture 80% nitrogen and 20% oxygen.

3.3.3 Transcription of repair related genes during recovery of cells

To gain insight into the processes necessary for recovery of the *D. radiodurans* R1 cells, the transcription of seven genes (Table 2.3) involved in stress response was monitored in cells after plasma treatment under the process conditions given in Table 3.4. For normalization, the transcript of DR1343, a housekeeping gene coding for glyceraldehyde-3-phosphate dehydrogenase was used whose expression had been shown to be stable in experiments with ionizing irradiation (Earl, *et al.* 2002a, Tanaka, *et al.* 2004). Since normalization to a single gene normally limits the accuracy of measured expression levels, the levels were used for the purpose of internal qualitative comparison only.

Table 3.4. Plasma process conditions used for investigation of the recovery phase of *D. radiodurans* R1 cells. All treatments were performed on the M450 plasma system at 70 mbar and 4 kW microwave power.

Process condition	Plasma exposure time (s)	Plasma gas composition N_2/O_2 % (v/v)	Optical filter
P1	5	80/20	none
P2	10	80/20	quartz glass
P3	5	10/90	none

As shown in Fig. 3.14, the survival of the cells after plasma treatment was comparable (0.7 to 1.4%) for each of the process conditions. Likewise, the recovery of sublethally injured cells treated under the different process conditions followed rather similar courses. Recovery was completed after 330 min (Fig. 3.14b and d) or 480 min (Fig. 3.14c) of incubation in DT medium after plasma treatment with process conditions P1 and P3, or P2, respectively.

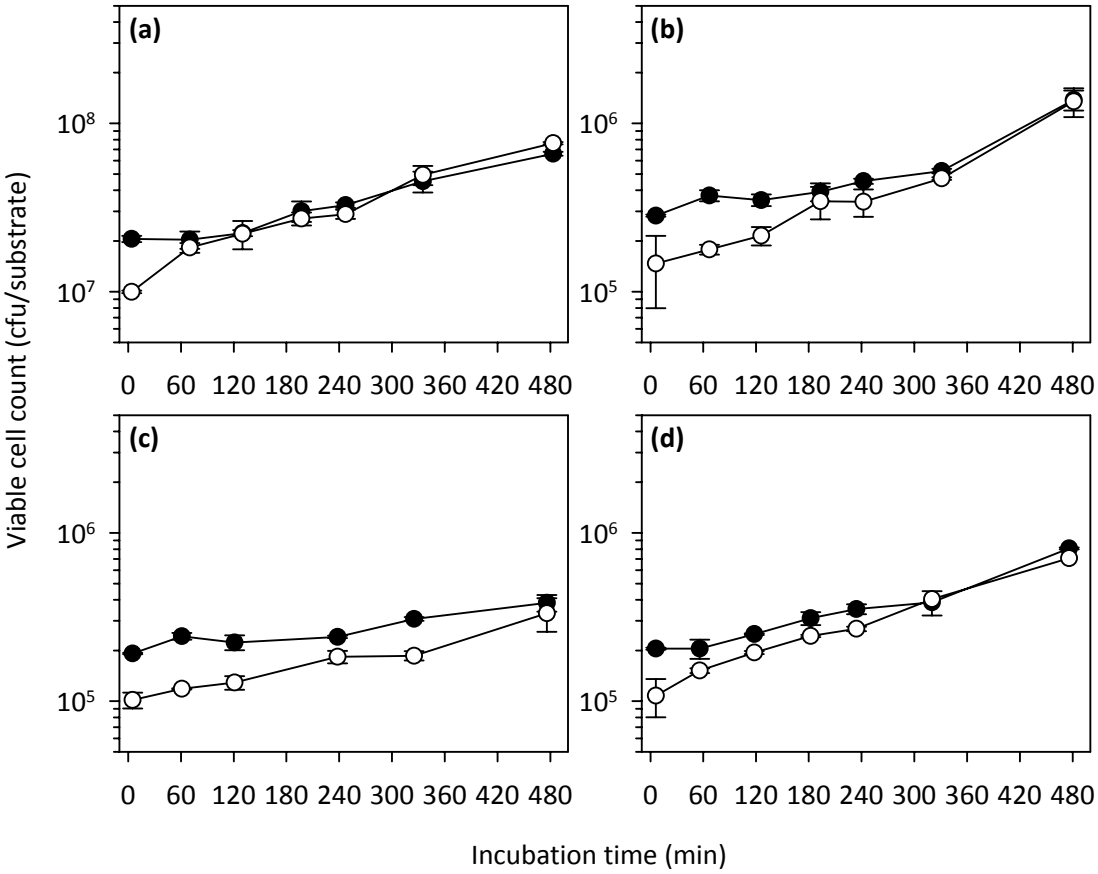


Fig. 3.14. Recovery of *D. radiodurans* R1 cells in DT medium after plasma exposure using process conditions P1 (b; see Table 3.4), P2 (c), or P3 (d) or without any treatment (a), as monitored by subsequent determination of the viable cell counts on stress (○) and standard agar (●). Error bars indicate ± one standard error.

With each of the three process conditions, an increase of transcription of the four genes DR2340 (*recA*), DR1913 (*gyrA*), DR2220, and DR0479 could be detected, however their transcriptional profiles differed substantially (Fig. 3.15). After plasma treatment P1, a significant up-regulation of these four genes was observed after 60 min of incubation reaching a maximum between 120 min and 180 min (Fig. 3.15a). At the end of recovery, the transcription of DR0479, DR1913, and DR2220 reached their initial levels, whereas the transcription of DR2340 remained increased, as it was also observed for plasma treatments P2 and P3. The highest increase of transcription was observed after plasma

treatment P2, with a rather late maximum between 240 min and 360 min of incubation (Fig. 3.15b). Plasma treatment P3 generally caused the lowest up-regulation, but the maximum transcription levels were detected earliest at 60 min for DR0479, DR 1913, and DR2220, and at 120 min for DR2340 (Fig. 3.15c). Under all plasma treatment conditions, no up-regulation of transcription was detectable for genes DR0261, DR1819, and DR2275. The experiments were repeated independently, leading to qualitatively comparable results.

