Differential tolerance of biofilms and planktonic cells of *Deinococcus geothermalis* to desiccation and to simulated space and Mars conditions

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„Forward ever –
backward never“

(Dennis Alcapone)

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ABBREVIATIONS

ACA	*Amaranthus caudatus* lectin
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCE	Before common era
BSA	Bovine serum albumin
CER	Cation-exchange resin
CFU	Colony-forming unit(s)
CLSM	Confocal laser-scanning microscope/microscopy
CME	Cellulose mixed-ester
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
DBA	*Dolichos biflorus* lectin
DLR	German Aerospace Center
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DSM	Strain indicator used by the German Collection of Microorganisms DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany)
e.g.	*exempli gratia* (Latin), 'for example'
eDNA	extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
EFM	Epifluorescence microscope/microscopy
EPS	Extracellular polymeric substances
Eq.	Equation
ESA	European Space Agency
ESEM	Environmental scanning electron microscopy

Fig.	Figure
FISH	Fluorescence *in situ* hybridisation
HAA	*Helix aspersa* lectin
i.e.	*id est* (Latin), 'that is'
ISS	International Space Station
LEO	Lower Earth orbit
p.a.	*pro analysi* (Latin), 'for analysis'
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PFD (water)	Particle-free de-ionised (water)
PSI	Planetary and Space Simulation facility
R2A	Reasoner's 2A agar
R2B	R2A broth
R_f	Retardation factor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
Tab.	Table
TCC	Total cell count(s)
TLC	Thin-layer chromatography
T_m	Phase transition temperature of the cell membrane
TRIS	Tris(hydroxymethyl)aminomethane
TRITC	Tetramethylrhodamine isothiocyanate
TSA	Tryptic soy agar
VBNC	Viable but non-culturable

SI units not listed.

ABSTRACT

The formation of biofilms is one of the most successful survival strategies of bacteria on Earth. Aggregated and embedded in a matrix of extracellular polymeric substances (EPS), biofilm cells are often more tolerant to environmental stress than single, planktonic cells of the same species. If microorganisms were to travel through space or to reside on a Mars-like planet, their survival in these hostile environments might be enhanced if they are organised in biofilms. The aim of this study was to test this hypothesis.

Deinococcus geothermalis DSM 11300 was chosen as a model organism due to its high intrinsic tolerance to both desiccation and radiation. Biofilms generated at the solid-air interface of membranes placed on R2A agar medium and, for comparison, membrane-deposited planktonic cells grown in R2A broth were air-dried overnight and exposed to various stressors relevant for space and Mars environments, including prolonged desiccation, vacuum, simulated Martian atmosphere, low and high temperatures, and UV radiation.

The EPS of *D. geothermalis* were isolated using a cation-exchange resin extraction method. They contained significant amounts of proteins and polysaccharides, both of which might promote the retention of water under dehydrating conditions, as well as relatively low amounts of extracellular DNA (eDNA), which could have a structural role within the EPS matrix. In biofilms, the EPS were of distinct spatial arrangement. Environmental scanning electron microscopy provided evidence for an EPS layer covering the uppermost layer of cells. Three different polysaccharide fractions – presumably galactosides – of different spatial distribution and arrangement were identified within the matrix using fluorescently-labelled lectins. When tryptic soy agar (TSA) was used as a nutrient source instead of R2A, biofilms of different morphology and EPS composition were formed. High peptone concentrations in the TSA medium (20 g l^{-1}) caused the cells to form highly cohesive aggregates. Dispersal of these cell aggregates could be achieved by treatment with proteinase K, suggesting the involvement of proteins, possibly in the form of adhesins or type IV pili, in the cell-to-cell attachment of the organism.

Following exposure to stress conditions, the viability of the organisms was assessed and compared to non-exposed cells. Since many bacteria are able to enter a viable but non-culturable (VBNC) state as a response to stress, cultivation-independent viability markers (membrane integrity, ATP levels, presence of 16S rRNA) were analysed in addition to the determination of colony counts. During prolonged desiccation, biofilms sustained viability significantly longer than planktonic cells: Compared to non-desiccated samples, a desiccation period of 56-61 days reduced the culturability of biofilms and planktonic cells to 5.6% and 0.8%, respectively. Membrane integrity was maintained to a high degree in biofilms, whereas more than 60% of planktonic cells showed signs of membrane damage following desiccation. Whilst biofilm cells sustained their initial ATP levels, planktonic cells experienced a 1-log reduction in ATP upon dehydration.

When desiccated biofilms and planktonic cells of *D. geothermalis* were exposed to vacuum, artificial Martian atmosphere, repeated thaw-freeze cycles, or extreme temperatures (-25 °C; +60 °C), their viability in terms of culturability and membrane integrity remained unchanged compared to dry but non-exposed controls. UV irradiation – either monochromatic (254 nm; ≥ 1 kJ m^{-2}) or polychromatic (200-400 nm; > 5.5 MJ m^{-2} for planktonic cells and > 270 MJ m^{-2} for biofilms) – significantly reduced the culturability of *D. geothermalis* in both its biofilm and planktonic form. Survival seemed to be insignificantly enhanced when the cells were irradiated in artificial Martian atmosphere instead of space-like vacuum.

Under both desiccation and UV irradiation, biofilms exhibited a decline in culturable cells whilst total cell counts and cultivation-independent viability parameters remained relatively stable. This suggests that a part of the population became VBNC as a response to these stressors. Induction of the VBNC state might confer an increased tolerance towards stress to *D. geothermalis*.

In conclusion, a significant fraction of the population of *D. geothermalis* sustained viability under all stress conditions tested, with biofilm cells often being more stress-tolerant than planktonic cells. It seems that the increased stress tolerance of biofilms is a result of the induction of a VBNC state and the protective effect of the EPS matrix. Judging from the results obtained in this study, *D. geothermalis* might survive in space or on Mars for a limited period of time, especially if shielded against the harmful extraterrestrial UV radiation.

1. INTRODUCTION

1.1 Lithopanspermia

The prospect of a spontaneous distribution of life throughout the universe has fascinated mankind for centuries. The idea traces back to Greek philosopher Anaxagoras (500-428 BCE), who laid the foundation for a theory widely known today as 'panspermia' (Greek, 'seeds everywhere'). According to panspermia, life can emerge anywhere in the universe provided that conditions are favourable, and mechanisms exist which may transport said life from one planetary body to another (Nicholson, 2009).

Various such transport mechanisms have been hypothesised. Arrhenius (1903) postulated that microorganisms present in the upper regions of a planet's atmosphere could be carried through space by the radiation pressure of nearby stars ('radiopanspermia'). His theory was criticised heavily, as the extraterrestrial radiation is considered lethal to unshielded organisms. Alternatively, a concept formed by Richter (1865), von Helmholtz (1871), and Thomson (1871) proposed rocky meteoroids as protective vessels for the transport of living organisms through space, a process later called 'lithopanspermia'. Such a process would not be driven by solar radiation but spallation[1], caused by the impact shock of a meteorite (Kamminga, 1982). The intended or unintended transfer of microorganisms throughout the solar system by man-made vehicles is another aspect of panspermia that has called for attention in the last few decades due to the emergence of human space travel. Nowadays, terrestrial microorganisms leave Earth on a regular basis – either as microbial contaminations of satellites, probes, and spacecraft, or associated with the skin and intestine of astronauts. These vectors may reach other celestial bodies much faster, under less stress, and with a higher probability than would meteoroids, thus posing a significant risk for the contamination of pristine extraterrestrial environments. So-called 'planetary protection' measures try to mitigate this forward-contamination as well as the backward-contamination of Earth by extraterrestrial microbial life forms (reviewed by Nicholson, 2009).

[1] "process in which fragments of material […] are ejected from a body due to impact or stress" (Nicholson, 2009)

1.1.1 The stages of lithopanspermia

A lithopanspermia scenario can be subdivided into three distinct stages: i) the ejection of life-bearing rock from a donor planet by spallation, ii) the passage through space as a meteoroid, and iii) the landing on a recipient planet that offers habitable conditions (Fig. 1.1; Nicholson, 2009).

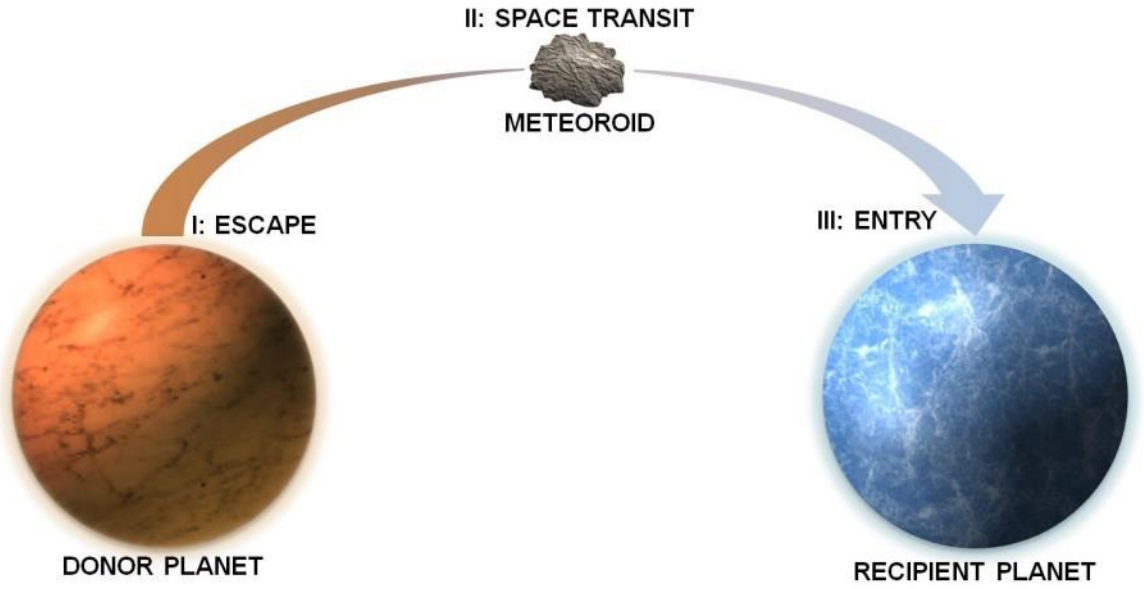

Figure 1.1. Schematic representation of a lithopanspermia scenario (after Nicholson, 2009).

Stage one, the ejection of rocky material from a planet into space, is an impact-driven process. When a solid planetary surface is hit by a meteorite, large amounts of near-surface soil and rock are spalled and thrown up. If velocity and size of the impacting object are of sufficient magnitude, the ejected matter may reach escape velocity and leave the host planet's gravitational field (Mileikowsky et al., 2000b). The pore spaces of near-surface rocks on Earth are frequently inhabited by endolithic (Greek, 'inside rock') microorganisms (Walker & Pace, 2007). One can only assume that this is also true for other inhabited planets. As a consequence, rocks ejected from such inhabited planets may carry endolithic organisms with them (Meyer et al., 2011). The impact of a meteorite on the donor planet causes the build-up of shock pressures in the range of 1-100 GPa (Burchell et al., 2004), which are needed for the thrown-up debris to reach escape velocity, e.g. 5.03 km s^{-1} for Mars (Mastrapa et al., 2001). The impact pressure causes the temperature of the affected rock to increase, what was historically believed to sterilise the ejecta during launch (Nicholson, 2009). More recent findings, however, suggest the possibility of a relatively mild spallation mechanism by which the shock wave directly translates into acceleration of the launched rocks (Melosh, 1984, 1985, 1989). In fact, analysis of Martian meteorites showed that during spallation these rocks were subjected

to heating in the range of 40-350 °C, i.e. some of them were never heated to temperatures of 100 °C or more (Shuster & Weiss, 2005; Weiss et al., 2000). Ejecta will experience additional heating due to friction, if the donor planet possesses an Earth-like or thicker atmosphere (Mileikowsky et al., 2000b). A low surface temperature of the donor planet would help to restrict said heating (Meyer et al., 2011).

In stage two, once ejected, the meteoroid starts to orbit around the Sun (or another star, for that matter). Orbiting may continue for up to millions of years until the meteoroid is either captured by a planetary body, or is hurled out of the solar system (Mileikowsky et al., 2000b). During space transit, the environmental conditions are highly detrimental to life as they are characterised by vacuum, desiccation, microgravity, extreme temperatures, and various types of radiation, including ionising and non-ionising solar UV radiation, diffuse X-rays, galactic cosmic rays (charged particles, gamma rays), and solar particles (Mileikowsky et al., 2000b; Nicholson, 2009). For the survival of the voyaging microbes, protection from these stressors is paramount. Endolithic microorganisms living inside the rock experience more favourable conditions than epilithic (Greek, 'on rock') lifeforms, as the meteoroid material shields them from radiation. Solar radiation poses by far the most harmful stressor in outer space, but it can be mitigated easily by shielding with a few micrometres of non-cracked rock (Mileikowsky et al., 2000b; Nicholson et al., 2005). A shield thickness of 0.5 g cm^{-2} and 30 g cm^{-2} is sufficient to shield from X-rays and solar particles, respectively. In order to shield from galactic cosmic rays, meteoroids would have to be the size of large boulders (~10 m), as this type of radiation penetrates deeper and the dose rates are amplified by generation of more particles within the rock itself (Mileikowsky et al., 2000b). Also, if the travelling organisms are embedded deep enough, vacuum exposure might be incomplete and friction-dependent temperature changes during escape or landing non-lethal (Melosh, 1988; Mendonça, 2014). According to speculations by Mileikowsky et al. (2000b), the time frame that permits survival in evacuated meteoroids is limited to a few decades due to desiccation and the decay of organic molecules such as DNA by vacuum. For microorganisms in non-evacuated rocks, on the other hand, flight times of up to 10^6 years would – theoretically speaking – be survivable.

In the third stage, the meteoroid reaches a planet – either by direct course or as it is captured by its gravitational field – and will land as a meteorite at velocities as high as 20 km s^{-1}. If the encountered planet possesses an atmosphere similar to Earth, the outermost layer of the meteorite will be subjected to frictional heating during landing (Nicholson, 2009). Small meteorites may overheat and evaporate before reaching the surface. Depending on its size, heating of the meteorite might be incomplete, i.e. metre-sized meteorites would remain cold inside, whilst the surface layer becomes glass. Similar to the spallation event, the meteorite faces shock pressures in the GPa range during impact on the recipient planet, accompanied by an increase in temperature and sudden deceleration (Mileikowsky et al., 2000b). Shattering

of the rock upon impact or during entry will release endolithic organisms into the new environment (Nicholson, 2009).

Attempts have been made to predict the likelihood of a successful lithopanspermia event, but the calculated results differ greatly due to the many factors involved (shock pressure magnitude, velocity and trajectory of the meteoroid, flight time, chance of survival, etc.) and most of them merely being assumptions (Nicholson, 2009). Whilst the interplanetary transfer of life within our solar system is considered probable (Mileikowsky et al., 2000b), it is very unlikely that life has ever been exchanged in between our and another solar system (Melosh, 2003).

1.1.2 Experimental support of the lithopanspermia hypothesis

Considerable experimental effort has been put into testing the feasibility of the lithopanspermia hypothesis from a biological point of view. In the course of a hypothetical lithopanspermia scenario the voyaging organisms are subjected to a cascade of extreme stresses. It seems difficult to prove the survivability of such a complex and protracted event in its entirety (Horneck et al., 2001), but multiple experiments have been carried out either in space or as simulations in the laboratory with focus on the individual stages of lithopanspermia. Bacterial spores, the dormant and persevering stage of microbes such as *Bacillus subtilis*, have been utilised as regular test candidates (Horneck et al., 2010).

Shock and acceleration experiments have presented substantial evidence that certain microorganisms would be able to survive stages 1 and 3 of lithopanspermia, i.e. impact-ejection and landing. Both stages are characterised by shock pressures in the range of 1-100 GPa and acceleration or deceleration to hypervelocity (Burchell et al., 2004). When embedded in or sandwiched between quartz, ceramics, or minerals, organisms such as *B. subtilis* (both spores and vegetative cells), *Rhodococcus erythropolis*, or the cyanobacterium *Chroococcidiopsis* sp. were able to survive shock pressures of 10-78 GPa, as determined by culture-dependent techniques exclusively (Burchell et al., 2004; Horneck et al., 2001; Horneck et al., 2008; Meyer et al., 2011). Both *B. subtilis* and *Deinococcus radiodurans* tolerated ultracentrifugation and ballistic experiments to simulate acceleration and jerk during ejection from Mars in the absence of heat or shock (Mastrapa et al., 2001). Cells and spores of *B. subtilis* and cells of *R. erythropolis* survived hypervelocities of 4.9-5.4 km s^{-1} (Burchell et al., 2004). Spores of *B. subtilis* mounted on a sounding rocket survived the hypervelocity (1.2 km s^{-1}) entry in Earth's atmosphere at a temperature of 145 °C with a reduction in culturability by two orders of magnitude (Fajardo-Cavazos et al., 2005).

The potential of microbes to survive space transit has been estimated experimentally in space and in space simulation facilities. Spores of *B. subtilis* remained culturable after residence in space for 6 years, especially when shielded against extraterrestrial UV radiation

(Horneck et al., 1994). Even tardigrades which are microscopic animals were able to withstand unshielded space exposure during a test period of 10 days (Jönsson et al., 2008). Recent findings simulated space exposure of several deinococci, *D. radiodurans* amongst others, in lower Earth orbit (LEO) for one year. In desiccated state, these organisms survived vacuum (10^{-1} Pa), monochromatic UV irradiation, a one-year dose of heavy ions, and 5,840 cycles of extreme temperature variations (Kawaguchi et al., 2013). The cyanobacterium *Chroococcidiopsis* sp. survived ground-based simulation of space and Mars environments, including space vacuum or artificial Martian atmosphere, UVC irradiation, and extreme temperature variations. Embedding of the cells in Martian mineral analogues facilitated survival (Baqué et al., 2013).

1.1.3 Lithopanspermia and Mars

The concept of the interplanetary transfer of microorganisms within rock fragments has gained rising attention as an explanation for the origin of life on Earth, implying that the sterile, early Earth was inoculated by meteorites bearing extraterrestrial life. Despite the fact that this explanation simply shifts the problem of how life evolved to another planet (Meyer et al., 2011), lithopanspermia must still be considered as a (very) hypothetical way of interchanging life forms in between planets, including not only the transport of life from Mars to Earth but also vice versa. Hence, it is of special interest with respect to life on Mars or Mars-like planets in other stellar systems, as Mars once may have offered habitable conditions similar to early Earth (Squyres et al., 2004). If so, exchange of rocky material between Mars and Earth in the past may have led to an interchange of life forms between the two planets.

Impacts on both celestial bodies were frequent in the past (Mileikowsky et al., 2000b), and small impact events on Mars supposedly still occur at regular intervals in the range of millions of years (Gladman, 1997; Mileikowsky et al., 2000b). Under the right circumstances – i.e. assuming optimal meteorite velocity, trajectory, and planetary alignment – these impacts would have led to the spall ejection of rocks which very well may have found their way to a nearby recipient planet (Gladman, 1997; Mileikowsky et al., 2000b). Flight times of meteorites from Earth to Mars and vice versa are believed to be most probably in the range of 10^7 years. A small fraction of arriving meteorites (approx. 1 out of 10^7), however, would travel in direct orbit, reducing the approximate flight time to a few months to years. It is these exceptional cases that are of special astrobiological interest, as the chances for organisms to survive space travel decrease with increasing flight times (Gladman, 1997; Gladman & Burns, 1996; Mileikowsky et al., 2000b).

For the success of microbial cross-contaminations between Earth and Mars, the arriving microorganisms would have to be able to survive the impact upon arrival and proliferate in their new environment (Nicholson, 2009). Conditions on present Mars largely seem hostile to life as we know it. The planet's surface is characterised by desiccated rock and soil enriched

in perchlorates that quickly oxidise any organic molecules which could act as nutrient sources for heterotrophic bacteria (de la Vega et al., 2007; Yen, 2000). Although a recent study presented evidence for the seasonal presence of downhill streams (Ojha et al., 2015), surface water is considered to be extremely scarce and – due to the low pressure (~10^3 Pa) and low surface temperature (-125 °C to -25 °C) of Mars – would have to be highly saline in order to be liquid (Bodnar, 2001; Martín-Torres et al., 2015; Squyres et al., 2004; Tosca et al., 2008). Additionally, the nature of Mars' surface mineralogy would cause the water to be highly acidic (Squyres et al., 2004) and/or enriched in perchlorates (Martín-Torres et al., 2015). Mars possesses a thin atmosphere which mostly contains carbon dioxide (> 95%), nitrogen, and argon, and only ineffectively shields from short-wave UV radiation (Nicholson, 2009). In the past, Mars is believed to once have offered more life-sustaining conditions, such as a thicker atmosphere, increased temperature and local warming by impacts and volcanic activity, and standing liquid water (Squyres et al., 2004; Westall et al., 2013). Though highly debatable, structures found by rover Curiosity on present Mars that resemble fossilised microbial mats on Earth suggest that Mars was inhabited at some point (Noffke, 2014).

1.2 The BOSS experiment

This study supplements the space experiment BOSS – Biofilm Organisms Surfing Space – which is carried out within the framework of the ESA (European Space Agency) mission EXPOSE-R2 on the International Space Station (ISS).

1.2.1 Hypothesis

The experiment BOSS investigates the potential of microorganisms to survive on Mars or Mars-like planets on one hand, and to survive travelling through space with respect to the lithopanspermia theory on the other hand. It is the hypothesis of the project that the survival of the test organisms is facilitated, if they are organised in the form of biofilms rather than being present as single cells. To test this hypothesis, samples of various microorganisms (biofilms and cells derived from planktonic cultures) have been transported to the ISS where they are exposed to the conditions of space in LEO and to simulated Mars conditions. After exposure for an estimated time period of 18 months, the samples will be returned to Earth and analysed for their survival. The survival rates of biofilms will be compared to the survival rates of planktonic cells.

1.2.2 EXPOSE-R2

The experiment BOSS is carried out within the EXPOSE-R2 facility, a hardware specifically constructed for the exposure of biological samples to space. The hardware comprises three trays, each one holding four sample carriers that are divided into two parts stacked on top of each other (Fig. 1.2). Each part of the sample carrier can accommodate up to 16 individual samples.

The upper part of the sample carrier is sealed with a window (quartz or magnesium fluoride) that permits the exposure of the accommodated samples to extraterrestrial UV radiation. The fluence and spectrum of the UV radiation can be adjusted by transmission filters and long pass cut-off filters (used to simulate the approximate light spectrum received by the surface of Mars). The lower part of the sample carrier is shielded from UV radiation in order to incorporate dark controls. The sample carrier is fitted with ventilation holes and valves. Once in space, the facility can be evacuated by opening the ventilation valves in order to expose the samples to pristine space conditions. In order to simulate Mars exposure, the carrier can be flooded with artificial Martian atmosphere.

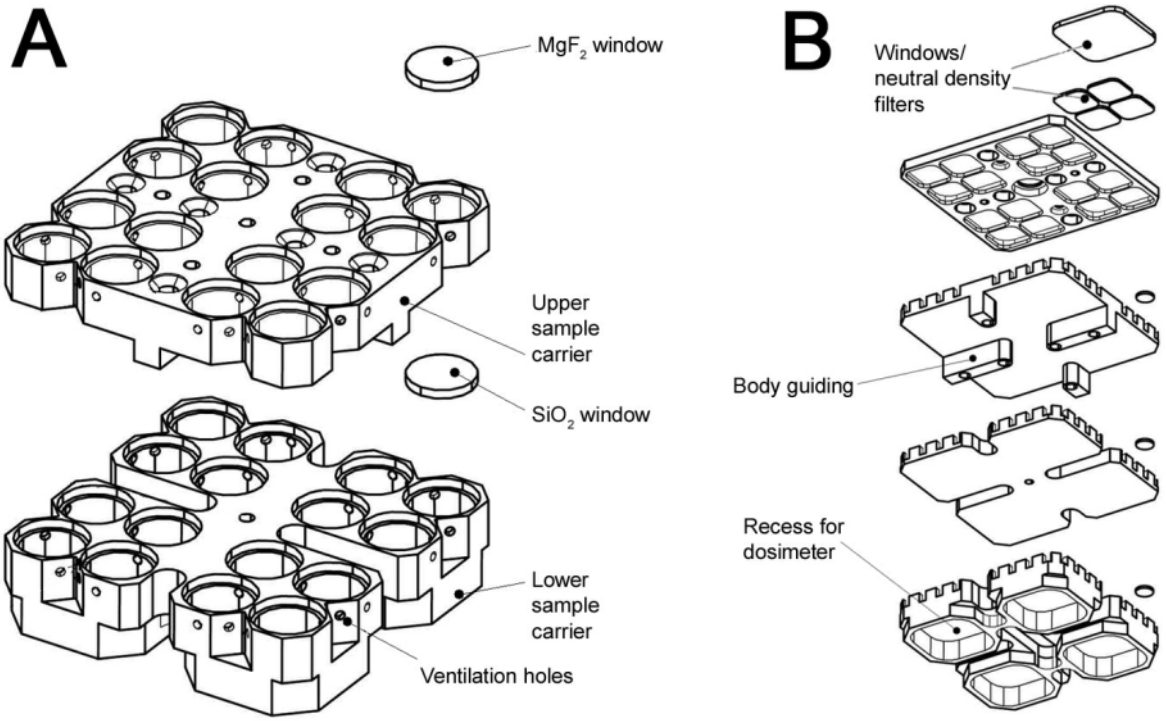

Figure 1.2. Schematics of a sample carrier of the EXPOSE-R2 hardware (courtesy of DLR Cologne). Each sample carrier comprises 2 modules (upper and lower sample carrier). Each module can accommodate 16 individual samples (**A**). Sample compartments are interconnected with ventilation holes to allow for evacuation of the carrier. The sample carrier sits below a window frame which can be equipped with quartz or magnesium fluoride glass and filters to reduce transmission or limit the transmitted spectrum to a certain wavelength range (**B**).

EXPOSE-R2 was launched into space on July 23th 2014 from Baikonur, Kazakhstan, using the spacecraft 56P Progress (Fig. 1.3) which docked with the ISS on July 24th. The EXPOSE-R2 facility was integrated into the supporting structure of the previous EXPOSE mission EXPOSE-R and mounted outside the ISS on August 18th. Exposure to space vacuum started on August 20th by evacuation of the respective sample carriers. On October 22nd the sun shield was removed in order to start exposure of the samples located in the upper sample carrier to extraterrestrial UV radiation (Fig. 1.4).

Figure 1.3. Spacecraft 56P Progress accommodating EXPOSE-R2 is being prepared for launch in Baikonur, Kazakhstan (courtesy of Roscosmos).

1.2.3 Ground-based simulation experiments

A mission ground reference control is run parallel to the space experiment at the Planetary and Space Simulation facilities (PSI) of the Institute of Aerospace Medicine at the German Aerospace Center (DLR) in Cologne, Germany. An equal set of samples is mounted in a duplicate sample carrier facility which is exposed to simulated space or Mars conditions. Using live telemetry data received from the ISS, the ground control samples are objected to the same fluctuations in temperature and irradiation as the samples in space. Further simulations were carried out beforehand as a preparation for the space mission. Whilst EXPOSE-R2 is still up and running at the point of writing, the results of the preparatory simulation experiments are presented in this work.

Figure 1.4. Photograph of the EXPOSE-R2 facility mounted outside the ISS (courtesy of ESA/NASA/RKA). It was taken by cosmonaut Aleksandr Samokutyaev on October 22nd 2014 during the removal of the sun shield. The event marked the beginning of exposure of the BOSS samples to extraterrestrial UV radiation.

1.2.4 Test organisms used in BOSS

In the BOSS experiment, various microorganisms are exposed to the life-threatening conditions of space and simulated Mars. The range of test organisms includes cyanobacteria of the genera *Gloeocapsa* and *Chroococcidiopsis*, spores of *B. subtilis*, mixed cultures of the halophile organisms *Halomonas muralis* and *Halococcus morrhuae*, and the bacterium *Deinococcus geothermalis*, with the latter one being used as a test organism in the present study. All test subjects share the ability to persist in extreme terrestrial habitats such as arid hot and cold deserts (Ferreira et al., 1997; Friedmann, 1980; Leuko et al., 2014; Olsson-Francis et al., 2010). In contrast to previous space experiments, in BOSS the test organisms are exposed as biofilms in which they are aggregated and embedded in extracellular polymeric substances (EPS) that are believed to facilitate survival of the organisms.

1.3 The test organism: *Deinococcus geothermalis* DSM 11300

D. geothermalis type strain DSM 11300 was chosen as a test organism for this project due to its exceptionally high tolerance to desiccation and ionising radiation (Tian & Hua, 2010), a feature common amongst the members of the *Deinococcaceae* (Minton, 1994). Thus it was supposed to possess an intrinsic advantage for surviving the harsh conditions of space which are characterised particularly by extreme desiccation and deleterious solar radiation.

1.3.1 Stress resistance of the *Deinococcaceae*

The genus *Deinococcus* presently comprises 51 species (Parte, 2014), which have been isolated from most different environments such as desert soil (de Groot et al., 2005), the Antarctica (Dong et al., 2014), marine sediments (Thorat et al., 2014), citrus leaves (Ahmed et al., 2014), or cleanrooms used for spacecraft assembly (Vaishampayan et al., 2014). *D. radiodurans*, firstly described as *Micrococcus radiodurans* in 1956 (Anderson et al., 1956), represents one of the most studied species of the genus and shows high phylogenetic similarity with the test organism *D. geothermalis* (Makarova et al., 2007). Deinococci are characterised by their extreme resistance against a multitude of environmental stressors (Tab. 1.1), particularly UV and gamma radiation (Makarova et al., 2007), which as a consequence are commonly used to isolate them from environmental samples. *D. geothermalis* tolerates radiation doses of 3,700 Gy whilst remaining fully culturable (Ferreira et al., 1997). As a comparison, the radiation-sensitive bacterium *Escherichia coli* is inactivated by doses as small as 30 Gy (Battista, 1997).

The reason for their radiation resistance remained ambiguous for years, as its evolutionary justification is obscure. Direct adaptation to radiation seems unlikely and would not implicate an evolutionary advantage, since deinococci are not exposed to high levels of radiation in their natural habitats. Their radiation resistance is considered more likely to be a result of the adaptation to changes in the water regime occurring in their natural habitat, e.g. the periodic desiccation of soil (Fredrickson et al., 2008; Mattimore & Battista, 1996). Deinococci have evolved potent repair systems to mend the extensive cellular damage that is caused by prolonged periods of desiccation. These repair mechanisms also help to cope with radiation stress, as it causes similar cellular injuries such as DNA double strand breaks (Mattimore & Battista, 1996), which are lethal if not repaired (Minton, 1994).

The genome of *D. geothermalis* (fully sequenced by Makarova et al., 2007) does not contain any unique or notable defence systems which would explain its extreme resistance (Liedert et al., 2012). It comprises a circular chromosome of 2,467,205 base pairs and two megaplasmids of 205,686 and 574,127 base pairs, respectively. Ionising radiation such as gamma radiation, X-rays, or short-wavelength UV light is known to induce double strand breaks in chromosomal and plasmid DNA. Deinococci seem to be as susceptible to this sort of DNA damage as are radiation-sensitive bacteria. In *D. radiodurans*, for example, a radiation dose as high as 5,000 Gy causes 200 DNA double strand breaks per genome (Battista, 1997; Slade et al., 2009). Since each cell possesses 8 to 10 genome copies, this accumulates to 1,600 to 2,000 DNA double strand breaks per cell (Cox & Battista, 2005). Despite that, radiated cells remain fully culturable and show no signs of mutation (Battista, 1997), suggesting that DNA repair in deinococci is highly efficient, accurate, and error-free (Cox & Battista, 2005; Makarova et al., 2007).

Table 1.1. List of stressors tolerated by selected members of the *Deinococcaceae*.

Stressor	Tolerated values	Species	Reference
Desiccation	85% survival after 6 weeks at < 5% relative humidity	*D. radiodurans*	[1]
	30% survival after 6 weeks	*D. radiodurans* R1	[2]
	10% survival after > 30 days	*D. geothermalis*	[3]
	13-63% survival after 42 days of desiccation in an exsiccator over $CaSO_4$	*D. radiodurans*	[4]
Gamma radiation	shoulder dose[a]: 5,000 Gy (corresponds to 200 DNA double strand breaks per genome); lethal dose: > 15,000 Gy; D_{37} dose[b]: 6,000 Gy (*E. coli*: 30 Gy)	*D. radiodurans* R1	[5]
	shoulder dose[a]: 3,700 Gy; D_{10} dose[c]: 5,100 Gy	*D. geothermalis* DSM 11300	[6]
Heavy metal ions	growth inhibited at 10 mM	*D. geothermalis* T27	[7]
	Fe(III), U(VI), Cr(VI)	*D. geothermalis* DSM 11300	[8]
Organic solvents	20% (v/v) e.g. toluene, ethyl acetate	*D. geothermalis* T27	[7]
	5% (v/v) benzene; 20% (v/v) n-decane, diethylphthalate	*D. geothermalis* DSM 11300	[7]
pH	5.0-9.0	*D. geothermalis* T27	[7]
	4.5-8.5	*D. geothermalis* DSM 11300	[6]
Reactive oxygen species	70% survival at 40 mM H_2O_2	*D. radiodurans* R1	[2]
Temperatures	< 55 °C	*D. geothermalis* T27	[7]
	30-57 °C	*D. geothermalis* DSM 11300	[6]
UV radiation	survives 1,000 J m^{-2}; D_{37} dose[b]: 550-600 J m^{-2} (*E. coli*: 30 J m^{-2})	*D. radiodurans* R1	[5]
	80% survival at 900 J m^{-2}	*D. radiodurans* R1	[2]
	0.01% survival at 4 kJ m^{-2} (254 nm)	*D. geothermalis*	[9]
	no loss of culturability at 500 J m^{-2}	*D. radiodurans*	[10]

[a] Dose of radiation that has no measurable effect on viability;
[b] Dose of radiation required to reduce the number of individuals of a population to 37%;
[c] Dose of radiation required to reduce the number of individuals of a population to 10%;
[1] Slade and Radman (2011), [2] Tian et al. (2007), [3] (Daly et al., 2004), [4] (Mattimore & Battista, 1996), [5] Battista (1997), [6] Ferreira et al. (1997), [7] Kongpol et al. (2008), [8] Brim et al. (2003), [9] (Makarova et al., 2007), [10] (Moseley & Mattingly, 1971)

Although the exact mechanisms are still unknown, recovery of deinococci from extensive DNA damage is likely the result of a combination of novel DNA repair systems and passive protective features, such as the presence of multiple genome copies per cell, the organisation of the nucleoid as a tightly-structured toroid unaffected by irradiation, and mechanisms which scavenge harmful reactive oxygen species (ROS; Cox & Battista, 2005; Daly et al., 2004; Englander et al., 2004; Mattimore & Battista, 1996).

The proteome of *D. geothermalis* E50051 was found to contain stress defence proteins, chaperons that re-establish the conformation of damaged proteins (Liedert et al., 2012), enzymes related to oxidative stress such as superoxide dismutase (sodA) and catalase (katA), a transketolase which is supposed to be involved in DNA excision repair after oxidative stress in *D. radiodurans* (Makarova et al., 2007), and the protein repair enzyme peptidylprolyl isomerase (Liedert et al., 2010). Further proteins of unknown functions have been discovered, putatively being involved in DNA repair, stress resistance, halo-tolerance, and oxidation reduction (Liedert et al., 2010). The expression of repair enzymes is highly upregulated in stressed cultures, but they are also abundant when stressors are absent, especially in stationary-phase cultures (Liedert et al., 2010; Liedert et al., 2012). *D. geothermalis* accumulates manganese ions whilst keeping iron ion levels low (Daly et al., 2004). High Mn(II)/Fe(II) ratios have been correlated with high tolerance towards desiccation and radiation. Whilst Fe(II) ions catalyse protein-damaging Fenton reactions, Mn(II) ions neutralise ROS and thus protect proteins from oxidative damage (Daly et al., 2007; Ghosal et al., 2005). Manganese ions are also cofactors in enzymes that battle oxidative stress such as superoxide dismutases and catalases (summarised by Liedert et al., 2012). In the absence of manganese, *D. geothermalis* is able to re-channel its carbon metabolism to produce ROS-scavenging metabolites such as succinate, whilst downregulating ROS-producing metabolic pathways (Liedert et al., 2012).

Deinococci possess a complex and unusual cell envelope that contributes to their tenacity. Although the organisms stain Gram-positive – usually an indication for a cell envelope comprising a plasma membrane surrounded by a peptidoglycan cell wall (Silhavy et al., 2010) – the deinococcal cell envelope shows features which suggest a distant relationship to Gram-negative-like ancestors (Brooks et al., 1980). Following the plasma membrane and an approximately 50 nm thick, porous peptidoglycan layer, the cell is engulfed in the so-called 'pink envelope' (Fig. 1.5). Starting from the inside, this array consists of a loosely-organised, compartmentalised layer, an outer cell membrane, a hydrophobic, protein-rich backing layer, and a surface (S-) layer of hexagonal arrangement coated with a superficial layer of carbohydrates (Farci et al., 2014; Gerber et al., 2015; Lister & Pinhero, 2001; Thompson & Murray, 1981; Thompson et al., 1982). In *D. radiodurans*, the highly expressed S-layer proteins 'Hexagonal Packing Intermediate' (HPI) and 'Surface Layer Protein A' (SlpA), are associated with environmental protection (Karlin & Mrázek, 2001; Lister & Pinhero, 2001). Evidence indicat-

ing the presence of an S-layer in *D. geothermalis* exists (Kolari et al., 2002; Liedert et al., 2010). Deinococci express carotenoid pigments (e.g. deinoxanthin in *D. radiodurans*) which are incorporated into cell envelope features such as the plasma membrane and the outer cell membrane (Thompson & Murray, 1981). These pigments are considered to play an important role in photoprotection and in the elimination of ROS (Gao & Garcia-Pichel, 2011; Tian & Hua, 2010; Tian et al., 2007).

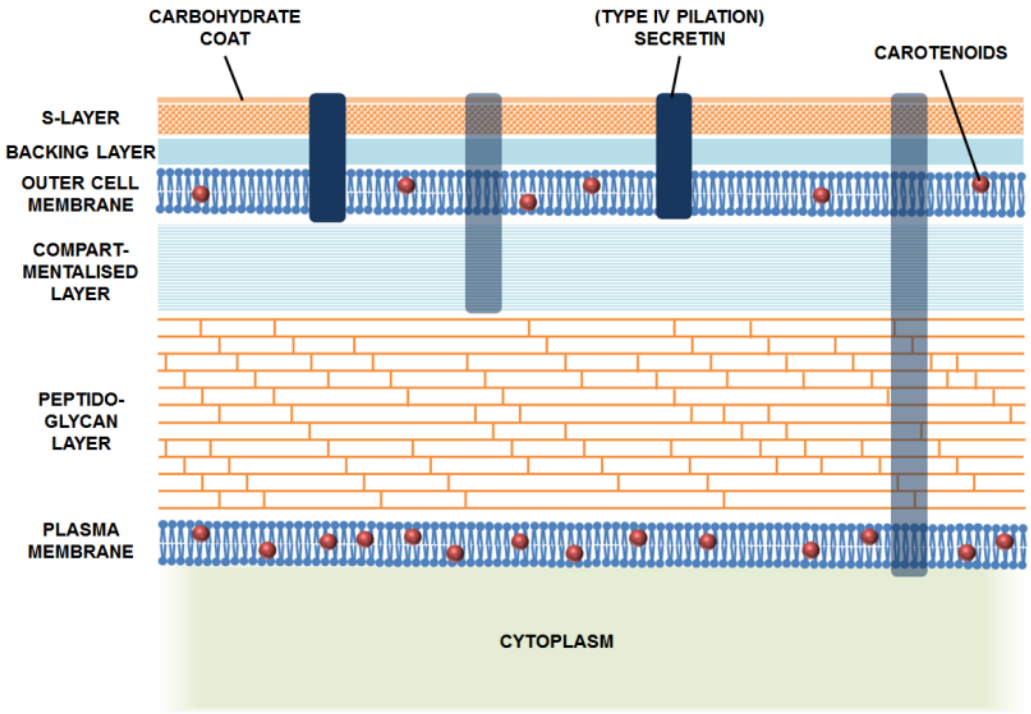

Figure 1.5. Simplified schematic representation of the deinococcal cell envelope comprising an inner plasma membrane and a peptidoglycan layer followed by a multi-layer arrangement termed 'pink envelope'. The hexagonally arranged S-layer is firmly attached to the outer cell membrane, likely by protein anchors. Secretins of the type IV pilation family extend from the S-layer to the outer cell membrane, or possibly even to the cytoplasm. Both membranes, particularly the plasma membrane, are enriched in carotenoids. In contrast to Gram-negative bacteria, the outer membrane of deinococci lacks lipopolysaccharides (after Farci et al., 2014; Gerber et al., 2015; Thompson & Murray, 1981; Thompson et al., 1982).

1.3.2 Physiology and metabolism of *D. geothermalis*

D. geothermalis is a moderately thermophilic bacterium with an optimum growth temperature of 45-50 °C and an optimum pH for growth of 6.5. Growth is restricted to a pH range of 4.5-8.5 (Tab. 1.1; Ferreira et al., 1997). The type strain *D. geothermalis* DSM 11300 was firstly isolated from hot springs at the Termi di Agnano, Naples, Italy (Ferreira et al., 1997), but later also found in biofilms growing in the splash zone of industrial paper mills (Väisänen et al.,

1998), in subterranean hot springs in Iceland (Marteinsson et al., 2001), in warm soil (Kongpol et al., 2008), and in marine subsurface sediments adjacent to hydrothermal vents (Kimura et al., 2003). *D. geothermalis* forms pink to orange-pigmented, non-motile, spherical cells with a diameter of 1.2-2.0 µm which commonly divide as diplococci or tetrads (Bornot et al., 2014b; Ferreira et al., 1997). The organism stains Gram-positive (Ferreira et al., 1997), although its cell envelope is far more complex than a positive Gram staining would typically suggest (see 1.3.1).

Only few reports regarding the nutritional requirements of *D. geothermalis* has been published so far (Bornot et al., 2014a; Bornot et al., 2014b; Brim et al., 2003; Ferreira et al., 1997; Kongpol et al., 2008). *D. geothermalis* has been cultured on a variety of nutrient media, most of them being nutrient-rich and containing at least one complex nutrient source such as tryptone, peptone, casamino acids, or yeast extract (summarised by Bornot et al., 2014b). These complex carbon and nitrogen sources may contain vitamins and amino acids and are preferred by deinococci to glucose (Bornot et al., 2014b; Venkateswaran et al., 2000). Whilst Ferreira et al. (1997) mentioned that yeast extract is not required for growth, recent findings showed that growth of *D. geothermalis* in the absence of yeast extract is linear and limited, whereas the addition of yeast extract to the nutrient medium enables exponential growth at a significantly higher growth rate (Bornot et al., 2014b). The organism is saccharolytic and can utilise a broad spectrum of carbon sources (Ferreira et al., 1997; Kolari et al., 2003; Väisänen et al., 1998) including hydrocarbon solvents (Kongpol et al., 2008). It is able to reduce various heavy metals such as Fe(III), U(VI), and Cr(VI), for which reason it is considered a suitable candidate for the bioremediation of radioactive sites (Brim et al., 2003; Kongpol et al., 2008).

1.3.3 Deinococcal biofilms

D. geothermalis is a primary biofilm former, notorious for contaminating industrial paper machines where it functions as a primer for secondary biofilm-forming organisms. It is able to adhere to and multiply on various submerged abiotic surfaces such as polystyrene, stainless steel, glass, or polyethylene, where it forms tenacious biofilms (Kolari et al., 2001; Kolari et al., 2002). In cultures of *D. geothermalis* E50051, Liedert et al. (2012) found evidence for the induction of proteins functionally related to adhesion and biofilm formation in *Pseudomonas aeruginosa* and *E. coli*.

As reported for *D. geothermalis* strain E50051, attachment to surfaces is mediated by highly adhesive, pseudopod-like structures distributed heterogeneously over the cell surface (Kolari et al., 2002). Reportedly, these structures, termed 'adhesion threads', occur exclusively in sessile cells (Fig. 1.6), irrespective of the nutrient medium used (Kolari et al., 2002). The adhesion threads emanate from the cell surface and span over a distance of up to 1 µm to mediate cell-to-surface as well as cell-to-cell adhesion (Kolari et al., 2002; Raulio et al., 2006;

Saarimaa et al., 2006). Isolation and N-terminal sequencing revealed the adhesion threads to consist of glycosylated proteins, the majority being a pilin highly similar to two putative type IV pilins in the genome of *D. radiodurans* R1. Consequently, the adhesion threads were characterised as type IV pili (Saarimaa et al., 2006), although they bear no morphological resemblance to type IV pili-related adhesion organelles observed in other microorganisms (Raulio et al., 2006). The finding of pili in strain E50051 seems inconsistent with observations made on type strain DSM 11300 by Ferreira et al. (1997), who stated that *D. geothermalis* possesses neither flagella nor pili. The functionality of type IV pili is associated with bacterial motility (Mattick, 2002; Wall & Kaiser, 1999) and DNA transfer (Dubnau, 1999), but also with the adhesion of organisms such as *P. aeruginosa* to biotic and abiotic surfaces (Giltner et al., 2006; Nudleman & Kaiser, 2004). Attachment of strain E50051 to abiotic surfaces was shown to be "firm but slippery" (Kolari et al., 2002), or in other words highly sticky. Kolari et al. (2002) were able to move single sessile cells along a surface using a glass capillary without interfering with attachment. This lateral flexibility of attachment was lost by air-drying, but re-established upon rehydration in water.