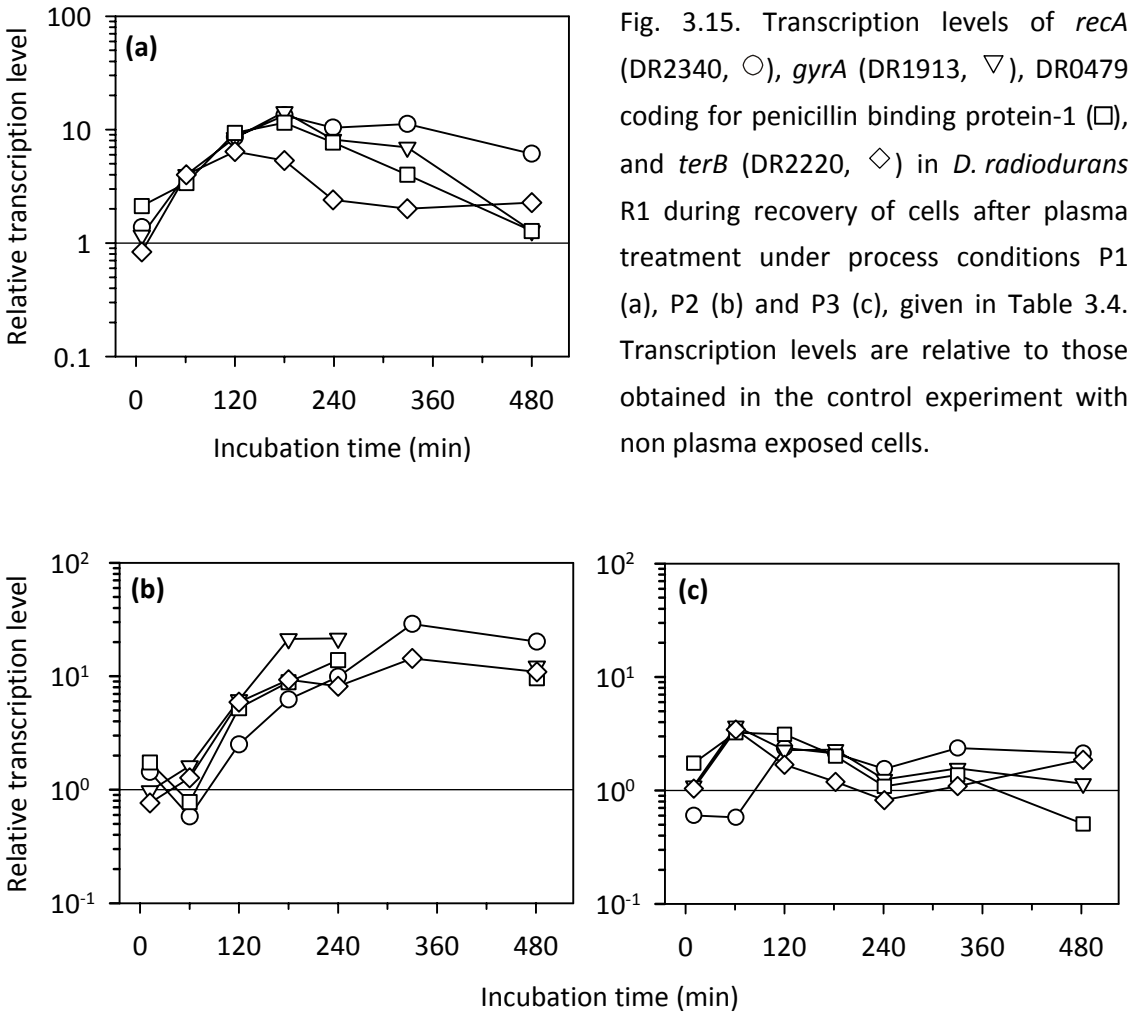


Fig. 3.15. Transcription levels of *recA* (DR2340, ○), *gyrA* (DR1913, ▽), DR0479 coding for penicillin binding protein-1 (□), and *terB* (DR2220, ◇) in *D. radiodurans* R1 during recovery of cells after plasma treatment under process conditions P1 (a), P2 (b) and P3 (c), given in Table 3.4. Transcription levels are relative to those obtained in the control experiment with non plasma exposed cells.

3.3.4 DNA repair during recovery of cells

The investigation of recovery of *D. radiodurans* cells after plasma treatment indicated that transcription of genes involved in DNA repair (*recA*, *gyrA*) were induced. To provide direct evidence for DNA repair, the degree of DNA damage was monitored during recovery of cells by determining the ratio q_{510}/q_{144} between the detectable copy numbers of a chromosomal target DNA fragment and an internal reference fragment using quantitative PCR. As shown in Fig. 3.16, the level of DNA damage caused by the plasma treatment depended on the process conditions applied.

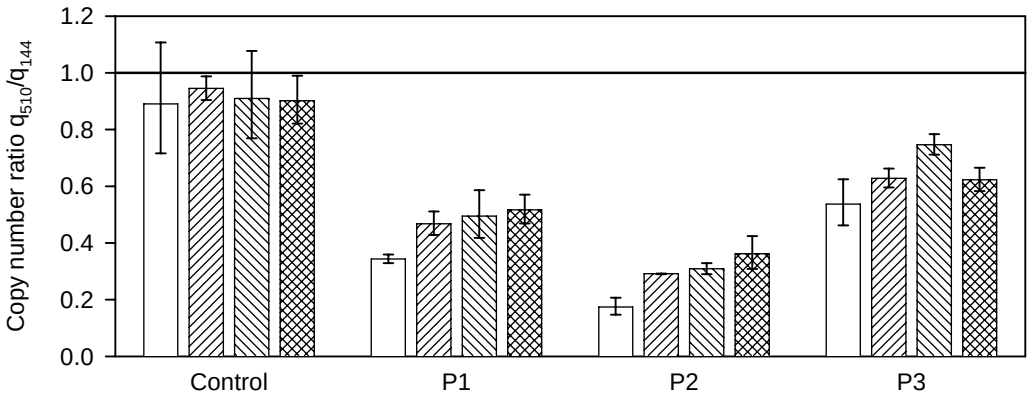


Fig. 3.16. Damage in chromosomal DNA extracted from *D. radiodurans* R1 during recovery of the cells in DT medium expressed as ratio q_{510}/q_{144} indicating the copy numbers of a 510 bp fragment and an internal 144 bp control fragment of the DR1343 locus detected by quantitative real-time PCR. The cells were plasma treated under process conditions P1, P2, and P3 (see Table 3.4) or remained without plasma treatment (Control). DNA was extracted after 0 min (□), 120 min (▨), 240 min (▧), and 480 min (▩) of incubation in DT medium. Error bars indicate ± one standard error of q_{510}/q_{144}.

The initial level of damage was highest for the process conditions P2 (as indicated by the lowest Q values), intermediate for P1 and lowest for P3. Moreover, for cells treated with the highly UV emitting plasmas generated under process conditions P1 and P2, a decrease of DNA damage was detectable in the first phase (120 min) of recovery. Cells subjected to treatment P3 plasmas

featuring high erosion rates but less UV intensity showed an only moderate reduction of DNA damage in a later phase (240 min) of recovery. For all process conditions, even after prolonged recovery for 480 min, the DNA damage did not revert to the levels obtained in the controls. While this observation is consistent with the transcription profiles for DR2340 (*RecA*) (see section 3.3.3), it is most likely caused by the irreversibly injured (killed) cells present in the recovery suspension.

4 Discussion

In the presented work, three major series of experiments were performed in order to characterize sterilization by low-pressure low-temperature nitrogen-oxygen plasmas and to elucidate the major effects caused by those plasmas on microorganisms.

In all experiments microwave-induced low-pressure low-temperature nitrogen-oxygen plasmas were used. Due to the plasma chemistry of the nitrogen-oxygen mixture, such plasmas produce a number of agents likely to be active in sterilization. The most important agents are radiation in the UV ranges A to C and below (vacuum UV with wavelength < 200 nm) as well as reactive particles such as nitric oxide, atomic oxygen, ozone, and radicals of peroxide, superoxide, or hydroxyl (Boudam, *et al.* 2006). The UV radiation is considered to be the main driving agent in plasma sterilization processes by directly causing damage in the nucleic acids of biologic contaminants (Moisan, *et al.* 2001). Such UV-induced damages include pyrimidine dimers, but also strand breaks (Friedberg, *et al.* 1995, Slieman and Nicholson 2000). Reactive particles were shown to be involved in inactivation of microorganisms by reacting with proteins (Gaunt, *et al.* 2006). In addition, plasmas can produce the less specific effect of erosion. It is reported that erosion is caused by two processes (Moisan, *et al.* 2001): First, photodesorption defined as cleavage of chemical bonds by high-energy photons resulting in formation of volatile compounds and second, etching defined as chemical reaction of plasma-generated reactive particles with biomolecules resulting in slow combustion into volatile products. Plasmas also

cause selective heating of biomaterials due to absorption of energy from plasma-emitted radiation as well as from exothermic reactions with plasma-activated particles on their surface (Cvelbar, *et al.* 2006). The properties of the plasma with respect to UV emission intensity and volume concentration of reactive particles can be varied by changing the relative composition of the nitrogen-oxygen mixture. For the plasma system used in the presented work, an optimum of UV emission intensity was achieved with 20%(v/v) of oxygen (Fig. 3.1). With higher amounts of oxygen, the amount of reactive oxygen species increases while the UV emission intensity decreases (Rossi, *et al.* 2006). Basing on the properties of these plasmas, Moisan *et al.* (2001) proposed a general model in order to explain the observed characteristic shapes of the inactivation kinetics featuring at least two phases with different inactivation rates. In this model, assuming that the biologic contamination is present in multiple layers, the occurrence of the first phase of rapid killing is explained by the primary action of UV radiation on the DNA of the organisms in the top layer, while in the subsequent phase the killing rate is limited by the comparably slow process of erosion to make the lower layers accessible for the UV radiation (for details, see Fig. 1.4). The results obtained in the present study are discussed with respect to this inactivation model.

In the *first series* (section 3.1), it was shown that plasma treatment is able to rapidly and efficiently sterilize a variety of generally resistant microbial life forms, including spores of *Bacillus* sp., conidia of *Aspergillus niger*, and cells of *Deinococcus radiodurans*. The inactivation kinetics of these organisms showed very dissimilar characteristics (Fig. 3.2). While a shoulder was observed for the deinococcal cells and a first order kinetics for the conidia, the spores exhibited an extremely fast inactivation at the beginning and a tailing at the end of the treatments. The characteristics of the inactivation kinetics can be related to the properties of the high-oxygen plasma used in these experiments. This plasma was initially optimized for depyrogenation and it features high erosion rates but has a relatively low UV emission intensity (Fig. 3.1). Assuming that the erosion rate is the limiting factor for the inactivation of microorganisms by these plasmas, the different characteristics of inactivation observed might

correlate to the amount of biomaterial to be removed before an individual germ is actually inactivated. The relation of the diameters of *A. niger* conidia and *D. radiodurans* tetrads (both 3 to 5 µm) to that of *Bacillus* spores (1 µm) might account for this phenomenon. A similar behavior is reported for heavily stacked spores, where inactivation is most efficient with highly eroding plasmas (Rossi, *et al.* 2006). The difference in heating of the substrates between pulsed (less heating) and continuous plasma operation (more heating) is also well reflected in the results: The operation mode did not influence the inactivation of the bacterial spores, which are well known for their high resistance to dry heat (Nicholson, *et al.* 2002), whereas the heat sensitive deinococcal cells and conidia did respond to the heating effects of the plasma by exhibiting an increase of their inactivation rates in continuous plasma mode (comparing Fig. 3.2 and Fig. 3.3). Bacterial spores were most rapidly inactivated though they are known to be highly UV resistant. This indicates that agents other than UV radiation are dominating this inactivation process. Such agent may include reactive particles present in the plasma.

Plastic vials were also successfully sterilized by plasma treatment. In these experiments, a plasma with the nitrogen-oxygen ratio optimized for maximum UV emission was used (Hägele 2005). The resulting inactivation kinetics featured a common characteristic: For all organisms concave shaped curves with an initial portion of rapid inactivation following by a second portion of slower inactivation were observed (Fig. 3.3). This behavior is in agreement with the model of Moisan *et al.* (2001) for sterilization by highly UV-emitting plasmas (see section 1.2). According to this model the organisms accessible to UV radiation are rapidly inactivated. Before organisms unreachable to UV radiation, e.g. stacked as layers or clumps or embedded in a biofilm, can be inactivated, removal of the covering layers by erosion is required. Erosion is a slow process limiting the inactivation rate. Thus, a second phase of slow inactivation ("tail") is observed. This model is confirmed by the observations in other plasma sterilization studies (Hury, *et al.* 1998, Moreau, *et al.* 2000, Feichtinger, *et al.* 2003). The tailing effect was most pronounced for *Bacillus* spores, because of their small diameter that allows a higher number to be

present in the top layer. The micrococci as well as the deinococci were present as tetrads or higher aggregates (Fig. 4.1) in which each of the single cells had to be inactivated in order to eliminate the formation of one colony. Cell aggregation in combination with stacking may also account for the apparent plateau phase observed in the kinetics of *D. radiodurans*.

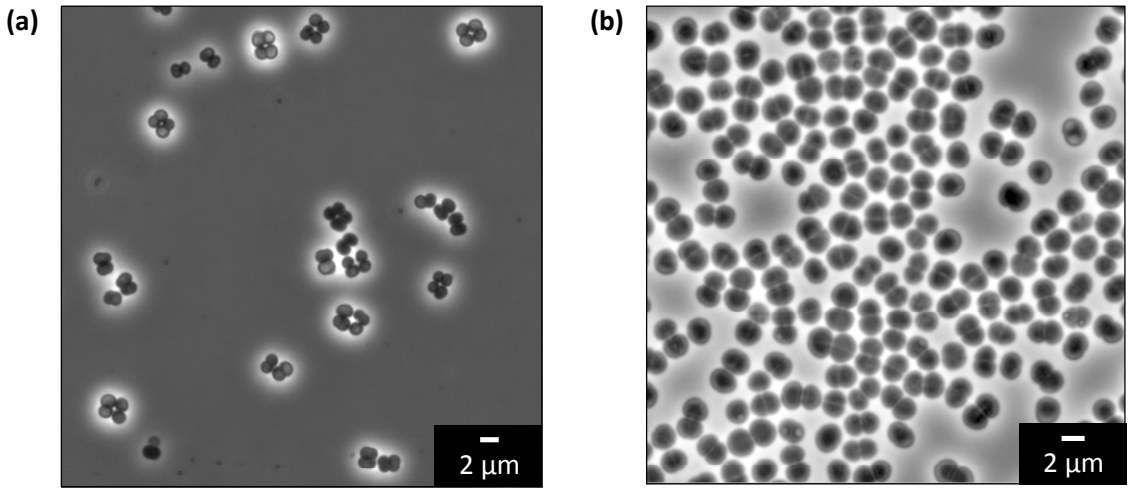


Fig. 4.1. Phase contrast micrographs of cell aggregates of *Micrococcus luteus* BG10 (a; original magnification 1000 fold) and *Deinococcus radiodurans* R1 (b; 1600 fold).

In the *second series* of experiments (section 3.2), it was shown that in low-pressure low-temperature nitrogen-oxygen plasma treatment, a combination of protein inactivation and DNA damage leads to efficient inactivation of *B. subtilis* spores. The plasma used in these experiments featured high radiation intensity in the UV-A+B+C range but also caused erosion of the biomaterial. The following results support the dominating role of UV radiation in the spore inactivation process. First, plasma-generated radiation between 215 nm and 280 nm produced the strongest inactivation of the spores (Fig. 3.8a). This behavior was also reported for plasma treatment of *B. atrophaeus* spores (Halfmann, *et al.* 2007) and *B. subtilis* spores (Feichtinger, *et al.* 2003). Second, spore inactivation was correlated with *in-vitro* detectable damages in the DNA (Fig. 3.9). Third, with spores of the following UV-sensitive mutants the highest inactivation was observed (Fig. 3.6, Table 3.2, Fig. 3.8a). In mutant 1A345, the

nucleotide excision repair (NER) pathway is defective, prohibiting efficient repair of various DNA damages after germination including UV induced ones (Munakata and Ikeda 1969). In spores of mutant 1S111, the α/β-type DNA-binding small acid-soluble proteins (SASP) are absent, leading to an increased sensitivity against harsh environmental conditions including UV radiation (Setlow 2006). On the other hand, spores of strain 1A488 deficient in the spore photoproduct lyase DNA repair pathway were less sensitive than spores of the nucleotide excision repair deficient strain. The spore photoproduct lyase pathway specifically repairs the spore photoproduct 5-thyminyl-5,6-dihydrothymine (Slieman, *et al.* 2000), which accounts for > 90% of the bipyrimidine photolesions induced by UV-C irradiation (Moeller, *et al.* 2007). The low sensitivity of spores of mutant 1A488 indicated that spore photoproduct is not the dominating type of DNA damage induced by plasma and that additional DNA damaging mechanisms exist. Though the strains used in this study were not isogenic and they were derived from different ancestors, the phenotypes of their spores are well-characterized in various inactivation studies (summarized in Setlow 2006) and thus, the interpretations of the results can be considered valid.

The results further indicate that proteins are also targets for the plasma-generated agents, as shown by the linear decrease of KatX activity in the first phase of spore inactivation (Fig. 3.5b). This is supported by the findings of other workgroups (Mogul, *et al.* 2003, Rossi, *et al.* 2006), who demonstrated a destruction of isolated proteins and enzyme inactivation by low-pressure low-temperature plasma. On the other hand, the results obtained in the present study with Δ*cotE* mutant strain PS3328 (Fig. 3.6, Fig. 3.8a) clearly showed that coat proteins function as protectors against plasma-generated reactive agents. Spores of strain PS3328 are lacking the outer coat protein layer and possess a loosely bound inner coat only (Driks 2002). Consistently, when directly exposed to plasma PS3328 spores exhibited a significantly lower survival than wild-type spores, whereas when shielded from access of plasma-generated reactive particles by optical filter plates their inactivation was comparable to that of the wild-type spores (Fig. 3.8a). Since an oxygen containing plasma was used in this

study, it is very likely that these particles are reactive oxygen species (ROS) such as atomic oxygen, radicals of peroxide, superoxide or hydroxyl, and ozone, which were shown to be involved in spore inactivation by reacting with proteins (Gaunt, *et al.* 2006). The assumption that the spore coat serves as a "reactive shield" to prevent access of reactive agents like ROS to the spore core is supported by the work of Riesenman and Nicholson (2000) and others (reviewed by Setlow 2006) showing that the coat layer is part of the spores' resistance mechanism against reactive chemicals.