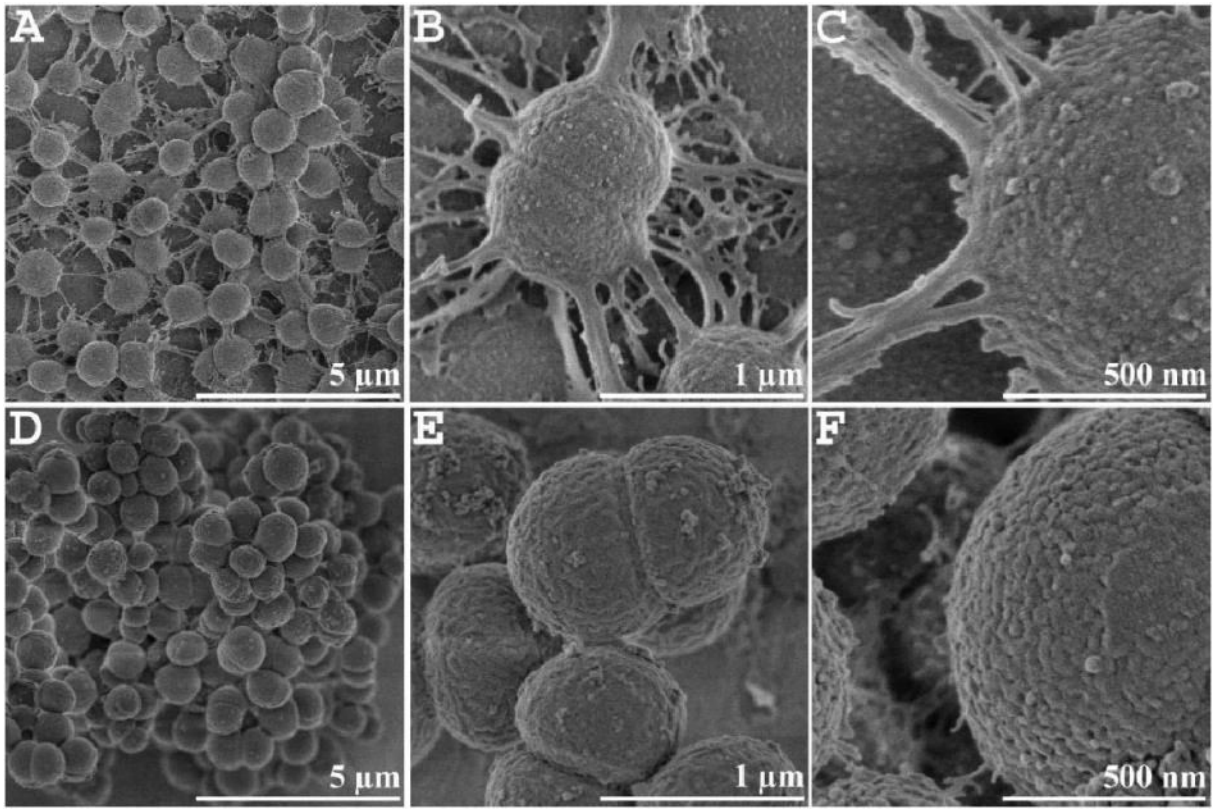

Figure 1.6. FESEM micrographs of sessile (**A**, **B**, and **C**) and planktonic (**D**, **E**, and **F**) cells of *D. geothermalis* strain E50051. Sessile cells (grown on glass slides submerged in 10-fold diluted tryptic soy broth) possess adhesion threads, which mediate cell-to-surface and cell-to-cell attachment. Planktonic cells lack adhesion threads (from Saarimaa et al., 2006).

Little is known about the EPS and other extracellular features of the type strain DSM 11300 investigated here, except that it forms a capsule rather than an extensive slime matrix (Ferreira et al., 1997). Both capsule and adhesion threads were not found on cells grown in liquid medium (Ferreira et al., 1997; Liedert et al., 2010; Saarimaa et al., 2006), suggesting the absence of EPS in the planktonic mode of existence. The EPS of strain E50051 have been studied to some extent. Apart from pilin-related proteins, a serine-like protease and three 5'-nucleotidases were identified in the EPS isolated from sessile cells. 5'-nucleotidases are suggested to play a role in peptidoglycan and exopolysaccharide synthesis. Six other proteins were recovered from EPS extracts but could not be identified (Saarimaa et al., 2006). The glycoconjugate nature of the EPS of *D. geothermalis* strain E50051 has been confirmed by using fluorescently-labelled lectins. CLSM observations acknowledged the lack of an extensive slime matrix of deinococcal biofilms, but revealed the presence of extracellular polysaccharides within the biofilm colony (Peltola et al., 2008; Saarimaa et al., 2006). Judging from lectin-dependent binding specificities, various target structures of heterogeneous distribution have been identified. Two distinct exocellular structures have been found within the core of the biofilm colony; a skeletal structure probably containing α-1,3-interlinked building blocks of N-acetylgalactosamine, and a matrix of multi-lectin specificity filling the intercellular void. Other lectins specific for galactose β-1,3-interlinked with N-acetylneuraminic acid identified a different target structure which was located on the cell surface and was associated with the adhesion threads described by Kolari et al. (2002) and Saarimaa et al. (2006).

1.4 Effects of extraterrestrial conditions on microbial viability

The environmental conditions of space and Mars are – by terrestrial standards – extremely hostile towards life (see 1.1). Both environments are characterised by a multitude of stressors that challenge microbial viability. The effect of two of these stressors, desiccation and solar UV radiation, on microbial life represents a main focal point of this study.

1.4.1 Desiccation-induced damage

Water is essential for life, as it is required to maintain physiological functions and cell membrane integrity of microbes (Billi & Potts, 2002; Potts, 2001). Microorganisms living in biofilms additionally depend on water as it is necessary for the transport of nutrients within the biofilm matrix (Schmitt & Flemming, 1999). In that sense, desiccation represents a severe stressor for microbial life.

Water is necessary to maintain the fluidity of the cell membrane (Billi & Potts, 2002). In hydrated state, membranes, i.e. phospholipid bilayers, are surrounded by a hydration shell. The amount of bound water to form the hydration shell may vary depending on the compo-

sition of the membrane (Crowe et al., 1987). The water molecules, infiltrating the space around the phosphate head groups of the phospholipids, cause the membrane to swell and diminish interactions between adjacent phospholipids and other membrane constituents (Crowe et al., 1987). In the event of desiccation, the hydration shell surrounding the phosphate head groups is lost. As a consequence, the phospholipids come in closer spatial proximity, leading to increased van der Waal's interactions amongst their hydrocarbon chains (Crowe et al., 1987). Accompanied by an elevated phase transition temperature (T_m), this causes a phase transition of the membrane from a liquid-crystalline phase to a gel phase at environmentally relevant temperatures (Billi & Potts, 2002; Crowe et al., 1987; Potts, 1994). In this state as well as during phase transition the cell membrane becomes susceptible to leakage (Crowe et al., 2001), either due to fusion of phospholipids (summarised by Crowe et al., 1987) or due to temporary membrane defects during the transition from liquid-crystalline phase to gel phase and vice versa (Lee, 1977). Upon rehydration, the cell membrane undergoes a further phase transition from gel phase back to liquid-crystalline phase, again possibly leading to leakage and thus cell lysis (Crowe et al., 1987; Potts, 1994).

Similar to membrane biopolymers, also intracellular proteins are directly affected by dehydration. In their functional state, proteins are hydrated and rely on their bound water in order to interact with ligands (Jaenicke, 1992). As the cell dehydrates and cellular water is lost, the bound water is stripped from proteins, causing conformational changes (Prestrelski et al., 1993) and loss of protein functionality (Jaenicke, 1992). Whilst enzymatic repair mechanisms are arrested, dysfunctional enzymes and electron transport chains lead to the build-up of ROS such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\bullet}$). These ROS accumulate during extended periods of desiccation and cause significant to lethal damage to lipids, proteins, and particularly DNA, when their concentrations exceed the antioxidant status of the cell (Brawn & Fridovich, 1981; França et al., 2007; Hansen et al., 2006). Other factors promoting the intracellular accumulation of ROS include the reduction of transport mechanisms within the cytoplasm, increased ionic strength, and altered cytoplasmic pH due to shrinkage of the cell (Senaratna & McKersie, 1986).

The oxidative damage caused by ROS is considered one the most deleterious effects of desiccation on living cells (França et al., 2007). Even desiccation-tolerant microorganisms such as certain cyanobacteria accumulate considerable amounts of ROS during dehydration (Potts, 1994). In yeast, desiccation was shown to cause a ten-fold increase in oxidation, confirming that loss of water induces oxidative stress (de Jesus Pereira et al., 2003). ROS cause peroxidation of lipids, contributing to the risk of membrane leakage (Vriezen et al., 2012). Furthermore, they damage proteins, e.g. by metal-catalysed oxidation. In this case, ROS react with Fe^{3+} ions, yielding Fe^{2+} and oxygen. The Fe^{2+} forms a complex with a metal-binding site of a protein which then reacts with hydrogen peroxide to form ferryl ions. Ferryl ions attack

nearby side chains, leading to carbonylation of the protein (França et al., 2007; Krisko & Radman, 2010; Potts, 1994). Oxidised proteins may exhibit increased instability, increased sensitivity towards proteolysis, and reduced to no activity (França et al., 2007; Stadtman & Oliver, 1991).

DNA and other nucleic acids represent the main targets of desiccation-induced damage (Asada et al., 1979; Mattimore & Battista, 1996). Mainly, this is due to the build-up of strand defects that would normally be repaired in hydrated cells but accumulate when repair mechanisms are arrested, as they are in dried cells (Potts, 1994; Setlow, 1992). Additionally, ROS generated upon dehydration of the cell also target nucleic acids (Brawn & Fridovich, 1981). Target structures include the phosphorus atom of the phosphodiester backbone or hydrogen atoms of the deoxyribose ring, and their cleavage leads to single strand DNA breaks (Imlay & Linn, 1988; Potts, 1994). Two closely-spaced single strand breaks on antiparallel DNA strands can cause double strand breaks and, thus, fragmentation of the DNA (Lieber, 2010). ROS may chemically alter DNA bases which then need to be removed enzymatically (Imlay & Linn, 1988). Maillard (browning) reactions modify DNA via cross-linking with other nucleic acids and proteins. The products of these amino-carbonyl reactions may show impaired functionality and accumulate with increasing periods of desiccation (Horneck et al., 2010; Potts et al., 2005).

DNA damage is lethal, if not readily repaired upon rehydration (Friedberg, 2003; Minton, 1994; Setlow, 1992). However, microorganisms can only repair a certain amount of DNA strand breaks. The capacity to mend DNA damage is species-specific and depends on the DNA repair and protection mechanisms utilised by the organism (see 1.4.3). Whilst desiccation-sensitive bacteria such as *E. coli* fail to repair less than ten double strand breaks (Daly, 2012), other, more desiccation-tolerant organisms such as cyanobacteria or deinococci abide twenty- to thirty-fold higher rates of DNA fragmentation (Cox & Battista, 2005; Daly, 2012).

1.4.2 UV radiation-induced damage

The cellular damage that is caused to microbes by UV and ionising radiation is similar to that caused by desiccation. Radiation-induced damage can be discriminated into direct and indirect damage (Daly, 2012). Direct damage occurs when photon energy is absorbed by a variety of biomolecules, thus causing deleterious photochemical reactions (Gao & Garcia-Pichel, 2011). In the case of DNA, excitation of the sugar-phosphate groups may lead to the destruction of the sugar moiety and eventually result in base release and breakage of the single strand (Ito, 1992). In proteins, photon absorption can cause changes in conformation (Gao & Garcia-Pichel, 2011), which reduce the functionality and effectivity of enzymes including those involved in combating and repairing oxidative damage (Daly, 2012). However, it is estimated that direct irradiation-induced DNA damage only accounts for about 20% of the total DNA

damage caused by UV radiation. The main source of cellular damage is due to the intracellular generation and accumulation of ROS (Ghosal et al., 2005) which is facilitated by desiccation and further amplified by exposure to light and other forms of radiation (França et al., 2007; Potts, 1994). As described above (1.4.1), ROS harm proteins, lipids, and nucleic acids, thus affecting cell membrane integrity (Potts, 1994), genome conservation (Imlay & Linn, 1988), and cellular maintenance and repair mechanisms due to reduced enzyme functionality (Daly, 2012; Krisko & Radman, 2010).

In terrestrial habitats, mainly UV-A radiation (315-400 nm) and UV-B radiation (280-315 nm) are of biological importance, as the most energetic wavelength range UV-C (100-280 nm) is filtered by the ozone layer (Elasri & Miller, 1999; Gao & Garcia-Pichel, 2011). In open space, however, unshielded microorganisms would experience the full spectrum of deleterious solar radiation – both ionising and non-ionising – accompanied by diffuse X-rays, galactic cosmic rays (charged particles, gamma rays), and solar particles (Cox & Battista, 2005; Mileikowsky et al., 2000b; Nicholson, 2009). Nevertheless, extraterrestrial UV seems to be the most deleterious stressor for life forms in space (Horneck & Rabbow, 2007). DNA represents a sensitive target for UV in space, as it readily absorbs the full spectrum (> 190 nm) of solar UV (Horneck et al., 2010). The extremely resistant spores of *B. subtilis* were reported to accumulate mutagenic and lethal DNA damage within seconds of exposure to solar UV in LEO (Nicholson et al., 2000). Also extreme UV radiation within the range of 10-190 nm was shown to inactivate bacteria such as *B. subtilis* or *D. radiodurans*, both known for their resistance to high doses of ionising radiation, during space exposure (Saffary et al., 2002). Extreme UV damages DNA (Ito, 1992), but as it is only transmitted through high vacuum, it might largely be absorbed by cell envelope structures, i.e. membrane lipids and proteins, of microorganisms in space (Saffary et al., 2002). The simultaneous exposure to vacuum and UV, as it is the case in space, further amplifies the mutagenic effects of UV radiation due to complete dehydration of the organisms (Horneck, 1993). Vacuum-induced DNA damage manifests in base modifications and strand breaks (Horneck & Rabbow, 2007). When exposed to space vacuum, spores of *B. subtilis* exhibited a ten-fold increased mutation rate compared to the spontaneous mutation rate of non-exposed spores (Horneck & Rabbow, 2007). Following long-term (559 days) exposure to space vacuum and full solar UV, mutation rates in *B. subtilis* spores had increased by almost four orders of magnitude (Moeller et al., 2012), demonstrating that DNA damage accumulates during exposure to extraterrestrial UV radiation (Horneck et al., 2010).

1.4.3 Microbial adaptation to desiccation and irradiation

Many terrestrial environments are characterised by extended periods of desiccation, intense solar irradiation, or a combination of both (e.g. intertidal systems, exposed rock surfaces in hot arid deserts). Numerous microorganisms such as cyanobacteria or deinococci have de-

veloped strategies that allow them to persist in these habitats (Chanal et al., 2006; Decho, 2000; Gao & Garcia-Pichel, 2011). These organisms have evolved a range of mechanisms that increase their tolerance against desiccation and oxidative damage. Such mechanisms include passive protection measures or active stress defences.

The intracellular accumulation of compatible solutes – either by biosynthesis of organic compounds such as betaines, sugars, or amino acids, or by selective intake of inorganic solutes such as potassium ions – helps to mitigate water stress by preventing osmotic dehydration of the cytoplasm (D'Souza-Ault et al., 1993; Larsen et al., 1987; Roberson & Firestone, 1992; van de Mortel & Halverson, 2004). The disaccharide trehalose seems to be one of the most effective and widely utilised compatible solutes in microorganisms, and its intracellular presence has been connected with increased desiccation tolerance (Crowe, 2014; França et al., 2007). Accumulation of trehalose and sucrose was found to be a response of drought-resistant cyanobacteria to desiccation (Hershkovitz et al., 1991), and also desiccation-sensitive bacteria such as *E. coli* may increase their tolerance against water stress by trehalose synthesis (Larsen et al., 1987; Zhang & Yan, 2012). Apart from acting as compatible solutes, these carbohydrates can help maintaining the fluidity of the cell membrane upon water loss (Crowe et al., 2001; Hershkovitz et al., 1991) and protect proteins during drying (Crowe et al., 1987) by replacing the water molecules in the hydration shell of these biomolecules (França et al., 2007; Leslie et al., 1995). Additionally, intracellular accumulation of trehalose leads to the formation of stable glasses (a process called 'vitrification') during desiccation (Crowe, 2014). The high viscosity of a glassy cytoplasm aids in maintaining the native folding of biomolecules and decreases the rates of (deleterious) chemical reactions (Buitink & Leprince, 2004). Trehalose might further act as an ROS scavenger, as it was shown to reduce oxidative damage in H_2O_2- or desiccation-stressed yeast cells (Benaroudj et al., 2001; de Jesus Pereira et al., 2003).

Many bacteria such as cyanobacteria or deinococci synthesise pigments, which act as microbial sunscreens and can scavenge ROS, therefore protecting against both UV radiation and desiccation (Fleming & Castenholz, 2007; Gao & Garcia-Pichel, 2011; Tian & Hua, 2010). Certain modifications to cell membrane constituents may contribute to a higher resistance to desiccation. For example, *cis/trans* isomerisation of lipopolysaccharides or an increased degree of unsaturation potentially influence membrane fluidity (Beney & Gervais, 2001; Hoekstra et al., 2001; Loffeld & Keweloh, 1996) and thus may help to suppress the dehydration-mediated rise in the T_m of the cell membrane (França et al., 2007). Other cell envelope structures such as S-layers may stabilise the cell in the case of inner volume changes caused by desiccation (Lister & Pinhero, 2001).

In addition to these protection mechanisms, repair and detoxification systems play a major role for the acquisition of desiccation oxidative stress tolerance in microorganisms (França et al., 2007). In fact, for some extremely resistant organisms such as deinococci, the

reason for their resilience seems to lie in their potential to efficiently repair rather than to protect their cellular functions (Makarova et al., 2007). DNA repair enzymes can target DNA modifications or strand breaks. Base mutations are eliminated by excision repair or by reversing the formation of pyrimidine dimers and methylated or alkylated bases. DNA single-strand or double-strand breaks are fixed by recombinational repair (Minton, 1994; Potts, 1994), which is only possible when multiple genome copies are present, as it is the case in *D. radiodurans* and certain cyanobacteria (Cox & Battista, 2005; Daly et al., 1994; Minton, 1994; Potts, 1994). Detoxifying mechanisms include enzymes such as catalases, peroxidases, and superoxide dismutases, which scavenge and eliminate ROS (Markillie et al., 1999). Fe-superoxide dismutase was found to be the third most abundant protein in desiccated cells of the cyanobacterium *Nostoc commune* and was still active after storage of the cells in dry state for 13 years (Shirkey et al., 2000). In order for these enzymes to function once metabolic activity is resumed, the protection of proteins from oxidative damage is essential, and can be facilitated by maintenance of high intracellular Mn(II)/Fe(II) ion ratios (Daly, 2012; Daly et al., 2004; Fredrickson et al., 2008; Krisko & Radman, 2010).

The physiological state of a cell at the event of drying is expected to have a direct influence on its desiccation tolerance (Potts, 1994; Vriezen et al., 2006). For example, rhizobia accumulate trehalose and the carbon storage polymer polyhydroxybutyrate during stationary-phase growth (Manna et al., 1999). When dried in stationary state, *Sinorhizobium meliloti* survived desiccation 2.9-fold better than cells of the same organism dried during exponential growth (Vriezen et al., 2006). Desiccation may induce dormancy, arresting the cell's metabolism. Low metabolic activity decreases the biosynthetic generation of toxic by-products such as free radicals, but simultaneously inhibits the induction of antioxidants and repair systems (Fredrickson et al., 2008). Certain microorganisms, most prominently *B. subtilis*, form dormant spores when environmental conditions become adverse. The water content of spores is lower than in vegetative cells, so that intracellular biomolecules are immobilised and metabolism is halted. In this state, they are highly resistant to desiccation, radiation, and other stressors (Setlow, 2007).

The production of EPS is another important microbial response to desiccation. EPS production is usually associated with biofilm formation and is triggered (or emphasized) when the cell experiences matric water stress. Bacterial EPS tend to be hygroscopic and have the potential to retain water several times their weight if the water availability in the bulk environment is declining (Chang et al., 2007; Or et al., 2007; Potts, 1994; Roberson & Firestone, 1992). By slowing the drying rate, hygroscopic EPS create a microhabitat in which survival through short periods of desiccation is facilitated (Flemming & Wingender, 2010), whilst giving its inhabitants the opportunity to physiologically and metabolically adapt to the changing hydraulic conditions (Or et al., 2007).

Microorganisms are able to actively respond to water stress by changing the composition and quantity of their EPS. For example, the soil bacterium *Pseudomonas* sp. decreases production of extracellular proteins but increases exopolysaccharide synthesis when being desiccated (Roberson & Firestone, 1992). Chang et al. (2007) reported that *Pseudomonas* spp. respond to desiccation by increasing the production of the exopolysaccharide alginate, resulting in a modified biofilm architecture of less surface area and thicker EPS layer at the biofilm-air interface, what is supposed to contribute to less evaporative water loss. Hydrophilic polysaccharides, e.g. uronic acid-containing compounds such as colanic acid produced by *Salmonella* spp., *Klebsiella* spp., and mucoid strains of *E. coli* (Ophir & Gutnick, 1994), represent the main constituent of EPS involved in desiccation tolerance (Flemming & Wingender, 2010; Roberson & Firestone, 1992). Their potential to retain water may be increased by heavy cross-linking (Decho, 2000). Also exocellular proteins could be involved in maintaining a hydrated environment within a biofilm (Flemming & Wingender, 2010).

Several studies have found evidence correlating EPS production with desiccation tolerance: Compared to mucoid cultures, EPS-deficient mutants of *Acinetobacter calcoaceticus*, *Erwinia stewartii,* and *E. coli* showed significantly increased sensitivity to desiccation (Ophir & Gutnick, 1994). The cyanobacterium *N. commune* maintained photosynthesis during desiccation when embedded in its EPS, but photosynthetic activity was lost and viability decreased when EPS-depleted cells were desiccated (Tamaru et al., 2005). The EPS of *Nostoc* sp. was also shown to inhibit fusion of membrane vesicles during desiccation (Hill et al., 1997) and to improve the desiccation tolerance of other organisms when added to cells of green and blue-green algae, respectively (Knowles & Castenholz, 2008). Evidence suggests that EPS to some extent protect microbial cells from free radicals which are a threat under exposure to desiccation or UV irradiation. In the nitrogen-fixing bacteria *Rhizobium* sp. and *Klebsiella pneumoniae* the enzymatic activity of nitrogenase is protected from ROS by several mechanisms, one of which is slime production (Postgate, 1982; Wilcockson, 1977). The removal of EPS from *Pseudomonas syringae* and other plant pathogens resulted in higher sensitivity to superoxide and hydrogen peroxide (Kiraly et al., 1997). Xu et al. (2012) found that EPS production facilitated survival of *P. aeruginosa* on ROS-contaminated oxide surfaces, suggesting quenching of the ROS by capsular and free EPS molecules. In some cases, EPS also play a role in attenuating exposure to UV radiation. Certain cyanobacteria integrate photon-absorbing pigments into their EPS (Gao & Garcia-Pichel, 2011). Elasri and Miller (1999) reported that an artificial biofilm matrix composed of alginate physically shields cells of *P. aeruginosa* embedded within against UV radiation.

1.5 Biofilms

Many microorganisms have the potential to form biofilms; in fact biofilms represent the predominant way of microbial life and possibly the oldest lifeform on Earth (Flemming & Wingender, 2010; Hall-Stoodley et al., 2004; Nisbet & Sleep, 2001). In biofilms, microbial cells are aggregated – usually at the interface of two phases – and transiently immobilised within a slimy matrix of self-developed EPS consisting mostly of proteins, polysaccharides, nucleic acids, and lipids (Flemming & Wingender, 2001). Natural biofilms represent complex multi-species communities that are organised into microhabitats, often of synergistic or symbiotic nature (Watnick & Kolter, 2000). Biofilms are formed extensively in aqueous environments such as surface waters, drinking water distribution systems, waste water pipes, or the human oral cavity (Hall-Stoodley et al., 2004; Wingender & Flemming, 2011). However, biofilms can also form in the absence of a fluid, in systems of temporal wetting such as soils or plant leaf surfaces (van de Mortel et al., 2004). These biofilms are not fully saturated with water, but, depending on the environmental conditions, often surrounded by a water layer of varying thickness (Chang & Halverson, 2003). Due to the reduced water availability, unsaturated biofilms show other morphologies and functional forms than saturated biofilms growing in fluids (Auerbach et al., 2000; Chang & Halverson, 2003; Chang et al., 2007; de Goffau et al., 2011), as the lack of a surrounding fluid attenuates the transport of nutrients, metabolic waste products, and cell signalling molecules within the biofilm (Chang & Halverson, 2003)

1.5.1 Biofilm formation

Biofilm formation is a multi-step process involving intercellular communication (Karatan & Watnick, 2009; Parsek & Greenberg, 2005; Stoodley et al., 2002). Biofilm formation is induced by single cells reversibly adhering to an interface (e.g. water-solid, water-air, solid-air; Fig. 1.7), to surface-associated cells, or to each other. The initial adhesion of microorganisms to inanimate surfaces is usually mediated by non-specific interactions, such as electrostatic, hydrophobic, and van der Waal's forces. Depending on the nature of the cell surface and the surface of the substratum (which may be conditioned by a range of molecules attracted from the surrounding phase, the so-called 'conditioning film'), contact can be enhanced or diminished (reviewed by Dunne, 2002). Microbial adhesins, i.e. proteins located on the cell surface or on cell appendages such as pili or fimbriae, can overcome otherwise repulsive conditions and facilitate attachment (Dunne, 2002; Pieters, 2007).

If the existing conditions favour growth, attachment of the cell to the surface becomes permanent during secondary adhesion, mediated by the production of adhesive EPS, by cell appendage-associated adhesins, or both. It is this stage, in which the initially adhered organisms reproduce and aggregate to form a microcolony, which is stabilised by the excretion of

further EPS (Dunne, 2002; Stoodley et al., 2002). The EPS facilitate intercellular cohesion. For example, secondary adhesion of the Gram-positive skin-associated bacterium *Staphylococcus epidermis* is characterised by the accumulation of so-called 'polysaccharide intercellular adhesins' (PIA) on the cell surface, which mediate cellular aggregation even before an extensive slime matrix is produced (Dunne, 2002). Eventually, the microcolony develops into a mature biofilm, in which the cells are embedded in and surrounded by a three-dimensional network of EPS (Flemming & Wingender, 2010). Other microorganisms, also those incapable of establishing biofilms on their own, will nest in the pre-existing biofilm, thus promoting species variety, synergistic effects, and interspecies competition (Elias & Banin, 2012; Ren et al., 2015).

Architecture and morphology of the formed biofilm are influenced by a range of parameters including the inhabiting species, the availability of nutrients and water, the composition of the EPS, and – in the case of submerged biofilms – the hydrodynamics of the aqueous phase. Biofilm morphology may resemble, for instance, mushroom-like structures surrounded by water channels (Fig. 1.7), or flat monolayers of cells spread out over the colonised surface (Flemming & Wingender, 2010).

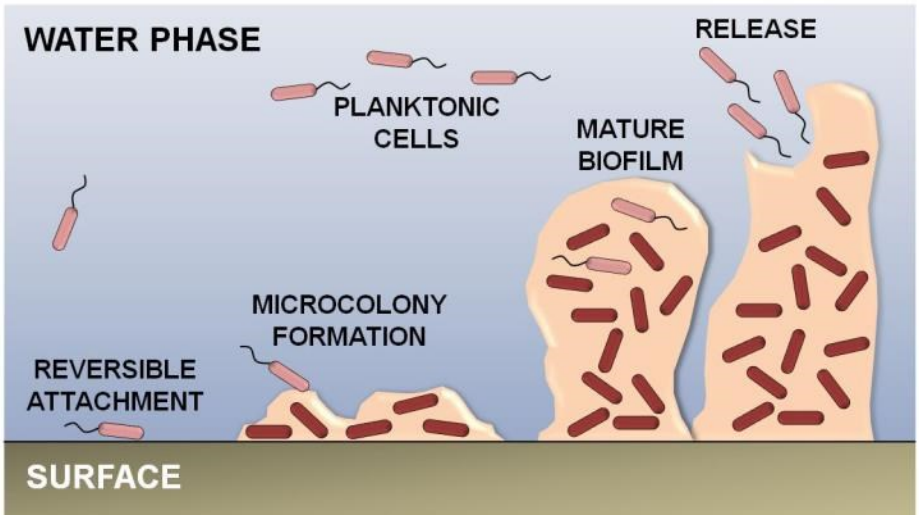

Figure 1.7. Classic model of biofilm formation on a submerged surface (after Monds & O'Toole, 2009; Stoodley et al., 2002).

Sessile microorganisms living in biofilms show a profile of gene transcription which is distinct from that of planktonic cells. Transition from planktonic to biofilm mode of life is commonly accompanied with an upregulation of genes coding for extracellular polymers whilst other genes such those regulating flagella synthesis are downregulated (Watnick & Kolter, 2000). Hence, microorganisms actively adjust to the biofilm lifestyle. Spontaneous release of biofilm-associated cells is possible and occurs when biofilm material is sloughed off by shear forces.

The inhabitants are also able to leave the biofilm when conditions become unfavourable by degrading the surrounding EPS (Stoodley et al., 2002; Watnick & Kolter, 2000).

1.5.2 The biofilm matrix

In most biofilms, the actual fraction of cells represents less than 10% of the dry mass, whilst the major fraction of the dry mass corresponds to the biofilm matrix (reviewed by Flemming & Wingender, 2010). The biofilm matrix is a complex mixture of EPS of microbial origin, mainly comprising polysaccharides, proteins, nucleic acids, and lipids (Flemming & Wingender, 2001). EPS may either be tightly-bound, i.e. associated with the cell, in which case they are also referred to as capsules or sheaths, or loosely-bound, in which case they are released into the environment (Flemming & Wingender, 2010; Helm & Potts, 2012). The EPS form an intricate three-dimensional network that is transiently stabilised by weak forces such as electrostatic interactions and hydrogen bonds, and physical entanglement of the polymers (Flemming et al., 2000; Mayer et al., 1999; Wloka et al., 2004).

Polysaccharides and proteins represent the dominant fraction of EPS. Depending on their monomer composition and the presence of organic or inorganic substituents, exopolysaccharides may be polyanionic, polycationic, or neutral of charge, what ultimately affects their physical and biological functions (Flemming & Wingender, 2010). For example, negatively-charged polysaccharides (e.g. the uronic acid-containing alginate in *P. aeruginosa*) may interact with each other via bridging with multivalent cations such as Ca^{2+} and thus contribute to the mechanical stability of the biofilm matrix (Körstgens et al., 2001; Mayer et al., 1999). Polysaccharide intracellular adhesin (PIA) is an example for a positively-charged exopolysaccharide, which, is involved in pathogenicity, attachment, and biofilm formation of *Staphylococcus* spp. (Jefferson, 2009; Mack et al., 1996). Cellulose represents a non-charged, water-insoluble exopolysaccharide synthesized by many bacteria that is involved in biofilm stability and hydrophobicity, and protection against chemical stress and desiccation (Saldaña et al., 2009; Serra et al., 2013; Solano et al., 2002; White et al., 2006; Zogaj et al., 2001).

Extracellular proteins comprise functional enzymes as well as structural proteins (Flemming & Wingender, 2010). Extracellular enzymes can be associated to the cell surface or accumulate in the EPS matrix, either by active secretion or as a result from cell lysis (Wingender et al., 1999). Whilst various enzymes classes (e.g. lipases, proteases, esterases) have been identified in bacterial EPS, their main function is the degradation of macromolecules into low-molecular-weight compounds that can serve as nutrients. In this way, the EPS matrix acts as an external digestive system (Flemming & Wingender, 2010). Degradation of the matrix itself can aid during dispersal or periods of nutrient limitation (McDougald et al., 2012). Structural proteins such as amyloids are a widespread architectural component of bacterial EPS. They mediate attachment to surfaces, confer structural integrity to flocs in acti-

vated sludge (Larsen et al., 2007), and interconnect cells within the matrix (Dueholm et al., 2010; Flemming & Wingender, 2010; Romero et al., 2010). Similar to polysaccharides, extracellular proteins can engage in electrostatic interactions with multivalent cations due to their high content of negatively charged amino acids, thus contributing to biofilm stability (Laspidou & Rittmann, 2002).

Extracellular DNA (eDNA) can make up for a significant proportion of EPS in bacterial biofilms. Though originally believed to be the result of cell lysis, eDNA seems to be an important structural component of biofilms that is actively secreted into the matrix. Besides contributing to the spatial arrangement of the EPS matrix by interactions with other EPS, eDNA is involved in the initial adhesion during biofilm formation (Böckelmann et al., 2006; Das et al., 2013; Flemming et al., 2007; Whitchurch et al., 2002).

The biofilm matrix offers a lifestyle that differs significantly from solitary planktonic growth. First and foremost, the EPS immobilise the cells by facilitating attachment to a substratum and keep them in close proximity, thus permitting intercellular communication by signalling molecules, exchange of genetic material by horizontal gene transfer, and the formation of synergistic microconsortia (Flemming & Wingender, 2010; Watnick & Kolter, 2000). The matrix can improve provision with nutrients. It entraps biodegradable molecules, either from the water phase or from lysed cells within the biofilm, that are recycled by other biofilm cells or digested by exocellular enzymes in order to increase their bioavailability. The EPS themselves can also act as a nutrient source, though, due to their complex composition, biodegradation is slow and requires a wide variety of enzymes (Flemming & Wingender, 2010). The matrix is a heterogeneous environment. Metabolic processes and respiratory activity lead to localised gradients within the biofilm; e.g. oxygen and nutrient concentrations and the pH may decrease with increasing biofilm depth. This in turn influences the spatial distribution of bacterial groups within the matrix by creating distinct ecological niches (Watnick & Kolter, 2000). The EPS matrix has protective properties. In a biofilm, the organisms are sheltered from certain toxic substances such as metal ions, some antibiotics, detergents, and disinfectants. The matrix protects against matric water stress and desiccation by maintaining favourable hydraulic conditions (Or et al., 2007), and shields the biofilm organisms from UV radiation and protozoal predators (Flemming & Wingender, 2010).

1.5.3 Biofilm analysis

The analysis of biofilms and their EPS is a challenging task. Due to the complex and species-dependent composition of microbial EPS, a universal method for the analysis of EPS does not exist (Flemming & Wingender, 2010). The analytical approaches used to describe biofilms can be classified into (i) techniques that study the EPS extracted from biofilms and (ii) non-destructive approaches to study biofilms *in situ* (Karunakaran et al., 2011).

1.5.3.1 Extraction and characterisation of EPS

In order to characterise the EPS of a biofilm, they need to be separated from the cells embedded within, either by physical or by chemical means. Due to the sheer variety of biofilms, there is no universal EPS isolation technique (Wingender et al., 1999). However, a range of different approaches exist that can be adapted to the biofilm of interest. Usually, biofilm material is collected and suspended in water or aqueous buffer, before being subjected to isolation treatments such as shaking, centrifuging, heating, filtration, or sonication. The addition of reagents to facilitate separation of the EPS from the cells is common and includes compounds such as complexing agents (e.g. ethylenediaminetetraacetic acid [EDTA] Liu & Fang, 2002), sodium hydroxide (Brown & Lester, 1980), or ion exchange resins (D'Abzac et al., 2010; Frølund et al., 1996; Jahn & Nielsen, 1995). The latter ones function by scavenging the cations which stabilise the EPS network through the bridging of negatively-charged groups of biopolymers. Depending on the biofilm investigated, these treatments may separate EPS from cells inefficiently or damage the cells, resulting in the leakage of cytoplasmic compounds into the EPS (D'Abzac et al., 2010). Hence, the quantitative isolation of EPS is complicated. Careful modifications of the isolation technique allow for the selective isolation of water-soluble or water-insoluble, and loosely-bound or tightly-bound (capsular) EPS, respectively (summarised by Flemming & Wingender, 2010; Michalowski, 2012). After isolation, salts and other low-molecular-weight compounds are removed, e.g. by dialysis (Denkhaus et al., 2007; Wingender et al., 2001).

Quantification of the isolated EPS according to sum parameters (e.g. proteins, polysaccharides, uronic acids, DNA) is usually done spectrometrically, by using colorimetric or fluorometric assays (e.g. Dubois et al., 1956; Filisetti-Cozzi & Carpita, 1991; Frølund et al., 1996). These methods rely on the basic principle of the target EPS component to react with a certain agent to form a product or complex that either absorbs light of a specific wavelength or emits fluorescence of a certain wavelength upon excitation. The use of appropriate standards enables quantification. For a more detailed, qualitative analysis of the isolated compounds, chromatographic or gel-electrophoretic approaches are applied. Gel electrophoresis (either one- or two-dimensional) is a common technique used to separate proteins by their molecular weight or by their weight and charge, respectively. Coupling of this method with a subsequent mass spectroscopic technique such as matrix-assisted laser desorption ionisation mass spectrometry (MALDI-MS) allows for single-protein analyses (Lahm & Langen, 2000). Chromatography-based approaches are used for the separation and purification of isolated proteins from other EPS components, and for the characterisation of polysaccharides in terms of molecular weight or monomer composition. In order to identify its monomers, the isolated polysaccharide has to be broken down into monosaccharides, what can be achieved by hydrolysis of the polymer using high temperatures and a hydrolytic agent such as hydrochloric acid. After hydrolysis, chromatographic methods such as thin-layer chromatography (TLC) or

size-exclusion chromatography can be used to separate and identify the monomers (Simon et al., 2009; Wingender et al., 2001).

1.5.3.2 Methods to study biofilms

Microscopy represents a widespread non-invasive approach that enables the study of biofilms *in situ* without disrupting the genuine biofilm architecture (Karunakaran et al., 2011). Both confocal laser scanning microscopy (CLSM) and epifluorescence microscopy (EFM) utilise fluorescent dyes or fluorescently-labelled probes to visualise cells and EPS components. For example, extracellular polysaccharides can be identified visually by fluorescent lectins such as concanavalin A, proteins can be localised by fluorescein isothiocyanate-labelled probes, and DNA can be labelled by a variety of dyes such as Syto® 9 or DAPI (Karunakaran et al., 2011; Strathmann et al., 2002). Fluorescence *in situ* hybridisation (FISH) coupled with CLSM or EFM allows the specific visualisation of ribosomal RNA (rRNA) by using oligonucleotide probes. The methods are based on the wavelength-specific excitation of the fluorescent probes and the subsequent detection of the emitted fluorescence. The use of EFM is restricted to thin biofilms, as fluorescence from all areas above and below the focal plane is collected, resulting in a blurry image in the case of thick biofilms. CLSM, however, collects only the fluorescence emitted at the focal plane, allowing for a more precise observation of biofilms along their vertical axis (Lawrence et al., 2007). Electron microscopy enables the study of the surface of biofilms (in the case of scanning electron microscopy; SEM) or cross-sections of biofilms (in the case of transmission electron microscopy) at considerably higher magnifications than EFM or CLSM, but requires sample preparation steps such as drying, fixing, and sputting (i.e. coating of the surface of the specimen with e.g. gold), and observation of the specimen in vacuum, all of which can alter the biofilm structure and produce artefacts (Surman et al., 1996). Environmental scanning electron microscopy (ESEM) as a modified form of SEM permits the observation of hydrated samples by operating at low pressures (Surman et al., 1996). Other non-disruptive approaches to study biofilms include atomic force microscopy (Surman et al., 1996), Fourier transform infrared spectroscopy (Karunakaran et al., 2011), or magnetic resonance imaging (Neu et al., 2010).

1.6 Survival of microbes

In order to assess survival, one must distinguish between living and dead organisms. In the case of microorganisms this is a problematic task, as the transition from life to death seems to involve many steps and the exact point of death often remains obscure (Davey, 2011). The survival of bacteria upon exposure to stress is usually determined by culture-dependent techniques (e.g. plate counts on nutrient agar), in which a lack of culturability would – according to conventional interpretation – indicate dead cells. Cultivation methods have many ad-

vantages, as they are usually cheap, convenient to handle, and ubiquitously used in microbiology, allowing for comparing different studies. Their solitary use, however, often underestimates the true proportion of viable organisms, as many bacteria are able to enter a dormant, so-called viable but non-culturable (VBNC) state (reviewed by Oliver, 2005).

1.6.1 The viable but non-culturable state

In the VBNC state, cells fail to grow on the medium they would usually grow on and show reduced to minute levels of metabolic activity. Yet, they are alive and some species are even able to resuscitate and become culturable again when environmental conditions become more favourable (Li et al., 2014; Oliver, 2005). Transition into the VBNC state is considered to be a response to deteriorating environmental conditions, allowing bacteria to survive otherwise lethal environmental conditions by becoming dormant (Ayrapetyan et al., 2015). This process is considered an active one, as VBNC cells show a pattern of gene expression distinct from culturable cells (Pinto et al., 2013). Dormancy is induced by a range of environmental stressors, including extreme temperatures, fluctuations in the availability of oxygen, nutrients, and water, pH changes, UV irradiation, toxic chemicals, or desiccation. In the VBNC state, cell division is arrested and other metabolic activities significantly reduced (Trevors, 2011). The cells may shrink (Clements & Foster, 1998) or alter their morphology from rod to a more coccus-like shape (Pinto et al., 2013; Trevors, 2011). Consequently, the DNA may become compressed when surrounded by dense cytoplasm (Trevors, 2011).

1.6.2 Detecting microbial viability

In order to identify VBNC cells and to differentiate them from dead cells, it is necessary to use methods that characterise the cell's state of vitality by aspects other than culturability, for example cellular integrity or metabolic activity (Hammes et al., 2011; Oliver, 2005). Common techniques include staining methods or molecular-based approaches (Trevors, 2011).

DNA-intercalating fluorescent dyes such as DAPI or Syto® 9 are used to determine the total cell count of the sample investigated (Oliver, 2005) and to detect the presence of (intact) nucleic acids within cells (Hammes et al., 2011). Membrane integrity can be assessed by using the Live/Dead® differential staining assay or the more recently developed real-time viability assay. The Live/Dead® assay measures the permeability of cell membranes to the membrane-impermeable fluorescent dye propidium iodide. Intact cells which exclude propidium iodide are counterstained with Syto® 9 (Hammes et al., 2011). The real-time viability assay measures the permeability of cell membranes to protons and a photoautomeric probe, whilst providing higher resolution than the Live/Dead® assay (Nocker et al., 2012).

Several methods exist that examine metabolic activity. FISH, as previously mentioned used to study the species composition of a biofilm, can also be used as a marker for active pro-

tein synthesis, since a positive FISH signal corresponds to the presence of rRNA (Moter & Göbel, 2000). A limitation of this method, however, is the fact that the intracellular rRNA concentration may vary depending on the species or the physiological state of the cell (Moter & Göbel, 2000). The direct viable count (DVC) method differentiates between active and inactive cells by preventing cell division using a gyrase inhibitor. Active cells fail to divide and thus elongate, allowing for their detection by microscopy (Kogure et al., 1979). Respiratory activity can be measured by using 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) or 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), both of which are microbially converted to intracellular deposits which can be identified by light microscopy or EFM, respectively (Rodriguez et al., 1992; Zimmermann et al., 1978). Enzymatic activity can be measured by using specific enzyme substrates that exhibit fluorescence upon conversion. For example, fluorescein diacetate is used to identify esterase activity (Joux & Lebaron, 2000).

Due to its ubiquitous occurrence in all known living organisms, adenosine triphosphate (ATP) is used as a biomarker to identify microbial contaminations in man-made environments such as clean room facilities (Venkateswaran et al., 2003), clinics, or food production (Stanley, 1989). The fact that ATP is present in live and growing cells but is released upon loss of cell membrane integrity (Venkateswaran et al., 2003) permits its use as a target molecule in distinguishing between dead or VBNC cells (Beumer et al., 1992; Federighi et al., 1998; Li et al., 2003). It is detectable by a range of commercially available luminometric luciferin/luciferase assays (Hammes et al., 2011; Stanley, 1989).

Further methods concentrate on characterising other aspects of cell viability such as membrane potential, efflux pump activity, or the intracellular pH (Tracy et al., 2010). Due to the complex and heterogeneous nature of microbial life and the ambiguous definitions of 'life' and 'death' in bacteria, a universal tool to accurately determine microbial viability does presently not exist. Therefore, a combination of different cultivation-independent viability markers suitable for the organism in question must be employed (Hammes et al., 2011).

1.7 Aims of this study

Earlier studies have shown that distinct microorganisms are able to survive the (simulated) conditions present in extraterrestrial environments such as LEO or Mars. However, these studies did not address the fact that bacterial tolerance to detrimental environments is greatly increased by the presence of an EPS matrix produced in the biofilm mode of life. Also, astrobiological studies repeatedly fail to utilise culture-independent techniques to assess survival, although they are necessary for the identification of viable but non-culturable cells. This study aims to introduce advanced methods for investigating the survival of biofilms of the test organism *D. geothermalis* DSM 11300 under desiccation as well as simulated space and Mars conditions, taking into account cultivation-independent viability markers such as membrane integrity, ATP concentration, and the presence of 16S rRNA.
In detail, the aims of this study are:

- To test the hypothesis that the biofilm mode of life allows *D. geothermalis* to survive in space
- To optimise cultivation conditions for the growth of biofilms of *D. geothermalis*
- To produce samples of biofilms and planktonic cells of *D. geothermalis* suitable for the exposition in space and to simulated space and Mars conditions
- To isolate, quantify, and characterise the EPS produced by *D. geothermalis*
- To assess the survival of *D. geothermalis* after long-term desiccation
- To assess the survival of *D. geothermalis* after exposure to simulated space and Mars conditions

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

The test organism used in this study was *D. geothermalis* DSM 11300 (type strain), formerly termed AG-3a (Ferreira et al., 1997).