In accordance with the model of Moisan *et al.* (2001), the inactivation kinetics of spores of all *B. subtilis* strains investigated in the present study followed a biphasic characteristic. In accordance with the model of Moisan *et al.* (2001), a progressive visible erosion of the spore material off the carriers occurred with increasing plasma exposure time. Remarkably, at least for wild-type spores a biphasic kinetics was still obtained when the spores were covered with UV-A+B+C transparent optical filters (Fig. 3.8b). The inactivation rate in the second phase was not influenced by the presence of the optical filters. Since the optical filters prevent etching by reactive particles in the plasma, it is indicated that in the second phase between the two erosion processes (see above) only photodesorption but not etching is relevant to spore inactivation. Considering the very short exposure times in the experiments, the normally slow process of photodesorption seems to be greatly enhanced by simultaneous action of effects indirectly caused by the plasma. Such effects may comprise selective heating of the spores due to absorption of energy from plasma-emitted radiation as well as from exothermic reactions with reactive particles on their surface. However, further investigation is needed to prove this speculation. These findings on the roles of erosion processes refine the model of Moisan *et al.* (2001) for spore inactivation by low-pressure plasma.

In the *third series* of experiments (section 3.3), it was demonstrated that low-pressure low-temperature nitrogen-oxygen plasma treatment kills vegetative bacterial cells by a mechanism comprising a combination of damages to their DNA, proteins and cell wall. These findings were established by investigating repair processes in bacterial cells required to recover from damages induced by

sublethal plasma treatment. As a model organism for vegetative bacteria, *D. radiodurans* was chosen because of its well-documented resistance against desiccation, high levels of UV radiation and reactive oxygen species (Makarova, *et al.* 2001, Daly 2006). Such conditions also prevail in low-pressure plasmas (Moisan, *et al.* 2001, Philip, *et al.* 2002). The properties of the plasma regarding the amounts of UV radiation as well as reactive oxygen species are mainly determined by the composition of the plasma gas mixture (Philip, *et al.* 2002, Rossi, *et al.* 2006) and had a direct impact on the predominance of each single mechanism (damage to DNA, to protein and to cell wall) in the total killing effect.

The conclusion that damage to DNA, protein and to cell walls are the main killing mechanisms is supported by the following results. First, the were unable to recover from sublethal damages induced by plasma treatment if biosynthesis of nucleic acids (transcription) and proteins (translation) as well as cell wall construction was prohibited (Fig. 3.13). Obviously, those processes were essential for the recovery of the cells. At least a part of the observed *de novo* protein synthesis may be due to induction of DNA repair pathways and replacement of damaged proteins. Thus, it can be inferred that the plasma treatment has caused damages to DNA, proteins and cell walls. Second, during the recovery phase, transcription of genes related to DNA-repair, oxidative damage removal, and cell wall synthesis was increased (Fig. 3.15). Third, plasma treatment caused a directly detectable amount of DNA damage that was continuously reduced in the course of the recovery phase (Fig. 3.16).

The properties of the plasma affected the predominance of the killing mechanisms in the following ways: DNA damage is the most important mechanism for killing of vegetative bacteria in highly UV emitting plasmas. In plasmas featuring a high oxygen content with at the same time low UV emission, bacteria are killed with the same efficiency, however mechanisms other than DNA damage are more predominant in the process. These conclusions are supported by the observation that the transcription levels of repair related genes were generally higher, if the cells were exposed to higher UV fluences during the plasma treatment, even though survival was identical in

all treatments (Fig. 3.15). In particular, the amount of DNA damage was correlated with the UV fluence of the plasma treatment (Fig. 3.16).

In the following, the results are discussed in more detail with reference to the conclusions on the killing mechanisms and their predominance mentioned in the above two paragraphs. To investigate the recovery, *D. radiodurans* cells were subjected to a short plasma treatment such that a part of the cells suffered injuries but remained viable. After the plasma treatment, these sublethally injured cells were incubated allowing repair of their injuries and the progress of the resulting recovery was monitored. To be able to differentiate between repair pathways (synthesis of components of the cell membranes and cell walls, translation, transcription), selective inhibitors were added to the incubation media. The plasma treatment with highly UV emitting plasma was survived by 6% of the cells and caused more than 80% of the survivors to be sublethally injured (Fig. 3.13). Growth of the survivors in DT recovery medium was stalled for several hours. During this period, the sublethally injured subpopulation recovered completely as indicated by the ability to form colonies on stress agar plates. The recovery was not inhibited by Cerulenin indicating that the recovering cells did not perform fatty acid synthesis. Taking the erosive nature of the plasma into consideration, it is very likely that the plasma did actually cause damages in the cell membrane (as is also the case for spores, see 3.2.1) and that these damages were not recoverable and therefore they were not detectable in the recovery experiment. The recovery of plasma treated cells was however abolished by penicillin, chloramphenicol and rifampicin. Thus, it can be concluded that a *de novo* synthesis of proteins and cell wall components is essential to repair the cell damages caused by plasma agents. The results are in agreement with studies demonstrating the degradation of the cell wall, DNA, and proteins when exposing bacterial cells to plasmas (Mogul, *et al.* 2003, Kim and Kim 2006). Moreover, *D. radiodurans* is also known to exhibit an extended lag-phase after ionizing irradiation, during which it recovers from various damages including damages to DNA and proteins (Liu, *et al.* 2003, Tanaka, *et al.* 2004). In studies about heat inactivation of *Staphylococcus aureus* and *B. subtilis* in which the same experimental approach was used, it was observed that

recovery of the organisms upon heat treatment is inhibited by transcription inhibitors but not by inhibitors of translation or cell wall formation (Iandolo and Ordal 1966, Miller and Ordal 1972).

To find out which genes are actually transcribed during the recovery and how the properties of the plasma influence this transcription, cells of *D. radiodurans* were plasma treated with plasmas of different nitrogen-oxygen mixtures producing either high UV radiation emission (processes P1 and P2; see Table 3.4) or a high amount of reactive oxygen particles (process P3). Subsequently, the transcription levels of genes involved in stress response were recorded in the sublethally injured *D. radiodurans* R1 cells. The stress related genes under investigation included genes involved in DNA repair, oxidative stress response, housecleaning during stress, and cell wall synthesis (Table 2.3). These genes were selected because it has already been shown by global transcriptional analysis that their expression is affected in the response of *D. radiodurans* to treatment with ionizing radiation and to desiccation (Liu, *et al.* 2003, Tanaka, *et al.* 2004). After plasma treatment, the expression of DR2340 (*recA*) and DR1913 (*gyrA*) was found to be highly up-regulated. This up-regulation was observed with all three different plasma processes (P1, P2, and P3). RecA is involved in recombinational DNA repair as well as regulation of DNA repair systems in *D. radiodurans* (Makarova, *et al.* 2001) and, like in other bacteria, disruption of *recA* leads to a highly UV radiation sensitive phenotype (Pogoda de la Vega, *et al.* 2005). GyrA is a subunit of DNA gyrase which relaxes supercoils in DNA caused by transcription, DNA replication and DNA repair processes. The up-regulation of these genes is consistent with not only the measured DNA damages (Fig. 3.16) but also with the finding that DNA is one of the major targets for plasma agents (Mogul, *et al.* 2003). A different transcription profile was observed for gene DR2220 coding for a homolog of the *Escherichia coli* TerB tellurite resistance protein. TerB likely reduces disulfide bonds in proteins (Turner, *et al.* 1999). In treatments P1 and P3 (see Table 3.4) DR2220 was only initially up-regulated, indicating that removal of oxidative protein damages takes place at the beginning of the recovery. Interestingly, this gene remained induced throughout recovery after treatment P2, where reactive oxygen species

from the plasma were shielded from access to the cells but UV radiation was still high. An explanation for this behavior could be that protein oxidation is mainly caused by photochemically activating proteins and/or oxygen molecules to enable the oxidation reaction. Oxidative stress is also known to be produced by ionizing irradiation in *D. radiodurans* and it causes an up-regulation of all of the genes investigated in this study (Liu, *et al.* 2003, Tanaka, *et al.* 2004). The gene DR0479 coding for penicillin binding protein 1 (PBP-1) was also up-regulated after plasma treatment. PBP-1 is involved in the final step of cell wall synthesis and cross-linking. In concordance with the results of the recovery inhibition study, this indicates that cell wall synthesis is necessary for the recovery from injuries caused by plasma treatment. The up-regulation of transcription of genes involved in DNA repair (*recA*, *gyrA*), protein oxidation removal (*terB*), and cell wall synthesis (DR0479) as well as the need of *de novo* protein synthesis during the recovery of plasma treated cells indicate that DNA, proteins, and the cell wall are primary targets of plasma action leading to the killing of *D. radiodurans* cells. These findings are in general consistent with those of Mogul *et al.* (2003). However, typical UV-photoproducts such as pyrimidine dimers seemed to play no primary role in the killing of *D. radiodurans* by plasma since no induction of *uvrB* and *uvsE* could be detected. On the other hand, the amount of DNA damage in the cells correlated with the UV intensity emitted under different plasma exposure conditions. When a plasma with a high oxygen content and low UV emission was used (P3), the shortest recovery phase, least DNA damage and the lowest induction of repair related genes was detected, while at the same time the killing effect was comparable to that obtained with highly UV emitting plasmas (P1 and P2). Thus, it is tempting to speculate that treatment with high-oxygen low-UV plasma induces other lesions in *D. radiodurans* which tend to be irreparable and lethal. Considering the high erosion rates to be expected in such a plasma, these lesions may likely affect the functions of essential components of the cell envelope such as membrane lipids or membrane proteins. However, it is also possible that the lower transcriptional induction observed after treatment with P3 plasma may

be due to a lower responsiveness of the investigated target genes to oxidative stress than to UV induced damages.

D. radiodurans cells exhibited a biphasic inactivation kinetics, as it had been observed for *B. subtilis* spores under the same plasma exposure conditions (see above). This suggests that the model for the mechanisms of action for plasma sterilization proposed by Moisan *et al.* (2001) is also applicable to *D. radiodurans*. In agreement with this model, the first phase *D*-values obtained for treatment with highly UV-emitting plasmas were higher for *D. radiodurans* (Fig. 3.11) than for *B. subtilis* spores (Table 3.2). Considering that *D. radiodurans* R1 is more resistant to UV-C irradiation compared to *B. subtilis* 168 spores (Pogoda de la Vega, *et al.* 2005, Moeller, *et al.* 2007, Bauermeister, *et al.* 2009), this confirms and stresses the importance of UV radiation in plasma sterilization. However, the oxidation of cell components by particles such as reactive oxygen species (Gaunt, *et al.* 2006) seems to play a more important role in killing *D. radiodurans* than in inactivating *Bacillus* spores with plasma. This suggestion is supported by the fact that, treatment of *D. radiodurans* with high-oxygen low-UV plasma (P3) resulted in a comparable killing efficiency while at the same time the DNA damage level was low. *D. radiodurans* cells differ from spores in a number of morphologic properties attenuating the intensity of UV radiation at the site of their DNA: they are more voluminous than spores, contain a radical scavenging carotenoid pigment (Zhang, *et al.* 2007), contain multiple copies of their genome per cell (Cox and Battista 2005), and grow in tetrads or even higher cell aggregates (Chou and Tan 1991).

5 Conclusion

Plasmas, as voluminous sources of UV-radiation and reactive particles, offer the potential to sterilize even three-dimensional objects while maintaining the integrity of heat sensitive high-tech materials. The absence of persisting residuals makes plasma processes ideal for medical or pharmaceutical applications. For high-volume applications like food packaging sterilization, plasma technology is advantageous due to its energy efficiency and very short processing times.

To establish plasma sterilization in medical, pharmaceutical or food production related environments, high levels of process safety are required. To ensure this, the design, the operating parameters, and process monitoring of plasma sterilization devices have to be set up appropriately. The results of this study enhance the knowledge about mechanisms how contaminating microorganisms are inactivated by low-pressure oxygen-nitrogen plasma and how they are able to survive plasma sterilization processes. Accordingly, a safe plasma process should provide a high level of UV emission and a high concentration of reactive oxygen species and the control of these properties should focus to ensure the generation of high levels of non-repairable damages not only in the DNA, but also in proteins, and cell membranes of contaminating microorganisms. Especially with highly UV emitting plasmas, the resulting inactivation is distinctly biphasic. With respect to this, it may be helpful to use a two-stepped plasma process. In the first step, the process should be controlled to yield high UV outputs, while in the second step the plasma should be shifted to provide

higher erosion rates. Moreover, the design of the sterilization process should consider the kind of microbial contamination (bacteria, spores, conidia, etc.) to be expected in a particular application. This is of particular significance considering that natural contamination occurs in the form of biofilms, where microbes are present embedded in additional biomaterials (e.g. exopoly-saccharides), or as aggregates of germs with dust particles. Any of these can limit the access of plasma agents to its targets.

It has to be stressed that the biphasic nature of microorganism inactivation by plasma processes is critical in order to assess sterilization safety. The deviation from log-linear characteristics practically prohibits a process validation basing on conventional sterility assurance levels (SAL). However, sterility requirements as defined by the European Pharmacopeia or by food safety standards are based on the SAL concept. Thus, the establishment of industrial plasma sterilization processes is greatly impeded (Yardimci and Setlow 2010). To overcome this problem, for non-heating sterilization processes, like plasma sterilization, alternative concepts such as the "tiered sterility assurance levels" have been proposed (von Woedtke and Kramer 2008, von Woedtke, *et al.* 2008). In contrast to conventional SAL, this concept considers the microbial contamination risks of the entire product life cycle – from production to packaging to storage to use. If all of these steps are performed under current good practices, the requirements to an appropriate sterilization process are often low enough to allow validation without the need to extrapolate experimental data.

Many researchers have published data on microbial inactivation obtained with very diverse plasma treatment conditions. While theories on the mechanisms of plasma sterilization have been established using the available data, still many aspects have so far not been proven experimentally. Moreover, there is still no established consensus about which methods and biological parameters to be used in order to investigate plasma sterilization making comparison of the data from different researchers difficult. The presented study has introduced new approaches and provided novel insights to plasma sterilization research. However, *further research* is required to obtain a more global view on the

response of microorganisms to the interaction with plasma. Such research could employ molecular biology methods like global expression screening with micro-arrays or physiological screening of live cells with fluorescent probes after plasma treatment. The results could provide information about which biological mechanisms in addition to DNA repair lead to survival or which components of the cells are most susceptible to plasma treatment, respectively. Moreover, by combining the PCR based ratio detection system (which was used for quantification of DNA damages) with the application of damage specific endonucleases (like *E. coli* endonucleases III and IV, T4 endonculease V, or formamidopyrimidine-DNA glycosylase which are commercially available) it should be possible to deliver information on the quality of DNA damages caused by the plasma.

6 Summary

Plastic based materials are increasingly used for packaging of pharmaceuticals (especially biologicals), food or beverages and production of medical devices. Their heat sensitivity requires safe and efficient non-thermal methods for decontamination. Plasma technology has the potential to provide a suitable means since it works at low temperatures and – in contrast to conventional methods like application of ionizing radiation or ethylene oxide exposure – is safe to operate, is free of residues and does not alter the bulk properties of the materials. Plasmas can generate various agents potentially active in decontamination like ultra-violet (UV) radiation, radicals and other reactive particles. To acquire an approval for plasma technology as a novel sterilization method, its process safety has to be proven. The research community has proposed hypotheses and models on its mechanisms of action, which are at least partially speculative. Still little is known about the details of the biologic effects of the combination of the various plasma agents on the components of microbial cells or spores. Especially, the question remains open which components of a cell or spore are the primary targets, and which of the agents are most effective in the inactivation process. The acquisition of such knowledge is necessary to identify parameters suitable to control, monitor, and assess the safety of plasma sterilization processes.

The aims of the presented work are to elucidate which components of a cell or spore are the primary targets in low-pressure plasma sterilization, and which of the putative agents contained in the plasma are most effective in the

inactivation process. To accomplish this, in the presented work suitable microbiological methods were established and the inactivation of bacterial spores and cells and fungal conidia by microwave induced low-pressure low-temperature nitrogen-oxygen plasmas was investigated. Moreover, two strategies were pursued that have hitherto not been applied in published plasma sterilization studies: (i) Using spores of *Bacillus subtilis* mutants to identify structural components serving as targets for sterilization with plasma and (ii) characterizing the response of *Deinococcus radiodurans* R1 cells to plasma treatment and identify repair processes during recovery from plasma induced damages in viable cells.

Plasmas producing a maximum of UV emission were most effective in inactivating bacterial cells and spores. The inactivation followed a biphasic kinetics consisting of a log-linear phase with rapid inactivation followed by a slow inactivation phase. A continuous model fit was applied to the experimental data allowing reliable calculation of decimal reduction values for both phases. Cells of *D. radiodurans* were found to be more resistant than spores of *B. subtilis*. For *B. subtilis* spores, in the course of plasma treatment damage to DNA, proteins and spore membranes were observed by monitoring the occurrence of auxotrophic mutants, inactivation of catalase (KatX) activity and the leakage of dipicolinic acid, respectively. Spores of the wild-type strain showed highest resistance to plasma treatment. Spores of mutants defective in nucleotide excision repair (*uvrA*) and small acid-soluble proteins (*ΔsspA ΔsspB*) were more sensitive than those defective in the coat protein CotE or spore photoproduct repair (*splB*). Exclusion of reactive particles and spectral fractions of UV radiation from access to the spores revealed that UV-C radiation is the most effective inactivation agent in the plasma, whereby the *splB* and *ΔcotE* mutant spores were equally and slightly less sensitive, respectively, than the wild-type spores. The extent of damages in the spore DNA as determined by quantitative PCR correlated with the spore inactivation.