2.1.2 Nutrient media

The nutrient media used in this study are listed in Tab. 2.1.

Table 2.1. Nutrient media used in this study.

Nutrient medium	Manufacturer	Reference
Reasoner's 2A agar (R2A)[a]	Difco™, BD	Reasoner and Geldreich (1985)
R2A agar (R2A)[b]	Fluka	
R2A broth (R2B)	self-made	
Thermus 162 medium, solidified	self-made	
Tryptic soy agar (TSA)[c]	Merck	

[a] Casamino acids (0.5 g l^{-1}), yeast extract (0.5 g l^{-1}), Proteose Peptone No. 3 (0.5 g l^{-1}), soluble starch (0.5 g l^{-1}), glucose (0.5 g l^{-1}), dipotassium hydrogen phosphate (0.3 g l^{-1}), magnesium sulphate (0.05 g l^{-1}), sodium pyruvate (0.3 g l^{-1}), agar (15.0 g l^{-1}); pH 7.2 ± 0.2 at 25 °C; 18.2 g in 1 l de-ionised water;
[b] Casein acid hydrolysate (0.5 g l^{-1}), yeast extract (0.5 g l^{-1}), proteose peptone (0.5 g l^{-1}), starch soluble (1.5 g l^{-1}), glucose (0.5 g l^{-1}), dipotassium hydrogen phosphate (0.3 g l^{-1}), magnesium sulphate (0.024 g l^{-1}), sodium pyruvate (0.3 g l^{-1}), agar (15.0 g l^{-1}); pH 7.2 ± 0.2 at 25 °C; 18.12 g in 1 l de-ionised water;
[c] Peptone from casein (15.0 g l^{-1}), peptone from soymeal (5.0 g l^{-1}), sodium chloride (5.0 g l^{-1}), agar (15.0 g l^{-1}); pH 7.3 ± 0.2 at 25 °C; 40 g in 1 l de-ionised water.

2.1.2.1 Liquid nutrient media

R2A broth (R2B) was prepared by mixing yeast extract (0.5 g l^{-1}), proteose peptone (0.5 g l^{-1}), casamino acids (0.5 g l^{-1}), soluble starch (0.5 g l^{-1}), sodium pyruvate (0.3 g l^{-1}), dipotassium hydrogen phosphate (0.3 g l^{-1}), magnesium sulphate heptahydrate (0.05 g l^{-1}), and glucose (0.5 g l^{-1}) in de-ionised water (Tab. 2.1). After adjusting the pH to 7.2 using crystalline dipotassium hydrogen phosphate, the solution was autoclaved at 121 °C for 20 min and stored at 8 °C.

2.1.2.2 Solid nutrient media

Two commercially available solid nutrient media were used: R2A agar (R2A) by two different manufacturers (Difco and Fluka, respectively), and tryptic soy agar (TSA; also known as CASO agar). They were prepared according to the manufacturer's instructions (Tab. 2.1) by suspending the provided dehydrated granulate in de-ionised water, autoclaving at 121 °C for 20 min, and pouring into portions of 25 ml into sterile petri dishes. Petri dishes without vents were used in order to prevent desiccation of the medium whilst incubation at 45 °C.

Thermus 162 medium was prepared by mixing yeast extract (1.0 g l^{-1}), tryptone (1.0 g l^{-1}), agar (28.0 g l^{-1}), nitrilotriacetic acid (0.1 g l^{-1}), calcium sulphate dihydrate (40.0 mg l^{-1}), magnesium chloride hexahydrate (0.2 g l^{-1}), and 0.01 M iron citrate solution (0.5 ml l^{-1}) with 0.5 ml of *Thermus* 162 trace element solution per litre of medium. After adjusting the pH to 7.2 using sodium hydroxide and autoclaving at 121 °C for 20 min, 100 ml of sterile *Thermus* 162 phosphate buffer were added to 900 ml of medium. The medium was poured into portions of 25 ml into sterile petri dishes without vents.

2.1.2.3 Manipulated nutrient media

R2A was spiked by adding 5.0 g l^{-1} sodium chloride or varying amounts of peptone (1.5-20 g l^{-1}) to the provided R2A granulate. The spiked R2A was prepared as described previously for non-spiked R2A.

2.1.3 Chemicals

Chemicals used in this study are listed in Tab. 2.2.

Table 2.2. Chemicals used in this study.

Substance	Chemical formula	Specifications	Manufacturer
Acetone	C_3H_6O	p.a.	AppliChem
Acetonitrile	C_2H_3N	99.9%	VWR
Agar, Bacto™			BD
Boric acid	H_3BO_3	p.a.	Merck
Bovine serum albumin (BSA)		protein standard	Sigma
Calcium chloride dihydrate	$CaCl_2 \times 2\ H_2O$	p.a., ≥ 99%	Fluka
Calcium sulphate dihydrate	$CaSO_4 \times 2\ H_2O$	p.a.	Sigma
Casamino acids, Bacto™			BD
Citifluor glycerol solution plus antifadient AF2			Science Services GmbH
Cobalt chloride hexahydrate	$CoCl_2 \times 6\ H_2O$	p.a.	Merck
Copper sulphate pentahydrate	$CuSO_4 \times 5\ H_2O$	99+%	Acros Organics
4',6-Diamidino-2-phenylindole dihydro-chloride (DAPI)	$C_{16}H_{15}N_5$		Sigma
D-fucose	$C_6H_{12}O_5$	98%	Sigma
D-galactose	$C_6H_{12}O_6$	min. 99%	Sigma
D-glucose	$C_6H_{12}O_6$	99.5% GC	Sigma
D-glucuronic acid	$C_6H_{10}O_7$		Sigma
D-mannose	$C_6H_{12}O_6$	mixed anomers	Sigma
D-xylose	$C_5H_{10}O_5$	99%	Sigma
Dipotassium hydrogen phosphate	K_2HPO_4	p.a.	Merck
Disodium 2,2'-bicinchoninate	$C_{20}H_{10}N_2O_4Na_2$		Sigma
Disodium carbonate	Na_2CO_3	p.a.	Merck
Disodium hydrogen phosphate dihydrate	$Na_2HPO_4 \times 2\ H_2O$	min. 99.5%	Riedel-de Haen
Disodium hydrogen phosphate dodeca-hydrate	$Na_2HPO_4 \times 12\ H_2O$	> 99%	Merck
Disodium molybdate dihydrate	$Na_2MoO_4 \times 2\ H_2O$	p.a.	Merck
Disodium tetraborate decahydrate	$Na_2B_4O_7 \times 10\ H_2O$	≥99.5%	Fluka
Dowex® Marathon® C		Na^+-form, strongly acidic	Sigma-Aldrich
Ethanol	C_2H_6O	absolute	VWR
Folin-Ciocalteu's phenol reagent		2 N	Sigma

Table 2.2. Chemicals used in this study (continued).

Substance	Chemical formula	Specifications	Manufacturer
Formaldehyde	CH_2O	37% in H_2O, 10-15% methanol	Fluka
Formamide	CH_3NO	de-ionised, ≥ 99.5%, p.a.	Roth
Hydrochloric acid	HCl	37%	Sigma
3-Hydroxybiphenyl	$C_{12}H_{10}O$		Sigma
Immersion oil		type N	Leica
Iron(III) citrate	$C_6H_5FeO_7$		Sigma
Isopropyl alcohol	C_3H_8O	99%	Sigma
L-fucose	$C_6H_{12}O_5$	min. 99%	Sigma
Lowry reagent			Sigma
L-rhamnose	$C_6H_{12}O_5$	min. 99%	Sigma
L-serine	$C_3H_7NO_3$	≥ 99.9%	Sigma
Magnesium chloride hexahydrate	$MgCl_2 \times 6\ H_2O$	p.a., ≥ 99%	Roth
Magnesium sulphate heptahydrate	$MgSO_4 \times 7\ H_2O$	p.a., > 99.0%	Fluka
Manganese sulphate monohydrate	$MnSO_4 \times H_2O$	≥98%	Merck
Methanol	CH_3OH	HPLC grade	VWR
Methyl-α-D-mannopyranoside	$C_7H_{14}O_6$	≥ 99%	Fluka
Molecular-grade water	H_2O	DEPC-treated	Roth
N-Acetylgalactosamine (NAc-galactosamine)	$C_8H_{15}NO_6$		Sigma
N-Acetylglucosamine (NAc-glucosamine)	$C_8H_{15}NO_6$		Sigma
N-Acetylmannosamine (NAc-mannosamine)	$C_8H_{15}NO_6$		Sigma
N-(1-naphthyl)ethylenediamine dihydrochloride	$C_{10}H_7NHCH_2CH_2NH_2 \times 2HCl$	> 98%	Sigma
Nitrilotriacetic acid	$C_6H_9NO_6$	≥ 99.0%	Sigma
Paraformaldehyde	$OH(CH_2O)_nH$		Merck
Pentanol	$C_5H_{11}OH$	≥ 99%	Sigma
Phenol	C_6H_6O	p.a., ≥ 99.0%	Sigma
Potassium chloride	KCl	p.a.	Merck
Potassium dihydrogen phosphate	KH_2PO_4	≥ 99.5%	Fluka
Potassium hydroxide	KOH	p.a.	J.T. Baker

Table 2.2. Chemicals used in this study (continued).

Substance	Chemical formula	Specifications	Manufacturer
Peptone, Bacto™			BD
R2A agar, Difco™			BD
R2A agar			Fluka
Rotipuran water	H_2O		Roth
SICAPENT® phosphorus pentoxide drying agent	P_4O_{10}	with indicator	Merck
Sodium acetate	CH_3COONa	p.a., min. 99%	Merck
Sodium chloride	NaCl	p.a.	Bernd Kraft
Sodium dihydrogen phosphate monohydrate	$NaH_2PO_4 \times H_2O$	p.a.	Merck
Sodium dodecyl sulphate (SDS)	$NaC_{12}H_{25}SO_4$	99%	Sigma
Sodium hydrogen carbonate	$NaHCO_3$	p.a.	Merck
Sodium hydroxide	NaOH	p.a., min. 99%	KMF
Sodium pyruvate	$C_3H_3NaO_3$	> 99%	Fluka
Starch	$(C_6H_{10}O_5)_n$	soluble	Sigma
Sulphamic acid	H_3NSO_3	≥ 99%	Sigma
Sulphuric acid	H_2SO_4	p.a., > 95%	Fisher Scientific
tetrasodium ethylenediaminetetraacetate (EDTA) dihydrate	$C_{10}H_{12}N_2Na_4O_8$	> 99%	Sigma
Tris(hydroxymethyl)aminomethane (TRIS)	$(HOCH_2)_3CNH_2$	99.9%	Sigma
Trisodium phosphate dodecahydrate	$Na_3PO_4 \times 12\ H_2O$	p.a.	Merck
Tryptic soy agar			Merck
Tryptone, Bacto™		pancreatic digest of casein	BD
Tween 80	$C_{64}H_{124}O_{26}$		Fluka
Yeast extract			Merck
Zink sulphate heptahydrate	$ZnSO_4 \times 7\ H_2O$	p.a.	Merck

2.1.4 Enzymes

Enzymes used in this study are listed in Tab. 2.3.

Table 2.3. Enzymes used in this study.

Enzyme	Specifications	Manufacturer
Cellulase	6 U mg^{-1}; from *Trichoderma reesei*;	Sigma
Proteinase K	600 U ml^{-1}; from *Engyodontium album*; ready-made solution	New England Biolabs
Pronase E	≥ 4 U mg^{-1}; from *Streptomyces griseus*	Sigma

2.1.5 Probes

Probes used in this study are listed in Tab. 2.4.

Table 2.4. List of fluorescently-labelled probes used in this study.

Probe	Specifications	Manufacturer
Amaranthus caudatus lectin (ACA)	Tetramethylrhodamine (TRITC)-conjugated; specificity: gal(β-1,3)galNAc[a], gal(β-1,3)Neu5Ac[b]; from tassel flower, Inca wheat	EY Laboratories
Dolichos biflorus lectin (DBA)	TRITC-conjugated; specificity: methyl-2-acetamido-2-deoxy-D-galactose[a], terminal α-galNAc[b], galNAc(α-1,3)galNAc[b]; from horse gram	EY Laboratories
EUB338 FISH probe	Cy3-conjugated; sequence: 5'-GCT GCC TCC CGT AGG AGT-3'; specificity: Eubacteria	Eurofins MWG Operon
Helix aspersa lectin (HAA)	TRITC-conjugated; specificity: galNAc[a,b], glcNAc[b]; from garden snail	EY Laboratories

[a] According to the manufacturer.
[b] According to Peltola et al. (2008).

2.1.6 Buffers and solutions

ATP stock solution (1 µM). 3 µl of 10 mM rATP standard (BacTiter-Glo™ Microbial Cell Viability Assay, Promega) were diluted in 30 ml of particle-free de-ionised (PFD) water.
BCA solution A. 5.43 g of disodium carbonate and 2.42 g of sodium hydrogen carbonate were dissolved in 90 ml of de-ionised water. 194.2 mg of disodium 2,2'-bicinchoninate were added

and the solution was filled up to 100 ml. The solution was stored in an amber glass bottle at 8 °C for up to 4 weeks.

BCA solution B. 1.248 g l^{-1} of copper sulphate pentahydrate and 1.262 g l^{-1} of L-serine were dissolved in de-ionised water. The solution was stored in an amber glass bottle at 8 °C for 4 weeks.

BSA stock solution (400 µg ml^{-1}). 2 mg of BSA were dissolved in 5 ml of de-ionised water. The solution was stored at 8 °C for up to 4 weeks.

Cellulase solution (60 U ml^{-1}). 10 mg ml^{-1} of cellulase (6 U mg^{-1}) were dissolved in cold de-ionised water. The solution was freshly prepared prior to use and sterilised by filtration[2].

DAPI solution. 25 µg ml^{-1} of DAPI (Sigma) were dissolved in 2% (v/v) formaldehyde. The solution was sterilised by filtration and stored at 4 °C in the dark.

Derivatisation agent. 30 mg of N-(1-naphthyl)ethylenediamine dihydrochloride were dissolved in 10 ml of 5% (v/v) sulphuric acid in methanol. The solution was freshly prepared prior to use.

EDTA solution (2%). 28.48 g l^{-1} of tetrasodium EDTA dihydrate were dissolved in de-ionised water. The solution was sterilised by autoclaving[3] and stored at room temperature.

EDTA solution (5 mM). 2.10 g l^{-1} of tetrasodium EDTA dihydrate were dissolved in de-ionised water. The solution was sterilised by autoclaving and stored at room temperature.

EDTA solution (10 mM). 4.20 g l^{-1} of tetrasodium EDTA dihydrate were dissolved in de-ionised water. The solution was sterilised by autoclaving and stored at room temperature.

EUB338 probe solution. A probe stock solution was prepared by dissolving 50 ng $µl^{-1}$ of lyophilised EUB338 probe in molecular-grade water. For a probe working solution, the probe stock solution was diluted 10-fold in hybridisation buffer.

Folin-Ciocalteu's phenol solution. 1 ml of Folin-Ciocalteu's phenol reagent (61.2% water, 12.2% lithium sulphate, 2% sodium tungstate dihydrate, 9.5% hydrochloric acid (≥ 25%), 6.9% phosphoric acid in water, 2% sodium molybdate dihydrate) was mixed with 5 ml of de-ionised water. The solution was freshly prepared prior to use.

Fucose stock solutions (10 mM). 1.64 g l^{-1} of D-fucose or L-fucose were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C.

Fucose stock solution (30 mM). 492 mg l^{-1} of L-fucose were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C.

Galactose stock solution (10 mM). 1.80 g l^{-1} of D-galactose were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C.

Galactose stock solution (30 mM). 540 mg l^{-1} of D-galactose were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C.

[2] Filtration through cellulose acetate syringe filters (0.2 µm pore size; Sarstedt).
[3] Autoclaving for 20 min at 121 °C.

Glucose stock solution (10 mg l^{-1}). 10 mg l^{-1} of D-glucose were dissolved in de-ionised water. The solution was stored at 8 °C.

Glucose stock solution (10 mM). 1.80 g l^{-1} of D-glucose were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C.

Glucose stock solution (30 mM). 540 mg l^{-1} of D-glucose were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C.

Glucose stock solution (200 µg ml^{-1}). 200 g l^{-1} of D-glucose were dissolved in de-ionised water. The solution was stored at 8 °C for several months.

Glucuronic acid stock solution (200 µg ml^{-1}). 200 g l^{-1} of D-glucuronic acid were dissolved in de-ionised water. The solution was stored at 8 °C for several months.

Hybridisation buffer. The composition of the hybridisation buffer is shown in Tab. 2.5.

Table 2.5. Composition of the hybridisation buffer (1 ml) used for FISH.

Component	Final concentration	Added volume (µl)
Rotipuran water	-	600
Sodium chloride solution (5 M)	0.9 M	180
TRIS buffer (1 M, pH 8.0)	20 mM	20
SDS solution (10%)	0.01%	1
Formamide[a]	20%	200

[a] Added prior to use.

Hydroxybiphenyl solution (0.15%). 15 mg of 3-hydroxybiphenyl were dissolved in 10 ml of 0.5% sodium hydroxide solution. The solution was stored at 8 °C for up to 1 week.

Lowry reagent. The content of each batch of Lowry reagent was dissolved in 40 ml of de-ionised water by stirring. The reagent was stored at 8 °C and used for up to 4 weeks.

Mannose stock solution (100 mM). 18.02 g l^{-1} of D-mannose were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C. Aliquots were diluted 10-, 20-, and 100-fold to produce mannose solutions with concentrations of 10 mM, 5 mM, and 1 mM, respectively.

Mannose stock solution (30 mM). 540 mg l^{-1} of D-mannose were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C.

Methyl-α-D-mannopyranoside stock solution (10 mM). 1.94 g l^{-1} of methyl-α-D-mannopyranoside were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C.

NAc-galactosamine stock solution (10 mM). 2.21 g l^{-1} of NAc-galactosamine were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C.

NAc-glucosamine stock solution (10 mM). 2.21 g l^{-1} of NAc-glucosamine were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C.

NAc-mannosamine stock solution (10 mM). 2.21 g l^{-1} of NAc-mannosamine were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C.

Particle-free de-ionised (PFD) water. De-ionised water was filtered through a Bottle Top Filter (pore size 0.2 µm; Nalgene) and sterilised by autoclaving.

Phenol reagent (5%; w/v). 50 g l^{-1} of phenol was dissolved in de-ionised water and stored at room temperature.

Phosphate buffer (PB; 6 mM; pH 7.0). 526 mg l^{-1} of sodium chloride, 760 mg l^{-1} of trisodium phosphate dodecahydrate, 75 mg l^{-1} of potassium chloride, and 552 mg l^{-1} of sodium dihydrogen phosphate monohydrate were dissolved in de-ionised water and the pH was adjusted to 7.0. The solution was sterilised by autoclaving and stored at room temperature.

Phosphate-buffered saline (PBS; pH 7.2; for FISH). 8.0 g l^{-1} of sodium chloride, 0.2 g l^{-1} of potassium chloride, 1.81 g l^{-1} of disodium hydrogen phosphate dihydrate, and 0.24 g l^{-1} of potassium dihydrogen phosphate were dissolved in de-ionised water and the pH was adjusted to 7.2. The solution was sterilised by autoclaving and stored at room temperature.

Phosphate-buffered saline (PBS; 50 mM, pH 7.5; for pronase E treatment). 0.857 g l^{-1} of potassium dihydrogen phosphate, 7.613 g l^{-1} of dipotassium hydrogen phosphate, and 8.766 g l^{-1} of sodium chloride were dissolved in de-ionised water. The pH was adjusted to 7.5. The solution was sterilised by autoclaving.

Potassium hydroxide solution (saturated). Potassium hydroxide was gradually dissolved in de-ionised water until no further dissolution was observed (approximately 120 g l^{-1}) and stored at room temperature.

Pronase E solution (30 U ml^{-1}). 7.5 mg ml^{-1} of pronase E (≥ 4 U mg^{-1}) were dissolved in PBS (50 mM; pH 7.5). The solution was freshly prepared prior to use and sterilised by filtration.

Proteinase K activation solution (10×; 10 mM). 1.47 g l^{-1} of calcium chloride dihydrate were dissolved in de-ionised water. The solution was sterilised by autoclaving.

Rhamnose stock solution (10 mM). 1.82 g l^{-1} of L-rhamnose were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C.

Rhamnose stock solution (30 mM). 492 mg l^{-1} of L-rhamnose were dissolved in de-ionised water. The solution was sterilised by filtration and stored at 8 °C.

Sodium acetate buffer (2×; 100 mM; pH 5.0). 8.2 g l^{-1} of sodium acetate were dissolved in de-ionised water and the pH was adjusted to 5.0. The solution was sterilised by autoclaving.

Sodium chloride solution (0.14 M). 8.18 g l^{-1} of sodium chloride were dissolved in de-ionised water. The solution was sterilised by autoclaving and stored either at room temperature or at 8 °C.

Sodium chloride solution (5 M; for FISH). 292.2 g l^{-1} of sodium chloride were dissolved in Rotipuran water. The solution was sterilised by autoclaving and stored at room temperature.

Sodium hydroxide solution (0.5%; w/v). 5 g l^{-1} of sodium hydroxide were dissolved in de-ionised water.

Sodium hydroxide solution (1 M). 40 g l^{-1} of sodium hydroxide were dissolved in de-ionised water. The solution was sterilised by autoclaving and stored at room temperature.

SDS solution (10%). 100 g l^{-1} of SDS were dissolved in Rotipuran water. The solution was sterilised by filtration and stored at room temperature.

Sulphamate reagent. 19.42 g of sulphamic acid were dissolved in 20 ml of de-ionised water by dropwise addition of saturated potassium hydroxide solution (approximately 8 ml). After cooling down, the pH was adjusted to 1.6 and the solution was filled up to 50 ml and stored at room temperature.

Sulphuric acid/tetraborate solution (75 mM). 28.6 g l^{-1} disodium tetraborate decahydrate were dissolved in concentrated sulphuric acid and stored at room temperature.

***Thermus* 162 phosphate buffer.** 5.44 g l^{-1} of potassium dihydrogen phosphate and 43.0 g l^{-1} of disodium hydrogen phosphate dodecahydrate were dissolved in de-ionised water. The pH was adjusted to 7.2. The solution was sterilised by autoclaving and stored at 8 °C.

***Thermus* 162 trace element solution.** 2.28 g l^{-1} of manganese sulphate monohydrate, 0.5 g l^{-1} of zinc sulphate heptahydrate, 0.5 g l^{-1} of boric acid, 0.025 g l^{-1} of copper sulphate pentahydrate, 0.025 g l^{-1} of disodium molybdate dihydrate, and 0.045 g l^{-1} of cobalt chloride hexahydrate were dissolved in de-ionized water. 0.5 ml l^{-1} of concentrated sulphuric acid were added. The solution was sterilised by autoclaving and stored at 8 °C.

TLC eluent. Acetonitrile, pentanol, and de-ionised water were mixed in a 3:1:1 ratio.

TRIS buffer (1 M; pH 8.0; for FISH). 121.14 g l^{-1} of TRIS were dissolved in Rotipuran water and the pH was adjusted to 8.0. The solution was sterilised by autoclaving and stored at room temperature.

TRIS-HCl buffer (2×; 40 mM; pH 8.0; for proteinase K treatment). 4.84 g l^{-1} of TRIS were dissolved in de-ionised water and the pH was adjusted to 8.0 using hydrochloric acid. The solution was sterilised by autoclaving.

Tween 80 stock solution (1%). 10 ml l^{-1} of Tween 80 were mixed with de-ionised water. The solution was sterilised by filtration and stored at room temperature.

Washing buffer. The composition of the washing buffer is shown in Tab. 2.6.

Xylose stock solution (30 mM). 450 mg l^{-1} of D-xylose were dissolved in de-ionised water. The solution was sterilised by filtration.

Table 2.6. Composition of the washing buffer (50 ml) used for FISH.

Component	Final concentration	Added volume (ml)
Sodium chloride solution (5 M)	250 mM	2.5
TRIS buffer (1 M, pH 8.0)	20 mM	1.0
SDS solution (10%)	0.01%	0.05
Rotipuran water	-	ad 50

2.1.7 Commercial kits

Commercial kits used in this study are listed in Tab. 2.7.

Table 2.7. Commercial kits used in this study.

Item	Manufacturer
BacTiter-Glo™ Microbial Cell Viability Assay	Promega
Live/Dead® *Bac*Light™ Bacterial Viability Kit L7012	Molecular Probes
Quant-iT™ PicoGreen® dsDNA Reagent Kit	Invitrogen

2.1.8 Consumables

The consumable materials used in this study are listed in Tab. 2.8. General laboratory consumables such as pipette tips are not listed.

Table 2.8. Consumable materials used in this study.

Item	Specifications	Manufacturer
Bottle Top Filter	non-fiber releasing membrane, surfactant-free cellulose acetate, 0.20 µm pore size	Nalgene
Cellophane sheets	35 × 50 cm	BioRad
Cellulose acetate syringe filters, Filtropur S plus 0.2	0.2 µm pore size	Sarstedt
Cellulose mixed-ester membrane filters, GN-6	white; gridded, 47 mm diameter, 0.45 µm pore size	Pall
Centrifuge tubes	50 ml, conical bottom, polypropylen	Sarstedt
Ceramic discs	12 mm diameter	provided by DLR Cologne
Cuvettes	PMMA, macro, 2.5 ml, 4 clear faces, disposable	Brand

Table 2.8. Consumable materials used in this study (continued).

Item	Specifications	Manufacturer
Cuvettes	PMMA, semi-micro, 1.5 ml, 2 clear faces, disposable	Brand
Diagnostic microscope slides	8-well, 6 mm, numbered	Thermo Scientific
Filter papers		Whatman
Glass beads	0.45-0.50 mm diameter	B. Braun Biotech International GmbH
Glass cuvettes	macro, 2.5 ml	Brand
Glass cuvettes	semi-micro, 1.5 ml	Brand
Glass discs	12 mm diameter	provided by DLR Cologne
Glass fibre filters, MN 85/220 BF	borosilicate glass, binder-free, 45 mm diameter	Macherey-Nagel
Glass fibre filters, MN 85/220	borosilicate glass, with organic binder, 45 mm diameter	Macherey-Nagel
Isopore™ polycarbonate membrane filters	brown, 30 mm diameter, 0.2 µm pore size	Millipore
Microcentrifuge tubes	1.5 ml	Sarstedt
Microcentrifuge tubes	2.0 ml	Sarstedt
Omnifix® syringes	single-use, 3-piece, 10 ml	B Braun
pH diagnostic dipsticks		Roth
Reaction chambers	for FISH	Vermicon
Spectra POR 3 dialysis tubes	3,500 Da molecular-weight cutoff	Spectra
Tissue culture plate	6-well, flat bottom with lid	Sarstedt
Tissue culture plate	24-well, flat bottom with lid	Sarstedt
Thoma cell counting chamber	0.100 mm depth, 0.0025 mm^2	Optik Labor
TLC silica gel 60 plates	10 × 20 cm	Merck

2.1.9 Equipment

The equipment used in this study is listed in Tab. 2.9. General laboratory equipment (e.g. pipettes, beakers) and equipment used by DLR during exposure experiments at the PSI facilities is not listed.

Table 2.9. Equipment used in this study.

Item	Specifications	Manufacturer
Alarm hygrometer testo 608-H2		Testo
Analytical balance BP 210 S		Sartorius
Analytical balance Extend ED4202S-CW		Sartorius
Bandelin Sonorex RK 103 H sonicator		Bandelin electronic
Biofuge Fresco cooling centrifuge		Heraeus Instruments
Cary 50Bio UV-Visible spectrometer		Varian
Cell density meter model 40		Fisher Scientific
DMLS light microscope, fitted with C Plan 40×/0.65 PH2 objective lens		Leica
Enterprise II UV laser, Enterprise RS232		Coherent
GFL-1092 shaking water bath		Gesellschaft für Labor-Technik mbH
GFL water bath		Gesellschaft für Labor-Technik mbH
Glomax 20/20 luminometer		Promega
Hera freeze freezer		Heraeus
Heratherm incubator		Thermo Scientific
Hybaid Shake 'n' Stack hybridisation oven		Thermo Scientific
Leitz Laborlux S epifluorescence microscope	PL Fluotar 100× / 1.32 oil objective, 2× Periplan 10× / 18 eye piece, HBO 50 UV unit	Zeiss
LSM 510 CLSM system	Axiovert 100M, AxioCam MRm, LSM 5 Control, MCU 28	Zeiss
Membrane filtration apparatus	3-branch manifold, stainless steel	Sartorius
Membrane vacuum filtration module	six-fold, stainless steel	Millipore
Nikon Eclipse Ni-E epifluorescence microscope	Intensilight C-HGFIE equipped with HG controller, DS-Qi1Mc camera, DS-Fi1 camera, Digital Sight DS-U3	Nikon
pH meter pH 540 GLP		WTW

Table 2.9. Equipment used in this study (continued).

Item	Specifications	Manufacturer
Quanta™ 400 FEG Environmental Scanning Electron Microscope (ESEM)		FEI
SFM 25 Bio-TEK fluorescence spectrometer		Kontron Instruments
Sorvall RC26PLUS cooling centrifuge		Sorvall
Thermomixer comfort		Eppendorf
TLC development chamber		Camag
TLC plate heater		Camag
Unitron shaking incubator		Infors
Vacuum centrifuge RVC 2-25		Christ
Vortex Genie 2 vortex mixer		Scientific Industries

2.1.10 Software

The software used in this study for data generation and evaluation is listed in Tab. 2.10.

Table 2.10. Software used in this study.

Software	Version	Manufacturer
Microsoft Excel	2010	Microsoft
Microsoft Word	2010	Microsoft
NiS Elements Imaging Software	4.10.01 64-bit	Nikon
LSM Image Browser	4.0.0.241	Zeiss
LSM 510		Zeiss
Simple Reads	2.0	Varian
Win UV Scan		Varian

2.2 Methods

2.2.1 Cultivation of *D. geothermalis*

Cryocultures of *D. geothermalis* DSM 11300 were stored at -70 °C. Preparatory cultures of *D. geothermalis* were maintained on R2A by incubation for 2 days at 45 °C.

2.2.1.1 Plate counts

100 µl of (diluted) bacterial suspension were plated in triplicates onto R2A agar plates. The plates were incubated for 2 days at 45 °C and colonies visible to the naked eye were counted. Only plates with a colony count ≥ 20 and < 300 were used for quantification. The number of colony-forming units (CFU) ml^{-1} of sample was calculated according to Eq. 2.1.

$$CFU\ ml^{-1} = CFU_{average} \times \frac{1}{0.1\ ml} \times \frac{1}{D}\ , \qquad \textbf{(Equation 2.1)}$$

with $CFU_{average}$ as the average number of CFU per dilution, and D as the dilution factor (e.g. 10^{-3}).

2.2.1.2 Generation *D. geothermalis* biofilms

Unsaturated biofilms of *D. geothermalis* were grown on a range of substrates, including cellulose mixed-ester (CME) membranes (GN-6 Metricel® MCE Membrane Disc Filters; 47 mm diameter; 0.45 µm pore size; Pall), glass discs (12 mm diameter; provided by DLR), ceramic discs (12 mm diameter; provided by DLR), borosilicate glass fibre filters with or without an organic binder of unknown composition (45 mm diameter, pore size not provided by the manufacturer; Macherey & Nagel), and cellophane discs (12 mm diameter) cut out from cellophane foil (pore size not provided by the manufacturer; BioRad).

CME membranes and glass fibre filters were inoculated by membrane filtration. The substratum was placed in a sterilised stainless-steel membrane filtration apparatus (Sartorius) and pre-wetted by filtering 10 ml of de-ionised water through it. Subsequently, 50 ml of de-ionised water containing 10^7 cells of *D. geothermalis* preparatory culture were filtered through in order to inoculate the substratum. The cell density of the suspension was controlled by counting the cells in a Thoma cell counting chamber (Optik Labor).

Glass discs, ceramic discs, and cellophane discs were sterilised by autoclaving in glass Petri dishes (for glass discs and ceramic discs) or in de-ionised water (for cellophane discs) and placed on the surface of a solid nutrient medium. Inoculation of the substrata was done by pipetting drops of a bacterial suspension of *D. geothermalis* preparatory culture cells in de-ionised water on top of them. The amount of cells added related to the surface area inocu-

lated, aiming for a cell density of 7.6×10^5 cells cm^{-2} (corresponding to the density of cells on CME membranes inoculated with 10^7 cells per membrane). If necessary, the suspension was spread out in order to cover the whole surface of the substratum. The droplets were allowed to air-dry briefly in a sterile bench.

For lectin staining, biofilms were grown on brown polycarbonate membranes (Isopore™; 30 mm diameter, 0.2 µm pore size; Millipore). Polycarbonate membranes were inoculated by membrane filtration using a six-fold vacuum stainless steel filtration module (Millipore), achieving an initial density of membrane-deposited cells of approximately 7.6×10^5 cells cm^{-2}.

The inoculated substrata were placed on the surface of a solid nutrient medium with their inoculated side facing upwards, and incubated for 2 days at 45 °C.

2.2.1.3 Optimisation of the cultivation conditions for biofilm formation

Cultivation conditions were varied in order to increase biomass production of *D. geothermalis* biofilms on CME membranes. Three sets of CME membranes were inoculated by membrane filtration with different inoculum sizes of 10^6, 10^7, and 10^8 cells per membrane. Each set was divided into three subgroups. For each subgroup, a different nutrient medium was used to incubate the CME membranes on: (i) R2A, (ii) TSA, and (iii) *Thermus* 162 medium. Each subgroup comprised 8 samples. Samples were incubated in duplicates for 24 h and 48 h at 37 °C and 45 °C, respectively. After incubation, one duplicate was used for wet weight determination, whilst the other duplicate was used to determine the total cell count of the biofilm. For wet weight determination, all biomass visible to the naked eye was scraped off the membrane and transferred to a piece of aluminium foil of known weight. The foil was weighted on an analytical balance and the wet weight of the sample was determined by subtracting the weight of the aluminium foil. For determination of the total cell count, all biomass was scraped off the membrane and transferred into 10 ml of 0.14 M sodium chloride solution. The suspension was homogenised by vigorous shaking for approximately 15 min, diluted 1,000-fold, and subjected to DAPI-staining (see 2.2.2.1) followed by microscopic enumeration of the cells.

2.2.1.4 Determination of biofilm dry weight

Prior to the experiment, a set of sterile CME membranes, each placed in a Petri dish with vents, was dried in a desiccator for 4 days. The weight of the membrane-containing dishes was determined. The membranes were then inoculated with *D. geothermalis* and incubated on either R2A or TSA according to the procedure described in 2.2.1.2. After incubation for 2 days the overgrown membranes were returned to their respective empty Petri dishes and placed in

a desiccator. The weight of the dishes was measured after 2 and 5 days of desiccation. The dry weight of the biofilm after 2 or 5 days of drying was calculated.

2.2.1.5 Generation of planktonic cultures of *D. geothermalis*

50 ml of R2B were poured into a 250 ml Erlenmeyer flask and inoculated with 5×10^6 cells ml^{-1} derived from a preparatory culture. The culture was incubated at 45 °C for 48 h under agitation at 160 rpm, either in a shaking water bath or a shaking incubator.

2.2.1.6 Analysis of planktonic growth characteristics of *D. geothermalis*

The growth characteristics of planktonic cultures of *D. geothermalis* were assessed by time-dependent cell density measurements over the first 48 h of growth. The experiment was carried out in two independent, time-delayed series in order to cover the whole time frame of the incubation period, including nights. Each series was run in triplicates. Each sample was prepared, inoculated, and incubated as described above (see 2.2.1.5). At the point of inoculation and hourly post-inoculation, 1 ml of culture was transferred to a semi-microcuvette and subjected to $OD_{600\ nm}$ measurement using a cell density meter (Fisher Scientific). If $OD_{600\ nm}$ values exceeded 0.5, the suspension was diluted tenfold in sterile R2B. Sterile R2B was used as a control. After 24 h and 48 h of incubation, the total cell count of the suspensions was determined microscopically by DAPI-staining of 1 ml of 1,000-fold diluted culture (for staining procedure, see 2.2.2.1).

2.2.2 Staining procedures and microscopy

2.2.2.1 DAPI staining and microscopy

The DNA-intercalating fluorescent dye 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma) was used to stain cells for microscopic enumeration and observation. For the staining of suspended cells of *D. geothermalis*, the bacterial suspension was diluted to a cell density of approximately 10^5-10^6 cells ml^{-1}. 4 ml of bacterial suspension were mixed with 1 ml of DAPI solution and incubated for 20 min in the dark. DAPI-stained suspensions were filtered through brown polycarbonate membrane filters (Isopore™; 30 mm diameter; 0.2 µm pore size; Millipore) using a six-fold vacuum stainless steel filtration module (Millipore). Another 5 ml of de-ionised water were filtered through. For the staining of cells immobilised on CME membranes or cellophane discs, 1 ml of 5-fold diluted DAPI solution (5 µg ml^{-1} DAPI in 2% formaldehyde) was carefully pipetted onto the support material and incubated for 20 min in the dark. The supernatant dye solution was removed by pipetting. Stained samples were stored at 4 °C in Petri dishes wrapped in aluminium foil until microscopic observation.

Microscopic evaluation was carried out at 1,000-fold magnification using an EFM (Leica/Nikon) equipped with a 100 × 100 µm counting grid. The filter was mounted on a glass

slide in antifading agent Citifluor AF2 (Science Services GmbH) and covered with a cover slip. Immersion oil (Leica) was applied onto the cover slip. For total cell counts, 20 fields of vision chosen randomly were evaluated for each filter.

2.2.2.2 Live/Dead® staining and microscopy

The Live/Dead® *Bac*Light™ Bacterial Viability Kit L7012 (Molecular Probes) comprises the membrane-permeable green-fluorescent nucleic acid dye Syto® 9 (3.34 mM in dimethyl sulphoxide; DMSO) and the membrane-impermeable red-fluorescent nucleic acid dye propidium iodide (20 mM in DMSO). Both dyes were mixed to create the Live/Dead® working dye solution. The manufacturer suggests mixing the dyes in a 1:1 ratio, but recommends adjusting their proportions for optimal discrimination. For staining of *D. geothermalis* cells in suspension, propidium iodide had to be diluted 100-fold in PFD water before mixing with undiluted Syto® 9 in a ratio of 1:1. The cell density of the bacterial suspension to be stained was adjusted to be in the range of 3-6 × 10^5 cells ml^{-1}. 1 ml of suspension was mixed with 3 µl of Live/Dead® working dye solution (containing 100-fold diluted propidium iodide) and incubated for at least 15 min in the dark before adding 4 ml of PFD water. Stained suspensions were filtered through brown polycarbonate membrane filters (Isopore™, 30 mm diameter, 0.2 µm pore size; Millipore) using a six-fold vacuum stainless steel filtration module (Millipore). Another 5 ml of PFD water were filtered through. Filters were recovered and stored at 4 °C in Petri dishes wrapped in aluminium foil. For *in situ* staining of *D. geothermalis* cells immobilised on CME membranes or cellophane, the undiluted stock solutions of the two dyes were mixed in a 1:1 ratio. 3 µl of Live/Dead® working dye solution were mixed with 1 ml of PFD water and carefully applied onto the substratum. Samples were incubated for at least 15 min in the dark before removing the supernatant dye solution by pipetting. Samples were stored at 4 °C in Petri dishes wrapped in aluminium foil until microscopic observation.

Isopropyl alcohol-damaged cells were used as a negative control. When working with cell suspensions, 1.5 ml of cell suspension was provided in a 2 ml microcentrifuge tube. The tube was centrifuged for 5 min at 6,000 × g and 4 °C. 900 µl of supernatant were replaced by 1.4 ml of 99% isopropyl alcohol, resulting in a final isopropyl alcohol concentration of 70%. The suspension was incubated at room temperature for 1 h, mixing every 15 min. The cell suspension was washed once or twice by centrifuging for 5 min at 6,000 × g and 4 °C, discarding the supernatant, and re-suspending the cell pellet in 1.5 ml of 0.14 M sodium chloride solution. 1 ml of the treated suspension was used for staining. Negative controls of samples of biofilms and immobilised planktonic cells were produced by applying 1 ml of 70% isopropyl alcohol onto the sample. After 1 h of incubation at room temperature the supernatant solution was removed by pipetting before proceeding to Live/Dead® staining. A washing step was

set aside as it harmed the integrity of the cell deposit. The use of a negative control was mandatory for CLSM.

For microscopy, filters and stained support materials were sandwiched between a glass slide and a cover slip, embedded in antifading agent Citifluor AF2 (Science Services GmbH). Immersion oil (Leica) was applied onto the cover slip. Cell enumerations were done at 1,000-fold magnification using an EFM equipped with a 100 × 100 µm counting grid. 10 randomly chosen fields of vision were evaluated for each dye and filter. Cells which stained only red or both red and green were considered membrane-damaged. Cells which stained only green were considered membrane-intact. Microscopic observations were done at 1,000-fold magnification using either EFM or CLSM. For CLSM, excitation of the two dyes at an excitation wavelength of 488 nm was performed using an argon laser. Emission by Syto® 9 was detected in the range of 505-530 nm using a band-pass filter. Emission by propidium iodide was detected at wavelengths ≥ 560 nm using a long-pass filter.

2.2.2.3 Lectin staining and microscopy

Lectins were purchased from EY Laboratories as ready-made stock solutions of purified lectin proteins labelled with tetramethylrhodamine isothiocyanate (TRITC) in buffer (10 mM phosphate, 0.15 M sodium chloride; pH 7.2-7.4). Protein concentrations of the stock solutions were 1 mg ml^{-1} for *D. biflorus* lectin (DBA) and for *H. aspersa* lectin (HAA) and 0.33 mg ml^{-1} for *A. caudatus* lectin (ACA). Lectin working solutions were prepared by diluting the stock solutions in phosphate-buffered saline (PBS; 6 mM) to a final concentration of 100 µg ml^{-1}, as suggested by the manufacturer. Biofilms of *D. geothermalis* were grown on R2A or TSA as described above (see 2.2.1.2). Brown polycarbonate membrane filters (Isopore™; 30 mm diameter; 0.2 µm pore size; Millipore) were used as a substratum to minimise background signals. For lectin staining, 1 ml of lectin working solution was carefully applied onto the biofilm. Samples were incubated for 20 min in the dark. Afterwards, the supernatant lectin solution was removed and the biofilm was carefully rinsed three times with 1 ml of 6 mM PBS. For counterstaining of the cells, 100 µl of DAPI solution were applied onto the biofilm and left to incubate for 20 min in the dark. Afterwards, the supernatant DAPI solution was removed and the sample was stored at 4 °C in a Petri dish wrapped in aluminium foil until microscopic observation.

For microscopy, samples were embedded in antifading agent Citifluor AF2 (Science Services GmbH) and sandwiched between a glass slide and a cover slip, using two additional cover slips as spacers in order to preserve the biofilm architecture. Immersion oil (Leica) was applied onto the uppermost cover slip. Evaluation was done at 1,000-fold magnification using either EFM or CLSM. For CLSM, excitation of TRITC at an excitation wavelength of 543 nm was performed using a helium/neon laser. Emission by TRITC was detected at wavelengths

≥ 560 nm using a long-pass filter. Excitation of DAPI at an excitation wavelength of 351 nm was performed using an UV laser. Emission of DAPI was detected in the range of 385-470 nm using a band-pass filter.

2.2.2.4 Fluorescence *in situ* hybridisation

Samples were fixed prior fluorescence *in situ* hybridisation (FISH). For fixation of the samples, 1 ml of bacterial suspension was provided in a 2 ml microcentrifuge tube. After centrifugation for 5 min at 6,000 × g and 4 °C, the supernatant was removed and the cell pellet was re-suspended in 2 ml of 4% (w/v) paraformaldehyde in PBS (for FISH; pH 7.2). The suspension was incubated for 1 h at 4 °C. The supernatant was removed by centrifugation (6,000 × g, 4 °C, 5 min). The cell pellet was washed in 2 ml of PBS and, following another centrifugation step for 5 min at 6,000 × g and 4 °C, was re-suspended in 250 µl of PBS. The suspension was mixed with 250 µl of ice-cold ethanol (absolute) and stored at -20 °C until hybridisation. For hybridisation, 10 µl of fixed cell suspension were applied into 6-mm-diameter wells of 8-well diagnostic microscopic slides (Thermo Scientific) and left to dry. Cells were dehydrated by a sequential 3-min immersion in 50%, 80%, and 96% ethanol (v/v in de-ionised water) and air-dried afterwards. 10 µl of EUB338 probe working solution (probe concentration 5 ng μl^{-1}; Eurofins MWG Operon) were added to each well. Slides were placed in a reaction chamber (Vermicon) saturated with hybridisation buffer and incubated at 46 °C in a pre-heated hybridisation oven (Thermo Scientific) for 90 min. Excess probe solution was removed by immersing the slides in 25 ml to 50 ml of washing buffer pre-heated to 46 °C, followed by incubation at 46 °C for 20 min. After washing, slides were rinsed with de-ionised water and allowed to dry in the dark. Cells were counter-stained with DAPI by adding 10 µl of DAPI solution (diluted in PBS to a concentration of 1 µg ml^{-1}) to each well and incubating for 20 min in the dark. Slides were rinsed with de-ionised water, air dried in the dark, and stored at 4 °C in a Petri dish wrapped in aluminium foil until microscopic evaluation.

For microscopy, a drop of antifading agent Citifluor AF2 (Science Services GmbH) was applied to each well before covering the samples with a cover slip. Immersion oil (Leica) was applied onto the cover slip and the samples were analysed at 1,000-fold magnification using an EFM equipped with a 100 × 100 µm counting grid. 20 randomly selected fields of vision or at least 200 individual cells were evaluated. The ratio of FISH-positive (orange-fluorescent) to DAPI-stained (blue-fluorescent) cells corresponded to the fraction of FISH-positive cells in a given sample.

2.2.2.5 Environmental scanning electron microscopy

For environmental scanning electron microscopy (ESEM), desiccated samples of biofilms and planktonic cells on CME membranes or cellophane were prepared as described in 2.2.7.1.

Samples were stored in desiccated state for 3 days before ESEM. Samples were sputtered with gold for 30 s, resulting in a gold layer with an approximate thickness of 6 nm. ESEM was carried out using a Quanta™ 400 FEG (FEI). Samples were observed at 5,000- to 20,000-fold magnification.

2.2.3 ATP measurements

2.2.3.1 Total ATP quantification

ATP was quantified using the luciferase-based BacTiter-Glo™ Microbial Cell Viability Assay (Promega). The assay was carried out in opaque 1.5 ml microcentrifuge tubes and executed according to the manufacturer's protocol, with an ATP standard curve ranging from 10 pM to 1 µM. Suspensions of *D. geothermalis* cells in 0.14 M sodium chloride solution corresponded to the sample solution. Sterile 0.14 M sodium chloride solution was used as a blank and for diluting the standard series. Luminescence was recorded 5 min after mixing sample and BacTiter-Glo™ reagent using a Glomax 20/20 luminometer (Promega). Excess BacTiter-Glo™ reagent was stored at -70 °C for up to 4 weeks.

2.2.3.2 Desiccation of ATP

A 1 µM ATP stock solution (Promega) was prepared in PFD water and aliquoted into volumes of 20 µl in 1.5 ml microcentrifuge tubes. The ATP concentration of the stock solution was verified by total ATP quantification as described in 2.2.3.1. ATP solutions were dried by vacuum centrifugation (Christ) for 3 h at 30 °C. Microcentrifuge tubes were closed and stored at room temperature in the dark for up to 63 days. At defined points in time, a triplicate of samples was analysed for their ATP content. To each microcentrifuge tube, 200 µl of PFD water were added. After incubating and mixing briefly, 100 µl of ATP solution were transferred into a new 1.5 ml microcentrifuge tube. The ATP concentration of both sub-samples was determined as described in 2.2.3.1 and averaged. The data was used to calculate the decay constant λ (Eq. 2.2) and the half-life $t_{1/2}$ of dried ATP (Eq. 2.3).

$$N(t) = N_0 e^{-\lambda t}, \qquad \textbf{(Equation 2.2)}$$

with $N(t)$ as the quantity of ATP at time t, N_0 as the initial quantity of ATP, λ as the decay constant, and t as the time.

$$t_{1/2} = \frac{ln(2)}{\lambda}, \qquad \textbf{(Equation 2.3)}$$

with $t_{1/2}$ as the half-life, and λ as the decay constant.

2.2.4 Detection of reducing sugars

Reducing sugars were detected in liquid cultures by using a bicinchoninic acid (BCA) assay (Waffenschmidt & Jaenicke, 1987). A stock solution of D-glucose (10 mg l^{-1}) was diluted in de-ionised water to produce standards with sugar concentrations of 2.5 µg ml^{-1}, 5.0 µg ml^{-1}, and 7.5 µg ml^{-1}. De-ionised water was used as a blank. A BCA working solution was prepared at the day of the experiment by mixing BCA solution A and BCA solution B in a 1:1 ratio. 0.5 ml of sample, standard, or blank (all in triplicates) were mixed with 0.5 ml of BCA working solution. After incubation at 100 °C for 15 min and cooling of the samples to room temperature (approximately 20 min), the absorption at 560 nm was measured using a UV/VIS spectrometer (Varian).

2.2.5 Isolation and analysis of EPS

2.2.5.1 EPS isolation

Prior to EPS isolation, cells were suspended in phosphate buffer (PB; 6 mM; pH 7.0). Biofilms were produced on CME membranes or cellophane as described in 2.2.1.2 and collected by scraping them off the substratum using a spatula; planktonic cells were cultured as described in 2.2.1.5 and collected by centrifugation for 20 min at 20,000 × g. In order to determine the total cell count, 500 µl of bacterial suspension were stained using DAPI as described in 2.2.2.1. Five different EPS isolation methods were tested, including heating, sodium hydroxide treatment in combination with formaldehyde, EDTA treatment, and shaking with or without the addition of the cation-exchange resin (CER) Dowex® (Sigma). The isolation procedures are described in Tab. 2.11. Following EPS isolation, the cells were removed by centrifugation for 20 min at 20,000 × g and 4 °C. If desired, a total cell count was performed by re-suspending the cell pellet in PB and staining the cells using DAPI (see 2.2.2.1). The supernatant, corresponding to the EPS solution, was sterilised by filtration using cellulose acetate syringe filters (pore size 0.2 µm; Sarstedt) and sequentially dialysed at 8 °C against 3 × 5 l of de-ionised water (twice for 1 h, once overnight) using dialysis tubes with a molecular weight cut-off of 3,500 Da (Spectra POR 3; Serva) in order to eliminate low molecular weight compounds. Dialysed EPS solutions were transferred into 50 ml centrifuge tubes and stored at -20 °C until analysis.

Changes in volume were noted after each step and considered when quantifying EPS constituents.

Table 2.11. EPS isolation methods employed in this study (after Michalowski, 2012).

Method	Procedure
Heating	Cell suspension was heated to 70 °C for 1 h in a water bath.
Shaking	Cell suspension was transferred to a 50 ml centrifuge tube mounted on a vortex shaker and shaken vigorously for 30 min.
Shaking in the presence of a CER	CER Dowex® (Marathon C, Na^+-form; Sigma) was hydrated and washed twice in PB (6 mM, pH 7.0) by stirring for 15 min per washing step. 0.2 g of hydrated Dowex® were added per ml of cell suspension. The suspension was shaken as described above. After the isolation treatment, the CER was allowed to sediment and the supernatant cell suspension was transferred into a new 50 ml centrifuge tube.
Sodium hydroxide/ formaldehyde	Cell suspension was pre-treated with formaldehyde by adding 6 µl of formaldehyde (37% in water) per ml of cell suspension. After incubation at 4 °C for 1 h, 400 µl of 1 M sodium hydroxide solution were added per ml of cell suspension. The suspension was stirred for 3 h at 4 °C.
EDTA	1 ml of 2% EDTA solution was added per ml of cell suspension. The suspension was stirred for 3 h at 4 °C.

2.2.5.2 Biochemical analysis of EPS and whole biofilms

Quantification of proteins. A commercial modified Lowry assay (Peterson, 1979) was used to quantify proteins in biofilms and in EPS isolated from *D. geothermalis*. Samples with high protein concentrations (> 60 µg ml^{-1}) were pre-diluted in de-ionised water. BSA (Sigma) was used as a standard. A 400 µg ml^{-1} BSA stock solution was diluted in de-ionised water to produce a 3-point standard series with concentrations ranging from 20 µg ml^{-1} to 60 µg ml^{-1}. De-ionised water was used as a blank. All samples were analysed in triplicates. 0.5 ml of sample or standard were mixed with 0.5 ml of Lowry reagent (Sigma) and incubated for 20 min at room temperature. Afterwards, 0.25 ml of Folin-Ciocalteu's phenol solution were added and the solution was incubated for another 30 min at room temperature before transferring it to a semi-micro glass cuvette with an optical path length of 10 mm. Absorbance was recorded at 750 nm against de-ionised water using a UV/VIS spectrometer (Varian).

Quantification of total carbohydrates. Total carbohydrate concentrations of biofilms and EPS isolated from *D. geothermalis* were determined using a sulphuric acid/phenol assay after Dubois et al. (1956). Samples with high carbohydrate concentrations (> 75 µg ml^{-1}) were pre-diluted in de-ionised water. D-glucose (Sigma) was used as a standard. A 200 µg ml^{-1} glucose stock solution was diluted in de-ionised water to produce a 3-point standard series with concentrations ranging from 25 µg ml^{-1} to 75 µg ml^{-1}. De-ionised water was used as a blank. All samples were analysed in triplicates. 0.5 ml of 5% (w/v) phenol reagent and 2.5 ml of con-

centrated sulphuric acid (Fisher Scientific) were quickly added to 0.5 ml of sample or standard. The solution was gently mixed and left to cool for 10 min at room temperature. Afterwards, samples were incubated in a water bath for 15 min at 30 °C and then left for 5 min at room temperature. Next, the solution was transferred to a glass cuvette with an optical path length of 10 mm. Absorbance was recorded at 490 nm against de-ionised water using a UV/VIS spectrometer (Varian).

Quantification of uronic acids. Uronic acids were measured according to Filisetti-Cozzi and Carpita (1991). D-glucuronic acid (Sigma) was used as a standard. A 200 µg ml^{-1} glucuronic acid stock solution was diluted in de-ionised water to produce a 3-point standard series with concentrations ranging from 50 µg ml^{-1} to 150 µg ml^{-1}. De-ionised water was used as a blank. All samples were analysed in triplicates. 0.5 ml of sample or standard were mixed with 50 µl of sulphamate reagent. 3 ml of sulphuric acid/tetraborate solution (75 mM) were added. The solution was mixed and incubated in a water bath for 20 min at 100 °C. After incubation, solutions were cooled to room temperature in an ice bath. 100 µl of 3-hydroxybiphenyl solution were added. The solutions were mixed and incubated for 10 min at room temperature before measuring the absorbance at 525 nm against de-ionised water using a UV/VIS spectrometer (Varian). If the assay was of brownish colour, the absorbance spectrum between 400 nm and 650 nm was recorded.

Quantification of DNA. The Quant-iT™ PicoGreen® dsDNA Reagent Kit (Invitrogen) was used to quantify DNA in biofilms and in EPS isolated from *D. geothermalis*. The procedure was carried out according to the manufacturer's instructions. λ DNA was used as a standard. A 100 µg ml^{-1} λ DNA stock solution was diluted in TE buffer (provided in the kit) to produce a 4-point high range standard series ranging from 2 ng ml^{-1} to 2 µg ml^{-1} and a 4-point low range standard series ranging from 0.05 ng ml^{-1} to 50 ng ml^{-1}, respectively. TE buffer was used as a blank. 1 ml of sample or standard were provided in PMMA macrocuvettes (Brand) and mixed with 1 ml of PicoGreen® reagent. After incubation for 2 min in the dark, the relative fluorescence emitted at 520 nm after excitation at 480 nm was recorded against the blank using a fluorescence spectrometer (Kontron Instruments).

2.2.5.3 Characterisation of extracellular polysaccharides by thin-layer chromatography

Following the isolation of EPS from biofilms of *D. geothermalis* grown on R2A or TSA using CER Dowex®, extracellular polysaccharides were degraded to monomers by acidic hydrolysis. Dialysed EPS solutions were concentrated in microcentrifuge tubes using a vacuum centrifuge (Christ) to accumulate an amount of EPS corresponding to approximately 120 µg of carbohydrates. Concentrated EPS solutions were dried by vacuum centrifuging overnight. For hydrolysis, dried EPS were rehydrated in 2 ml of 0.1 M hydrochloric acid (Sigma), transferred to glass reagent tubes with screw caps, and incubated for 24 h or 48 h at 100 °C using a heat

block (VWR). After incubation, solutions were neutralised using a 1 M sodium hydroxide solution. Neutral pH was confirmed by using pH test sticks (Roth). The solution was concentrated by vacuum centrifugation to a final volume of 100 µl. If desired, acetone was used to precipitate salts and proteins. For precipitation, 100 µl of hydrolysed EPS were mixed with 100 µl of acetone and incubated for 24 h at 4 °C. Samples were stored at -20 °C until analysis. Prior use, acetone-treated samples were centrifuged for 45 min at 20,000 × g and 4 °C. The supernatant was used for thin-layer chromatography (TLC).

TLC was performed in a TLC development chamber (Camag) filled with TLC eluent to a level of approximately 1 cm. Filter papers (Whatman) were added to the chamber at least 1 h prior to the TLC procedure in order to facilitate saturation of the gas phase with TLC eluent. TLC was carried out on TLC silica gel 60 plates (10 × 20 cm; Merck). 30 mM stock solutions of various monosaccharides (D-galactose, D-glucose, D-mannose, D-xylose, L-fucose, L-rhamnose; all Sigma) were used as standards. 2 µl of EPS sample or 1 µl of standard were applied as spots onto the silica gel plate, 1.5 cm apart from the bottom end of the plate and 1 cm apart from each other. Spots were dried with the aid of a hair-dryer. Plates were placed into the TLC development chamber and developed in 3 sequential 30-min steps. After each step the plate was allowed to air-dry for 20-30 min. After the third drying step, the plate was carefully sprayed with approximately 10 ml of derivatisation agent and incubated in a fume hood for 10 min at 100 °C using a TLC plate heater (Camag). The plate was allowed to cool and the chromatogram was recorded using a flat-bed scanner. The retardation factor R_f was calculated for each spot as the ratio of the distance travelled by the centre of a spot to the distance travelled by the eluent front.

2.2.6 Dispersal of TSA-grown biofilms

Various approaches were devised in order to facilitate dispersal of TSA-grown biofilm aggregates, including physico-chemical treatments and treatments involving the addition of carbohydrates or enzymes.

2.2.6.1 Physico-chemical treatments

Biofilms of *D. geothermalis* were produced on CME membranes placed on TSA (see 2.2.1.2) and suspended in de-ionised water or dispersal agent. Tab. 2.12 lists the various physico-chemical procedures tested. After treatment, samples were stained with DAPI (see 2.2.2.1) and evaluated qualitatively for relief of aggregation using EFM.

Table 2.12. List of various physico-chemical methods applied in order to disperse aggregates of TSA-grown biofilms of *D. geothermalis* suspended in aqueous solution.

Method	Procedure
Shaking	Biofilm suspension was shaken vigorously on a vortex mixer at full speed for 60 min.
Ion scavenging	Biofilm was suspended in 5 mM or 10 mM EDTA solution and shaken vigorously for 5 min.
Surfactant	Biofilm was suspended in 0.001%, 0.01%, 0.1%, or 1% Tween 80 solution and shaken vigorously for 5 min.
Sonication	Biofilms were suspended in de-ionised water, EDTA solution, or Tween 80 solution as described above, shaken vigorously for 5 min, and sonicated at 50-60 Hz for up to 5 min.

2.2.6.2 Carbohydrate treatments

TSA-grown biofilms were suspended in 10 ml of de-ionised water and shaken thoroughly. 1 ml of biofilm suspension was transferred to a microcentrifuge tube and centrifuged for 10 min at 9,500 × g. The supernatant was discarded. The cell pellet was re-suspended in 1 ml of carbohydrate stock solution (D-fucose, D-galactose, D-glucose, D-mannose, L-fucose, L-rhamnose, NAc-galactosamine, NAc-glucosamine, NAc-mannosamine, and methyl-α-D-mannopyranoside; all at 10 mM; D-mannose was additionally administered at concentrations of 1 mM, 5 mM, and 100 mM). As a control, 1 ml of de-ionised water was added instead. pH was measured and samples were incubated for 24 h at 4 °C. Following carbohydrate treatment, samples were stained with DAPI (see 2.2.2.1) and the width of 100 randomly chosen aggregates was measured using the NiS Elements Imaging Software (Nikon). Width was always measured horizontally in order to prevent subjective errors. All tests were done in duplicates.

2.2.6.3 Enzymatic treatments

Enzymatic treatments involved the addition of cellulolytic enzyme cellulase and two proteolytic enzymes pronase E and proteinase K, respectively. TSA-grown biofilms were suspended in 10 ml of de-ionised water and shaken thoroughly. 1 ml of biofilm suspension was transferred to a microcentrifuge tube and centrifuged for 10 min at 9,500 × g. The supernatant was discarded. The cell pellet was subjected to enzymatic treatment. All tests were done in duplicates.

Cellulase. The cell pellet was re-suspended in 400 µl of de-ionised water and mixed with 500 µl of 2× sodium acetate buffer (100 mM, pH 5.0). 100 µl of cellulase solution (60 U ml^{-1}; from *T. reesei*) were added, resulting in a final enzyme concentration of 6 U ml^{-1}. De-ionised water was used instead of enzyme solution for an enzyme-free control. The sus-

pension was mixed gently and the pH was measured. The suspension was incubated for 24 h at 37 °C.

Pronase E. The cell-pellet was re-suspended in 1,000 µl of pronase E solution (30 U ml^{-1}; from *S. griseus*), resulting in a final enzyme concentration of 30 U ml^{-1}. Heat-inactivated pronase E (incubated in a water bath at 95 °C for 15 min) was used for a negative control. PBS (50 mM, pH 7.5) was used instead of enzyme solution for an enzyme-free control. The suspension was mixed gently and the pH was measured. The suspension was incubated for 24 h at 37 °C.

Proteinase K. The cell pellet was re-suspended in 350 µl of de-ionised water. 500 µl of 2× TRIS-HCl buffer (40 mM, pH 8.0) and 100 µl of 10× proteinase K activation solution (10 mM Ca^{2+}) were added and the solution was mixed. 50 µl of proteinase K solution (600 U ml^{-1}; from *E. album*; New England Biolabs) were added, resulting in a final enzyme concentration of 30 U ml^{-1}. Heat-inactivated proteinase K (incubated in a water bath at 95 °C for 15 min) was used for a negative control. De-ionised water was added instead of proteinase K solution for an enzyme-free control. The suspension was mixed gently and the pH was measured. The suspension was incubated for 24 h at 37 °C.

Following enzymatic treatment, samples were stained with DAPI (see 2.2.2.1) and the total aggregate count was determined microscopically. Additionally, the width of 100 randomly chosen aggregates was measured using the NiS Elements Imaging Software (Nikon). Width was always measured horizontally in order to prevent subjective errors. Colony counts were performed by plating on R2A, followed by incubation for 2 days at 45 °C.

2.2.7 Desiccation and rehydration of *D. geothermalis*

2.2.7.1 Preparation of desiccated samples

Desiccated samples of both biofilms and planktonic cells, immobilised on either CME membranes or cellophane discs, were prepared with respect to desiccation experiments and space and Mars simulations. Biofilms were grown directly on top of the support. Planktonic cells were deposited on top of the support. For the deposition of planktonic cells, cells grown in liquid culture were harvested by centrifugation (1,900 × g, 20 °C, 15 min) and re-suspended in 0.14 M sodium chloride solution. Cells were then deposited either on CME membranes by membrane filtration or on cellophane discs by pipetting drops of bacterial suspension on top of the material. The drops were allowed to air-dry. The amount of cells deposited corresponded to a cell density of approximately 3×10^8 cells cm^{-2}, mimicking the cell density observed in matured biofilms of *D. geothermalis.* Cellophane discs carrying biofilms or planktonic cells were placed in empty Petri dishes with vents and air-dried overnight in the dark at approximately 59% relative humidity. CME membranes carrying biofilms or planktonic cells were

placed in a Petri dish. Using a cork borer, up to 5 circular pieces with a diameter of 12 mm were cut out of each CME membrane. The small CME discs were transferred to empty Petri dishes with vents and dried under the same conditions as described for the cellophane samples.

2.2.7.2 Rehydration and detachment of desiccated cells

General procedure. For rehydration, sample discs carrying desiccated biofilms or planktonic cells of *D. geothermalis* were transferred to a microcentrifuge tube containing 1 ml of 0.14 M sodium chloride solution and approximately 300 mg of glass beads (0.45-0.50 mm diameter; B Braun Biotech International GmbH). Samples were left to rehydrate for 30 min. Rehydrated cells were detached by vigorous shaking on a vortex mixer for 2 min.

Variations of the rehydration procedure. Variations were made to the time period of rehydration and to the rehydration temperature. Desiccated biofilms of *D. geothermalis* grown on CME membranes placed on R2A were rehydrated for 0 h, 0.5 h, 4.5 h, and 24 h before starting the detachment process. Samples rehydrated for 0 h were immediately subjected to the detachment procedure once introduced into the rehydration medium. The experiment was carried out in two series in order to test the effect of temperature during rehydration. For the first series, the rehydration medium was kept at room temperature (corresponding to the conditions described above in 'General procedure'). For the second series, the rehydration medium was pre-heated to 45 °C, and during rehydration samples were incubated at 45 °C. Both series were carried out in triplicates. After rehydration, total cell counts (see 2.2.2.1), plate counts (see 2.2.1.1), and quantification of the ATP content (see 2.2.3.1) were performed. The ratio of plate counts to total cell counts was calculated to obtain the proportion of culturable cells.

Sequential detachment. Desiccated samples of biofilms and planktonic cells of *D. geothermalis* on CME membranes were rehydrated as described above in 'General procedure' and shaken vigorously on a vortex mixer for 2 min. The bacterial suspension was recovered and the substratum was transferred to a new microcentrifuge tube containing 1 ml of 0.14 M sodium chloride solution and 300 mg of glass beads. After thorough shaking for 2 min, the supernatant was recovered and the detachment procedure was repeated a third time. Supernatants from all three detachment steps were stained with DAPI (see 2.2.2.1) and total cell count was performed using EFM. CME membranes were stained with DAPI and evaluated qualitatively by EFM.

2.2.8 Stress experiments

2.2.8.1 Long-term desiccation

Samples of biofilms and planktonic cells of *D. geothermalis* were prepared on CME membranes and desiccated as described in 2.2.7.1. Samples were stored at room temperature in the dark for a time period of at least 61 days. Temperature and relative humidity of the surrounding air were monitored using a hygrometer (Testo). At defined points in time, samples were rehydrated (see 2.2.7.2) and analysed for survival. Prior analysis, cells were rehydrated and detached from the substratum as described in 2.2.7.2. Total cell count was performed using DAPI (see 2.2.2.1). Cell viability was assessed in terms of culturability using plate counts (see 2.2.1.1), membrane integrity (see 2.2.2.2), ATP content (see 2.2.3.1), and presence of 16S rRNA (see 2.2.2.4). Freshly-prepared, i.e. non-dried samples were used as a reference.

2.2.8.2 Exposure to simulated space and Mars conditions

Experiments simulating the conditions of space and Mars were performed in the PSI laboratory at DLR Cologne (Germany). The PSI allows for mimicking extraterrestrial conditions such as vacuum, temperature, and solar radiation of spaceflight, or the surface conditions of other celestial bodies like, for instance, Mars. Two sets of exposure experiments were carried out, one analysing the effects of single extraterrestrial stressors on the viability of *D. geothermalis*, the other testing survival of the organism under a multitude of stressors to simulate exposure in space or on Mars, respectively. The experiments functioned as a preparation for the space experiment BOSS, carried out under authentic space conditions on board the ISS. All stress experiments simulating space and Martian conditions were carried out at least in duplicates. For each test, at least three untreated samples per condition were stored at DLR in the dark and used as a control.

Single-stressor exposure experiments. Samples of biofilms and planktonic cells of *D. geothermalis* were prepared on CME membranes and cellophane discs and desiccated as described in 2.2.7.1. At DLR, samples were subjected to individual stressors, including vacuum, artificial Mars atmosphere, temperature cycles, peak temperatures, and monochromatic UV radiation. For vacuum/atmosphere experiments, samples were mounted inside a duplicate EXPOSE facility which was placed in the vacuum facility of PSI. The facility was evacuated and vacuum exposure was performed as described in Tab. 2.13. After exposure, the facility was flooded with nitrogen, opened, and samples were removed. For exposure to Martian atmosphere, the sample-loaded EXPOSE facility was flooded with artificial Mars gas (see Tab. 2.13 for gas composition; provided and mixed by Praxair Deutschland GmbH) and pressurised to approximately 10^3 Pa. Temperature exposure was performed in a temperature-controlled (cryostat, Proline series, Lauda) vacuum facility flooded with argon in order to

avoid precipitation of atmospheric water on the samples during cold phase. After temperature exposure under the conditions shown in Tab. 2.13 the EXPOSE facility was allowed to equilibrate with room temperature before it was opened and samples were removed. For monochromatic UV irradiation, samples were placed within a homogeneously irradiated field under a monochromatic UV source (mercury low pressure lamp, NN 8/15, Heraeus) with a main emission wavelength of 254 nm. UV fluence rates were measured with a calibrated UV-C radiometer (Ultra Violet Products) and the spectral irradiance was determined using a double monochromator (DM 300, Bentham). The distance between lamp and samples was adjusted to ensure a radiation intensity of 80 μW cm^{-2} at sample position. Fluences are shown in Tab. 2.13. Prior analysis, cells were rehydrated and detached from the substratum as described in 2.2.7.2. Total cell count was performed using DAPI (see 2.2.2.1). Cell viability was assessed in terms of culturability, by plating on R2A and incubating for 2 days at 45 °C, and membrane integrity (see 2.2.2.2). Dried but otherwise untreated controls were used as a reference.

Table 2.13. Test conditions of single-stressor exposure experiments.

Stressor	Performed test conditions
Vacuum	$3.5 \times 10^{-2} \pm 1.2 \times 10^{-1}$ Pa, 7 days
Mars atmosphere[a]	$6.5 \times 10^{2} \pm 1.2 \times 10^{-1}$ Pa, 7 days
Temperature cycles	2 h at -10 ± 1 °C; 2 h at +45 °C 48 cycles, 8 h each
Temperature peaks	-25 ± 0.5 °C, 1 h +60 ± 0.5 °C, 1 h
Monochromatic UV irradiation (254 nm) in air at room temperature	0 J m^{-2} 10 J m^{-2} 100 J m^{-2} 1,000 J m^{-2} 10,000 J m^{-2}

[a] Gas composition of artificial Mars atmosphere: 95.5% carbon dioxide, 2.7% nitrogen, 1.6% argon, 0.13% oxygen, 0.07% carbon monoxide.

Multi-stressor exposure experiments. Samples of biofilms and planktonic cells of *D. geothermalis* were prepared on cellophane discs and desiccated as described in 2.2.7.1. At DLR, samples were subjected to the simulated conditions of either space or Mars. For each test condition and sample type, samples were arranged in stacks of five and mounted in the sample carrier of a duplicate EXPOSE facility. Samples at stack positions 2 ('top' position, superimposed by 1 sample) and 4 ('bottom' position, superimposed by 3 samples) were analysed by the author. Samples were exposed to simulated space and Martian conditions for a time

period of 28 days. During this time, space samples were stored in vacuum (3×10^{-4} Pa), whilst Mars samples were stored in an artificial Mars atmosphere with a gas composition of 95.5% carbon dioxide, 2.7% nitrogen, 1.6% argon, 0.13% oxygen, and 0.07% carbon monoxide at a pressure of 10^3 Pa. Simultaneously, samples were irradiated with polychromatic UV (200-400 nm) with a fluence of 5.5×10^5 kJ m^{-2} using a metal halide high pressure lamp (SOL 2000; Dr. Hönle AG, UV-Technologie). By using magnesium fluoride (space) or quartz (Mars) neutral density filters, transmission of the lamp was reduced to 0.1%, resulting in a UV fluence of 5.5×10^2 kJ m^{-2}. The spectral irradiance was measured at the samples' location using a double monochromator (DM 300, Bentham). Irradiation was performed discontinuously for 120 h, distributed equally over 18 weekdays. The UV dose corresponded to the expected UV dose received during a 12-months space mission. Temperature was controlled to not exceed 10 °C during irradiation. Between irradiation, samples were cooled to -25 °C. Non-irradiated samples were used as dark controls. At the end of the experiment, the sample carrier was equilibrated to room temperature and flooded with nitrogen before removing the samples. Prior analysis, cells were rehydrated and detached from the substratum as described in 2.2.7.2. Total cell count was performed using DAPI (see 2.2.2.1). Cell viability was assessed in terms of culturability by plating on R2A and incubating for 2 days at 45 °C, membrane integrity (see 2.2.2.2), ATP content (see 2.2.3.1), and presence of 16S rRNA (see 2.2.2.4). Dried but otherwise untreated samples were used as a reference.

2.2.8.3 Inactivation of *D. geothermalis*

Air-dried samples of biofilms and planktonic cells were desiccated for 1 day or 2 months prior to inactivation of the cells using either heat or isopropyl alcohol in order to produce 'dead controls' (Davey, 2011). For heat treatment, 1 ml of 0.14 M sodium chloride solution was added per sample. Samples were incubated for 1 h at 95 °C in a water bath and allowed to cool to room temperature afterwards. Following the standard rehydration procedure (see 2.2.7.2), the cells were dislodged from the support material and analysed for viability. For isopropyl alcohol treatment, the dry samples were incubated in 70% (v/v) isopropyl alcohol for 1 h. Samples were centrifuged twice for 5 min at 6,000 × g. After each centrifugation step, the supernatant was discarded and 1 ml of 0.14 M sodium chloride solution was added. Afterwards, the cells were recovered from the support material as described in 2.2.7.2. Following both inactivation procedures, the viability of the dead controls was assessed in terms of culturability, membrane integrity, ATP content, and presence of 16S rRNA and compared to the viability of non-treated samples desiccated for the same period of time.

3. RESULTS

The main goal of this study was to investigate the survival of biofilms and planktonic cells of *D. geothermalis* following exposure to desiccation and to simulated space and Mars conditions as a preparation for the ESA space mission BOSS. *D. geothermalis* was chosen as a test organism due to its high tolerance to desiccation and UV radiation (Makarova et al., 2007), both of which represent important stressors in extraterrestrial environments. The first part of the study required to establish a model biofilm which could be used for the exposure experiments carried out within the local laboratory facilities, at DLR Cologne, as well as aboard the EXPOSE-R2 sample carrier located outside the ISS. For this it was necessary to find the cultivation conditions suitable for the generation of *D. geothermalis* biofilms on a transportable support. These biofilms were then compared to planktonic cells of the same organism in terms of their EPS and their tolerance to desiccation and to various other stressors simulating aspects of space and Mars.

3.1 Cultivation of *D. geothermalis*

D. geothermalis type strain DSM 11300 was used as a test organism in this study. As described in literature, it had so far been cultivated in the laboratory with the aid of various solid and liquid nutrient media, including R2A, tryptic soy agar (TSA), solidified *Thermus* 162 medium, and R2A broth (R2B; Ferreira et al., 1997; Kolari et al., 2001; Kolari et al., 2002; Liedert et al., 2012; Peltola et al., 2008; Raulio et al., 2006; Saarimaa et al., 2006). Here, R2B was used for growing planktonic cultures of *D. geothermalis*, whereas R2A, TSA, and *Thermus* 162 medium were tested for their suitability in the generation of biofilms. The cultivation conditions were varied in order to find optimum conditions for the generation of biofilms.

3.1.1 Generation of *D. geothermalis* biofilms

For the space experiment BOSS and the preceding space and Mars simulation tests, all biological samples needed to be transportable and of uniform size for them to be introduced into

the EXPOSE sample carrier. This required some sort of mobile support that would accommodate the test organisms and could be integrated into the EXPOSE facility. Biofilms, in a general way of speaking, grow whilst attached to a substratum. Separation of the biofilm from the substratum would destroy its genuine architecture. This, however, was undesired, as the aim of the experiment was to expose biofilms in their unaffected morphology. Therefore, the substratum used for biofilms to grow on simultaneously had to function as a mobile support.

A model was developed that allowed for the production of water-unsaturated biofilms[4] of *D. geothermalis* at the air-substratum interface (Fig. 3.1). The bacteria were applied onto the surface of the substratum/support which in turn was placed on nutrient agar. Porosity of the substratum allowed for the diffusion of nutrients from the medium through the material into the biofilm colony. In the first experiments, CME membranes were chosen as a support for their easy handling, their widespread application in the cultivation of microorganisms, and their porous nature (mean pore size 0.45 µm). After incubation for 2 days at 45 °C, confluent biofilms of *D. geothermalis* were obtained.

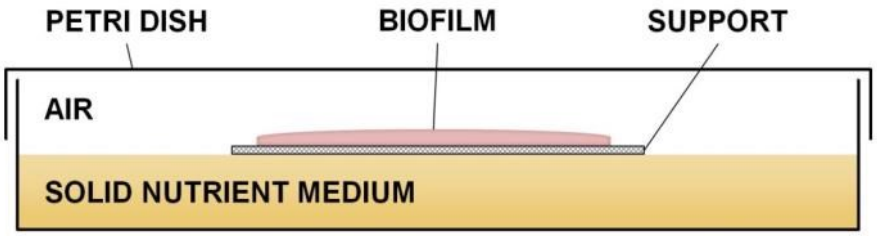

Figure 3.1. Schematic representation of a water-unsaturated biofilm growing at the interface between air and substratum.

In order to find optimum conditions for the production of biofilms on CME membranes, a preliminary experiment was carried out, evaluating the growth of *D. geothermalis* as a function of different cultivation parameters such as incubation time, incubation temperature, type of nutrient medium, and size of the inoculum. The biofilm yield was quantified in terms of wet weight per cm^2 and total cell counts per cm^2 of biofilm surface area.

Three types of nutrient agar were used: R2A, TSA, and *Thermus* 162 medium. *D. geothermalis* formed biofilms on all three media. Biofilms formed on R2A and *Thermus* 162 medium were thin and of light pink colour, and exhibited a smooth, matt surface (Fig. 3.2). When grown on TSA, *D. geothermalis* produced glossier, orange-coloured biofilms with a rougher surface. To the naked eye, these biofilms appeared to be thicker than biofilms formed on R2A or *Thermus* 162 medium. In fact, measurement of the dry weight confirmed that the biofilm yield was significantly higher on TSA compared to R2A (Fig. 3.3).

[4] Biofilms grown in humid air instead of liquids.

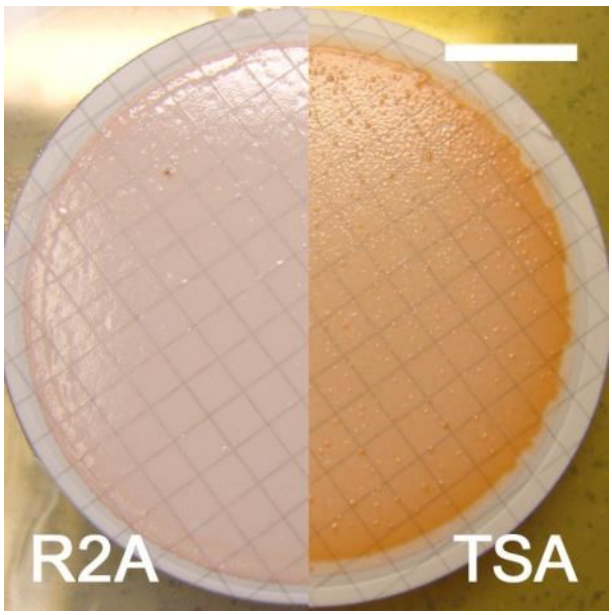

Figure 3.2. Merged photograph of biofilms of *D. geothermalis* on CME membranes grown either on R2A (left) or TSA (right) after cultivation for 2 days at 45 °C. Bar: 1 cm.

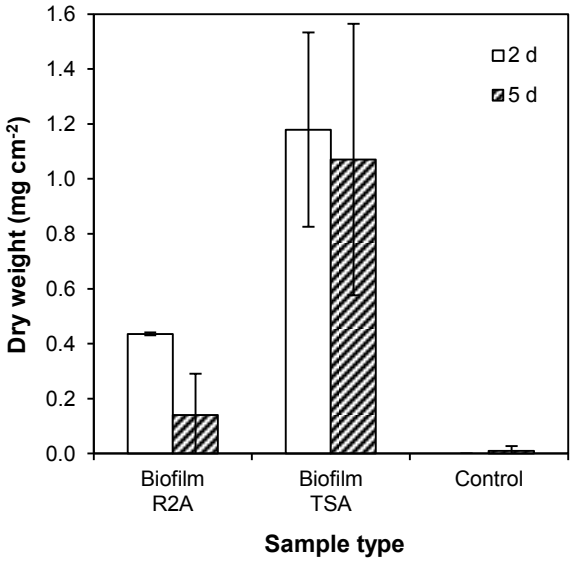

Figure 3.3. Dry weight of biofilms of *D. geothermalis* grown on CME membranes placed either on R2A or TSA. A CME membrane wetted with sterile de-ionised water and incubated as the biofilms was used as a control. Samples were weighed after storage in a desiccator for 2 and 5 days, respectively. n = 3.

All three biofilm types were found to be sticky when manipulated with an inoculation loop or pipette tips, to which they regularly stuck during routine laboratory procedures. TSA-grown biofilms additionally showed slimy properties. However, significant differences in their adhesive properties were obvious. R2A-grown biofilms exhibited strong cell-to-substrate adhesion that impeded the complete detachment of the biofilm cells from CME membranes, as shown by DAPI-staining of scraped membranes (results presented in chapter 3.2.4). Yet, once suspended in aqueous solution, they were easily dispersed by thorough mixing on a vortex mixer.

Microscopy confirmed the majority of detached cells to be present as single cells or small aggregates of 2 or 4 cells (diplococci or tetrads, respectively), typically observed for *Deinococcus* (Holt & Bergey, 1994), allowing for the quantification and further microscopic evaluation of these samples (Fig. 3.4). Similar observations were made for biofilms grown on *Thermus* 162 medium. For TSA-grown biofilms, on the other hand, cell-to-substrate adhesion was found to be marginal, thus cells were easily detached by scraping the biomass from the CME membrane with a spatula. The low mechanical stability of TSA-grown biofilms obstructed microscopic observations *in situ*: Covering the biofilm with a staining solution (e.g. DAPI, Live/Dead® dye mix) caused large parts of it to slough off. Complete dispersal of TSA-grown biofilms, however, proved to be difficult, as cell aggregation was very pronounced. Suspended in aqueous solution, TSA-grown cells remained as irregularly-shaped aggregates that were resistant to further dispersal by common laboratory practice (e.g. thorough shaking

using a vortex mixer; Fig. 3.4). Most of the cells in these aggregates were concealed by an amorphous matrix which seemed to be stained by DAPI (see chapter 3.7 for more details). Due to this, cell enumeration was impossible. Depending on their size, which ranged between 1.9 µm and 15.5 µm, aggregates were estimated to contain approximately 4-50 cells.

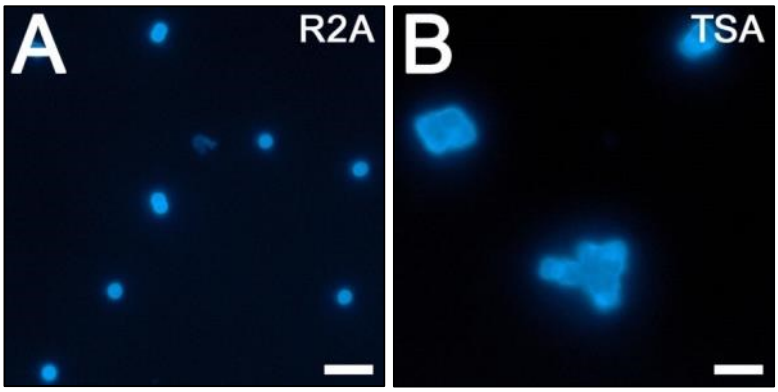

Figure 3.4. EFM micrographs of DAPI-stained biofilm cells of *D. geothermalis* dispersed in aqueous solution by thorough shaking on a vortex mixer. Biofilms were grown on R2A (**A**) and TSA (**B**), respectively. Bar: 5 µm. 1,000× magnification.

Two different incubation temperatures were chosen: (i) 45 °C, which is the growth optimum of *D. geothermalis* (Ferreira et al., 1997) and is commonly used for cultivation of this species in the laboratory (e.g. Bornot et al., 2014b; Peltola et al., 2008; Saarimaa et al., 2006), and (ii) 37 °C, which has been used by Kolari et al. (2002) to produce biofilms of *D. geothermalis* in polystyrene multi-well plates, and by Bornot et al. (2015; 2014b) to characterise the growth of *D. geothermalis* in culture. Growth of *D. geothermalis* biofilms was overall more pronounced at 45 °C compared to 37 °C (Fig. 3.5). This was most evident for biofilms cultivated on TSA. Similar wet weight and total cell count between R2A-grown biofilms incubated for 24 h and 48 h, respectively, suggested that biofilms grow and mature faster at 45 °C compared to 37 °C. Two different incubation times, 24 h and 48 h, were compared. In most cases, the biofilm yield in terms of wet weight and total cell counts increased with increasing incubation times.

The inoculum sizes tested were 10^6, 10^7, and 10^8 cells per CME membrane filter. Biofilm yield generally increased with increasing size of the inoculum. TSA-grown biofilms were strongly affected by the inoculum size. At 37 °C, the sample inoculated with 10^6 cells showed no measurable growth. The same inoculum size caused biofilms on *Thermus* 162 medium to grow more slowly than larger inocula. On R2A, *D. geothermalis* seemed to be less affected by the size of the inoculum, as all samples reached the same level of cell density after 48 h of incubation.

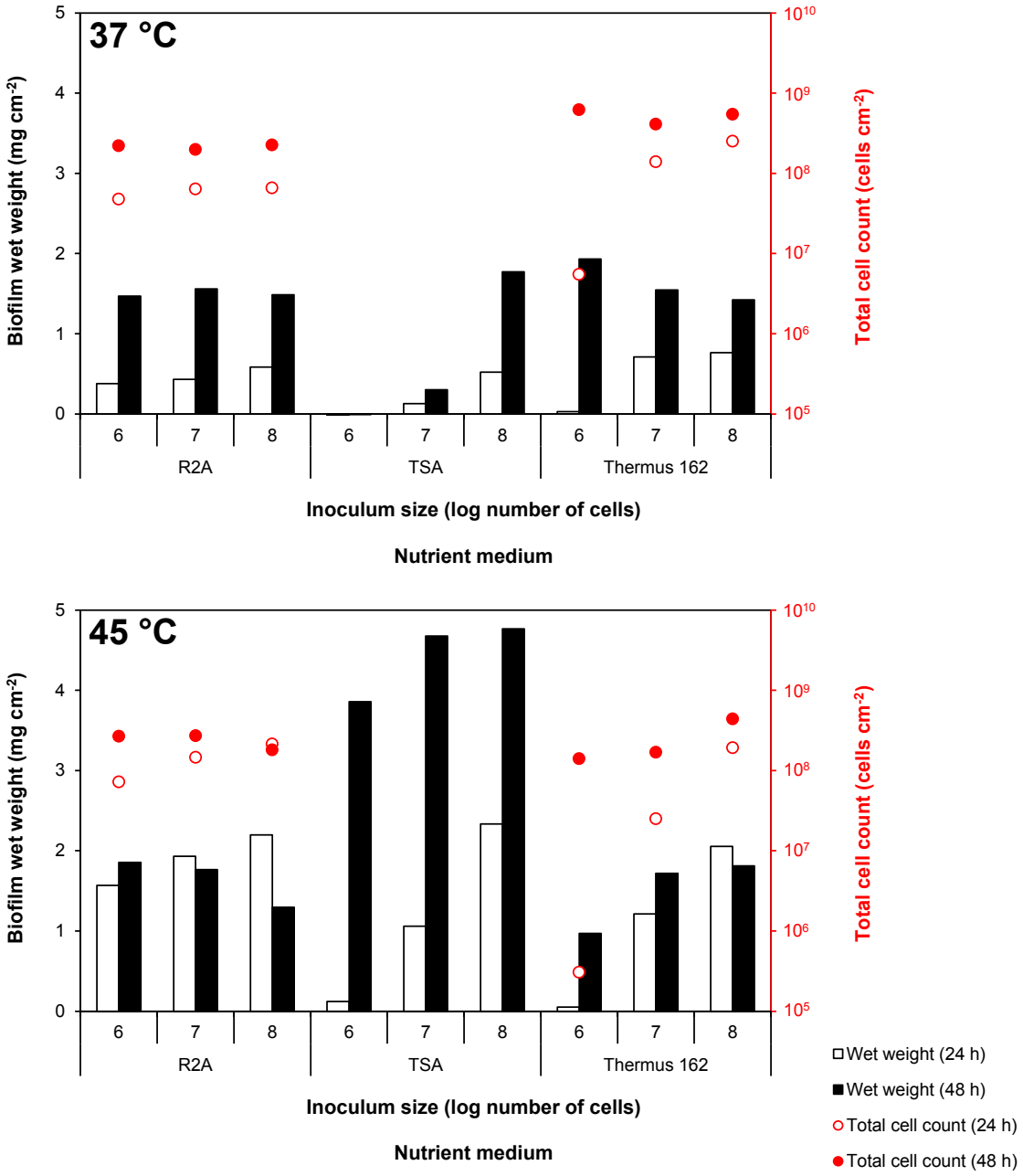

Figure 3.5. Biofilm production of *D. geothermalis* in terms of wet weight (columns) and total cell count (circles), depending on incubation temperature (37 °C, upper chart, or 45 °C, lower chart), incubation time (24 h or 48 h), size of the inoculum (10^6 to 10^8 cells per filter), and nutrient medium (R2A, TSA, *Thermus* 162 medium). Total cell count of biofilms grown on TSA could not be determined due to the cells being present in aggregates. n = 1.