Spore inactivation was effectively mediated by a combination of DNA damage and protein inactivation. DNA was identified to be the primary target for spore

inactivation by UV radiation emitted by the plasma. Coat proteins were found to constitute a protective layer against the action of the plasma.

For the investigation of the recovery from plasma-induced damages, cells of *D. radiodurans* R1 were subjected to short plasma treatments with various plasmas. A part of the survivors was sublethally injured as determined by their ability to form colonies on standard medium but not on stress medium and by the observation of a prolonged lag phase. Incubation of the cells in a recovery medium after plasma treatment allowed a part of the survivors to recover their ability to grow on stress medium. This recovery strongly depended on transcriptional and translational processes and cell wall synthesis, as revealed by addition of specific inhibitors to the recovery medium. Genes involved in DNA repair, oxidative stress response and cell wall synthesis were induced during recovery, as determined by quantitative RT-PCR. Damage to chromosomal DNA caused by plasma agents and *in-vivo* repair during recovery was directly shown by quantitative PCR. Plasmas with less UV radiation emission were also effective in killing *D. radiodurans* cells but resulted in less DNA damage and lower induction of the investigated genes.

The response of *D. radiodurans* to plasma indicated that DNA, proteins and cell wall are primary targets of plasma, whose damage initially leads to the cells' death. Protein oxidation was more important for the killing of *D. radiodurans* cells than of *B. subtilis* spores. Thus, the plasma process parameters must regard the expected contaminating biological material in order to obtain a high-level sterilization.

The results provide new insight into the interaction of non-thermal low-pressure plasmas with microorganisms. This knowledge supports the definition of suitable parameters for novel plasma sterilization equipment to control process safety. For example, monitoring the UV intensity below 280 nm and spectrometric online measurement of bands related to excited reactive gas particle species during the process is recommended.

7 Zusammenfassung

Polymerbasierte Materialien werden zunehmend für die Verpackung von Arzneimitteln (vor allem für Biologika), Lebensmittel oder Getränke und die Produktion von medizinischen Geräten eingesetzt. Ihre Hitzeempfindlichkeit erfordert sichere und effiziente nicht-thermische Dekontaminationsverfahren. Die Plasmatechnologie hat das Potenzial, solchen Anforderungen gerecht zu werden, da sie bei niedrigen Temperaturen arbeitet und – im Gegensatz zu herkömmlichen Methoden wie ionisierende Bestrahlung oder Ethylenoxid-begasung – sicher zu bedienen ist, keine Rückstände hinterlässt und keine Veränderungen an den Volumeneigenschaften der Materialien hervorruft. Plasmen können verschiedene Agenzien erzeugen, die potenziell für die Dekontamination aktiv sind, zum Beispiel ultraviolette (UV) Strahlung, Radikale und andere reaktive Teilchen. Um eine Zulassung für ein neuartiges Plasma-Sterilisationsverfahren zu erlangen, muss dessen Prozesssicherheit nachgewiesen werden. Einschlägig tätige Forschungsgruppen haben Hypothesen und Modelle für die Wirkmechanismen der Plasmasterilisation vorgeschlagen, die teilweise noch spekulativ sind. Bisher sind kaum Details über die biologische Auswirkung der Kombination von verschiedenen Plasma-Agenzien auf die Komponenten von mikrobiellen Zellen oder Sporen bekannt. Insbesondere bleibt die Frage offen, welche Komponenten einer Zelle oder Spore die primären Wirkorte sind, und welche der Agenzien am effektivsten sind. Diese Kenntnisse sind notwendig, um geeignete Parameter zur Steuerung,

Überwachung und Bewertung der Sicherheit von Plasma-Sterilisationsprozessen zu ermitteln.

Das Ziel der vorliegenden Arbeit ist die Aufklärung, welche der Komponenten einer Zelle oder Spore die primären Wirkorte bei der Niederdruck-Plasmasterilisation sind, und welche der im Plasma enthaltenen Agenzien am effektivsten bei der Inaktivierung von Mikroorganismen sind. Hierfür wurden in der vorliegenden Arbeit geeignete mikrobiologische Methoden evaluiert und etabliert und die Inaktivierung von bakteriellen Sporen und Zellen sowie Schimmelpilzkonidien durch mikrowelleninduzierte Niederdruck-Niedertemperatur-Stickstoff-Sauerstoff-Plasmen untersucht. Weiterhin wurden zwei Strategien verfolgt, die bisher noch nicht in veröffentlichten Arbeiten zur Plasmasterilisation verwendet worden sind: (i) Verwendung von Sporen von *Bacillus subtilis*-Mutanten zur gezielten Untersuchung von Komponenten, die als Wirkorte für die Plasmasterilisation in Frage kommen. Und (ii), Charakterisierung der Reaktion von *Deinococcus radiodurans* R1 Zellen auf verschiedene Plasmabehandlungen, um biologische Prozesse *in lebenden Zellen* zu identifizieren, die bei der Reparatur von plasmainduzierten Schäden eine Rolle spielen.

Plasmen, die eine maximale UV-Emission erzeugen, waren am effektivsten bei der Inaktivierung von Bakterienzellen und Sporen. Die Inaktivierung folgte einer biphasischen Kinetik, die sich aus einer log-linearen Phase schneller Inaktivierung gefolgt von einer Phase langsamer Inaktivierung zusammensetzt. Für die experimentell ermittelten Datenpunkte wurde eine Kurvenanpassung mit einem stetigen biphasischen Modell durchgeführt. Dies ermöglichte die zuverlässige Berechnung von dezimalen Reduktionswerten (*D*-Werte) für beide Phasen. Dabei erwiesen sich Zellen von *D. radiodurans* R1 als resistenter als Sporen von *B. subtilis*. Während der Plasmabehandlung von *B. subtilis* Sporen wurde das Auftreten auxotropher Mutanten, die Inaktivierung der Katalase (KatX)-Aktivität sowie die Freisetzung von Dipicolinsäure untersucht. Dabei wurden Schäden an der DNA, an Proteinen beziehungsweise an den Sporenmembranen beobachtet. Sporen des Wildtyp-Stamms wiesen die höchste Resistenz gegen Plasmabehandlung auf. Sporen von Stämmen mit Mutationen

an einem DNA-Reparatursystem (*uvrA*) und bei der Synthese der DNA-bindenden Proteine SASP (*ΔsspA ΔsspB*) waren empfindlicher als Sporen mit veränderter Coat-Schicht (*cotE*) oder als solche, denen ein funktionierendes Enzym zur direkten Reparatur des Spore-Photoproduct (*splB*) fehlt. Eine Abschirmung der Sporen vor reaktiven Teilchen und Teilen des UV-Spektrums zeigte, dass die UV-C Strahlung das effektivste Agens für die Inaktivierung im Plasma ist. Im Vergleich zu Wildtyp-Sporen erwiesen sich dabei die Sporen der Mutanten *splB* als gleich und *ΔcotE* als etwas weniger empfindlich. Das Ausmaß der Schäden in der Sporen-DNA wurde mittels quantitativer PCR bestimmt und korrelierte mit der Inaktivierung der Sporen.

Die Inaktivierung von Sporen wurde effektiv durch eine Kombination aus DNA-Schädigung und Proteininaktivierung bewirkt. Die DNA wurde als primärer Wirkort für die Sporeninaktivierung durch im Plasma erzeugte UV-Strahlung identifiziert. Die Coat-Schicht der Sporen bildet eine Schutzschicht gegen die Wirkung des Plasmas.