In conclusion, biofilm production was highest when cultivating *D. geothermalis* on TSA for 48 h at 45 °C, with an initial inoculum size of 10^7 to 10^8 cells per CME membrane. However, the strong tendency of TSA-grown biofilms to form tenacious cell aggregates that could not be dispersed by common laboratory means impeded further analysis of these samples, rendering them useless for exposure experiments. Still, due to their prominent adhesive behaviour, TSA-grown biofilms of *D. geothermalis* remained of interest for other experiments such as EPS

studies. Biofilms grown on R2A and on *Thermus* 162 medium were comparable in terms of colour, morphology, and yield. When searching for an alternative biofilm model for exposure experiments, R2A-grown biofilms exceeded those grown on *Thermus* 162 medium, as their growth appeared to be more robust with respect to changes in incubation parameters. Based on these results, it was decided to produce biofilm samples by incubation on R2A for 48 h at 45 °C, with an initial inoculum size of 10^7 cells 13.2 cm^{-2} (corresponding to the surface area of CME membranes where biofilm growth occurred) for all further experiments, as these conditions reproducibly yielded confluent, water-unsaturated biofilms with a thickness of approximately 5-20 µm, as determined by CLSM (Fig. 3.6).

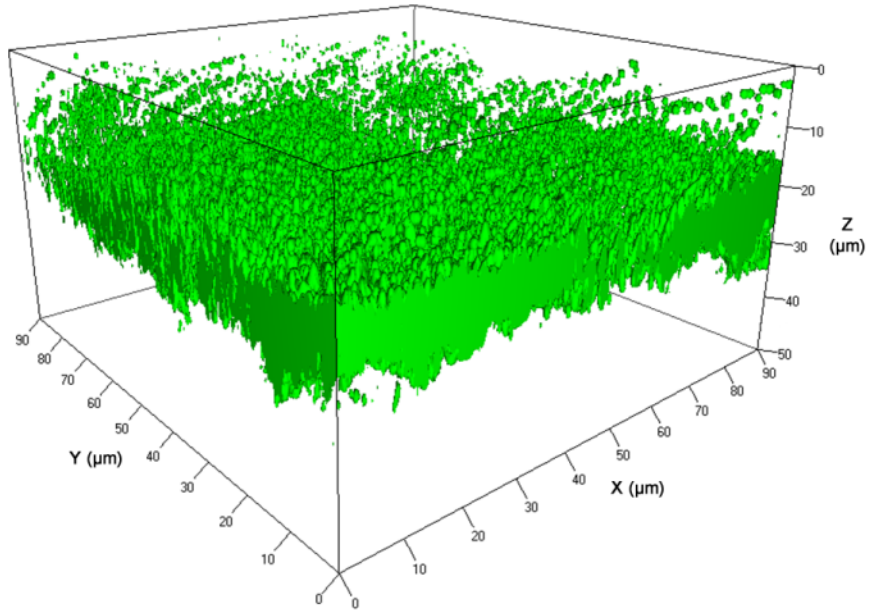

Figure 3.6. 3D model of CLSM micrographs of a Syto® 9-stained biofilm of *D. geothermalis* grown for 2 days at 45 °C on a CME membrane placed on R2A. The biofilm exhibited a thickness of 5-20 µm.

3.1.2 Planktonic growth of *D. geothermalis*

In order to characterise the planktonic growth of *D. geothermalis*, the optical density at 600 nm ($OD_{600\ nm}$) of liquid cultures in R2B medium growing under agitation at 45 °C was collected over a period of 2 days. The obtained growth curve showed a sigmoidal shape, reaching exponential growth after 4-5 h of incubation (Fig. 3.7). Stationary phase was reached after about 12 h of incubation. The total cell counts of the suspension were determined by DAPI-staining after 24 h and 48 h of incubation, respectively. After 24 h, the mean total cell count was 2.03×10^8 cells ml^{-1}, after 48 h it was 1.86×10^8 cells ml^{-1} (n = 3). With respect to the exposure experiments carried out in this study, planktonic cells were harvested, deposited on membranes, and air-dried after approximately 48 h of incubation. Thus, the majority of planktonic cells exposed would have been in the late stationary phase of growth.

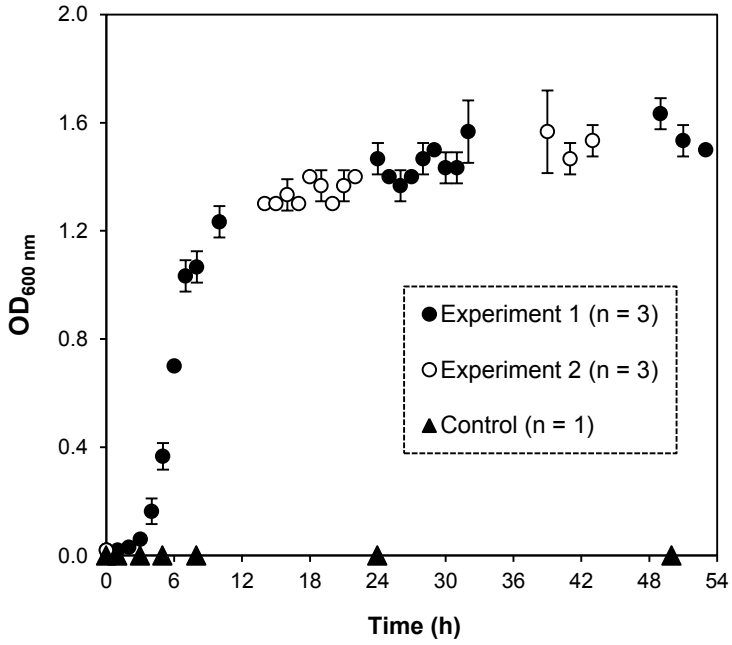

Figure 3.7. Planktonic growth of *D. geothermalis* in liquid medium (R2B) under agitation (160 rpm) at 45 °C. The experiment was carried out in two independent series (closed and open circles) in order to cover the whole time frame. Sterile R2B was used as a control.

3.2 Preparation and handling of samples

For exposure experiments, samples of biofilms and planktonic cells of *D. geothermalis* had to be prepared as transportable sample discs. These sample discs comprised a circular, 12-mm-diameter support with *D. geothermalis* grown (biofilms) or precipitated (planktonic cells) on top. After preparation, the samples were desiccated, exposed to single stressors or a combination of multiple stressors, and rehydrated to be analysed for survival.

3.2.1 Finding an alternative carrier material for UV exposure

During space simulation experiments at DLR Cologne it was discovered that CME membranes were unsuitable for the exposure to UV irradiation with fluences of ≥ 1,000 J m^{-2}, as it caused embrittlement and discolouration of the material. Therefore, various other materials (glass discs, ceramic discs, glass fibre filters, and cellophane) resistant to the UV fluences expected during exposure experiments were tested for their suitability as a substratum for biofilms. The advantage of glass fibre filters was that their large pore size allowed them to be inoculated by membrane filtration like it was done with CME membranes. This was not possible for the other materials tested, as they lacked pores (glass discs, ceramic discs) or were rapidly blocked when using membrane filtration (cellophane). Alternatively, these materials (glass discs, ceramic discs, cellophane) were placed on the surface of the nutrient medium and inoculated by pipetting small volumes of bacterial suspension on top of them. Doing so, the biofilms could be produced on all materials tested.

Yet, with respect to sample analysis, some materials revealed disadvantages. Detachment of the cells proved to be difficult and incomplete when using glass fibre filters and ceramic discs, as confirmed by macro- and microscopic observations. Both materials exhibited a rough surface which supposedly shielded the cells located in deeper regions from being detached by shaking on a vortex mixer. When using glass beads in order to facilitate shaking-mediated detachment, glass fibre filters were disrupted. This caused clogging of pipette tips what complicated analysis of these samples. The uneven surface structure of glass fibre filters and ceramic discs also impeded *in situ* microscopic observations of biofilms grown on them, as the biofilm layer was often not parallel to the focal plane of the microscope. Hence, both materials were discarded. Glass discs were excluded, since biofilm formation on this material was patchy and cell density one to two orders of magnitude lower than on the other materials.

Cellophane exhibited a flat surface which was densely colonised, indicating transport of nutrients through the small pores of the material. The pore size of cellophane was not provided by the manufacturer. Judging from ESEM observations, the pore diameter was in the range of 10-100 nm. Detachment by vigorous shaking was almost complete, as only few cells were found to remain on the surface afterwards. The average cell density of biofilms on cellophane was 2.9×10^8 cells per cm^2, very similar to the cell density of biofilms on CME membranes ($2\text{-}4 \times 10^8$ cells per cm^2). As a consequence, cellophane was chosen as a substratum for biofilms used in UV exposure experiments. CME membranes remained the substratum of choice for experiments that did not involve UV irradiation such as desiccation experiments.

3.2.2 Preparation of desiccated samples

With respect to the space experiment BOSS and the preceding stress simulation tests at DLR Cologne, samples had to be dried prior to exposure. In agreement with the project coordinator it was decided to desiccate the samples by air-drying. The rate of drying by air was compared to drying the samples in an exsiccator. The exsiccator was loaded with phosphorus pentoxide as a drying agent but not evacuated in order to limit humidity without altering the pressure regime. The drying rates of sample dried in air and of samples dried in an exsiccator were found to be similar (Fig. 3.8). When drying to constant weight, though, total water loss was higher (approximately 10 mg per sample) for samples dried in an exsiccator (Fig. 3.8).

The EXPOSE sample carrier provided circular cavities for the implementation of biological samples with a diameter of approximately 12 mm. A cork borer was used to punch out circular samples from biofilms homogeneously grown on CME membranes or cellophane sheets (Fig. 3.8).The sample discs seemed dry after over-night storage in Petri dishes with vents under ambient laboratory conditions with a mean air temperature of 20.9 °C and a rela-

tive air humidity of approximately 59% (Fig. 3.10). Drying caused the carrier material to bend slightly (Fig. 3.8).

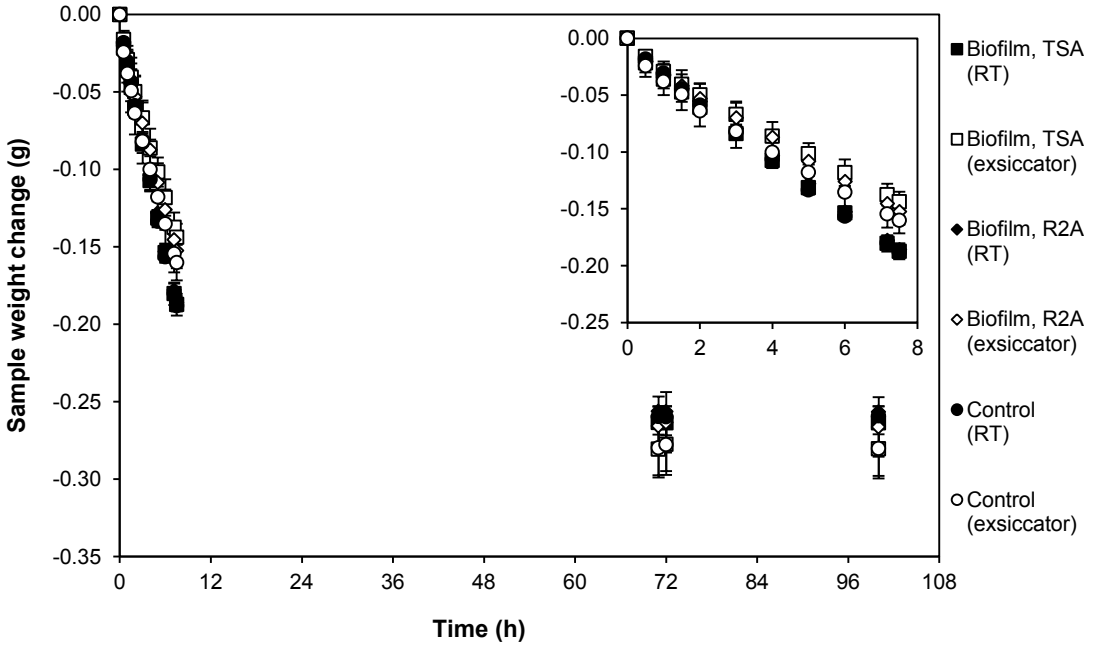

Figure 3.8. Comparison of the relative weight loss when drying biofilms of *D. geothermalis* on CME membranes by either air-drying at room temperature (RT; closed symbols) or by storage in a non-evacuated exsiccator loaded with phosphorus pentoxide (open symbols). The control samples were wet CME membranes prepared by membrane filtration with sterile de-ionised water. n = 3.

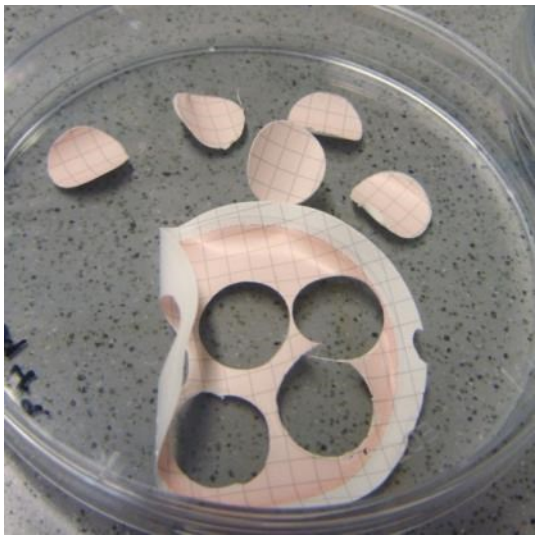

Figure 3.9. Air-dried and punched-out samples of a *D. geothermalis* biofilm on a CME membrane grown on R2A.

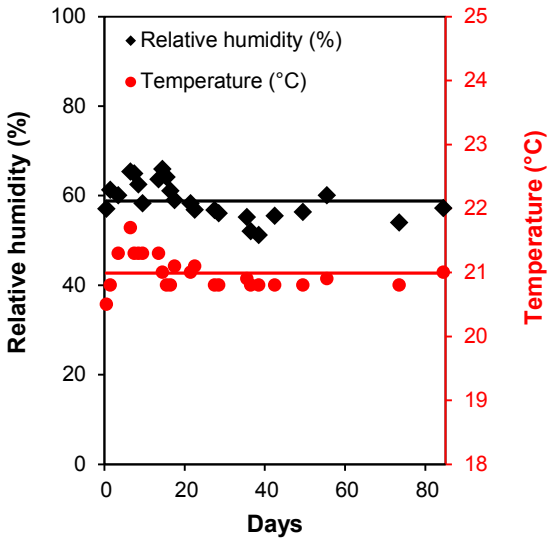

Figure 3.10. Relative humidity (black diamonds) and ambient temperature (red circles) measured over a period of 85 days within the cupboard used for storage of desiccated samples. Lines represent the respective mean values.

3.2.3 Rehydration of samples

In order to analyse the samples after exposure, the cells needed to be detached from the carrier material. For this, it was necessary to rehydrate the samples. Physiological sodium chloride solution was chosen as a rehydration agent in order to minimise water stress during the rehydration event. A preliminary experiment showed that a rehydration period of 30 min was sufficient for the samples to swell so that the cells could be scraped off. Longer rehydration periods did not result in a higher recovery of cells as measured by total cell counts (data not shown).

An experiment was carried out to determine the extent to which a 30-min rehydration period would affect cell viability. Detrimental or beneficial effects of the rehydration treatment were considered undesirable, as they would bias the assessment of cell viability after exposure experiments. In order to investigate the effect of the duration of the rehydration period, samples rehydrated for 30 min were compared to samples rehydrated for shorter or longer periods of time, respectively. To investigate the effect of temperature during rehydration, the experiment was carried out at room temperature as well as at 45 °C, which is the growth optimum of *D. geothermalis* (Ferreira et al., 1997). Viability was measured in terms of ATP concentration as a marker of metabolic activity, and in terms of colony-forming ability to identify recovery of stressed cells.

Both viability markers presented no evidence for reduced or increased viability of *D. geothermalis* after a 30-min rehydration period, compared to samples which were not allowed to rehydrate (0 h rehydration period). Prolonged periods of rehydration even seemed to deteriorate cell viability, as both the ATP concentration and the fraction of culturable cells decreased when samples were rehydrated for 24 h (Tab. 3.1). Increasing the rehydration temperature to 45 °C caused a significant decrease in culturability of samples rehydrated for 24 h. The results suggested that a rehydration of the samples for 30 min at 22 °C did not negatively affect cell viability.

3.2.4 Efficacy of detachment of cells from the carrier material

In order to analyse exposed samples, it was necessary to detach the rehydrated cells from the support material. This was usually achieved by thorough shaking on a vortex mixer, optionally in the presence of approximately 300 mg of glass beads (0.45-0.50 mm diameter) per ml of sample. Whilst the detachment of cells from cellophane was achieved almost completely by a single detachment step (see 3.2.1), the same treatment – even with the addition of glass beads – did not recover all cells attached to CME membranes.

Table 3.1. Effect of the rehydration temperature and the duration of the rehydration period on the culturability and ATP concentration of desiccated samples of *D. geothermalis*. The term 'rehydration period' refers to the time for which the samples were left within the rehydration agent before they were subjected to the detachment process (2 min of thorough shaking in the presence of glass beads). n = 3.

Rehydration period (h)	Culturable cells (%)		ATP (log mol cell^{-1})	
	22 °C	45 °C	22 °C	45 °C
0	26.4 ± 6.0	21.6 ± 5.1	-18.6 ± 0.1	-18.8 ± 0.0
0.5	27.2 ± 11.1	16.1 ± 3.5	-18.7 ± 0.1	-18.8 ± 0.1
4.5	30.6 ± 4.4	20.9 ± 13.4	-18.6 ± 0.1	-18.7 ± 0.4
24	13.8 ± 4.6	< 0.007[a]	-19.4 ± 0.2	-19.9 ± 0.3

[a] Below limit of detection.

Sequential detachment procedures (three in total) were carried out in order to determine the efficacy of the initial detachment step. Compared with the amount of cells recovered during the first detachment step, a fraction of cells of less than 1% was detached during the second step (Fig. 3.11). During a third detachment step, a fraction of cells corresponding to 0.2% of the initially detached cells was obtained. Since the amount of cells recovered during the second and third detachment procedure was negligibly small, only one detachment step was employed in further experiments.

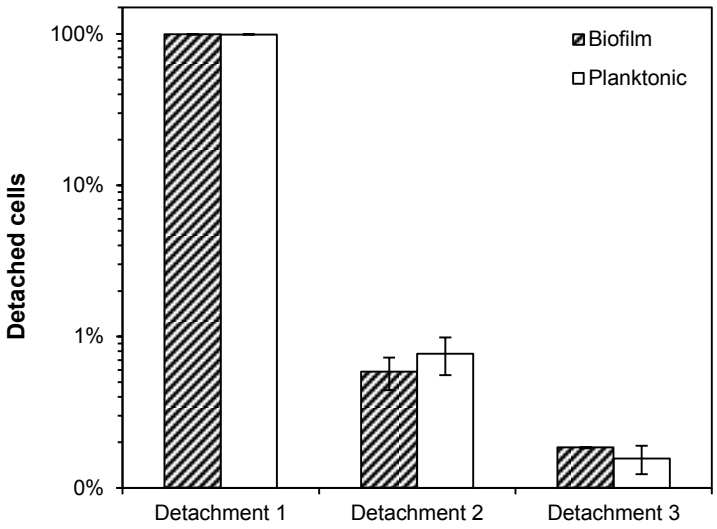

Figure 3.11. Efficacy of the procedure used for detachment of rehydrated biofilms and planktonic cells of *D. geothermalis* from CME membranes by thorough mixing in the presence of glass beads. Of all cells detached during three sequential detachment procedures (= 100%), over 99% were recovered during the first detachment step. n = 3.

Microscopic observation of DAPI-stained CME membranes showed that even after three detachment steps, cell recovery was still incomplete (Fig. 3.12). The fraction of cells not to be recovered from the CME membrane could not be determined quantitatively, but was estimated to be low compared with the initial total cell number. Microscopy showed that already after one detachment step most of the cells had been detached, as multilayers of cells were not observed and a large proportion of the surface of the membrane was void of cells. For biofilms, small patches of more densely packed cells were observed occasionally. The remaining cells which could not be detached as well as the cells in the planktonic samples were found to be scattered randomly over the membrane (e.g. Fig. 3.12 D). It was concluded that the proportion of cells detached after a one-step treatment could be regarded as representative for the sample as a whole.

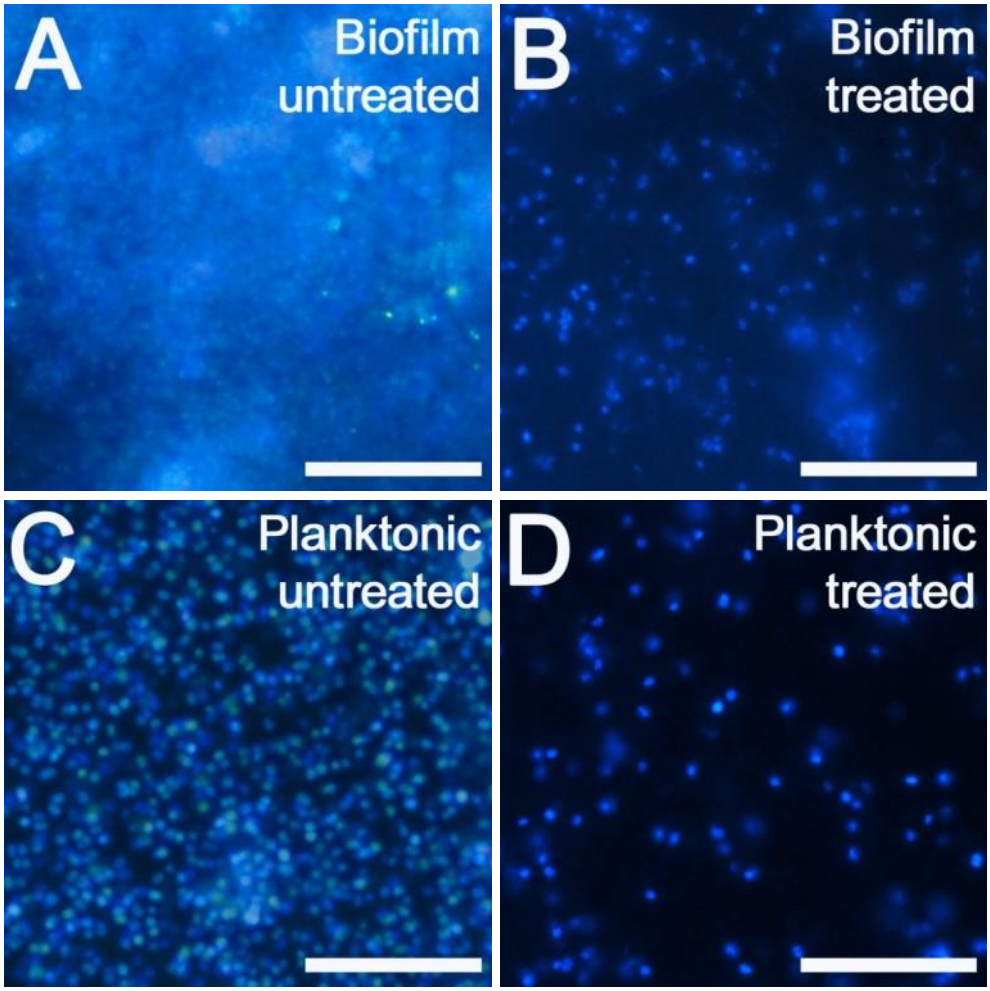

Figure 3.12. DAPI-stained samples of biofilms (**A**, **B**) and planktonic cells (**C**, **D**) of *D. geothermalis* on CME membranes before (left column) and after (right column) three sequential detachment treatments. Bar: 20 µm. 1,000× magnification.

3.3 Visualisation of the biofilm morphology by ESEM

Depending on the nutrient medium provided, biofilms formed by *D. geothermalis* differed in morphology and adhesion properties (see 3.1.1). ESEM was used in order to find ultrastructural evidence for this phenomenon, and to illustrate morphological differences on a microscopic level. Planktonic cells deposited on a support were for comparison.

In all cases, biofilms appeared as tightly packed aggregates of cells (Fig. 3.13). The surface of R2A-grown biofilms appeared smoother than the surface of TSA-grown biofilms which exhibited a rough, relief-like configuration (Fig. 3.13 D). This may be influenced by the type of cell aggregates formed on the respective nutrient medium: In R2A-grown biofilms, cells were mainly present as single cells or diplococci, whereas tetrads or larger conglomerations of cells dominated in TSA-grown biofilm. Planktonic cells appeared to be similar to R2A-grown cells, as they occurred primarily as single cells or diplococci. The thread-like cell appendages reportedly occurring in sessile cells of *D. geothermalis* (Peltola et al., 2008; Raulio et al., 2006; Saarimaa et al., 2006) were not detected in any of the samples.

The presence of extracellular material, presumably representing EPS, was evident for all biofilm types observed. For R2A-grown biofilms, the EPS layer was highly amorphous and seemed to superimpose the uppermost layer of cells, covering them like a veil (Fig. 3.13 A, C, E). At the border of the biofilm, the EPS layer extended for approximately 5-10 µm into the CME membrane material were cells seemed to be absent (Fig. 3.13 E). TSA-grown biofilms seemed to produce more EPS compared to R2A-grown biofilms. When cellophane was used as a support, the top layer of TSA-grown biofilms was covered in an amorphous EPS matrix (Fig. 3.13 B) that appeared to be much thicker than the surficial EPS layer of R2A-grown biofilms. However, when using CME membranes as a support the cells were still surrounded by a relatively large amount of EPS, but the matrix seemed gritty rather than slimy, forming small clumps adhering to the cells (Fig. 3.13 D). Veil-like patches that could represent EPS were also found in samples of planktonic cells. However, these patches were distributed heterogeneously and did not stretch over the sample surface, leaving the topmost layer of cells largely exposed (Fig. 3.13 F).

To summarise, judging from ESEM observations, an amorphous matrix presumably representing EPS was present in all sample types investigated. The visible quantity of EPS differed with the sample type, with TSA-grown biofilms showing the highest and planktonic cells showing the least amount of EPS. TSA nutrient medium seemed to promote the aggregation of cells to tetrads or bigger aggregates, whilst single cells or diplococci dominated in the other sample types. Furthermore, the carrier material used seemed to influence the surface structure of the biofilm, as biofilms grown on CME membranes exhibited an increased roughness compared to those grown on cellophane.

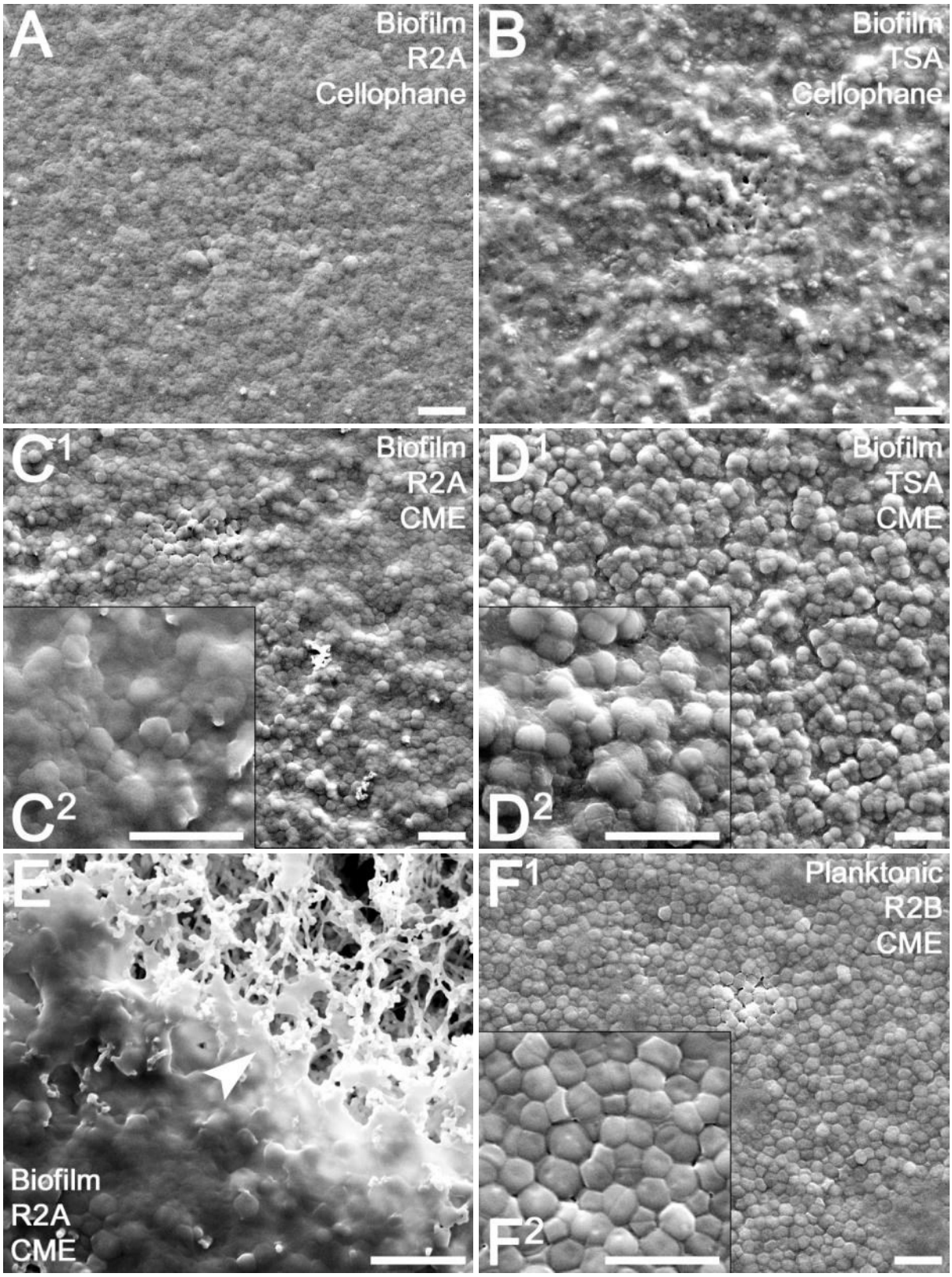

Figure 3.13. ESEM micrographs of air-dried samples of biofilms (**A**-**E**) and planktonic cells (**F1**, **F2**) of *D. geothermalis.* Biofilms were grown either on R2A or on TSA, using cellophane or CME membranes as a substratum. **E** shows the border of the biofilm, at which the presence of EPS extending into the membrane material becomes apparent (arrow). Bar: 5 µm.

3.4 EPS isolation and quantification

In order to find evidence for the role of EPS in the extreme stress tolerance of *D. geothermalis*, the EPS of biofilms and planktonic cells were isolated and analysed. Five different EPS extraction methods were compared in terms of EPS yield and their impact on cell integrity. The EPS were then quantified according to their biochemical composition in terms of proteins, polysaccharides, eDNA, and uronic acids.

3.4.1 Comparison of EPS isolation methods

Various methods exist that permit the extraction of EPS from bacterial cultures or biofilms. These methods may differ in their extraction efficacy depending on the type of EPS present in the sample, and in their impact on cell integrity (Wingender et al., 1999). Five EPS isolation methods (heat, shaking, CER, formaldehyde/sodium hydroxide, EDTA; for details see Tab. 2.11) were chosen and tested for their suitability in extracting EPS from *D. geothermalis* biofilms grown on R2A or TSA, using CME membranes as a support. The amount of EPS yielded by the individual methods was compared and their possibly detrimental effect on the cells investigated.

Cell lysis during EPS isolation may lead to the contamination of the isolated EPS with intracellular material and consequently to an overestimation of the true EPS quantity (McSwain et al., 2005). In order to detect possible detrimental effects of the five different EPS isolation methods on biofilms of *D. geothermalis*, an experiment was carried out to compare both total cell counts (cells per cm^2 of biofilm surface) and plate counts (CFU per cm^2 of biofilm surface) before and after each EPS isolation procedure.

Prior to EPS isolation, total cell counts were in the range of 2.1-3.5 × 10^8 cells cm^{-2}. Plate counts were approximately two orders of magnitude lower and in the range of 2.7-4.2 × 10^6 CFU cm^{-2}. EPS isolation by heat and by using formaldehyde/sodium hydroxide caused the plate counts to decrease by more than five orders of magnitude to values below the limit of detection (75.8 CFU cm^{-2}; Fig. 3.14), presumably indicating cell lysis. Due to this, both isolation methods were excluded from further investigations. The remaining EPS isolation procedures did not significantly affect the culturability of *D. geothermalis*. EPS isolation by heat and by using EDTA caused a slight reduction (40.0% and 31.7%, respectively) in total cell counts, what might indicate loss of cells due to cell lysis.

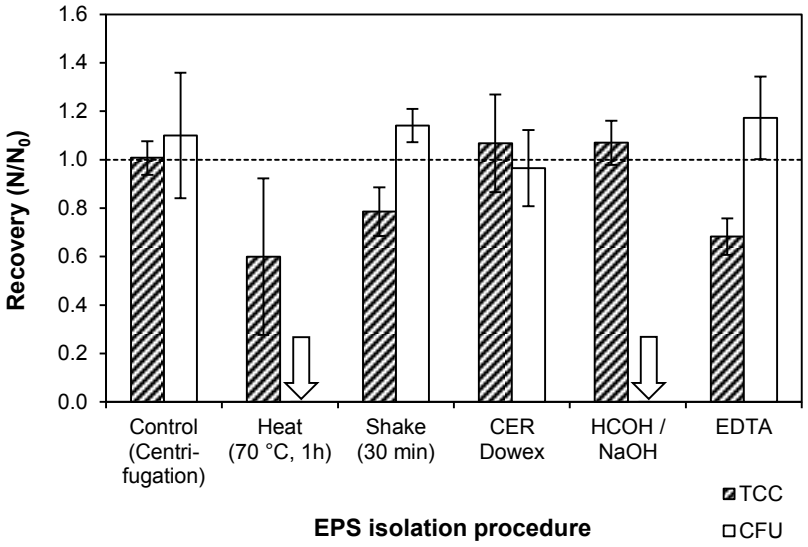

Figure 3.14. The effect of five different EPS isolation techniques on R2A-grown biofilms of *D. geothermalis*, measured in terms of total cell counts (shaded bars) and plate counts (light bars) before (N_0) and after (N) treatment. Centrifuged samples were used as a control. Arrows indicate plate counts below the limit of detection (75.8 CFU cm^{-2}). n = 3 (total cell counts), 2 (plate counts).

All five methods resulted in the extraction of proteins, polysaccharides, and eDNA from both R2A- and TSA-grown biofilms (Fig. 3.15). However, the total EPS yield as well as the ratio of the yield between R2A- and TSA-grown biofilms differed with the isolation method applied: The highest protein yield of 66.7 µg cm^{-2} and 113.7 µg cm^{-2} (values relating to the biofilm surface area) for R2A- and TSA-grown biofilms, respectively, was obtained by using EDTA. The other extraction methods yielded proteins with concentrations in the range of 0.7-3.0 µg cm^{-2} (R2A) and 31.8-58.9 µg cm^{-2} (TSA). For polysaccharides, isolation by heat and by formaldehyde/sodium hydroxide was most efficient, as yield was significantly higher (10.5-12.8 µg cm^{-2} for R2A-grown biofilms; 24.3-27.8 µg cm^{-2} for TSA-grown biofilms) compared to the other methods (3.7-5.3 µg cm^{-2} for R2A-grown biofilms; 10.7-11.3 µg cm^{-2} for TSA-grown biofilms). The highest amount of eDNA isolated from R2A-grown biofilms was obtained by using formaldehyde/sodium hydroxide yielding 0.46 µg cm^{-2}. For isolating eDNA from TSA-grown biofilms, CER Dowex® seemed to be most effective with a yield of 1.6 µg cm^{-2}. Uronic acids could only be detected in the EPS isolated from TSA-grown biofilms by using formaldehyde/sodium hydroxide, but their concentrations were significantly lower than the limit of quantification (25.4 µg cm^{-2}). In every case, the EPS yield of TSA-grown biofilms was higher than the EPS yield of R2A-grown biofilms: Depending on the isolation method applied, the yield of TSA-grown biofilms was elevated by a factor of 1.7-46.0 for polysaccharides, 2.0-3.1 for proteins, and 2.5-32.0 for eDNA. Although it did not yield the highest amounts of EPS

compared to the other methods, the CER Dowex® EPS isolation method was chosen for all further EPS isolation approaches since it did not seem to cause extensive cell lysis.

In order to study the effect of EPS isolation by CER Dowex® on planktonic cells of *D. geothermalis*, liquid cultures were subjected to CER treatment. The procedure caused an insignificant reduction of the mean total cell count and plate count from 2.9×10^8 cells ml^{-1} to 2.2×10^8 cells ml^{-1} and from 3.6×10^7 CFU ml^{-1} to 3.1×10^7 CFU ml^{-1}, respectively, further demonstrating the mildness of this method.

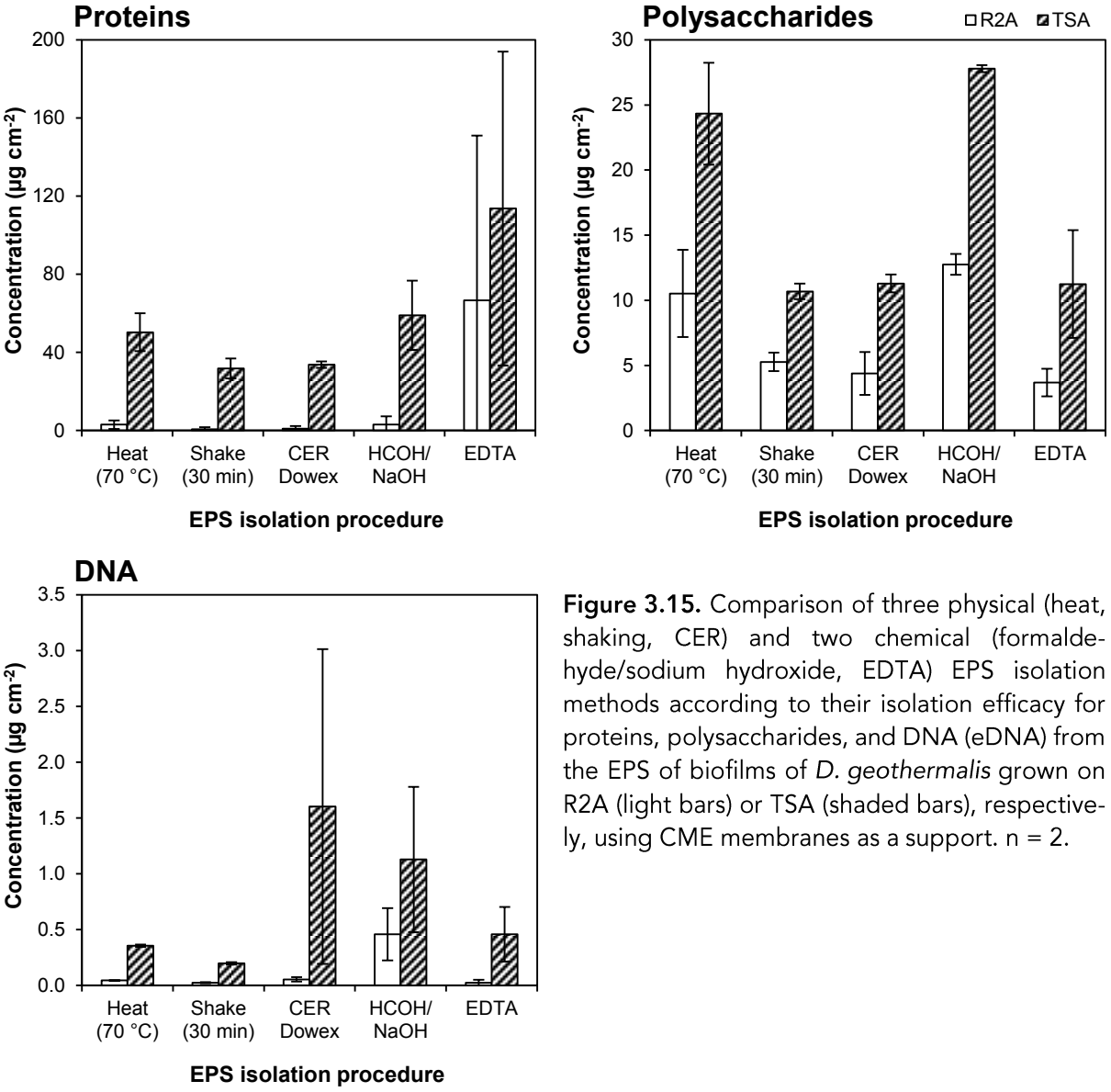

Figure 3.15. Comparison of three physical (heat, shaking, CER) and two chemical (formaldehyde/sodium hydroxide, EDTA) EPS isolation methods according to their isolation efficacy for proteins, polysaccharides, and DNA (eDNA) from the EPS of biofilms of *D. geothermalis* grown on R2A (light bars) or TSA (shaded bars), respectively, using CME membranes as a support. n = 2.

3.4.2 Biochemical composition of *D. geothermalis* biofilms

In order to relate the amount of EPS isolated by CER Dowex® to their total concentration in the biofilm, untreated suspensions of biofilms grown on R2A or TSA were quantified in terms of total proteins, total polysaccharides, total uronic acids, and total DNA.

Also here, TSA-grown biofilms showed higher concentrations of the sum parameters measured compared with R2A-grown biofilms (Tab. 3.2). In both biofilm types, proteins were by far the dominant fraction with a concentration of 84.0 µg cm^{-2} and 564.3 µg cm^{-2} for R2A and TSA, respectively. The second largest fraction measured were polysaccharides with a concentration of 32.3 µg cm^{-2} for R2A-grown biofilms and 81.9 µg cm^{-2} for TSA-grown biofilms. DNA was found to be present at concentrations of 1.0 µg cm^{-2} for R2A and 9.1 µg cm^{-2} for TSA.

Table 3.2. Total concentrations of proteins, polysaccharides, DNA, and uronic acids of biofilms (grown either on R2A or TSA) and of the EPS isolated from them using CER Dowex®.

Parameter (µg cm^{-2})	R2A		TSA	
	Biofilm	EPS	Biofilm	EPS
Proteins	84.0 ± 14.8	3.3 ± 0.6	564.3 ± 25.7	37.6 ± 5.1
Polysaccharides	32.3 ± 1.2	4.5 ± 1.2	81.9 ± 11.6	9.5 ± 1.7
DNA/eDNA	1.0 ± 0.4	0.3 ± 0.1	9.1 ± 2.9	0.8 ± 1.0
Uronic acids	0.2 ± 0.2[a]	0.1 ± 0.2[a]	9.6 ± 9.2[a]	0.1 ± 0.1[a]

[a] Below limit of quantification (50 µg ml^{-1} D-glucuronic acid).

Uronic acid could be detected in the biofilm suspensions, but their concentrations were below the limit of detection of 50 µg ml^{-1}. When processed for uronic acid determination, a brownish discolouration of the biofilm suspensions was observed. As suggested by Filisetti-Cozzi and Carpita (1991), the spectra of the samples between 400 nm and 650 nm were recorded (Fig. 3.16). In both cases (R2A- and TSA-grown biofilms), no characteristic absorbance maximum of the reaction product of the assay was detected at a wavelength of 525 nm, which was used for uronic acid quantification. Also, the absorbance of the biofilm samples at 525 nm was lower than the absorbance of the lowest standard used for calibration of the assay (50 µg ml^{-1} D-glucuronic acid). These findings suggested that *D. geothermalis* biofilms – as generated in this study – contain little to no uronic acids.

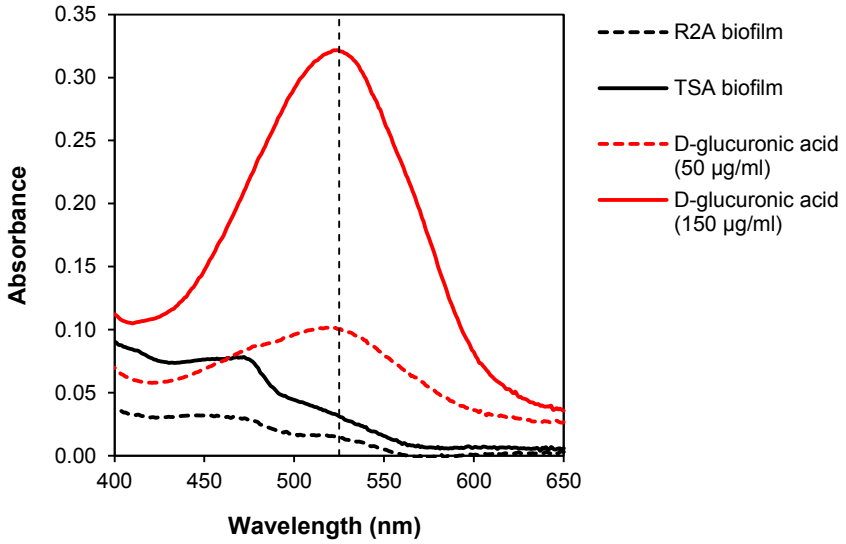

Figure 3.16. Absorbance spectra of R2A-grown (dashed black line) and TSA-grown (black line) biofilms of *D. geothermalis* treated for uronic acid quantification according to Filisetti-Cozzi & Carpita (1991). Standard solutions of D-glucuronic acid (50 µg ml^{-1} and 150 µg ml^{-1}) were treated in the same way and used to obtain reference absorbance spectra (red lines). The vertical dashed line represents the wavelength of 525 nm at which absorbance was read for uronic acid quantification.

3.4.3 EPS composition of biofilms and planktonic cells of *D. geothermalis*

In all exposure experiments conducted, R2A-grown biofilms and R2B-grown planktonic cells of *D. geothermalis* were used as samples, using either CME membranes or cellophane discs as a support. In order to characterise and quantify the EPS in these samples, the EPS of membrane-associated biofilms and planktonic cells were isolated using CER Dowex® and quantified in terms of proteins, polysaccharides, eDNA, and uronic acids. To investigate the effect of the support used for generating unsaturated biofilms, the EPS of biofilms grown on CME membranes and cellophane, respectively, were analysed.

Concentrations of EPS were slightly higher in samples of planktonic cells compared to biofilm samples (Fig. 3.17). Proteins were found to be the dominant fraction of EPS in planktonic cells (22.6 fg $cell^{-1}$), but significantly less in biofilm cells (9.7-11.7 fg $cell^{-1}$; depending on the support material used). Similar amounts of polysaccharides were extracted from biofilms and planktonic cultures, their concentrations being in the range of 12.7-15.3 fg $cell^{-1}$. The protein/polysaccharide ratio of biofilms and of planktonic cells was 0.7-0.9 and 1.5, respectively. The concentration of eDNA in the EPS of planktonic cells was higher (2.5 fg $cell^{-1}$) than in the EPS of biofilms (0.5-0.8 fg $cell^{-1}$). If detected, concentrations of uronic acids were significantly below the limit of quantification (corresponding to the concentration of the lowest standard used; 50 µg ml^{-1} D-glucuronic acid). In general, the measured concentration values of EPS

never exceeded those of whole biofilms (see 3.4.2) and, thus, seemed plausible. The type of carrier material used as a support for the generation of biofilms (CME membranes, cellophane discs) did not have a significant effect on the gross composition of the EPS.

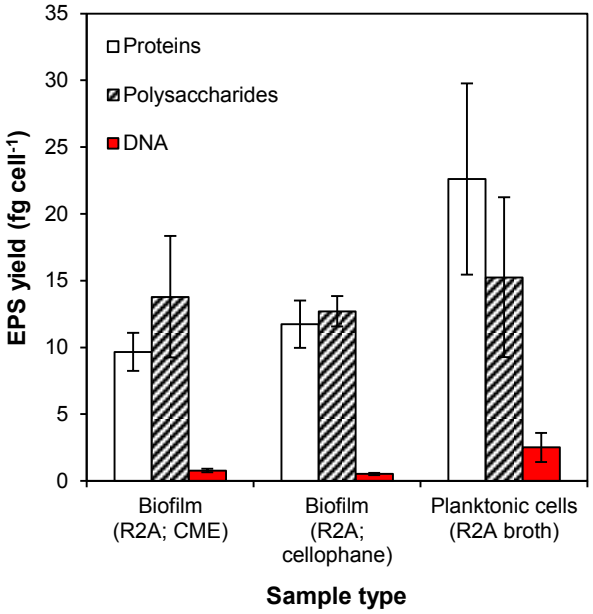

Figure 3.17. Quantitative comparison of the EPS isolated from R2A-grown biofilms (n = 5, 3) and planktonic cells (n = 5) of *D. geothermalis* using CER Dowex®.

3.4.4 Comparison of the EPS of R2A- and TSA-grown biofilms

Growing biofilms of *D. geothermalis* on TSA caused the cells to aggregate in small flocs of approximately 4-50 cells which came apparent when the biofilm was suspended in aqueous solution (see 3.1.1). These aggregates were highly resistant to dispersion, hampering further analysis. In order to understand the contribution of the EPS to this intercellular adhesion, the EPS of TSA-grown biofilms were isolated using CER Dowex®, and quantitatively compared in terms of with the EPS isolated from R2A-grown biofilms which consistently did not exhibit cellular aggregates. Since total cell counts of cell suspensions of TSA-grown biofilms were impossible due to the cells being present in aggregates, the two biofilm types were compared in terms of EPS yield per surface area of biofilm.

Of all parameters tested, proteins were found to be by far the most dominant fraction in the EPS of TSA-grown biofilms (37.6 µg cm^{-2}) and exceeded the protein concentration per cm^2 of biofilm area of R2A-grown biofilms by more than 10-fold (Fig. 3.18). The concentration of polysaccharides in the EPS of TSA-grown biofilms was found to be 2.6 times higher as in R2A-grown biofilms. Thus, the EPS of TSA-grown biofilms exhibited a protein/polysaccharide ratio significantly higher (3.9) compared with the EPS of R2A-grown biofilms (0.7). eDNA was detected in the EPS of TSA-grown biofilms in concentrations similar to R2A-grown biofilms (0.8 µg cm^{-2} for TSA; 0.3 µg cm^{-2} for R2A). Concentrations of uronic acids

were always below the limit of quantification (corresponding to the concentration of the lowest standard used; 50 µg ml^{-1} D-glucuronic acid). For both R2A-grown and TSA-grown biofilms, concentrations of EPS were always lower as those of untreated whole biofilms (Tab. 3.2).

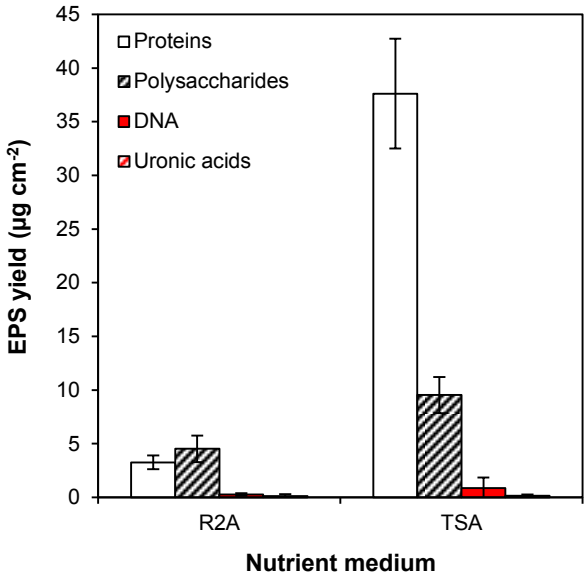

Figure 3.18. Quantitative comparison of the EPS isolated from biofilms of *D. geothermalis* grown on R2A or TSA, respectively. CME membranes were used as a carrier material. n = 5.

In conclusion, EPS analysis showed that TSA-grown biofilms produced significantly more EPS – especially extracellular proteins – than R2A-grown biofilms. An increased amount of EPS could be a reason for the strong intercellular adhesion observed in suspensions of TSA-grown biofilms.

3.4.5 EPS production of planktonic cells

Compared to biofilms, planktonic cells of *D. geothermalis* seemed to produce significant amounts of EPS (Fig. 3.17). An experiment was carried out in order to investigate, whether this could be an indication for the contamination of the isolated EPS by constituents of the liquid nutrient medium used for cultivation (R2B). R2B contains several compounds of interest (i.e. compounds with a molecular mass ≥ 3.5 kDa that are not removed by dialysis), including starch, peptone, and yeast extract. Cultures of planktonic cells were subjected to EPS isolation using CER Dowex®, and proteins, polysaccharides, and eDNA were quantified after each step involved in the isolation procedure.

The presence of proteins and polysaccharides in the nutrient medium was clearly demonstrated, as they could be detected in sterile, dialysed R2B (Fig. 3.19). When R2B was inoculated with *D. geothermalis* and incubated for 2 days at 45 °C, the amount of proteins and polysaccharides in the medium decreased from 221.8 µg ml^{-1} to 168.9 µg ml^{-1} and from

442.3 µg ml^{-1} to 100.1 µg ml^{-1}, respectively, indicating utilisation of the nutrient medium constituents by the cells to an extent unknown. The cell fraction of the bacterial culture contained 129.7 µg ml^{-1} of proteins, 29.9 µg ml^{-1} of polysaccharides, and 1.7 µg ml^{-1} of eDNA. These values decreased to 101.6 µg ml^{-1}, 21.4 µg ml^{-1}, and 1.5 µg ml^{-1} after CER Dowex® treatment of the cells, indicating an extraction of these biopolymers from the cells. In the EPS-containing supernatant of CER-treated cell suspensions, 8.2 µg ml^{-1} of proteins, 5.1 µg ml^{-1} of polysaccharides, and 0.8 µg ml^{-1} of eDNA were recovered, suggesting a loss of proteins and polysaccharides and an increase in eDNA due to the EPS isolation procedure.

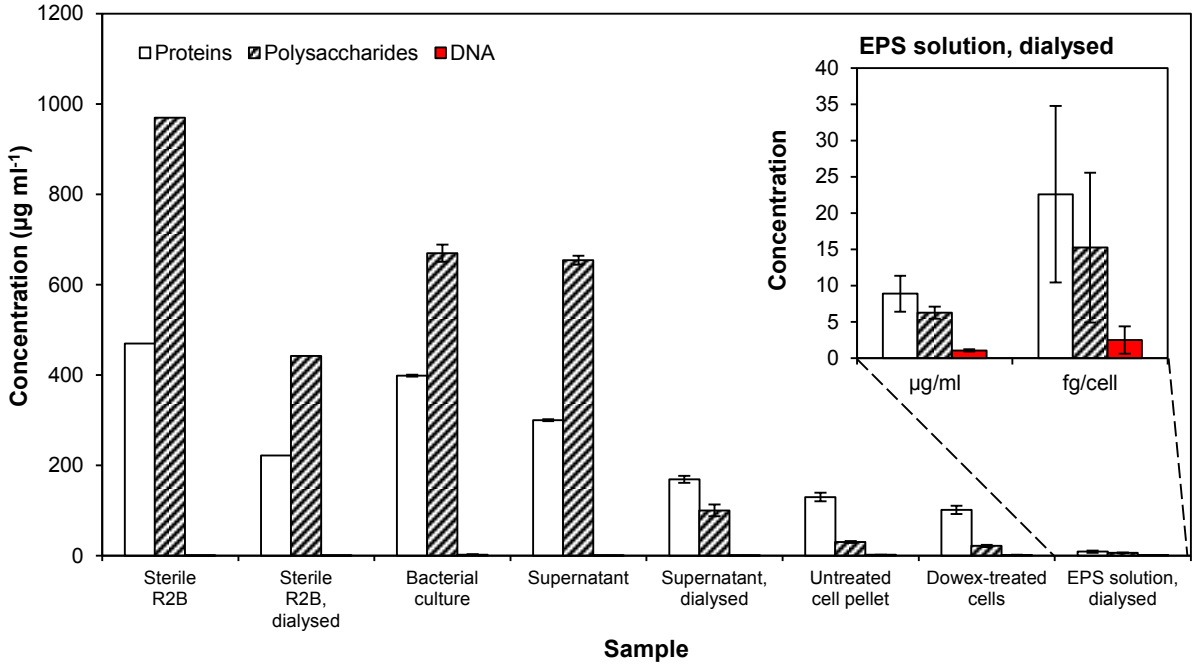

Figure 3.19. Quantification of proteins, polysaccharides, and eDNA in a liquid culture of *D. geothermalis* grown in R2B during all stages of EPS isolation (n = 3; n = 5 for dialysed EPS solution). Sterile R2B, both dialysed and non-dialysed, was used as a control (n = 1).

In order to investigate the extent to which *D. geothermalis* utilises the polysaccharides provided in the liquid nutrient medium R2B (starch or constituents of yeast extract), the growth of *D. geothermalis* in R2B deprived of glucose as a simple carbon source was compared to growth in standard, glucose-containing (0.50 g l^{-1}) R2B. The concentration of reducing sugars in the medium was monitored in order to detect products of the degradation of polysaccharides. *D. geothermalis* was found to grow in glucose-deficient R2B, although growth was less pronounced compared to growth in the presence of glucose (Fig. 3.20). In both cases, *D. geothermalis* reached stationary growth after about 14-20 h of incubation. In glucose-deficient cultures, the concentration of reducing sugars increased within 24 h from 36.4 µg ml^{-1} to 176.0 µg ml^{-1}, indicating the degradation of starch (initial concentration 500 µg ml^{-1}) or other polysaccharides (initial concentration unknown). The method did not

allow for estimating whether the starch had been utilised completely within 48 h. When cultivating *D. geothermalis* in R2B containing 500 µg ml^{-1} of glucose, the reducing sugar concentration dropped from 543 µg ml^{-1} to 303 µg ml^{-1} within 48 h, but was not consumed completely. A plateau phase was observed between 11 h and 24 h of incubation, suggesting the degradation of starch in the presence of freely available glucose.

To conclude, *D. geothermalis* was able to grow in liquid culture using starch or yeast extract as a carbon source when glucose was absent. Degradation of starch and yeast extract within 48 h of incubation might have been incomplete. Thus, these polymeric compounds might have contaminated the EPS extracted from planktonic cultures as they are not removed during dialysis. Contamination of the EPS by nutrient medium constituents would lead to overestimated values when quantifying EPS.

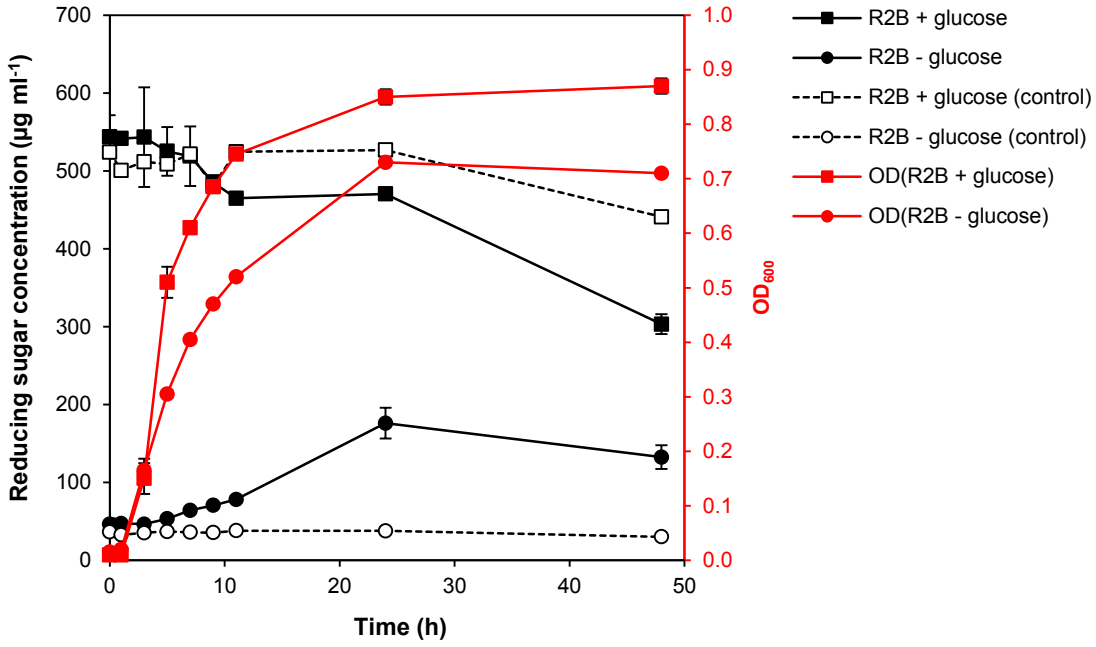

Figure 3.20. Growth of liquid cultures of *D. geothermalis* at 45 °C in R2B with (squares) or without (circles) supplementation of the medium with D-glucose (0.50 g l^{-1}). The concentrations of reducing sugars (µg ml^{-1}) in the medium are indicated on the left y-axis (black symbols). Sterile medium was used as controls (open symbols). The optical density (600 nm) of the cultures was used to measure growth, as indicated on the right y-axis (red symbols). n = 2; 1 for controls.

3.4.6 Summary

Out of five different methods tested, CER Dowex® was chosen for the isolation of the EPS from biofilms and planktonic cells of *D. geothermalis*. Compared to the other method, EPS isolation by CER yielded moderate amounts of EPS, whilst damage to cells was low. With respect to all growth conditions tested, *D. geothermalis* produced EPS containing proteins, polysaccharides, and eDNA. The concentrations of uronic acids in the EPS were minute and

always below the limit of quantification. Depending on the mode of growth and the nutrient medium provided, the amount and composition of EPS differed significantly. TSA-grown cells produced by far the highest amount of EPS per cm^2 of biofilm surface in terms of proteins and polysaccharides, whilst R2A-grown biofilms produced less EPS, with concentrations similar to planktonic cells. Proteins were found to be the dominant fraction in TSA-grown biofilms and planktonic cells, whereas polysaccharides dominated in the EPS isolated from R2A-grown biofilms.

3.5 Characterisation of extracellular polysaccharides by thin-layer chromatography

Thin-layer chromatography (TLC) was used in order to investigate the composition of the polysaccharide fraction of the EPS of *D. geothermalis* biofilms. The EPS of biofilms grown on R2A or TSA were isolated using CER Dowex® and concentrated by vacuum centrifugation. Acid hydrolysis (0.1 M hydrochloric acid, 100 °C, 24 h or 48 h) was used to release monosaccharides from the polysaccharides. Optionally, the hydrolysate was treated with acetone in order to precipitate any high molecular weight contaminants (e.g. proteins, lipids). The supernatant was used for TLC. Various neutral monosaccharides were used as references.

Vacuum centrifugation yielded dry pellets of EPS. EPS pellets of R2A-grown biofilms were of light yellowish colour and were easily solubilised when adding 0.1 M hydrochloric acid. EPS pellets of TSA-grown biofilms were of brownish colour and could not be solubilised in 0.1 M hydrochloric acid, even with the aid of thorough mixing. After hydrolysis, however, all samples were clear and colourless. When dried by vacuum centrifugation, the hydrolysed EPS of R2A-grown cells appeared as white crystals. The hydrochloric acid-treated EPS of TSA-grown cells appeared as an amorphous pellet of brownish colour. Acetone treatment caused precipitation of the hydrolysates: For EPS extracted from R2A-grown cells, the precipitate was white and of minute quantity. For TSA-grown cells, the amount of the formed precipitate was more substantial, white to yellow of colour, and of cotton-like appearance.

Following TLC and staining of the TLC plate with N-(1-naphthyl)ethylenediamine, each of the EPS preparations produced a single, irregularly shaped spot of pink colour (Fig. 3.21). The pink colouration of the spots was more intense for EPS preparations made from TSA-grown biofilms compared to those made from R2A-grown biofilms. Regarding their travelled distance, all spots were found to be in the same range with R_f values of 0.31-0.35 (Fig. 3.21). No significant differences were observed between samples hydrolysed for 24 h and 48 h, respectively. Acetone treatment seemed to cause a slight increase of the R_f value, possibly because impurities were removed from the EPS extract. The R_f values of the samples were found to be significantly lower compared to those of the neutral references monosaccharides.

The identification of the sample spots using sugar monomer reference standards was not possible. The method requires further optimisation of the hydrolysis conditions and the TLC procedure (type of plate, eluent, and derivatisation agent; choice of reference sugars).

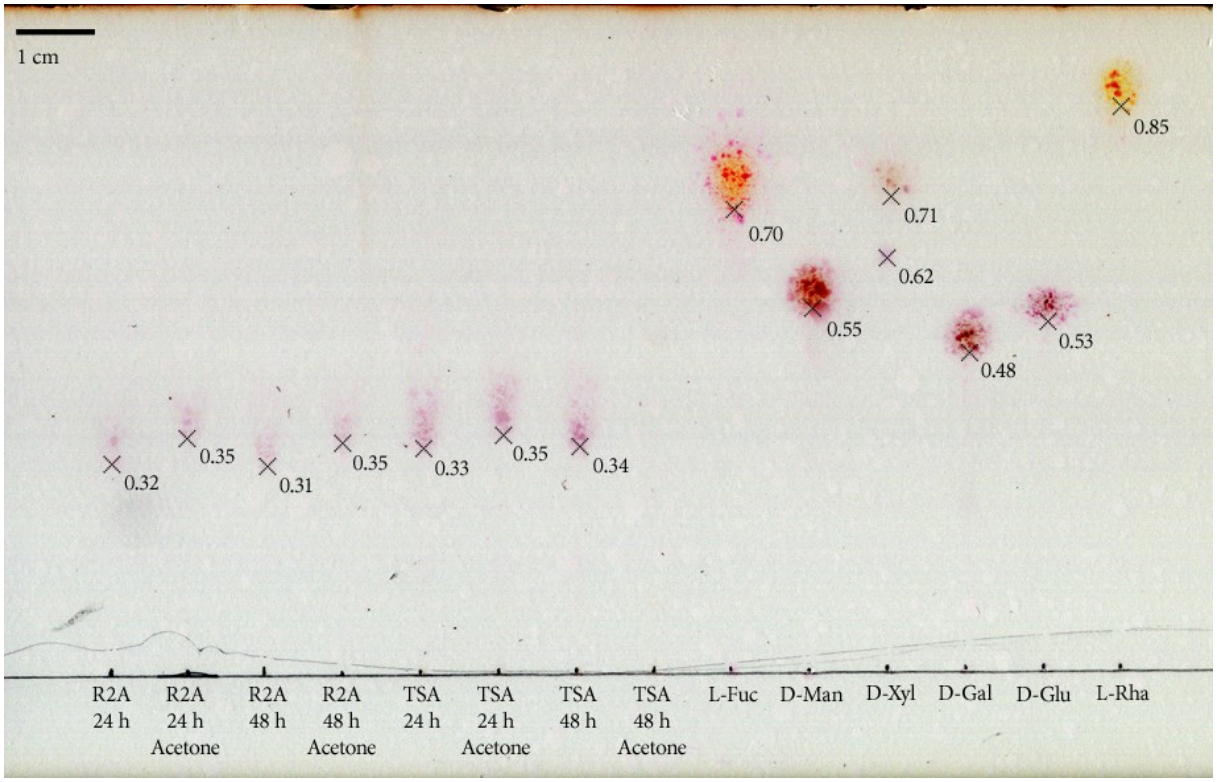

Figure 3.21. Thin-layer chromatogram of the EPS of R2A- and TSA-grown biofilms of *D. geothermalis*, hydrolysed for 24 h or 48 h at 100 °C using 0.1 M hydrochloric acid, with or without subsequent acetone treatment. Standard solutions (30 mM) of L-fucose, D-mannose, D-xylose, D-galactose, D-glucose, and L-rhamnose were used as references. 2 µl of sample and 1 µl of standard solution were applied, respectively. The retardation factor R_f is indicated for each spot.

3.6 Visualisation of the EPS distribution using fluorescently-labelled lectins

Fluorescently-labelled lectins were used to investigate the spatial distribution of the EPS within *D. geothermalis* biofilms *in situ*, and to demonstrate the presence of certain sugar moieties within the EPS based on lectin-glycoconjugate-specific interactions. Three different lectins, known to specifically bind to different target structures in biofilms produced by *D. geothermalis* E50051 (Peltola et al., 2008), were tested for their potential to bind to biofilms of *D. geothermalis* DSM 11300. Biofilms grown on both R2A and TSA were examined in order to consider differences in the composition and distribution of the EPS of these two biofilm types. Here, in contrast to other experiments, biofilms were generated on polycarbonate membranes in order to minimise unspecific binding of lectin to the support material.

On polycarbonate, *D. geothermalis* produced biofilms similar to those produced on CME or cellophane. R2A-grown biofilms exhibited a tightly packed, homogeneous arrangement of cells and a total biofilm thickness of 16-26 µm. In TSA-grown biofilms, the cells were present in aggregates of 5-10 µm in diameter. These aggregates became dislodged from the polycarbonate membrane during the staining procedure, as cell-to-substrate adhesion was poor. Thus, the spatial arrangement of cells and EPS within these biofilms, as investigated here by CLSM and EFM, was altered considerably.

The lectin (ACA) has been reported to be specific for galactose β-1,3-linked to N-acetylneuraminic acid (Neu5Ac; Peltola et al., 2008) and galactose β-1,3-linked to N-acetyl-galactosamine[5] (see Tab. 2.4). In R2A-grown biofilms, ACA was found to bind to a meshwork-like structure (Fig. 3.23 A) exclusively present in the deeper zones of the biofilm, as observed by CLSM. There, it seemed to stretch over the entire base of the biofilm, although signal strength was generally low. No binding of ACA occurred within the upper region of the biofilm. Binding of ACA to individual cells in R2A-grown biofilms was very rare and usually not observed. In TSA-grown biofilms, ACA adhered exclusively to cell surfaces (Fig. 3.23 B). However, ACA seemed to interact only to a small fraction of cells (approximately 20%), and also here lectin-cell interaction mostly occurred in the deeper region of the biofilm.

Lectin DBA was reported to be specific for methyl-2-acetamido-2-deoxy-D-galactose[5], terminal α-linked N-acetylgalactosamine, and α-1,3-interlinked N-acetylgalactosamines (Tab. 2.4; Peltola et al., 2008). In R2A-grown biofilms, binding of DBA to two main target structures with different morphologies was observed: (i) amorphous patches (Fig. 3.22), and (ii) thread-like structures which were of different length (up to approximately 20 µm) and sometimes came in radiant or parallel alignment to each other (Figs. 3.22, 3.23 C). Distribution of the structures relating to the biofilm area was highly heterogeneous and was most prominent in the deeper region of the biofilm. In TSA-grown biofilms, DBA reacted with amorphous structures that seemed to be in immediate contact with the cells, engulfing and interconnecting the cells in cell aggregates (Fig. 3.23 D). However, DBA did not bind to the cell surfaces.

Lectin HAA is supposed to recognise N-acetylglucosamine and N-acetyl-galactosamine[5] (Tab. 2.4; Peltola et al., 2008). In R2A-grown biofilms, it bound to structures widely distributed inside the biofilm that seemed to fill the intercellular space (Fig. 3.23 E). Thread-like structures exhibiting comparatively higher signal intensities were distributed heterogeneously throughout the biofilm. Interaction of HAA with TSA-grown biofilms was weak. HAA recognised thread- or tube-like structures, possibly linked to the cell aggregates (Fig. 3.23 F).

[5] According to the manufacturer (EY Laboratories).

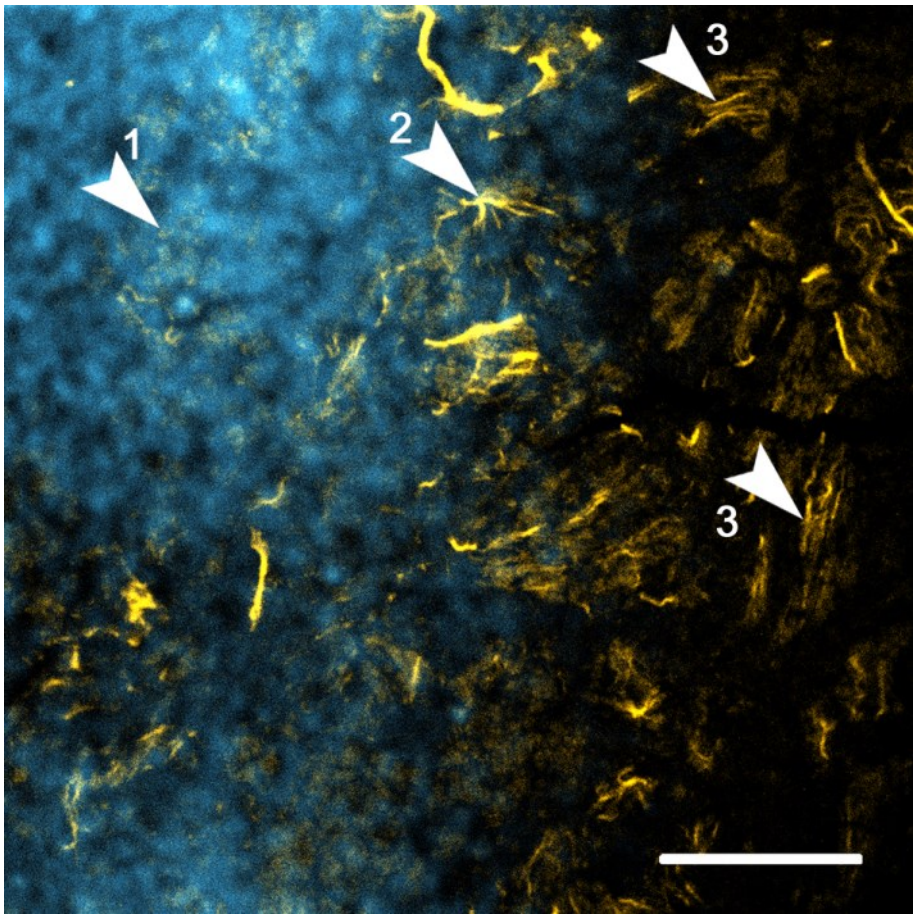

Figure 3.22. CLSM micrograph of a R2A-grown biofilm of *D. geothermalis* stained with fluorescently-labelled lectin DBA (yellow) and DAPI (blue). Heterogeneously distributed target structures of different morphologies were observed: amorphous patches (arrow 1) and thread-like structures which sometimes showed radiant (arrow 2) or parallel alignment (arrows 3). Instrument deficiency caused irregular illumination of the field of vision, resulting in a decreased intensity of the DAPI signal on the right hand side. Bar: 20 µm. 1,000× magnification.

In conclusion, all three lectins tested were found to bind to both biofilm types, yet to different target structures. In R2A-grown biofilms, homogeneously distributed network-like structures were recognised by ACA and HAA, respectively. In TSA-grown biofilms however, ACA reacted solely with cell surface structures, and HAA with defined structures associated with the cell aggregates. DBA bound to a target structure that was widespread in TSA-grown biofilms where it surrounded cells and cell aggregates. In R2A-grown biofilms, the target structures of DBA were scattered heterogeneously and were of varying morphology.

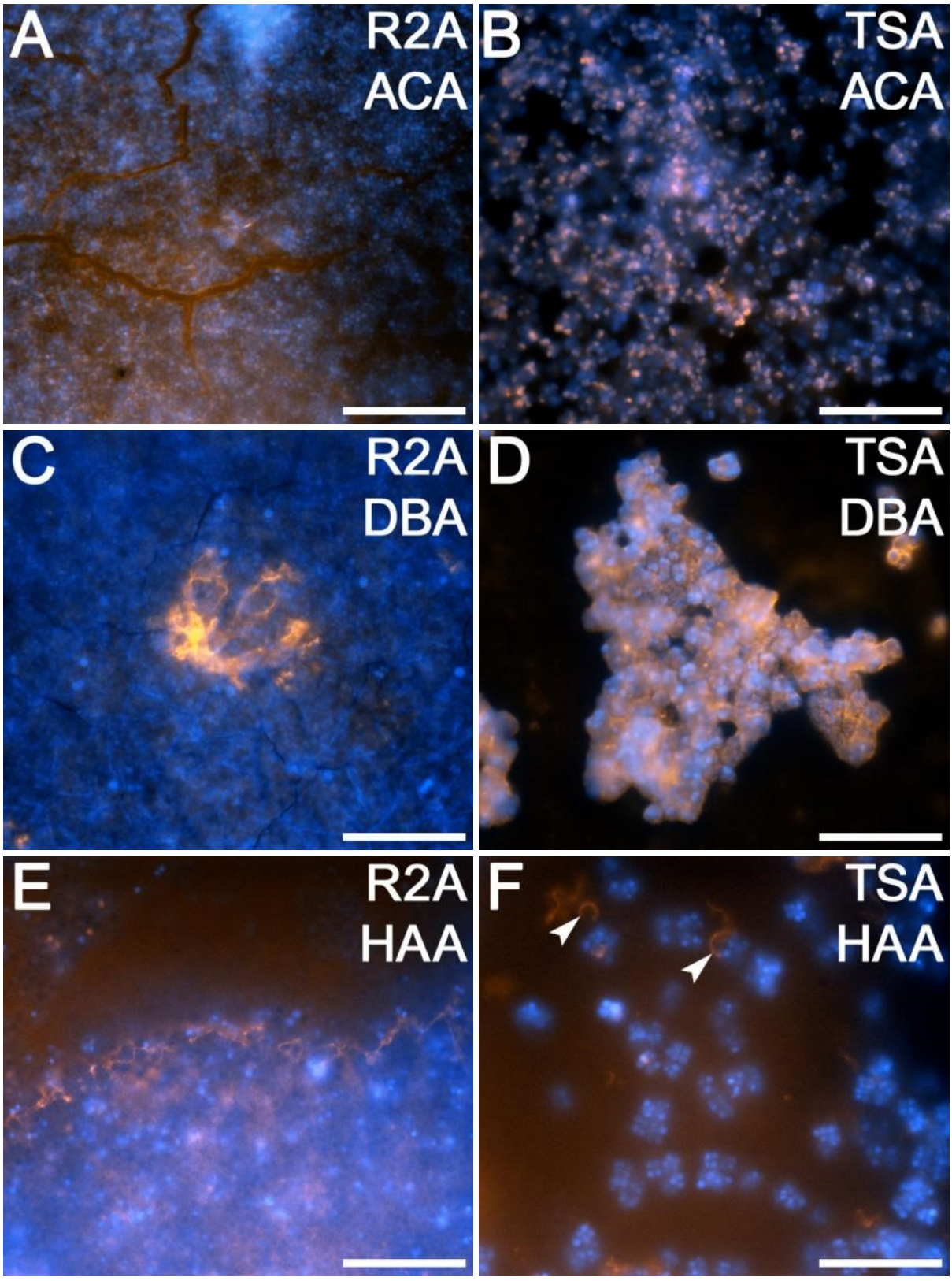

Figure 3.23. EFM micrographs of 2-day-old biofilms of *D. geothermalis* grown at 45 °C on R2A (**A**, **C**, **E**) or TSA (**B**, **D**, **F**), using polycarbonate membranes as a support. Biofilms were stained with TRITC-labelled lectins ACA (**A**, **B**), DBA (**C**, **D**), and HAA (**E**, **F**), whilst DAPI was used to stain the cells. Orange signal: lectin; blue signal: DAPI. Arrows: Thread-like structures recognised by HAA lectin in TSA-grown biofilms. Bar: 20 μm. 1,000× magnification.

3.7 Dispersal of TSA-grown cell aggregates

Biofilms grown on TSA dispersed into smaller cell aggregates of approximately 4-50 cells when suspended into aqueous solution, but further dispersal of these aggregates could not be achieved mechanically by common laboratory practice (thorough shaking on a vortex mixer; see 3.1.1). Due to this, cell enumeration and other microscopic analyses were impaired, rendering TSA-grown samples useless for stress experiments. Cell aggregation was not observed when using R2A as a nutrient medium. Here, thorough shaking allowed for the complete dispersal of biofilms into single cells and diplococci.

In order to find out how the composition of the nutrient medium influences the formation of cell aggregates, R2A was spiked with either sodium chloride (5.0 g l^{-1}) or peptone, both being constituents of TSA (see Tab. 2.1). Peptone was added in concentrations ranging from 1.5 g l^{-1} to 20 g l^{-1} (with 20 g l^{-1} corresponding to the peptone concentration in TSA) in order to detect concentration-dependent effects. Spiking R2A with sodium chloride did not have a significant effect on cell aggregate size (Fig. 3.24), as most cells were present as single cells or diplococci. The addition of peptone, however, facilitated cell aggregation. Tetrads and small aggregates could be observed with peptone concentrations as low as 1.5 g l^{-1}. The mean aggregate size was found to increase with increasing peptone concentrations (Fig. 3.24). With the highest peptone concentration applied (20 g l^{-1}), a mean cell aggregate size of 3.7 µm was observed, which was in the range of aggregate sizes found for TSA-grown cells (mean aggregate size 4.1 µm). Evidence for the amorphous material that was regularly seen to surround TSA-grown cells was now also found in R2A-grown aggregates with a peptone supplementation of 10 g l^{-1} or 20 g l^{-1} (Fig. 3.25).

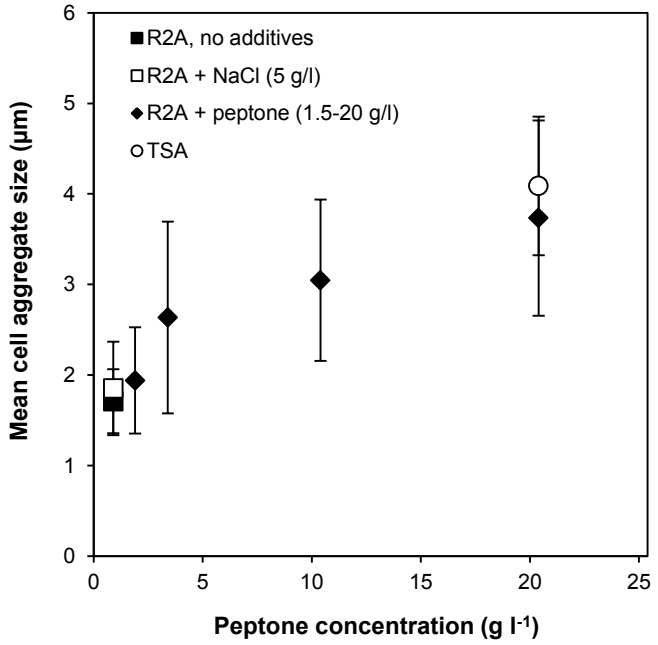

Figure 3.24. Correlation of the mean cell aggregate size and the peptone concentration of the nutrient medium. R2A was spiked with increasing concentrations of peptone (closed diamonds). R2A without the addition of peptone was used as a negative control (closed square). R2A was supplemented with sodium chloride to investigate its contribution to cell aggregate formation (open square). TSA was used as a positive control (open circle). Error bars indicate the range of cell aggregate sizes in n = 1 sample.

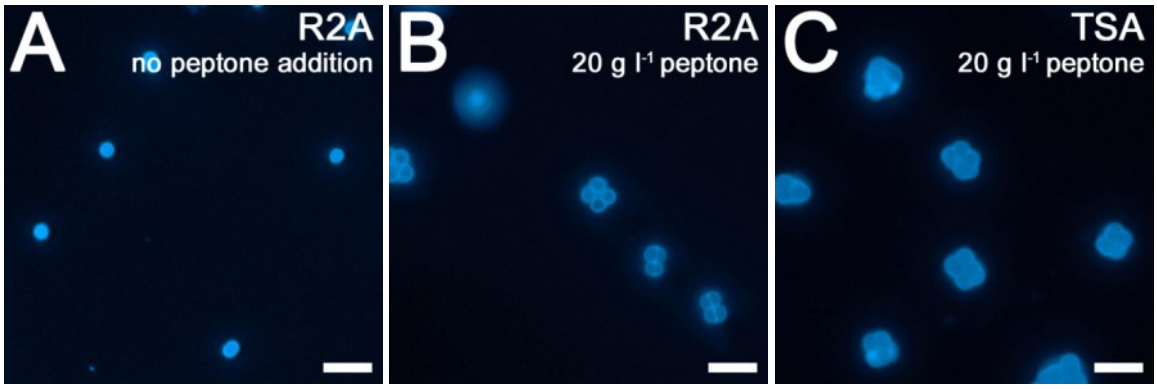

Figure 3.25. EFM micrographs of DAPI-stained biofilm cells of *D. geothermalis* dispersed in 0.14 M sodium chloride solution after growth under different nutrient conditions: R2A without the addition of peptone (**A**), R2A spiked with 20 g l^{-1} peptone (**B**), and TSA containing 20 g l^{-1} peptone (**C**). Bar: 5 µm. 1,000× magnification.

Numerous attempts were made to disperse TSA-grown cell aggregates, as a successful dispersal would shed light on the mechanisms involved in the strong cell-to-cell adhesion. Biofilm suspensions were prepared in 0.14 M sodium chloride solution or de-ionised water and subjected to dispersion. Dispersal procedures included shaking, sonication, the use of the complexing agent EDTA and the surfactant Tween 80 (see Tab. 2.12), as well as the addition of various monosaccharides and enzymes (Tab. 3.3).

Table 3.3. Monosaccharides and enzymes used for the dispersal of TSA-grown cell aggregates and the pH values of the respective assays.

Assay (carbohydrates)	pH	Assay (enzymes)	pH
Water, de-ionised (sugar control)	6.22	Proteinase K	8.06
D-fucose (10 mM)	6.34	Proteinase K, inactivated enzyme	7.97
L-fucose (10 mM)	6.40	Proteinase K, no enzyme	8.01
D-galactose (10 mM)	6.15	Pronase E	7.39
D-glucose (10 mM)	6.16	Pronase E, inactivated enzyme	7.28
D-mannose (1 mM)	6.67	Pronase E, no enzyme	7.55
D-mannose (5 mM)	6.59	Cellulase	5.00
D-mannose (10 mM)	6.22	Cellulase, no enzyme	5.02
D-mannose (100 mM)	6.11		
L-rhamnose (10 mM)	6.27		
NAc-galactosamine	6.05		
NAc-glucosamine	6.14		
NAc-mannosamine	6.23		
Methyl-α-D-mannopyranoside	6.23		

Cell aggregates were highly resistant to shaking, to sonication, and to the application of surfactant Tween 80. Interestingly, cell aggregates also withstood the various treatments used for the isolation of EPS (see Tab. 2.11). It was then hypothesised that cell aggregation is maintained by EPS. Hence, aggregation might be relieved by (i) weakening the interactions between the EPS, or (ii) by degrading the EPS constituents involved in intercellular adhesion. The complexing agent EDTA was used to extract divalent cations such as Ca^{2+} from the biofilm matrix, as they might contribute to EPS stability by interconnecting negatively charged polymers. However, EDTA treatment did not affect the size of the cell aggregates, suggesting that the intercellular adhesion of TSA-grown cells does not rely on EPS-crosslinking by cations.

A range of monosaccharides was employed to destabilise the EPS by weakening potential protein-carbohydrate interactions due to an excess of the respective target-sugar moieties. Of all sugars tested (listed in Tab. 3.3), only D-mannose was found to have an effect on the cell aggregate size. When administered to biofilm suspensions at a concentration of 10 mM, the mean aggregate size was reduced from 4.1 µm to 3.2 µm, with > 75% of the cell aggregates being smaller than 4.1 µm (Fig. 3.26). Still, complete dispersal was not achieved. With a pH of 6.22, the pH value of the D-mannose assay was equal to the pH value of the control (deionised water without the addition of exogenous sugar; Tab. 3.3), ruling out a pH-dependent effect. In order to find the optimum concentration of D-mannose, it was administered in concentrations ranging from 1 mM to 100 mM. However, apart from the 10 mM assay, all other concentrations proved to be ineffective in facilitating the dispersal of the cell aggregates (Fig. 3.26). Mannose-related glycosides NAc-mannosamine and methyl-α-D-mannopyranoside proved to be ineffective at the concentration tested (10 mM).

Enzymatic treatments were performed using cellulase, pronase E, and proteinase K in order to degrade potential EPS constituents responsible for cell aggregation. Untreated suspensions of TSA-grown biofilms were used as a reference. For negative controls, the enzymes were either heat-inactivated ('inactivated enzyme control') or not added to the assay ('no enzyme control').

Cellulase treatment for 24 hours at 37 °C did not relieve aggregation. Treatment with proteolytic enzyme pronase E for 24 hours at 37 °C caused a reduction of the total aggregate count (as the total number of aggregates per ml of biofilm suspension) by one order of magnitude and a reduction of the plate count by over two orders of magnitude, both indicating cell lysis (Fig. 3.27). This effect was weakened by decreasing incubation time from 24 h to 3 h. In this case, colony count was reduced by only one order of magnitude. Total aggregate count was increased compared to the controls, indicating a greater degree of dispersal. Microscopic evaluation of the samples revealed that pronase E seemed to disrupt the cell aggregates, as large amounts of loose, amorphous residues were present in those samples that had been

treated with pronase E. Due to this, microscopic enumeration of DAPI-stained cells was strongly impeded.

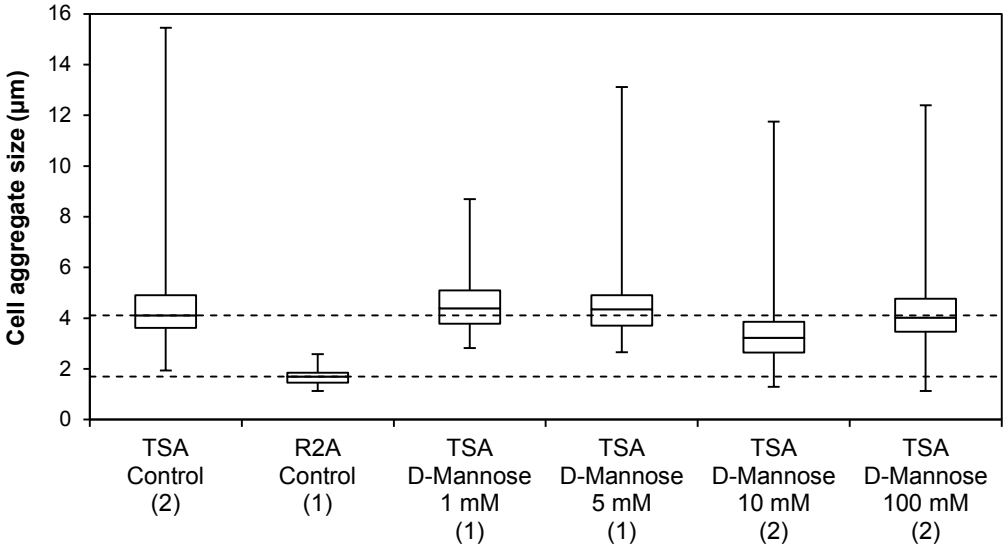

Figure 3.26. Cell aggregate size distribution of TSA-grown biofilms of *D. geothermalis* after treatment with D-mannose in varying concentrations (1-100 mM). Untreated suspensions of TSA- and R2A-grown biofilms, respectively, were used as controls. The mean aggregate sizes of these controls were used as a reference for measuring success of dispersion, and were 4.1 µm for TSA-grown biofilms and 1.7 µm for R2A-grown biofilms (dashed lines). Numbers in parentheses indicate the amount of trials performed. For each trial, 50-100 aggregates were measured.