Um die Reparatur von plasmainduzierten Schäden zu untersuchen, wurden Zellen von *D. radiodurans* R1 kurzen Behandlungen mit unterschiedlichen Plasmen unterzogen. Ein Teil der Überlebenden konnte Kolonien auf Standard-Medium, nicht aber auf Stressmedium bilden, wobei zusätzlich eine verlängerte Lag-Phase beoabachtet wurde. Diese Überlebenden wurden als subletal geschädigt gewertet. Durch Inkubation der Zellen in einem Recovery-Medium konnte ein Teil der Überlebenden sich so weit erholen, dass sie ihre Fähigkeit, auf Stressmedium zu wachsen, wieder erlangten. Diese Erholung war maßgeblich abhängig von Transkriptions- und Translationsprozessen sowie von der Zellwandsynthese. Dies zeigte sich durch die Zugabe von entsprechenden spezifischen Inhibitoren zum Recoverymedium. Eine Analyse mittels quantitativer RT-PCR zeigte, dass Gene während der Erholungsphase induziert waren, die an der DNA-Reparatur, an der Resistenz gegen oxidativen Stress sowie an der Zellwandsynthese beteiligt sind. Sowohl durch das Plasma verursachte Schäden an chromosomaler DNA als auch deren Reparatur *in-vivo* während der Erholungsphase wurden durch direkte Messung mit quantitativer PCR demonstriert. Plasmen mit weniger UV-Emission waren ebenfalls effektiv

bei der Abtötung von *D. radiodurans* Zellen, sie erzeugten jedoch weniger DNA-Schädigung und eine niedrigere Induktion der untersuchten Gene.

Das Verhalten von *D. radiodurans* Zellen nach Plasmabehandlung weist darauf hin, dass die DNA, Proteine und Zellwand primäre Wirkorte des Plasmas sind, deren Schädigung zuerst zum Absterben der Zellen führt. Die Proteinoxidation hatte mehr Einfluss auf die Sterilisation von *D. radiodurans* Zellen als bei *B. subtilis* Sporen. Die Auslegung der Plasmaprozesse muss folglich die Art des in der jeweiligen Anwendung zu erwartenden kontaminierenden biologischen Materials berücksichtigen, um eine hohe Sterilisationssicherheit zu erzielen.

Die Ergebnisse liefern neue Einblicke in die Wechselwirkungen von nicht-thermischen Niederdruck-Plasmen mit Mikroorganismen. Dieses Wissen unterstützt die Definition von geeigneten Parametern für neuartige Plasma-Sterilisationsanlagen zur Kontrolle der Prozesssicherheit. Zum Beispiel ist eine Überwachung der UV-Intensität unterhalb von 280 nm sowie eine spektrometrische Online-Messung von Emissionsbanden angeregter reaktiver Teilchen während des Prozesses zu empfehlen.

8 Appendix

8.1 Culture media and buffers

All ingredients were obtained from Merck (Darmstadt, Germany) unless stated otherwise. All substances were dissolved in deionized water. Media and buffers were autoclaved at 121°C for 15 min. Adjustments for pH were done at 25°C with diluted hydrochloric acid or sodium hydroxide solution.

Table 8.1. Culture media (in alphabetical order).

Dextrose tryptone agar (DTA)	Glucose	5 g/l
	Casein peptone (Difco)	10 g/l
	Yeast extract (Difco)	1 g/l
	Bacto-agar (Difco)	15 g/l
	Adjust to pH 7.0. Autoclave.	
Dextrose tryptone agar stress medium	Glucose	5 g/l
	NaCl	8.8 g/l
	Casein peptone (Difco)	10 g/l
	Yeast extract (Difco)	1 g/l
	Bacto-agar (Difco)	15 g/l
	Adjust to pH 7.0. Autoclave.	
Dextrose tryptone broth (DT)	Glucose	5 g/l
	Casein peptone (Difco)	10 g/l
	Yeast extract (Difco)	1 g/l
	Adjust to pH 7.0. Autoclave.	

Difco sporulation medium agar (DSM) (Harwood and Cutting 1990)	Peptone (Difco)	5.0 g/l
	Beef extract (Difco)	3.0 g/l
	KCl	1.0 g/l
	$MgSO_4 \cdot 7\ H_2O$	0.12 g/l
	Bacto-agar (Difco)	20.0 g/l
	Adjust pH to 7.0 to 7.2. Autoclave.	
	After autoclaving, cool down to 50°C and add the following filter-sterilized solutions:	
	1 M $Ca(NO_3)_2$	1 ml/l
	0.01 M $MnCl_2$	1 ml/l
	1 mM $FeSO_4$	1 ml/l
Rose Bengal agar for colony counting of molds (Jarvis 1973, modified)	Peptone	5.0 g/l
	Glucose	10.0 g/l
	KH_2PO_4	1.0 g/l
	$MgSO_4 \cdot 7\ H_2O$	0.5 g/l
	Rose bengal (Sigma)	0.05 g/l
	Agar-agar	15.0 g/l
	Adjust to pH 7.2 ± 0.2. Autoclave. Store plates refrigerated (+2 to +8°C) and in dark for no longer than one week.	
Spizizen's minimal medium agar (Harwood and Cutting 1990)	$(NH_4)_2SO_4$	2 g/l
	K_2HPO_4	14 g/l
	KH_2PO_4	6 g/l
	Na_3-Citrate $\cdot$ 2 H_2O (Sigma)	1 g/l
	$MgSO_4 \cdot 7\ H_2O$	0.2 g/l
	Bacto-agar, washed (Difco)	15 g/l
	Autoclave.	
	After autoclaving, cool down to 50°C and add the following as filter-sterilized solutions:	
	50% (w/v) Glucose	10 ml/l
	5 mg/ml L-Tryptophan	10 ml

Wort agar for cultivation of *A. niger* and generation of conidia.	Universal peptone ("Neopeptone") (Difco)	0.75 g/l
	Malt extract (Sigma)	15.0 g/l
	Maltose (Sigma)	12.75 g/l
	Dextrin	2.75 g/l
	Glycerol	2.35 g/l
	KH_2PO_4	0.4 g/l
	NH_4Cl	1.0 g/l
	Agar-agar	20.0 g/l
	Adjust to pH 5.0. Autoclave.	

Table 8.2. Buffers for general use.

Phosphate buffered saline (PBS)	NaCl	8 g/l
	Na_2HPO_4	1.44 g/l
	KH_2PO_4	0.24 g/l
	Adjust pH to 7.4	
Resuspension buffer (to resuspend organisms from plastic vials)	Peptone	1 g/l
	KH_2PO_4	0.3 g/l
	$Na_2HPO_4 \cdot 2H_2O$	0.6 g/l
	Tween 80	0.5 g/l
	Adjust pH to 7.0. Autoclave, then add a filter sterilized solution of sucrose to a final concentration of 300 mM	
Tris-EDTA (TE)	Tris-HCl (Biomol)	100 mM
	Na_2EDTA (Biomol)	10 mM
	Adjust pH to 7.4	
Tris-acetate-EDTA (TAE), Electrophoresis buffer	Tris-Acetate (Biomol)	40 mM
	Na_2EDTA (Biomol)	1 mM
	Adjust pH to 8.0	
NaCl-Tris-EDTA (STE)	Tris-HCl, pH 8 (Biomol)	10 mM
	Na_2EDTA (Biomol)	10 mM
	NaCl	150 mM
	Adjust pH to 8.0	

Table 8.3. Buffers and reagents for nucleic acid preparation

Lysis buffer for RNA preparation	Tris-HCl	30 mM
	EDTA	1 mM
	Adjust pH to 8.0. Autoclave. Add immediately before use:	
	Lysozyme for chicken egg white (Serva)	15 mg/ml
	Proteinase K (Roche)	2 mg/ml
	Other reagents required:	
	β-Mercaptoethanol (Fluka)	
	80% (v/v) Ethanol, non-denatured (Roth)	
Spore decoating solution (Setlow and Setlow 1998)	Tris-HCl, (Biomol)	50 mM
	Na_2EDTA (Biomol)	10 mM
	Sodium dodecyl sulfate (Serva)	1% (w/v)
	Urea (Calbiochem)	8 M
	Dithiothreitol (Sigma)	50 mM
	Adjust to pH 8. Filter sterilize.	
Spore lysis solution (Setlow and Setlow 1998, modified)	Potassium phosphate buffer, pH 7	10 mM
	Na_2EDTA (Biomol)	10 mM
	NaCl	150 mM
	Lysozyme from chicken egg white (Serva)	2 mg/ml (270 000 U/ml)

Table 8.4. Reagents for the determination of catalase activity (Goldblith and Proctor 1950, modified)

Reaction buffer (10 mM phosphate, pH 7)	Na_2HPO_4	1.44 g/l
	KH_2PO_4	0.24 g/l
Substrate solution	Hydrogen peroxide	2 mM
	Dissolve in reaction buffer immediately before use. Store in dark and on ice.	
Dye solution	$KMnO_4$	0.5 mM
	Dissolve in deionized water.	
Stopping reagent	H_2SO_4	5 N
Catalase solution	Catalase from bovine liver (Sigma C-9322)	100 U/ml
	Dissolve in reaction buffer immediately before use. Store on ice.	