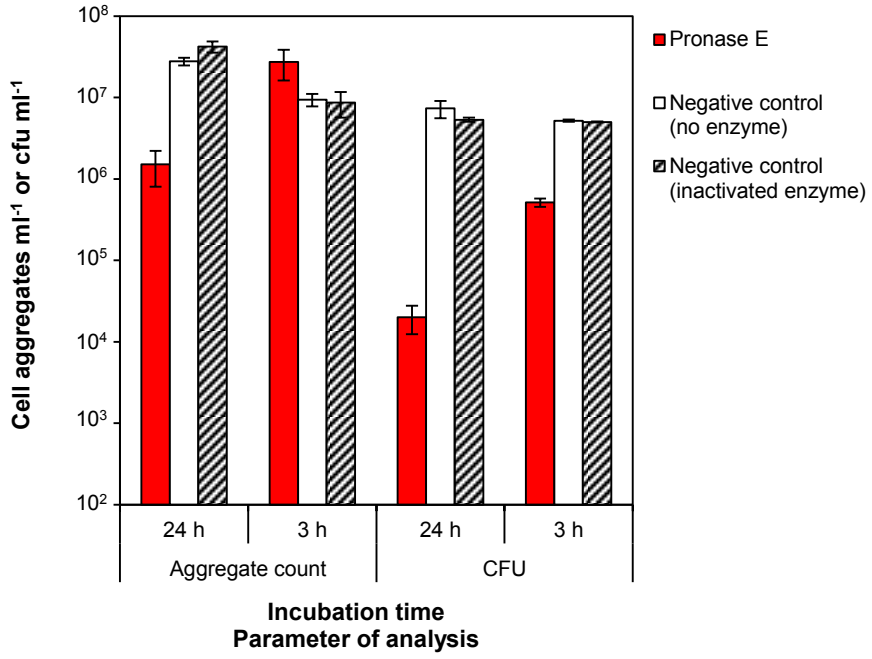

Figure 3.27. Effect of pronase E treatment for 24 h and 3 h on total aggregate counts and plate counts of TSA-grown cells of *D. geothermalis*. For negative controls, the assay was performed without the addition of pronase E or with the addition of the heat-inactivated enzyme. n = 2.

The disruption of cell aggregates by pronase E activity suggested the involvement of proteinaceous EPS in cell-to-cell adhesion in *D. geothermalis*. In order to verify this assumption, proteinase K was employed as a second proteolytic enzyme. Proteinase K treatment for 24 h at 37 °C proved to be successful in dispersing TSA-grown cell aggregates (Fig. 3.28). After treatment, the majority of cells was present as single cells, diplococci, or tetrads, with a mean cell aggregate size of 2.2 µm (as opposed to 4.1 µm and 1.7 µm for untreated biofilm suspensions of TSA- and R2A-grown cells, respectively. In contrast to samples treated with pronase E, no evidence for loose residues of EPS material was found. Instead, treatment with proteinase K seemed to cause the matrix which surrounded TSA-grown cells to disappear. Since the effect was not observed after heat-inactivation of the enzyme, it was likely to be caused by proteolytic activity. Treatment with proteinase K did not decrease culturability.

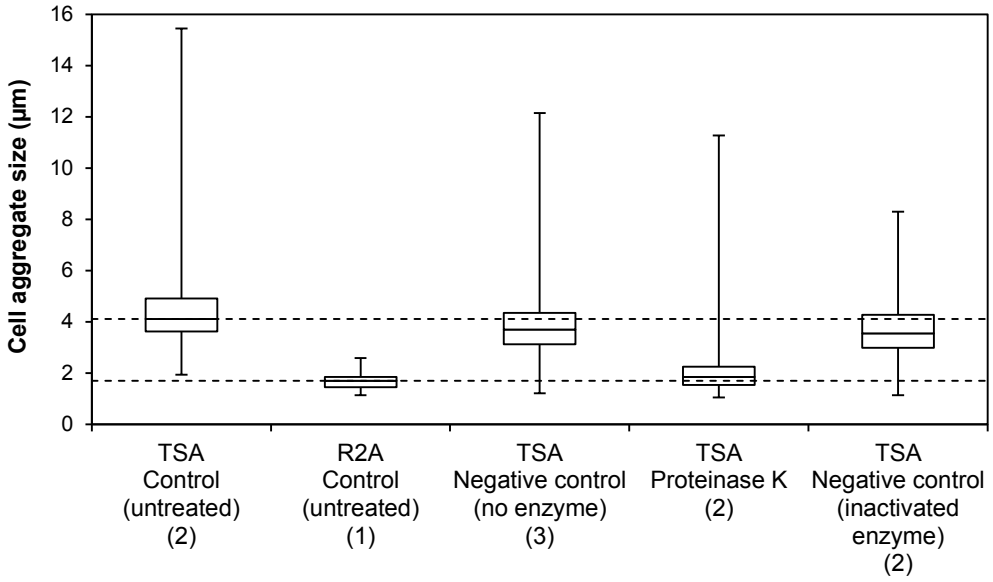

Figure 3.28. Size distribution of cell aggregates of suspended TSA-grown biofilms of *D. geothermalis* after treatment with the proteolytic enzyme proteinase K. Untreated suspensions of TSA- and R2A-grown biofilms were used as references. The mean aggregate sizes of these controls were used as a reference for measuring success of dispersion, and were 4.1 µm for TSA-grown biofilms and 1.7 µm for R2A-grown biofilms (dashed lines). For negative controls, the assay was performed without the addition of proteinase K or with the addition of the heat-inactivated enzyme. Numbers in parentheses indicate the number of trials performed. For each trial, 50-100 aggregates were evaluated.

To conclude, the formation of cell aggregates of *D. geothermalis* was shown to be caused by the presence of peptone in the nutrient medium provided, with the mean cell aggregate size increasing with the concentration of peptone. TSA-grown aggregates resisted dispersal by a range of measures, including shaking, sonication, and various physico-chemical and enzymatic treatments. Cell-to-cell adhesion was considered to be EPS-mediated. Abstraction of divalent cations from the EPS by EDTA did not destabilise the cell aggregates, suggesting that

crosslinking of the EPS by cations is not important for aggregation. The addition of D-mannose at 10 mM slightly decreased the mean aggregate size, indicating that protein-carbohydrate interactions could be involved in cell aggregation. Proteolytic enzymes successfully caused dispersal of the cell aggregates: Pronase E disrupted the aggregates but at the same time seemed to cause cell lysis, whilst treatment with proteinase K led to the removal of the EPS envelope surrounding the cell aggregates. All results considered, the involvement in proteinaceous EPS in the cell-to-cell adhesion of TSA-grown cells was clearly demonstrated.

3.8 Stress simulation experiments

A main focus of this study was to test whether biofilms of *D. geothermalis* could survive the life-threatening conditions present in space and on Mars. Several experiments were carried out which simulated certain aspects of these environments. Stressors included desiccation, space vacuum or Martian atmosphere and pressure, UV radiation, and temperature extremes, which were applied either individually or in combination. For each stress experiment, air-dried samples of R2A-grown biofilms and planktonic cells of *D. geothermalis* were exposed. CME membranes were used as a support for desiccation experiments, whilst cellophane was chosen for the remaining exposure experiments. Following exposure, the survival of the test organisms was assessed by cultivation and by cultivation independent viability markers (membrane integrity, presence of 16S rRNA, ATP content).

3.8.1 Tolerance of *D. geothermalis* to long-term desiccation

In order to study the effect of desiccation on *D. geothermalis*, biofilms and planktonic cells deposited on CME membranes were air-dried and stored in desiccated state at room temperature for at least 2 months (see 3.2.2). At defined points in time, the survival of the samples was assessed by cultivation and cultivation-independent methods.

Total cell counts obtained by staining the cells with DAPI changed little over time. On average, biofilm samples comprised $2.7 \times 10^8 \pm 7.7 \times 10^7$ cells cm^{-2}. The mean cell count of planktonic samples was $3.6 \times 10^8 \pm 6.8 \times 10^7$ cells cm^{-2}. Both biofilms and planktonic cells of *D. geothermalis* were able to survive long-term desiccation for 61 days when stored under ambient conditions. However, biofilms maintained viability to a significantly higher extent than planktonic cells, as indicated by the viability markers culturability, membrane integrity, and ATP (Fig. 3.29).

The fraction of culturable cells in non-desiccated samples was low, with 19.8% of biofilm cells and 28.4% of planktonic cells being culturable. Desiccation for 2 months further reduced the culturability of the samples to 5.6% and 0.8% for biofilms and planktonic cells, respectively (Fig. 3.29 A). Whilst culturability of planktonic cells dropped by approximately

one order of magnitude already within the first days of desiccation, biofilm cells seemed to maintain higher levels of culturability for an extended period of time.

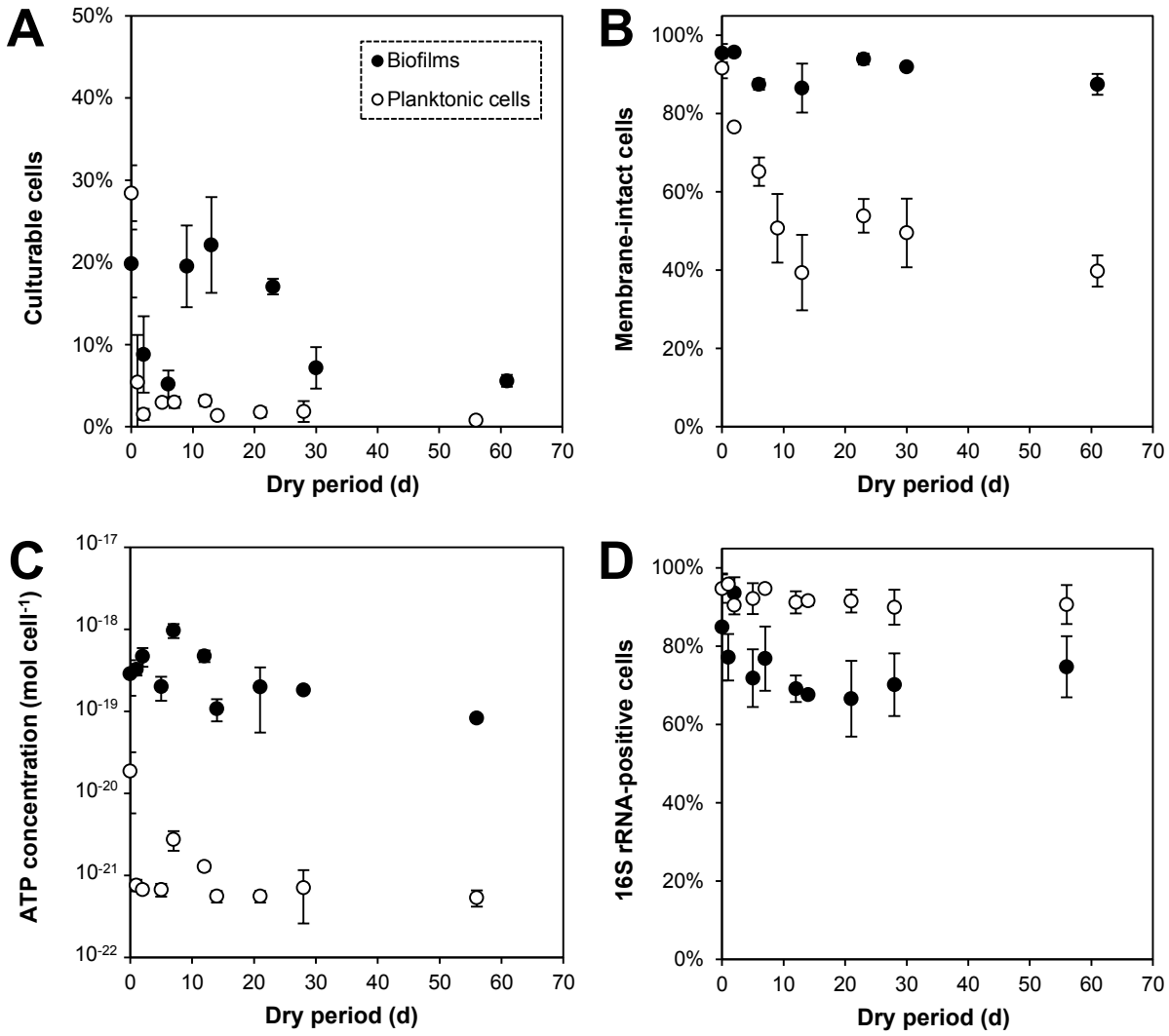

Figure 3.29. Viability of air-dried biofilms (closed circles) and planktonic cells (open circles) of *D. geothermalis* during desiccation for up to 61 days at room temperature and 59% relative humidity. Viability was assessed in terms of culturable cells (**A**) as the fraction of CFU in total cell counts (by DAPI staining), membrane-intact cells (**B**) as the fraction of propidium iodide-negative cells in total cell counts (by Live/Dead® staining), total ATP concentrations (**C**) correlated to total cell counts (by DAPI staining), and 16S rRNA-positive cells (**D**) as the fraction of FISH-positive cells in total cell counts (by DAPI staining). n = 3.

Prior to desiccation, both biofilms and planktonic cells exhibited high levels of membrane integrity, with 95.5% of biofilm cells and 91.6% of planktonic cells stained propidium iodide-negative (i.e. membrane-intact). A desiccation period of 61 days reduced the proportion of membrane-intact cells in biofilms and planktonic samples to 87.5% and 39.8%, respectively (Fig. 3.29 B), clearly demonstrating that biofilms were less affected by dehydration. CLSM observations of Live/Dead®-stained biofilms and planktonic cells desiccated for 90 days

supported these findings. Although no quantitative data was obtained, it was unequivocal that *D. geothermalis* biofilm cells maintained membrane integrity to a significantly higher extent than planktonic cells did, as indicated by differences in the presence of propidium iodide-stained (red) cells (Fig. 3.30). Membrane-damaged cells were found to be distributed homogeneously throughout the samples, i.e. a pattern indicating localised membrane damage such as a top-to-bottom gradient of membrane-damaged cells could not be observed.

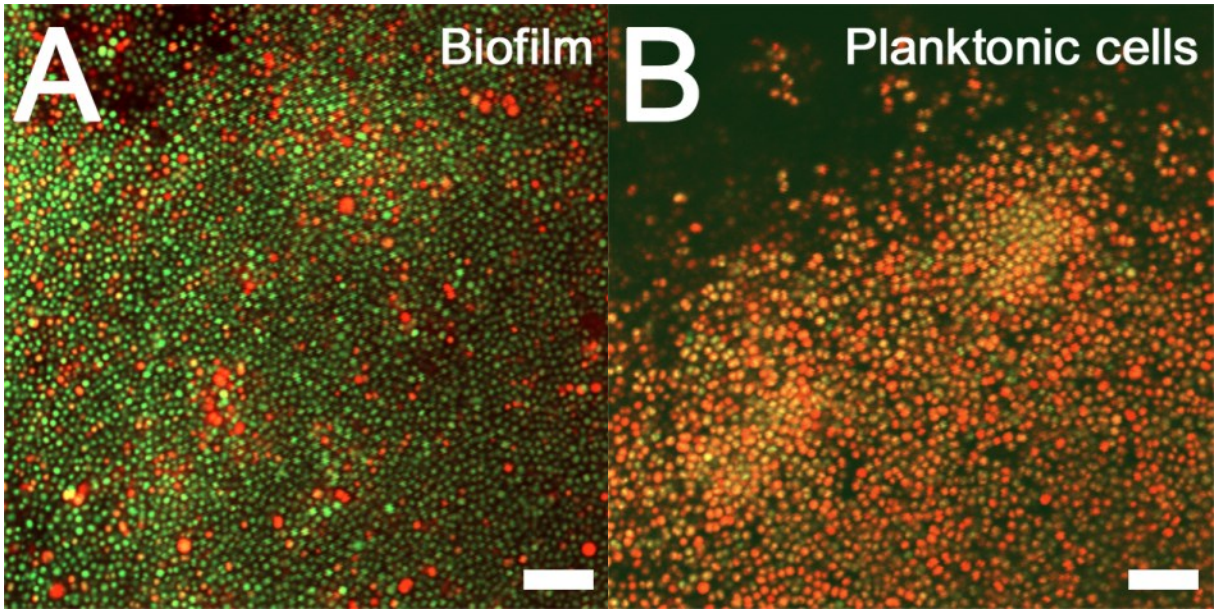

Figure 3.30. CLSM micrographs of Live/Dead®-stained cells of *D. geothermalis* as a biofilm (**A**) and as planktonic cells deposited on a CME membrane (**B**). Samples were stained after being stored in air-dried condition for 90 days. 1,000× magnification. Bar: 10 µm.

Non-dried biofilms exhibited an average ATP concentration of 2.9×10^{-19} mol cell^{-1}. In planktonic cultures, the initial average ATP concentration was over one order of magnitude lower (1.9×10^{-20} mol ATP cell^{-1}), possibly due to the procedures involved in sample preparation (centrifugation and membrane filtration), which might have removed significant amounts of extracellular ATP from planktonic samples. When desiccated, biofilm cells maintained relatively stable ATP levels, although a trend for ATP concentrations to decline throughout the dry period of 2 months could be observed (Fig. 3.29 C). Eventually, the average ATP concentration decreased to 8.3×10^{-20} mol cell^{-1}. ATP levels in planktonic samples decreased immediately after the desiccation event by about one order of magnitude, but afterwards remained fairly stable throughout the dry period, with concentrations ranging from 5.4×10^{-22} mol cell^{-1} to 2.7×10^{-21} mol cell^{-1} (Fig. 3.29 C).

In order to test the stability of ATP under desiccation conditions, a stock solution of ATP in PFD water with a theoretical ATP concentration of 1.0×10^{-7} M was dried by vacuum centrifugation and stored in dry state for up to 63 days. The measured ATP concentration of the freshly prepared stock solution was 1.1×10^{-7} M. The same concentration was measured

following a 3-h-drying process, after which no visible fluid residue was observed in the samples. During a subsequent desiccation period of 63 days, ATP concentrations decreased over time to 1.6×10^{-8} M (Fig. 3.31). Based on a trend curve fitted to the data ($R^2 = 0.90$), the decay of dried ATP could be described as exponential, with a decay constant $\lambda = 0.035$. The half-life of dried ATP was estimated to be 19.8 days. Thus, after a dry period of 2 months, total ATP had decayed to approximately 12% of its initial quantity.

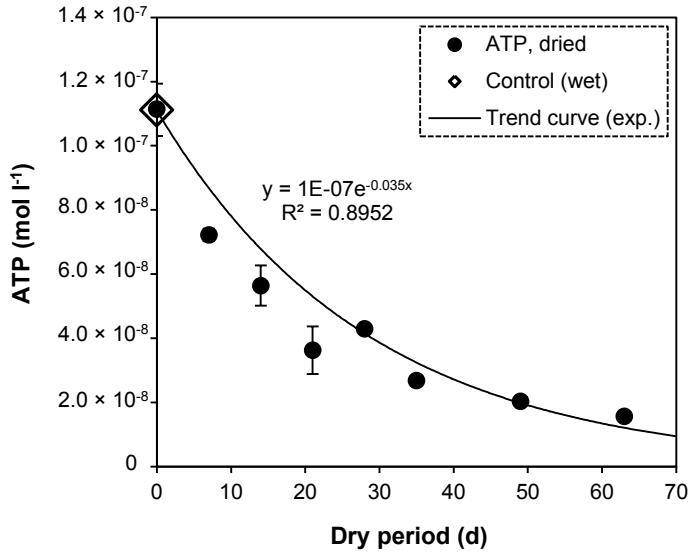

Figure 3.31. Exponential decay observed for vacuum-dried ATP (closed circles) stored in closed microcentrifuge tubes at room temperature and 59% relative humidity over a period of 63 days. The ATP content was measured prior to desiccation as a reference (open diamond). The calculated decay constant $\lambda = 0.035$ corresponded to a half-life of ATP of $t_{1/2} = 19.8$ days. n = 3.

In order to determine the presence of ribosomes as an indicator for possible protein biosynthesis, cells were marked with a 16S rRNA-targeting probe using FISH. The fraction of FISH-positive cells in both biofilms and planktonic samples changed only little during desiccation (Fig. 3.29 D), suggesting that dehydration did not affect the presence of 16S rRNA in *D. geothermalis*. Interestingly, however, planktonic samples contained significantly more FISH-positive cells compared to biofilm samples: On average, 92.3% ± 2.0% of planktonic cells but only 75.3% ± 8.5% of biofilm cells were FISH-positive, which indicates that in biofilms a significant proportion of cells was metabolically inactive.

In order to obtain a general overview of the effect of desiccation on different aspects of viability, the results were plotted as radar charts (Fig. 3.32). Using multiple logarithmic axes, each chart considers the parameter total cell counts, culturability, membrane integrity, and the presence of ATP and 16S rRNA in both biofilms and planktonic cells. A normalisation of the data was necessary in order to fit the different, unit-dependent parameters into a single chart. Normalisation was achieved by calculating the dimension-independent ratio N/N_0 for each parameter, with N representing the state of viability after desiccation and N_0 representing the state of viability of non-desiccated samples. Thus, values of $N/N_0 < 1$ correspond to negative changes in viability, whereas values of $N/N_0 > 1$ would indicate a positive change in

viability following exposure to desiccation. When these ratios were plotted, a distinct pattern was generated which expresses the overall viability status of the stress-exposed organism. By using the same arrangement of viability markers amongst different charts, cells exposed to different stress conditions can be compared according to multiple parameters. The data illustrated in Fig. 3.32 unequivocally confirms that biofilms were more desiccation-resistant than planktonic cells.

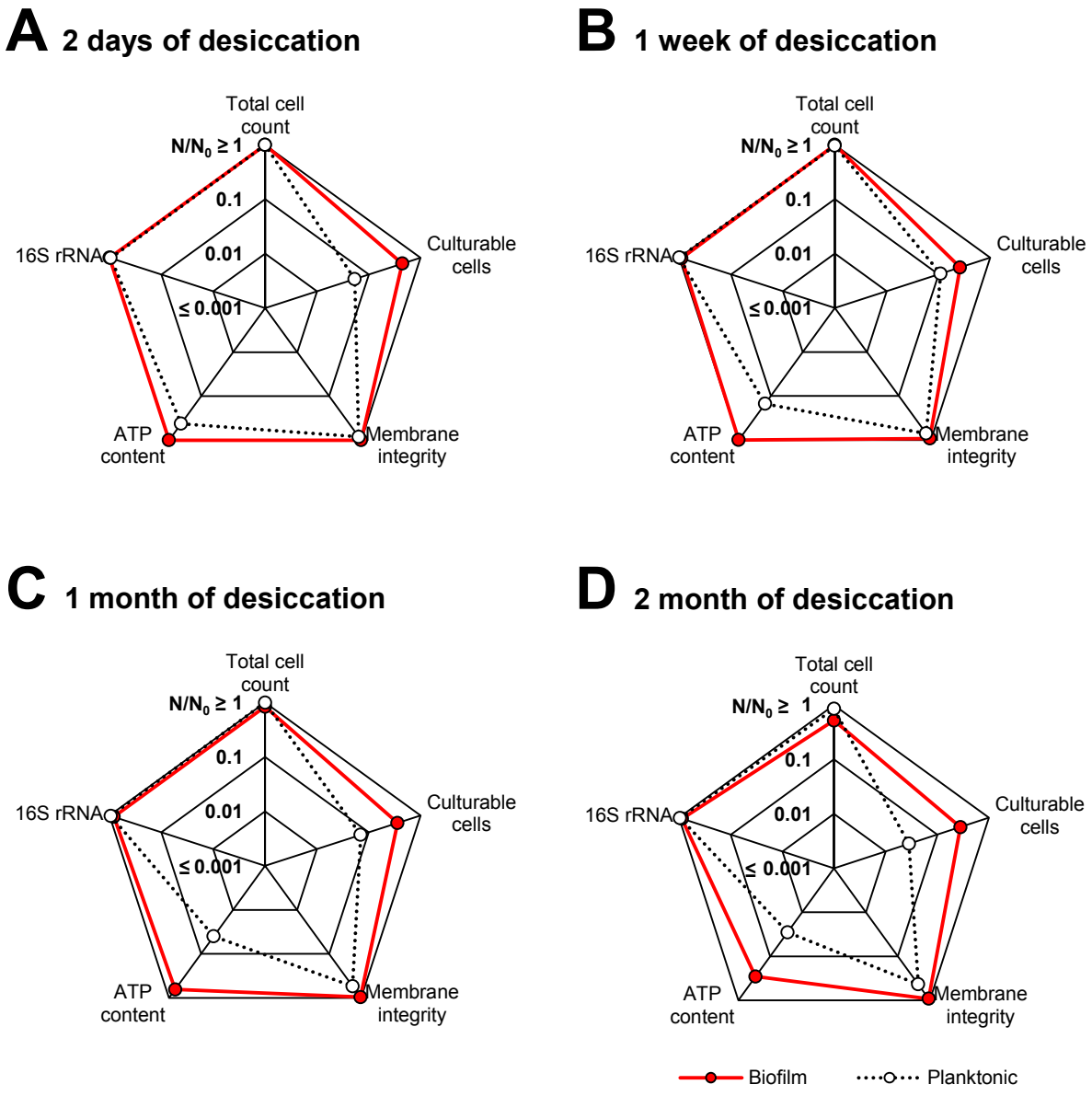

Figure 3.32. The effect of desiccation for 2 days (**A**), 1 week (**B**), 1 month (**C**), and 2 months (**D**) on the viability of biofilms (red circles) and planktonic cells (white circles) of *D. geothermalis*. Culturability, membrane integrity, ATP content, and presence of 16S rRNA were used as viability markers. Results were normalised (N/N_0) with respect to non-dried controls ($N_0 = 1$). n = 3.

To conclude, *D. geothermalis* survived storage in desiccated state at room temperature and 59% relative humidity for at least 2 months in both planktonic and biofilm mode. Judging from four independent viability markers (colony-forming ability, membrane integrity, ATP levels, and the presence of 16S rRNA indicating protein synthesis), biofilms were clearly more desiccation-tolerant than planktonic cells. Compared to planktonic cells, biofilms maintained culturability, membrane integrity, and ATP levels during desiccation for a significantly longer period of time. All of these three parameters were found to only slowly decrease over time in biofilms, whilst planktonic cells displayed a sudden drop in viability directly after the drying event. Tested under similar conditions, vacuum-dried free ATP decayed exponentially with a half-life of about 20 days. Desiccation did not seem to affect the presence of 16S rRNA, as the amount of FISH-positive cells in both sample types remained relatively stable over time. However, compared to planktonic cells, biofilms exhibited a significantly smaller average percentage of FISH-positive cells.

3.8.2 Effect of single space-relevant stressors on the viability of *D. geothermalis*

In order to evaluate the impact of selected space-relevant stressors on survival, biofilms and planktonic cells of *D. geothermalis* dried on cellophane or CME membranes were exposed to monochromatic UV irradiation (254 nm) with fluences ranging from 10-10,000 J m^{-2}, to vacuum, to simulated Mars gas atmosphere, to repeated thaw-freeze cycles, and to the peak temperatures (-25 °C; +60 °C) expected during the space mission EXPOSE-R2 (see Tab. 2.13 for detailed test conditions). Viability after exposure was assessed in terms of culturability and membrane integrity and compared to the viability of desiccated but non-exposed controls, which were stored at room temperature in the dark for the duration of the experiment.

Samples produced using CME membranes as supports were destroyed during exposure to monochromatic UV radiation of fluences of ≥ 10^3 J m^{-2}, as UV irradiation caused discolouration and embrittlement of the support material. Analysis of these samples was impossible. The samples produced by using cellophane as a support, on the other hand, remained intact during the exposure to the various stressors tested. Thus, the results presented below refer only to cellophane-deposited cells.

Due to their large quantity, all samples were divided into three separate sets, which were analysed independently. Thus, at the time of analysis, the samples had been in desiccated state for 46, 64, and 82 days, respectively. The state of viability of non-exposed controls was used as a reference. The fraction of culturable cells in non-exposed biofilms ranged from 8.6-34.3%. The proportion of culturable cells was smaller in planktonic samples, where their fraction ranged from 4.0-7.9% (Fig. 3.33). All non-exposed cells exhibited a high degree of membrane integrity. A mean fraction of 92.6% of biofilm cells was membrane-intact. With

respect to results obtained from long-term desiccation experiment, in which a 61-day-period of desiccation caused a reduction of the percentage of membrane–intact cells to 39.8% (see 3.8.1), membrane integrity of planktonic samples was relatively high. Even after 82 days of desiccation, 85.8% of the cells observed were still membrane-intact (Fig. 3.33).

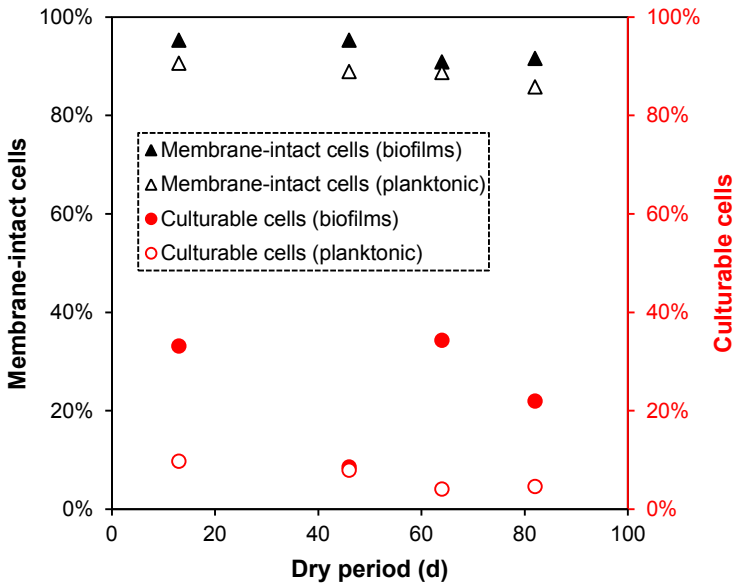

Figure 3.33. Viability of non-exposed controls (biofilms and planktonic cells of *D. geothermalis* on cellophane) used for single-stress experiments. One set of controls was analysed prior to the start of the exposure experiment (day 13 of the dry period). After exposure, the remaining controls were analysed in three separate sets at days 46, 64, and 82. Results are presented as the fraction of membrane-intact cells (black triangles) and culturable cells (red circles), respectively, with respect to total cell counts (not shown). n = 1.

Exposure to vacuum, different temperatures, and Martian atmosphere and pressure did not cause a reduction in viability (culturability and membrane integrity) of both biofilms and planktonic cells of *D. geothermalis* compared to non-exposed controls (Fig. 3.34). Irrespective of exposure to stress, the culturability of biofilms was approximately one order of magnitude higher ($1.1\text{-}2.2 \times 10^8$ CFU cm^{-2}) than the culturability of planktonic cells ($2.3\text{-}4.5 \times 10^7$ CFU cm^{-2}; Fig. 3.34). Membrane integrity was preserved at high levels in both samples types regardless of exposure to stress (> 91.3% ± 6.1% for biofilms and > 83.8% ± 12.0% for planktonic samples; Tab. 3.4).

Irradiation with monochromatic UV of fluences > 10^2 J m^{-2} significantly affected the culturability of biofilms and planktonic cells of *D. geothermalis*, although biofilms seemed to be more sensitive to monochromatic UV radiation than planktonic cells (Fig. 3.35). The highest fluence applied (10 kJ m^{-2}) decreased the culturability of biofilms by two orders of magnitude (99.2%), whilst the culturability of planktonic cells was reduced by only 72.9% compared to non-irradiated controls. Total cell counts were not affected by UV radiation and ranged from 4.4×10^8 cells cm^{-2} to 7.6×10^8 cells cm^{-2} in biofilms and from 5.2×10^8 cells cm^{-2} to 6.6×10^8 cells cm^{-2} in planktonic samples. Membrane integrity was preserved in irradiated biofilm cells. In planktonic cells, a UV fluence of 10 kJ m^{-2} slightly decreased the amount of intact cells from 87.8% in the non-exposed control to 78.6% (Fig. 3.35; Tab. 3.4).

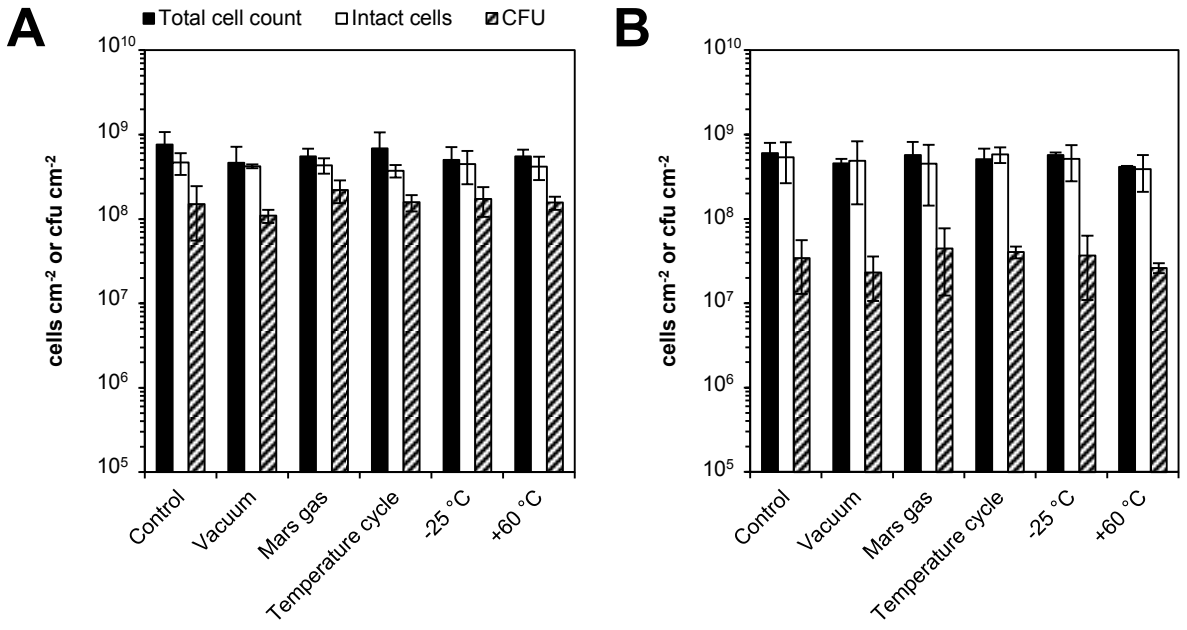

Figure 3.34. Total cell counts (black bars), membrane-intact cells (shaded bars), and colony-forming units (white bars) of desiccated biofilms (**A**) and planktonic cells (**B**) following exposure to vacuum, artificial Mars atmosphere, temperature cycles, and peak temperatures (-25 °C and +60 °C). Controls were dried but non-exposed samples of biofilms or planktonic cells, respectively. n = 3.

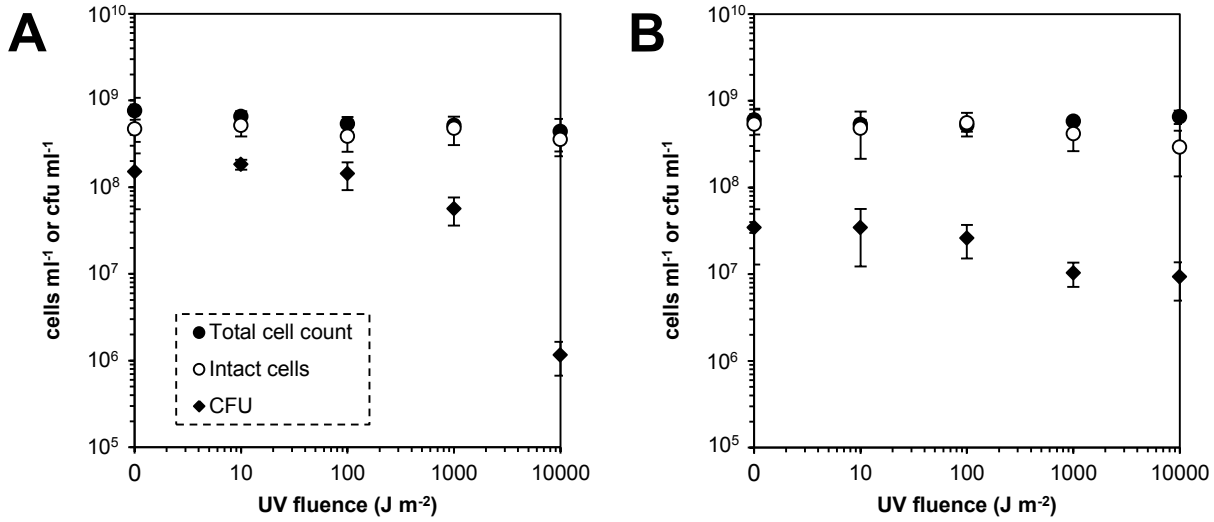

Figure 3.35. Effect of monochromatic (254 nm) UV radiation on total cell counts (closed circles), membrane-intact cell counts (open circles), and plate counts (diamonds) of biofilms (**A**) and planktonic cells (**B**) of *D. geothermalis.* n = 3.

Table 3.4. Fractions of culturable cells and membrane-intact cells in samples of biofilms and planktonic cells of *D. geothermalis* after exposure to selected space-relevant stressors. n = 3.

Stressor	Culturable cells (% of total cell counts)		Membrane-intact cells (% of total cell counts)	
	Biofilms	Planktonic cells	Biofilms	Planktonic cells
Vacuum	28.2 ± 13.9	5.2 ± 2.9	94.1 ± 3.2	86.5 ± 9.5
Mars atmosphere	39.5 ± 2.8	6.8 ± 3.6	94.8 ± 2.3	87.6 ± 2.8
Temperature cycle	30.1 ± 22.8	8.6 ± 2.8	91.3 ± 6.1	89.0 ± 2.2
-25 °C	43.1 ± 34.8	6.7 ± 5.1	95.0 ± 4.0	90.4 ± 3.2
+60 °C	28.3 ± 2.3	6.3 ± 0.9	96.0 ± 1.2	83.8 ± 12.0
UV (10^1 J m^{-2}, 254 nm)	28.4 ± 5.3	6.2 ± 3.7	92.8 ± 2.4	83.6 ± 8.3
UV (10^2 J m^{-2}, 254 nm)	26.5 ± 7.2	5.0 ± 2.0	93.2 ± 4.3	87.3 ± 5.3
UV (10^3 J m^{-2}, 254 nm)	11.2 ± 4.9	1.8 ± 0.6	94.2 ± 2.4	84.0 ± 3.4
UV (10^4 J m^{-2}, 254 nm)	0.3 ± 0.2	1.5 ± 0.9	91.7 ± 2.4	78.6 ± 11.0
Non-exposed control	21.6 ± 12.9	5.5 ± 2.1	92.6 ± 2.4	87.8 ± 1.7

In summary, *D. geothermalis* survived exposure to single stressors which simulated certain aspects of space or Martian environments. Neither of the stressors applied seemed to affect total cell counts or the membrane integrity of *D. geothermalis.* Culturability was significantly reduced when *D. geothermalis* was exposed to monochromatic UV radiation at 254 nm with fluences of 1-10 kJ m^{-2}.

3.8.3 Effect of simulated space and Mars conditions on the viability of *D. geothermalis*

In order to simulate the exposure of *D. geothermalis* to space and Martian-like environments, the organism was exposed to a combination of stressors relevant for extraterrestrial environments. Desiccated biofilms and planktonic cells deposited on cellophane were exposed to vacuum (3×10^{-4} Pa) or artificial Mars atmosphere at low pressure (10^3 Pa) to simulate space and Mars climate, respectively. In contrast to the previous irradiation experiment, samples were now irradiated with polychromatic UV radiation (200-400 nm) with a maximum fluence of 5.5×10^2 kJ $m^{-2,}$ corresponding to the fluence received by the samples of EXPOSE-R2 during a 12-months stay in LEO.

Samples were arranged in stacks of five so that a greater amount of samples could be accommodated in the limited space of the EXPOSE sample carrier. Due to the stacking and thus the shielding effect by superimposing sample layers, UV exposure decreased from top to bottom. For each stack, samples at position 2 ('top') and 4 ('bottom') were analysed within the scope of this study. In addition to that, another set of samples was exposed to vacuum or Mar-

tian atmosphere but completely shielded from UV radiation ('dark samples'). The fluences received by the individual sample types are listed in Tab. 3.5. Planktonic cells received an approximately 2-fold higher fluence than biofilms, indicating that planktonic samples were more penetrable for UV radiation than were biofilm samples. In between space and Mars conditions, fluences were of comparable magnitude. Irradiation was performed discontinuously for 120 h in total. The temperature was +10 °C during irradiation and -25 °C between irradiations.

Table 3.5. Fluences of polychromatic UV (200-400 nm) as received by samples of biofilms and planktonic cells depending on their position in the sample stack during the exposure to simulated space and Martian conditions. n = 1.

Sample position	UV fluence received (kJ m^{-2})			
	Space		Mars	
	Biofilms	Planktonic cells	Biofilms	Planktonic cells
top	1.2×10^2	2.2×10^2	1.1×10^2	2.1×10^2
bottom	4.5×10^1	8.3×10^1	4.4×10^1	8.1×10^1
dark	0	0	0	0

Following exposure to single space and Mars stressors, the viability of the cells was assessed in terms of culturability, membrane integrity, ATP levels, and the presence of 16S rRNA. The viability of the exposed cells was compared to the viability of desiccated but non-exposed controls, which were stored at room temperature in the dark for the duration of the experiment. For analysis, the samples were subdivided into three separate sets, each one including non-exposed controls if available. At the date of rehydration, samples had been kept in desiccated state for 162, 167, and 174 days, respectively, during which exposure to simulated space and Mars-like conditions was carried out for 31-32 days. When they were rehydrated and analysed after this period of time, all samples showed signs of viability.

Non-exposed biofilms and planktonic cells (i.e. controls) exhibited comparable levels of viability (Fig. 3.36). Colony-forming ability was conserved in 9.8% of the biofilm cells and in 6.8% of the planktonic cells. Similar to the single-stressor experiments (see 3.8.2) membrane integrity was maintained to a high degree. 87.7% of biofilm cells and 78.7% of planktonic cells stained propidium iodide-negative, thus seemed to be membrane-intact. The majority of cells showed evidence for the presence of 16S rRNA. 95.8% of biofilm cells and 95.1% of planktonic cells were FISH-positive. The mean ATP concentration in the biofilm samples was 1.4×10^{-19} mol $cell^{-1}$. ATP concentrations of planktonic samples ranged from 9.2×10^{-20} mol $cell^{-1}$ to 6.7×10^{-19} mol $cell^{-1}$, with a mean ATP concentration of 2.8×10^{-19} mol $cell^{-1}$.

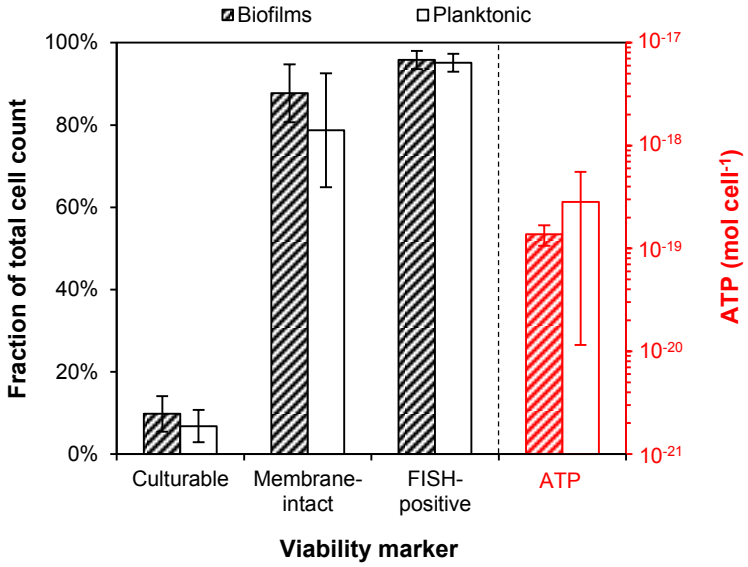

Figure 3.36. Viability of dried but non-exposed biofilms (shaded bars) and planktonic cells (white bars) of *D. geothermalis* used as references in the space and Mars simulation experiment.
Culturable cells, membrane-intact cells, and FISH-positive cells are illustrated as their fraction of total cell counts (black). ATP values are given as the absolute ATP concentration per cell (red). n = 4.

Exposure to simulated space conditions or Martian atmosphere in the absence of UV irradiation did not affect the survival of both biofilms and planktonic cells in comparison with non-exposed controls. In biofilms, the culturability of dark samples exposed to simulated space vacuum or Martian atmosphere was even higher (35% and 33% for space and Mars conditions, respectively) than the culturability of non-exposed controls (10%; Fig. 3.37).