Table 8.5. Reagents for determination of dipicolinic acid (Scott and Ellar 1978).

For adjusting pH	NaOH	1 M
Sample complexing reagent	$CaCl_2$	100 mM
Reference complexing reagent	EGTA	50 mM
	Adjust to pH 7.0 with NaOH solution.	
Calibration stock solution	Dipicolinic acid (Sigma)	1 mM

8.2 Oligo nucleotide sequences

Table 8.6. PCR primers for quantification of DNA damages in *B. subtilis*. Primers were constructed based on the published genome sequences of *B. subtilis* 168 (accession no. NC_000964)

Name	**Sequence (5'–3')**
Bs_dnaK1154f	ACACGACGATCCCAACAAGC
Bs_dnaK1254r	AGACATTGGGCGCTCACCT
Bs_dnaK855f	CACAATGGGTCCTGTCCGTC

Table 8.7. PCR primers for quantification of DNA damages in *D. radiodurans*. Primers were constructed based on the published genome sequences of *D. radiodurans* R1 (Genbank accession no. AE000513).

Name	**Sequence (5'–3')**
DR1343f	GGCAAGTACAAAGGCATCA
DR1343f2	CACATCATCTCCAATGCCA
DR1343r	GTTGTCGTACCACGAGAAGAA

Table 8.8. PCR primers and sequence specific probes for determination of relative transcription levels of damage and stress related genes in *D. radiodurans* by quantitative real-time RT-PCR. Probes were 3' labelled with either FAM or Yakima Yellow and featured 5' non-fluorescent quenchers. Target IDs refer to the annotation of the *D. radiodurans* R1 genome sequence.

Target ID	**5'–3' Sequence of**	
	forward primer/ reverse primer	**probe**
DR1343	GGCAAGTACAAAGGCATCA GTTGTCGTACCACGAGAAGAA	TACACCGAAGACCCCAT
DR0261	GCATAACAGCTTGCTCTGAA TTATGCCAACGGCCTGTG	ACCTCCCCACTCTCCT
DR0479	GCCGCTACAAAGACTTCA GATTTGCCCGTCTTCTACCA	TACCGCCCGCTGATGA
DR1819	GCTCTACCGACTGAGTTC CAGTTGCGGCGCTAGATG	CCTTTTTCCCATGCTC
DR1913	CCCAGATCATCATCAGCGAA AATCTTGCCCGCCTTGTA	CAACAAGACCAACCTCC
DR2220	GACTTCGGGAAAATCGAG GTGAATCACTTCGCGCAT	AGGTCATCGGCAAGGT
DR2275	TTCATCTCCTACTACGACTAC GCGCAATCTCTCGATTTCCT	ACCAACCCGAAGCATAC
DR2340	ATTGATGTGGTGGTCGTG TGCCGGTCTTGGAGAGAA	CTCGGTGGCTGCTCTG

8.3 PCR systems for checking the genotypes of the *Bacillus subtilis* mutants

The genotypes of the *B. subtilis* mutant strains were checked with specific PCR systems. Basing on the published genome sequence of *B. subtilis* 168 (Kunst, *et al.* 1997) primers targeting the mutation locations were constructed (Table 8.9). The strains were streaked from cryocultures on DTA plates containing the appropriate antibiotics. Genomic DNA was purified with the High Pure PCR Template Preparation Kit (Roche). One nanogram of DNA was applied as template in PCR reactions using the following temperature profile: 94°C/240 s; 35 cycles of 94°C/30 s, 48°C/30 s, 72°C/120 s; post-cycle 72°C/300 s. All reactions were performed with Taq-S reagents (Genaxxon Bioscience, Ulm, Germany) according to manufacturer's instructions. Primers concentrations were 200 nM. PCR products were analyzed by agarose gel electrophoresis, ethidium bromide staining, and fluorescent detection. Genotypes were evaluated according to the expected PCR product sizes (Table 8.10, Table 8.11). To check the point mutation in *splB1*, the PCR product was subjected to a restriction digest with *Hin*dIII endonuclease (Fermentas) before electrophoresis.

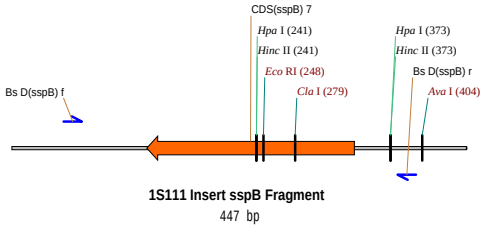
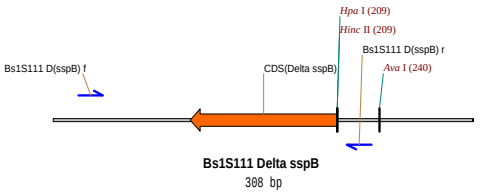
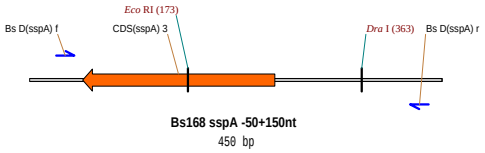
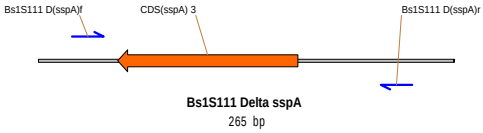
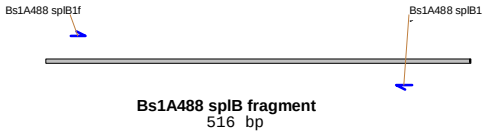
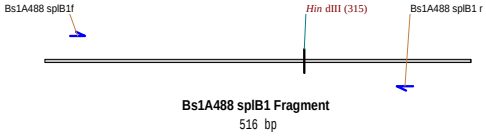
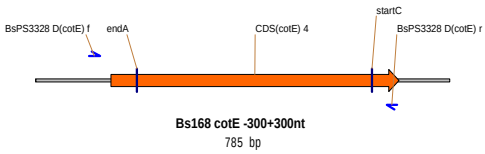
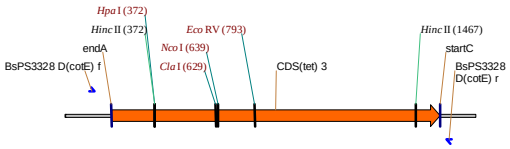
Table 8.9. PCR primer sequences for checking the genotypes of the *B. subtilis* mutant strains.

Name	**Sequence (5'–3')**	
Bs_splB1	forward:	CTTCATCTAATTCGAGCTTC
	reverse:	CATTGAACATTTTGGMCA
Bs_D(sspA)	forward:	ACTCCTAAGCCATTATGTGA
	everse: GCGGTAGATAACATTGAGAC	
Bs_D(sspB)	forward:	TAGGATATGTGGAGCAGG
	reverse:	TTCACAGATTGGTGCAAC
Bs_D(cotE)	forward:	CAGCATAAGATAACACGAAGA
	reverse:	TTCTTCAGGATCTCCCACTA

Table 8.10. PCR primer combinations for checking of the genotypes of the *B. subtilis* mutant strains.

Strain	PCR primer pairs	PCR product length (bp)	Reference
1A757/168 (wild type)	Bs_splB1 Bs_D(sspA) Bs_D(sspB) Bs_D(cotE)	416 407 347 585	
1S111	Bs_D(sspA) Bs_D(sspB)	217 215	(Connors*, et al.* 1986, Mason and Setlow 1986)
1A488	Bs_splB1	284 + 132 (after restriction with *Hin*dIII)	(Fajardo-Cavazos and Nicholson 1995)
PS3328	Bs_D(cotE)	approx. 2300	(Paidhungat*, et al.* 2001)

Table 8.11. Illustrations of the genomic locations relevant for genotype checking of the *B. subtilis* mutant strains.

Wild-type (control)	**Mutation**
sspB^{+} (PCR product length 347 bp)	Δ*sspB* (PCR product length 215 bp)
	
sspA^{+} (PCR product length 407 bp)	Δ*sspA* (PCR product length 217 bp)
	
splB^{+} (PCR product length 416 bp, without *Hin*dIII restriction site)	*splB1* (PCR product length 416 bp, *Hin*dIII fragments 284 bp and 132 bp)
	
cotE^{+} (PCR product length 585 bp)	Δ*cotE::tet* (PCR product length 2.3 kbp)
	

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