Irradiation with polychromatic UV (200-400 nm) affected the culturability of *D. geothermalis*, but none of the other viability markers tested. Culturability clearly decreased with increasing UV fluences (Fig. 3.38). In biofilms, the highest UV fluence (110-120 kJ m^{-2}) caused the fraction of culturable cells to decrease from 35.2% to 1.3% under space conditions, and from 33.2% to 2.4% under Mars conditions. In planktonic cells, the highest UV fluence applied (210-220 kJ m^{-2}) reduced the fraction of culturable cells from 10.2% to 0.7% and from 13.3% to 0.7% under space and Mars conditions, respectively. Whilst the data may suggest that biofilms were more UV-tolerant than planktonic cells (Figs. 3.37, 3.39), it must be emphasised that planktonic samples received 1.8- to 2.6-fold higher fluences compared to biofilm samples (Tab. 3.5; Fig. 3.38). Apart from that, *D. geothermalis* seemed to have tolerated Mars conditions better than space conditions, although the observed differences were not significant (Figs. 3.37; 3.39).

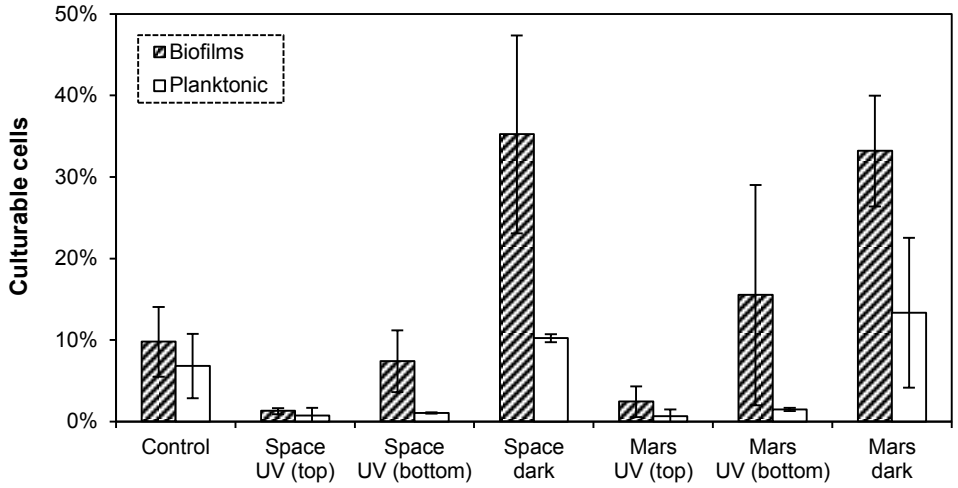

Figure 3.37. Fraction of culturable cells in biofilms (shaded bars) and planktonic cells (white bars) of *D. geothermalis* after desiccation and exposure to simulated space or Martian climate with or without irradiation with polychromatic UV (200-400 nm). The control was dried but non-exposed. n ≥ 2.

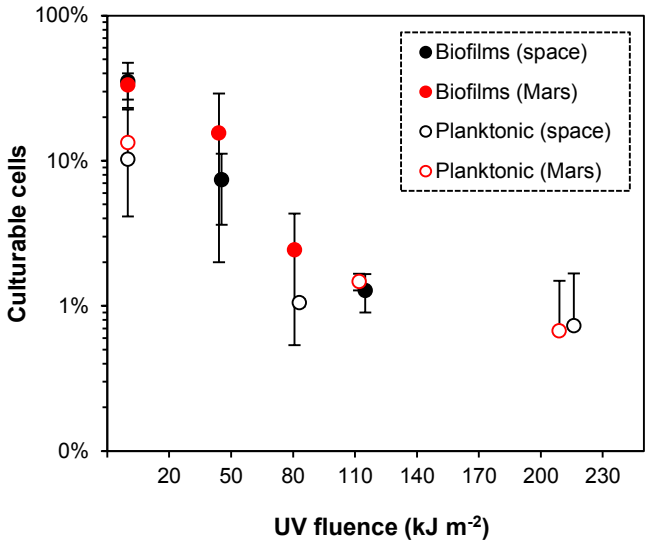

Figure 3.38. Correlation of the fluence of polychromatic UV radiation (200-400 nm) and the culturable fraction of biofilms (closed circles) and planktonic cells (open circles) of *D. geothermalis* exposed to simulated space (black symbols) or Mars (red symbols) conditions, respectively. n ≥ 2.

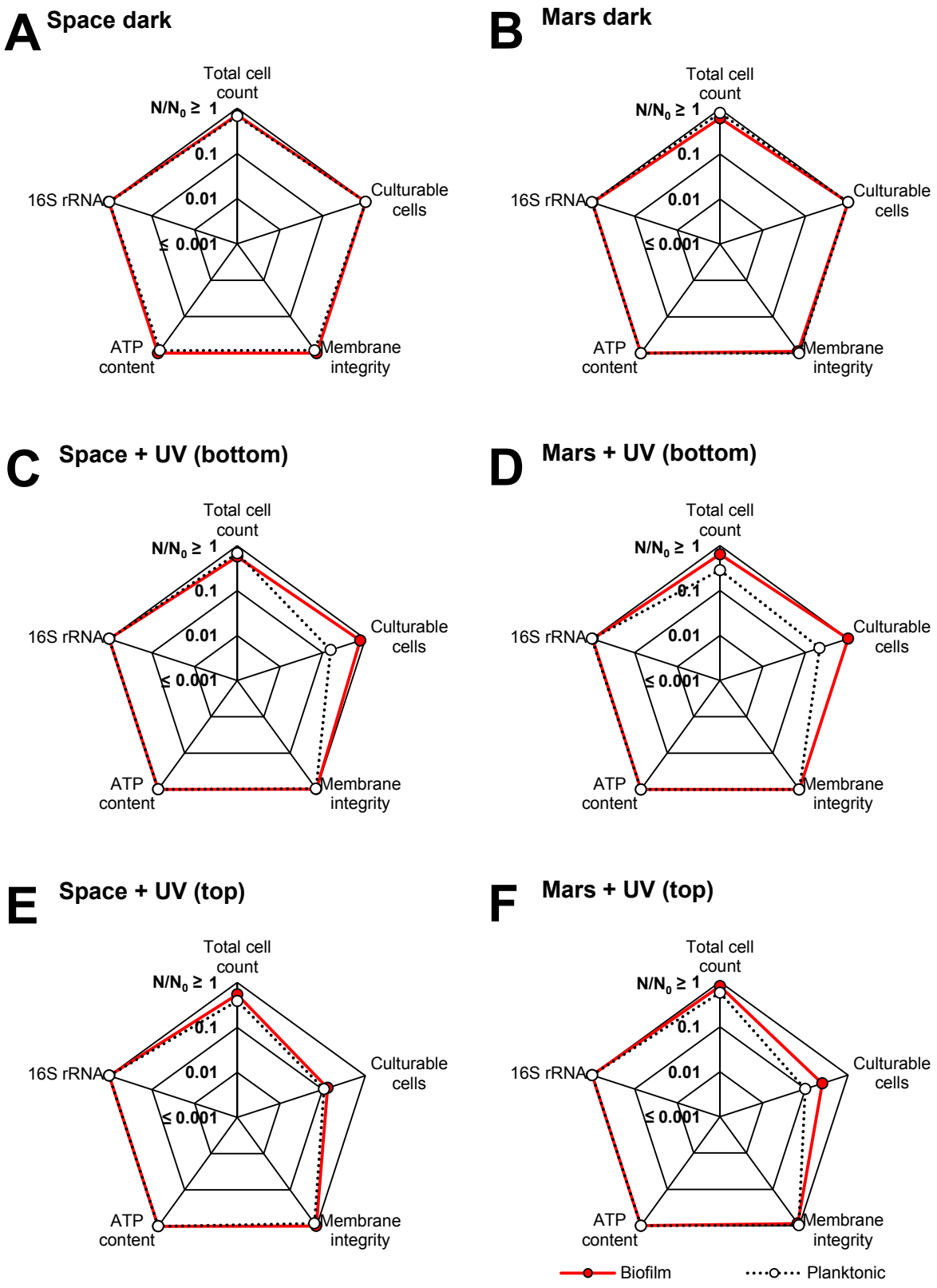

Figure 3.39. The effects of simulated space (left column) and Mars (right column) conditions on the viability of desiccated samples of biofilms (red circles) and planktonic cells (white circles) of *D. geothermalis* deposited on cellophane. Colony-forming ability, membrane integrity, ATP content, and presence of 16S rRNA were used as viability markers. Results were normalised (N/N_0) with respect to dried but non-exposed controls ($N_0 = 1$). $n \geq 2$.

In summary, *D. geothermalis* survived long-term desiccation for up to 174 days and – in addition to that – exposure to conditions simulating a 12-month stay in space or on Mars. Desiccation alone led to low levels of culturability in both biofilms and planktonic cells. Desiccated cells of *D. geothermalis* were found to be resistant to space-like temperatures and vacuum and to Mars-like atmospheric conditions, as viability did not further decrease upon exposure to these stressors. Polychromatic UV irradiation, however, caused a significant, fluence-dependent reduction in culturable cell numbers of both biofilms and planktonic cells, whilst cultivation-independent parameters indicated viability (VBNC). By trend, *D. geothermalis* seemed to be slightly less susceptible to UV radiation when exposed under Martian conditions compared to exposure under space conditions.

3.8.4 Viability of inactivated cells of *D. geothermalis* ('dead controls')

In order to relate signals of low viability obtained from stress experiments (see 3.8.1 to 3.8.3) to the actual state of viability, 'dead controls' (Davey, 2011) were generated: Samples of biofilms and planktonic cells of *D. geothermalis* were dried for 1 day or 2 months and treated with either heat (95 °C) or isopropyl alcohol (70% v/v). Afterwards, their viability was assessed in terms of culturability, membrane integrity, ATP content, and presence of 16S rRNA.

When cells of *D. geothermalis* dried for 1 day were killed, treatment with isopropyl alcohol proved to be more effective than treatment with heat, as lower levels of viability (particularly membrane integrity and ATP levels) were achieved (Fig. 3.40). When cells dried for 2 months were killed, treatment with isopropyl alcohol was even more effective in reducing viability, suggesting that desiccation increased the susceptibility of *D. geothermalis* to cellular damage by isopropyl alcohol. With respect to the heat treatment, differences in viability between cells dried for 1 day and cells dried for 2 months were inconspicuous. Whilst biofilms by trend seemed to be more strongly affected by the inactivation procedures than planktonic cells, differences in the viability of both cell types were insignificant.

Total cell counts remained unaffected by either inactivation treatment (Tab. 3.6). Both treatments, however, led to a loss of culturability. In all cases, the number of CFU ml^{-1} decreased from 10^7-10^8 to below the limit of detection of 10^2 CFU ml^{-1}. Membrane integrity was lost in > 99% of both biofilm and planktonic cells treated with isopropyl alcohol. Heat treatment was less effective than treatment with isopropyl alcohol: 2.2-3.8% of biofilm cells and 4.1-11.3% of planktonic cells remained membrane-intact after the treatment. Isopropyl alcohol treatment was also more effective in diminishing ATP levels. Whilst heat treatment caused a reduction of total ATP by one order of magnitude compared to an untreated control, treatment with isopropyl alcohol decreased the concentration of ATP by two (samples dried for 1 day) or three (samples dried for 2 months) orders of magnitude. Neither heat nor isopropyl alcohol treatment affected the number of FISH-positive cells, suggesting that the presence of 16S

rRNA remained unaffected by either inactivation procedure. Therefore, it seems as though 16S rRNA is an unsuitable target molecule for the detection of viability in *D. geothermalis*.

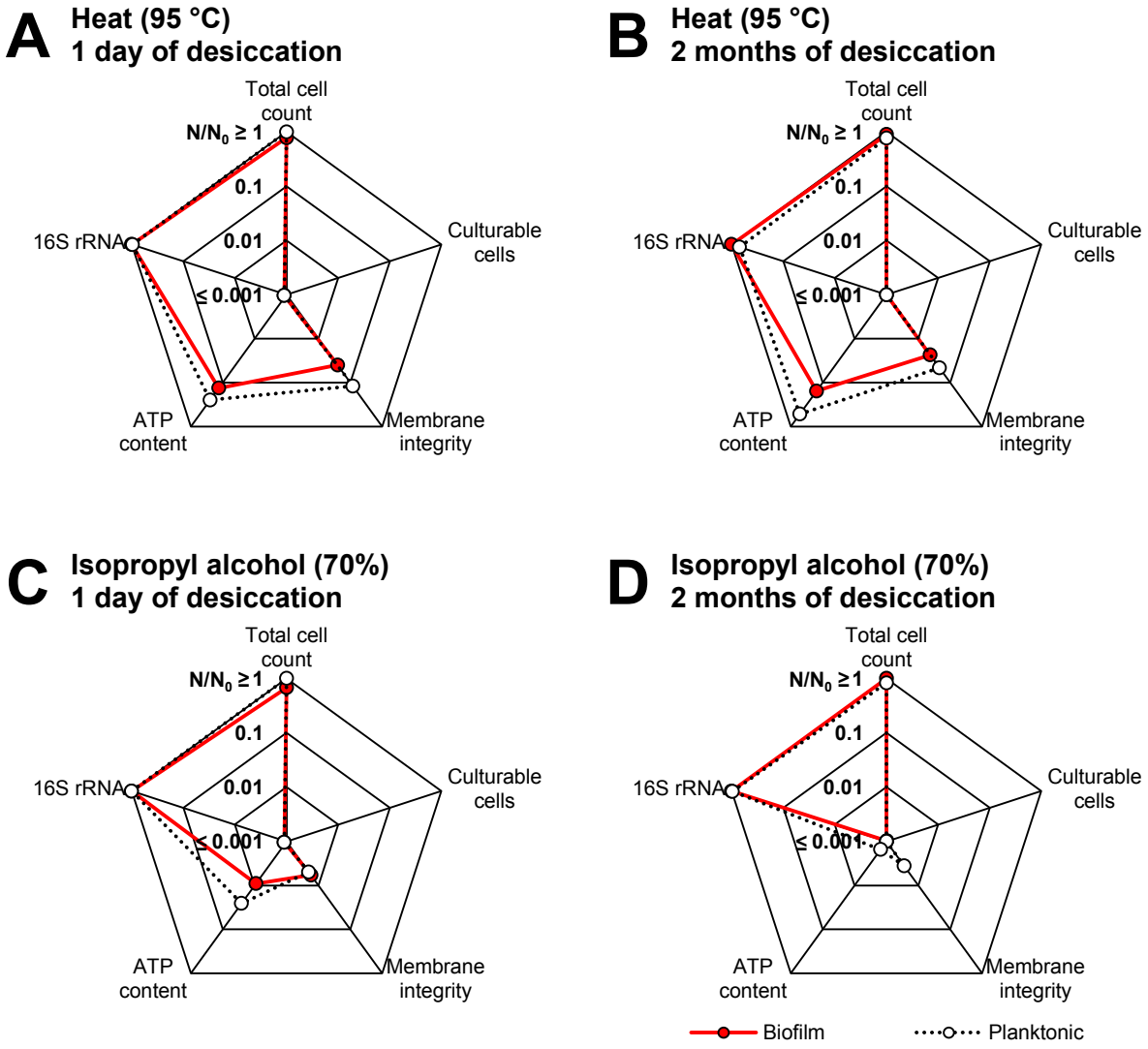

Figure 3.40. Viability of dead controls of biofilms (red circles) and planktonic cells (white circles) of *D. geothermalis* inactivated by treatment with heat (95 °C; **A**, **B**) and isopropyl alcohol (70%; **C**, **D**). Samples were desiccated for 1 day (left column) or 2 months (right column) prior to the inactivation procedure. Culturability, membrane integrity, ATP content, and presence of 16S rRNA were used as viability markers. Results were normalised with respect to non-treated controls dried for the same period of time as the treated samples (N_0 = 1). n = 3.

Table 3.6. Average viability of dead controls of biofilms and planktonic cells of *D. geothermalis*, dried for 1 day or 2 months, and treated with heat (95 °C) or isopropyl alcohol (70%). n = 3.

	Treatment	Control (untreated)		Heat (95 °C)		Isopropyl alcohol (70% v/v)	
	Desiccation period	1 day	2 months	1 day	2 months	1 day	2 months
Biofilms	Total cell counts (cells ml^{-1})	3.6×10^8	2.0×10^8	2.8×10^8	1.8×10^8	2.4×10^8	2.4×10^8
	Plate counts (cfu ml^{-1})	1.6×10^8	5.3×10^7	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$
	Membrane-intact cells (%)	94.5	93.7	3.8	2.2	0.6	0.0
	ATP (mol $cell^{-1}$)	7.8×10^{-19}	6.3×10^{-19}	1.0×10^{-19}	9.8×10^{-20}	7.1×10^{-21}	6.1×10^{-22}
	FISH-positive cells (%)	92.2	88.2	98.6	91.5	96.3	91.1
Planktonic cells	Total cell counts (cells ml^{-1})	3.2×10^8	3.7×10^8	3.5×10^8	2.9×10^8	3.6×10^8	3.1×10^8
	Plate counts (cfu ml^{-1})	4.5×10^7	1.9×10^7	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$
	Membrane-intact cells (%)	93.6	89.7	11.3	4.1	0.5	0.3
	ATP (mol $cell^{-1}$)	9.1×10^{-19}	4.7×10^{-19}	2.3×10^{-19}	2.5×10^{-19}	2.4×10^{-20}	7.2×10^{-22}
	FISH-positive cells (%)	94.8	91.9	91.8	66.6	95.7	91.9

4. DISCUSSION

The aim of this study was to test the hypothesis that the survival of *D. geothermalis* DSM 11300 is facilitated under desiccation and in simulated space and Martian environments, if the organism is organised in biofilms rather than being cultivated as individual, planktonic cells. In biofilms, microorganisms exhibit certain traits such as an elevated tolerance to environmental stressors such as dehydration (Anderson et al., 2012; Or et al., 2007; Roberson & Firestone, 1992; Tamaru et al., 2005), which are missing in planktonic cells of the same species (van de Mortel et al., 2004). Many properties that are unique to biofilm-inhabiting microorganisms can be attributed to the presence of a self-developed EPS matrix which surrounds the cells (Flemming & Wingender, 2010).

Biofilms are complex and dynamic systems characterised by spatial and temporal heterogeneity (Stewart & Franklin, 2008). The first goal of this study was to generate single-species model biofilms of *D. geothermalis*, which could be reproduced reliably. It was decided to grow water-unsaturated biofilms at the solid-air interface rather than submerged, fully-hydrated biofilms, as they represent more likely candidates for life within meteorites or in Martian environments where liquid water is scarce. Different cultivation conditions and support materials were tested in order to find optimum conditions for biofilm growth and sample handling. Biofilms were eventually grown for 2 days at 45 °C on R2A, using CME membranes or cellophane discs as a support. More biomass was obtained when using TSA instead of R2A as a nutrient medium. However, under these conditions, *D. geothermalis* formed tenacious cell aggregates which impeded further analysis and could only be dispersed by treatment with proteinase K.

The EPS of *D. geothermalis* were isolated using the CER Dowex®. Proteins and polysaccharides were abundant, but also eDNA was found in relatively low amounts in the EPS. Evidence obtained from ESEM suggested that the EPS matrix covered the uppermost layer of cells in biofilms. Although significant amounts of EPS were extracted from planktonic cultures, no such superficial EPS layer was evident in membrane-deposited planktonic cells. Lectin staining showed that at least three galactoside-containing polysaccharide fractions of

different distribution and arrangement were present within the biofilms, as concluded from the inhibition sugars of those lectins.

Biofilms and planktonic cells of *D. geothermalis* were desiccated in ambient air. Dried cells were further exposed to a range of individual stressors relevant for space and Mars, including vacuum, artificial Mars atmosphere, and extreme temperatures, and to simulated space and Mars conditions. Following exposure, the survival of the cells was determined in terms of plate counts and the cultivation-independent viability markers membrane integrity, ATP, and 16S rRNA (see Figs. 3.32, 3.39). *D. geothermalis* remained viable under all conditions tested, including desiccation for at least 2 months. Desiccation and UV radiation were identified as the most harmful stressors, as both caused a significant reduction in the culturability of the organism. Following exposure to stress, biofilms maintained significantly higher levels of viability than planktonic cells.

4.1 Biofilms of *D. geothermalis* – growth and characteristics

D. geothermalis was used as a test organism in this study due to its extraordinary tolerance to desiccation and radiation (Tian & Hua, 2010), which makes it an eligible candidate for surviving in space and Martian environments where these stressors are dominating (Horneck et al., 2010; Nicholson, 2009; Westall et al., 2013). The first goal of this study was to develop a model biofilm of *D. geothermalis* to fit both the experimental setup and the project hypothesis.

Firstly, the samples had to be suitable for the exposure to ground-based space and Mars simulations on the one hand, and to genuine space conditions with respect to the space experiment BOSS on the other hand. This turned out to be a challenging task, as the substratum used for the biofilm to grow on simultaneously had to function as a transportable carrier material supporting the overlying biofilm during exposure experiments. Particularly during exposure to UV radiation – either in the laboratory or in LEO – the support had to meet several requirements (e.g. UV resistance, non-toxicity, suitability as a substratum for biofilm formation), what limited the choice of materials available.

Secondly, the samples had to represent a model for microbial life travelling through space during a lithopanspermia event. In theory, a lithopanspermia event starts with the spallation of life-bearing rock into space (Nicholson, 2009), implying that endolithic microorganisms living inside the ejected rock would be the most likely candidates to be dispersed into space (Meyer et al., 2011). On Earth, microorganisms living in soil and rock are commonly organised in unsaturated biofilms (Auerbach et al., 2000; Chang & Halverson, 2003), which, thus, could represent a valid model for microbial life travelling inside meteoroids. During space travel, the model biofilm would be dehydrated due to vacuum and the absence of liquid water (Mileikowsky et al., 2000b). Microbial growth would therefore cease to occur once the organisms are ejected from the donor planet (not taking into account the re-

activation of the organisms after the landing on a life-sustaining recipient planet). The same theoretical considerations apply to life on Mars. The conditions on the surface of present Mars are considered highly hostile to life. Intense solar radiation and high concentrations of surface-associated perchlorates quickly destroy organic molecules (de la Vega et al., 2007; Nicholson, 2009; Yen, 2000), forcing microbial life if at all to exist in the subsurface (Gómez et al., 2010). Due to both low surface temperature and pressure, liquid water is estimated to be only transiently available – either in the form of downslope run-offs or as brines in the uppermost few centimetres of the subsurface (Bodnar, 2001; Martín-Torres et al., 2015; Ojha et al., 2015; Squyres et al., 2004; Tosca et al., 2008) – suggesting that Martian biofilms would more likely exist in an unsaturated and endolithic state in the subsurface.

Previous studies have reported that *D. geothermalis* forms biofilms on a range of abiotic surfaces, including polystyrene (Kolari et al., 2001; Kolari et al., 2002), stainless steel (Kolari et al., 2003; Kolari et al., 2001; Kolari et al., 2002; Peltola et al., 2008; Raulio et al., 2006), glass (Kolari et al., 2002; Peltola et al., 2008; Raulio et al., 2006; Saarimaa et al., 2006), and titanium (Raulio et al., 2006). However, these studies exclusively produced water-saturated biofilms by submerging the above listed materials in liquid nutrient media such as R2B or tryptic soy broth (e.g. Kolari et al., 2002; Saarimaa et al., 2006). To the author's knowledge, so far no attempt has been made to generate biofilms of *D. geothermalis* in an unsaturated environment.

In order to produce unsaturated biofilms of *D. geothermalis*, CME membranes were inoculated by membrane filtration and placed on nutrient agar. Incubation caused the growth of colonies on the upper surface of the CME membrane which eventually coalesced into a confluent biofilm. The use of CME membranes in combination with membrane filtration is a common method for the identification of bacterial contaminations in drinking water (Schindler, 2008), and it was used to isolate *D. geothermalis* from spring water in the first place (Ferreira et al., 1997). The present study demonstrated that the organism is able to form unsaturated biofilms at the solid-air interface of these membranes. Additionally, the formation of biofilms on ceramics, glass, glass fibre filters, polycarbonate membranes, and cellophane was detected, broadening the range of abiotic surfaces *D. geothermalis* has been known to adhere to and multiply on.

4.1.1 Growth of *D. geothermalis* under selected cultivation conditions

Almost two decades after its first description by Ferreira et al. (1997), the physiology and growth behaviour of *D. geothermalis* is still not comprehensively understood. In order to find optimum conditions for the generation of unsaturated biofilms, different cultivation conditions were tested.

Two different incubation temperatures, 37 °C and 45 °C, were tested for the generation of *D. geothermalis* biofilms. Growth was faster at an incubation temperature of 45 °C com-

pared to 37 °C. This is not surprising, as the growth optimum of *D. geothermalis* was reported to be in the range of 45 °C to 50 °C (Ferreira et al., 1997). This observation was confirmed in two recent studies by Bornot et al. (2015; 2014b), who almost doubled the growth rate (from 0.33 h^{-1} to 0.64 h^{-1} and from 0.47 h^{-1} to 0.79 h^{-1}, respectively) by increasing the incubation temperature from 37 °C to 45 °C.

The initial cell density of the inoculum that was applied to the support materials prior to biofilm formation was varied. Biofilm yield after 48 h of incubation increased with increasing inoculum size, i.e. biofilms seemed to grow faster if the initial cell density was higher. Many microbial actions are cell density-dependent, as they are controlled by cell-to-cell communication using signalling molecules (Li & Tian, 2012). Intercellular signalling can induce morphological or structural changes in biofilms. For example, it is involved in the formation of aerial hyphae in the soil bacterium *Streptomyces coelicolor* and the development of fruiting bodies in *Myxococcus* (Kaiser & Losick, 1993). In many bacteria such as *P. aeruginosa* (Davies et al., 1998), *Vibrio cholerae* (Hammer & Bassler, 2003), and streptococci (Parsek & Greenberg, 2005; Suntharalingam & Cvitkovitch, 2005) it influences biofilm formation and development. In *D. geothermalis*, a higher initial cell density could positively influence biofilm formation due to increased concentrations of signalling molecules, particularly in unsaturated environments in which the transport of these compounds is attenuated in comparison to aqueous environments (Chang & Halverson, 2003).

The biofilm formation of *D. geothermalis* was compared on the three nutrient media R2A, TSA, and *Thermus* 162 medium, all of which have been used before for the cultivation of the organism (Bornot et al., 2015; Ekman et al., 2007; Ferreira et al., 1997; Kolari et al., 2001; Peltola et al., 2008). *D. geothermalis* formed unsaturated biofilms on all three media. Biofilm formation and yield, however, differed with the nutrient media used. On R2A, biofilms grew fast as they reached their maximum yield after 24 h of incubation at 45 °C, and biomass was not increased by further incubation. On *Thermus* 162 medium, biofilms seemed to grow slower; an incubation period of 48 h at 45 °C was necessary to obtain a yield comparable with R2A-grown biofilms grown at the same temperature within 24 h. Initial growth was also slow on TSA. After 24 h of incubation, biofilm yield was in most cases significantly less compared to biofilms grown on R2A. After 48 h, however, more biomass was produced on TSA than on any of the other media tested. All media tested had a similar pH in the range of 7.2 to 7.3, ruling out pH-dependent effects. It is conceivable that the extent of biomass production was dependent on the composition of the respective nutrient medium. All three media contain one or more complex sources of carbon and nitrogen, and growth factors such as amino acids and vitamins, but their total composition is quite different. With respect to complex carbon and nitrogen sources, R2A contains yeast extract (0.5 g l^{-1}), peptone (0.5 g l^{-1}), and casamino acids (0.5 g l^{-1}), TSA contains peptone from casein and soymeal (20 g l^{-1}), and *Thermus* 162

medium contains yeast extract (1.0 g l^{-1}) and tryptone (1.0 g l^{-1}). All these compounds have been found suitable for the cultivation of *D. geothermalis* (Bornot et al., 2014a; Bornot et al., 2015; Bornot et al., 2014b; Ferreira et al., 1997; Liedert et al., 2012).

Apart from three different complex nutrient sources, R2A medium contains glucose, starch, and pyruvate, which can be utilised by *D. geothermalis* (Bornot et al., 2014a; Bornot et al., 2014b; Ferreira et al., 1997). Particularly the presence of glucose and pyruvate, which were shown to be readily taken up by *D. geothermalis* during the first few hours of growth (Bornot et al., 2014b; Liedert et al., 2012), could explain the fast biofilm formation on R2A. Starch is not consumed until late exponential growth (Liedert et al., 2012), but may serve as an alternative carbon source during stationary phase-growth. Kolari (2003) showed that supplementation of white water growth medium with both glucose and starch increased the biofilm yield of *D. geothermalis* E50051 by up to 56%. As another constituent of R2A, yeast extract was reported to be preferred carbon source of *D. geothermalis* and the sole nutrient that allows exponential and unlimited growth in fed-batch liquid cultures (Bornot et al., 2014a; Bornot et al., 2015; Bornot et al., 2014b). Under the cultivation conditions used here, however, growth was limited to a maximum cell density of approximately 2-4 × 10^8 cells cm^{-2}, possibly due to localised nutrient deprivation, the build-up of metabolic waste products, and/or nutrient transport limitations (Chang & Halverson, 2003). The effect of the mineral content of the medium (dipotassium phosphate and magnesium sulphate) on the growth of *D. geothermalis* remains unknown.

Thermus 162 medium contains yeast extract and tryptone, both acting as complex nutrient sources for carbon, nitrogen, and growth factors. Yet, growth on *Thermus* 162 medium was slower than on R2A, possibly due to the absence of simple carbonaceous compounds such as glucose. Although yeast extract has been reported to be one of the carbon sources favoured by *D. geothermalis* (Bornot et al., 2014b), its uptake was shown to be retarded during diauxic growth. When yeast extract was offered as a complex carbon source next to glucose to a liquid culture of *D. geothermalis* DSM 11302, the uptake of yeast extract began after 1.4-2.6 h of culture, then increasing exponentially, whereas glucose was utilised almost immediately after start of the experiment (Bornot et al., 2014b). The same study emphasised that both glucose and yeast extract are necessary to ensure optimal growth. *Thermus* 162 medium does contain citrate, a possible carbon source and an intermediate of the citric acid cycle, but the compound is not utilised by the test organism (Ferreira et al., 1997). The medium further contains a broad range of mineral trace elements such as manganese sulphate, boric acid, copper sulphate, and cobalt chloride (see Tab. 2.1), none of which were reported to facilitate growth of *D. geothermalis* (Bornot et al., 2014a). Medium supplementation with manganese was even shown to inhibit growth (Bornot et al., 2014a), which is interesting considering the importance of high intracellular manganese concentrations in deinococcal stress resistance (Daly

et al., 2007; Daly et al., 2004; Ghosal et al., 2005; Liedert et al., 2012). Although the *Thermus* 162 medium was initially recommended for the cultivation of *D. geothermalis* (DSMZ, 2015; Ferreira et al., 1997), it seems less suitable than R2A. A recent study which tested the growth of three different strains of *D. geothermalis* (DSM 11300, DSM 11301, DSM 11302) in *Thermus* 162 broth confirmed this observation (Bornot et al., 2015).

Peptone represents the sole nutrient source in TSA, its other constituents being sodium chloride and agar. The comparatively high concentration of peptone could explain the large amount of biomass produced on this medium, as nutrient deprivation is less likely compared to R2A and *Thermus* 162 medium. Similar to *Thermus* 162 medium, the absence of readily utilisable nutrients, in particular glucose (Bornot et al., 2014b), could explain the slow initial growth of *D. geothermalis* on TSA, as the cells would have to adjust to the offered substrates. It also has to be taken into consideration, that all preparatory cultures were maintained on R2A, possibly leaving the organisms primed for this particular medium. So far, yeast extract has been considered a necessity for growing *D. geothermalis* exponentially, and growth was reported to be poor without yeast extract supplementation (Bornot et al., 2014a; Bornot et al., 2014b; Kongpol et al., 2008). Interestingly, in this study *D. geothermalis* produced the largest amounts of biomass on TSA, the only medium tested which lacks yeast extract but provides an excess of peptone, whereas biofilm growth seemed indeed limited on those media which contain yeast extract, i.e. R2A and *Thermus* 162 medium. The results suggest that the complex peptone mixture from casein and soybeans contains growth factors similar to those contained in yeast extract (e.g. amino acids, vitamins, peptides). They are unlikely to be essential for growth of *D. geothermalis*, as cultivation on defined minimal media is possible (Bornot et al., 2014a). Growth of *D. geothermalis* does not strictly depend on provision with exogenous amino acids (Brim et al., 2003), but it might be stimulated by it (Ferreira et al., 1997). For *D. radiodurans*, a rich supply with amino acids was shown to support growth and increase the radiation resistance of the organism (Venkateswaran et al., 2000). The growth factor(s) contained in peptone might facilitate growth by (i) acting as a catalyst, (ii) by scavenging toxic compounds in the culture, and/or (iii) by providing precursor molecules for enzymes (Bornot et al., 2015; Koser & Saunders, 1938).

This study represents the first protocol for the generation of artificial unsaturated biofilms of D. geothermalis on different abiotic materials. In agreement with observations made by Bornot et al. (2014b), the provision with both glucose and yeast extract was shown to improve the growth of *D. geothermalis* (as it was the case for R2A) compared to growth on yeast extract without glucose addition (as it was the case for *Thermus* 162). However, biomass production was even higher when only peptone was provided, suggesting that the growth factors contained in yeast extract that facilitate growth of *D. geothermalis* are also abundant in peptone, or that peptone offers even better conditions for the growth of *D. geothermalis* than does

yeast extract. This represents a novel observation, as so far yeast extract has been considered necessary for the proliferation of *D. geothermalis* under laboratory conditions (Bornot et al., 2014a; Bornot et al., 2015; Bornot et al., 2014b).

4.1.2 Effect of nutrient supply on biofilm phenotype

Depending on whether R2A or TSA was used as a nutrient medium, *D. geothermalis* formed biofilms that differed significantly in (i) their colouration, (ii) their tendency to form cell aggregates, (iii) their adhesive properties, (iv) the quantitative composition of their EPS, and (v) the spatial arrangement of their EPS.

The distinctive colour of *D. geothermalis* biofilms, which appeared as light pinkish when cultivated on R2A, but in a more intense orange when cultivated on TSA, is likely due to carotenoid pigments such as the deinoxanthin in *D. radiodurans*, that are incorporated into the cell envelope of the organism (Thompson & Murray, 1981; Tian & Hua, 2010). Whilst *D. geothermalis* seems to possess several genes necessary for carotenoid biosynthesis (Makarova et al., 2007; Tian & Hua, 2010), the pigment(s) produced by the organism have not been characterised, yet. Thus, it cannot be concluded, whether the differential pigmentation of *D. geothermalis* cultivated on either R2A or TSA is due to the fact that different pigments are be expressed on the two media, or due to other reasons which may result in a different colouration (e.g. different pigment concentrations, colour alteration by overlying EPS layers). Since the deinococcal pigments are considered to protect the cells from UV radiation and ROS (Gao & Garcia-Pichel, 2011; Tian & Hua, 2010; Tian et al., 2007), the two biofilm phenotypes observed here might exhibit differential stress tolerances in that respect.

Both biofilm types exhibited distinctive adhesive properties. In both cases, biofilms were sticky when manipulated with standard laboratory tools, which has been observed before in *D. geothermalis* E50051 (Kolari, 2003; Kolari et al., 2002; Raulio et al., 2006). In this case, the stickiness of deinococcal cells has been attributed to adhesive structures distributed heterogeneously over the cell surface (Kolari et al., 2002), later to be characterised as type IV pili (Saarimaa et al., 2006). R2A- and TSA-grown biofilms differed in their adhesive behaviour in that R2A-grown biofilms – in contrast to TSA-grown biofilms - adhered tenaciously to the substratum, whereas TSA-grown biofilms showed a high degree of cell-to-cell adhesion, leading to the formation of amorphous cell aggregates resistant to dispersal by common laboratory means such as shaking on a vortex mixer.

In many bacteria, including human pathogens such as *E. coli*, *P. aeruginosa*, or *Staphylococcus aureus*, adhesion is mediated by adhesive proteins, so called adhesins or lectins (Johnson, 1999; Pieters, 2011). These adhesion factors are located on the cell surface or on cell appendages such as pili or fimbriae and induce adhesion by interaction with receptors containing specific carbohydrate moieties (Millsap et al., 1998; Pieters, 2007). Bacteria may

produce multiple adhesins, some of which can be regulated on a transcriptional level (Dunne, 2002), allowing the organism to switch from planktonic to sessile mode, but also to use different adhesins under different environmental conditions (Dunne, 2002). *V. cholerae*, for example, is able to switch between toxin-coregulated type IV pili and a mannose-sensitive hemagglutinin pili for the adhesion to different substrates (Watnick et al., 1999). Due to differences in the amount and quality of nutrients provided, *D. geothermalis* could express different adhesins, resulting in the differential adhesion behaviours observed.

In submerged biofilms of *D. geothermalis* E50051, cell-to-cell as well as cell-to-substrate adhesion seem to be mediated by thread-like cell appendages described as type IV pili, which consist of glycosylated proteins (Peltola et al., 2008; Raulio et al., 2006; Saarimaa et al., 2006). These adhesion threads could not be observed in this study when analysing the surface morphology of dried unsaturated biofilms of *D. geothermalis* DSM 11300 using ESEM. Evidence for the presence of adhesion threads, however, was obtained when *D. geothermalis* biofilms were stained with fluorescently-labelled lectins. Lectin ACA, which is specific for galactose β-1,3-linked to N-acetylneuraminic acid and to N-acetylgalactosamine, has been reported to exclusively stain adhesion threads in *D. geothermalis* E50051 (Peltola et al., 2008; Saarimaa et al., 2006). In this study, ACA lectin identified a dense meshwork of thread-like EPS at the base of R2A-grown biofilms, that was absent in upper regions of the biofilm. This could indicate the presence of adhesion threads, connecting the lowermost layers of cells to the substratum, which would explain the tenacious adhesion of R2A-grown biofilms to CME and cellophane. In TSA-grown biofilms, ACA lectin stained the surface of the cells, suggesting the presence of a target structure involved in intracellular adhesion in close proximity to the cell envelope.

The addition of compatible carbohydrates in adequate concentrations can lead to a destabilisation of protein-carbohydrate interactions, thus diminishing adhesin-mediated bacterial attachment (Pieters, 2011). When mannose at a concentration of 10 mM was administered to suspensions of TSA-grown *D. geothermalis*, aggregation was slightly reduced, suggesting that mannose might play a role in the intercellular adhesion of the organism. Mannose-specific adhesins are, for example, associated with type I pili in *E. coli*, which in this case are involved in the attachment to host tissue (Jones et al., 1995; Krogfelt et al., 1990). Staining of *D. geothermalis* biofilms with ACA lectin identified the presence of galactosides (galactose-β-1,3-N-acetylneuraminic acid, galactose-β-1,3-N-acetylgalactosamine) on the surface of TSA-grown cells. Galactosides are ligands commonly involved in bacterial aggregation (Millsap et al., 1998). In *P. aeruginosa*, galactose-β-1,4-N-acetylgalactosamine represents the ligand specific for type IV pili-based attachment (Schweizer et al., 1998). *P. aeruginosa* utilises type IV pili to adhere to human lung tissue, but also to abiotic surfaces such as stainless steel or polystyrene (Giltner et al., 2006). In a previous study, hyaluronic acid and polygalacturonic

acid inhibited the cell-to-cell attachment of *D. geothermalis* E50051, whereas mannose did not (Kolari, 2003). The author proposed that the intercellular adhesion of *D. geothermalis* is carbohydrate-mediated, and that uronic acids may be the essential ligands responsible for adhesion. The presence of uronic acids in biofilms of *D. geothermalis* DSM 11300, however, could not be verified in the present study. Strain-to-strain differences might be an explanation for differential modes of adhesion. *D. geothermalis* E50051 has been isolated from industrial paper mill water (Väisänen et al., 1998), whereas *D. geothermalis* DSM 11300 has been found in hot springs in Italy (Ferreira et al., 1997). Despite belonging to the same species, *D. geothermalis* strains can differ significantly in their physiological properties (Bornot et al., 2015).

When peptone was provided as a nutrient source – either in form of TSA (20.0 g l^{-1}) or as peptone-spiked R2A (1.5-20.0 g l^{-1}) – the cells in *D. geothermalis* biofilms formed highly cohesive aggregates whilst neglecting adhesion to the substratum (particularly when grown on TSA). The spatial arrangement of TSA-grown biofilms was feeble; pipetting drops of water onto the biofilm already caused significant proportions of the biomass to slough off from the substratum. Hence, although not being cultivated in liquids, TSA-grown biofilms seemed to exhibit traits of planktonic flocs such as an elevated hydrophobicity and lack of substrate attachment (Flemming et al., 2000; Mahendran et al., 2012). Interestingly, Kolari (2003) mentioned that nitrogen-containing compounds such as peptone or tryptone diminished biofilm formation but enhanced planktonic growth in *D. geothermalis*, and that peptone concentrations of > 3.3 g l^{-1} caused the transition of all cells from biofilm to planktonic mode. Vice versa, the nutrient-poor medium R2(B) reportedly facilitates biofilm growth of *D. geothermalis* in contrast to rich media (Liedert et al., 2012). Also in *B. subtilis*, nutrient limitation was shown to stimulate biofilm formation (Zhang et al., 2014). It seems that the availability of nitrogen sources or amino acids might be an important factor in the differentiation of *D. geothermalis*.

This study clearly demonstrated that proteins are involved in the cohesion of TSA-grown cell aggregates, since treatment of the aggregates with the proteolytic enzymes pronase E and proteinase K diminished aggregation. Treatment with proteinase K was significantly more effective and – in contrast to treatment with pronase E – did not lyse the cells, suggesting that the proteins essential for aggregation were present extracellularly (i.e. in the form of EPS or cell appendages). In line with that, Kolari et al. (2002) briefly mentioned that the adhesion threads of *D. geothermalis* E50051 were sensitive to subtilisin-type proteases. Also in other bacteria such as *Bacillus cereus*, *Listeria monocytogenes*, *P. aeruginosa*, *S. aureus*, or *S. epidermis*, proteases have successfully been used to interfere with attachment or to disperse pre-existing biofilms (Boles & Horswill, 2011; Morvay et al., 2011; Nguyen & Burrows, 2014). Altogether, these results support the theory that proteinaceous EPS or adhesins associated with type IV pili or the cell surface mediate the cell-to-cell attachment of *D. geothermalis*.

This has been presumed for strain *D. geothermalis* E50051 (Kolari et al., 2002; Saarimaa et al., 2006), but has not been observed yet in the type strain *D. geothermalis* DSM 11300 investigated here.

4.2 The EPS of *D. geothermalis*

4.2.1 EPS isolation

One objective of this work was to isolate and analyse the EPS of *D. geothermalis*. To the author's knowledge, no information is available on the successful isolation of EPS from type strain DSM 11300, investigated here. The same applies to other species of the *Deinococcaceae*. Solely for *D. geothermalis* E50051, Saarimaa et al. (2006) successfully isolated extracellular, cell surface-associated proteins and cell appendages characterised as type IV pili by suspending the cells in buffer containing 1 mM EDTA, followed by vigorous shaking, centrifugation, and precipitation of proteins.

The nature and biochemical composition of EPS is influenced by a range of factors such as the EPS-producing species, the present environmental conditions (e.g. availability of nutrients and water), or the age of the biofilm. Thus, a universal EPS isolation method does not exist. Various procedures allow for the separation of EPS and the cells embedded within by physical or by chemical means (Wingender et al., 1999). Chemical EPS isolation methods usually provide higher extraction yields than do physical methods, but tend to damage bacterial cells, leading to an overestimation of the EPS by intracellular material. Furthermore, the chemicals used as extraction agents can contaminate or chemically alter EPS, thus limiting the possibilities to quantify these EPS (D'Abzac et al., 2010; Liu & Fang, 2002). Choosing a suitable technique for the isolation of the EPS in question is paramount, as the efficacy of the EPS isolation step determines the outcome of subsequent analyses (Michalowski, 2012).

Five physical and chemical EPS isolation methods commonly used to extract EPS from bacterial cultures, activated sludge, or drinking water biofilms (D'Abzac et al., 2010; Douterelo et al., 2014; Liu & Fang, 2002; Michalowski, 2012; Wingender et al., 2001; Zhang et al., 1999) were tested for their suitability in isolating EPS from *D. geothermalis* DSM 11300: Heating (70 °C for 1 h), shaking (30 min), shaking in the presence of CER Dowex®, EDTA treatment, and combined formaldehyde/sodium hydroxide treatment, all followed by high-speed centrifugation (20,000 × g). Centrifugation itself represents a physical EPS isolation method (Wingender et al., 2001) used to separate loosely-bound EPS from bacterial cultures (Nielsen & Jahn, 1999), but further treatments are usually necessary to extract the tightly-bound capsular EPS (Wingender et al., 2001). Proteins, polysaccharides, and eDNA were found in the isolated EPS. As observed before (D'Abzac et al., 2010), the concentrations of each sum parameter were found to vary with the type of isolation method used.

Heat. Heat treatment showed the highest extraction efficacy amongst the physical extraction methods with respect to proteins and polysaccharides. Similar observations were made by D'Abzac et al. (2010), who found that heating was superior to sonication or CER treatment when isolating EPS from sludge. Heating may disrupt the EPS of *D. geothermalis* more effectively than shaking or CER, what might be an explanation for the comparatively higher values (D'Abzac et al., 2010). On the other hand, heating is considered a relatively harsh extraction method, as it promotes cell lysis (McSwain et al., 2005). Increased cell lysis is undesirable, because the contamination of EPS with cell constituents leads to an overestimation of EPS (D'Abzac et al., 2010). Nucleic acids are regularly used as a marker to identify abnormal cell lysis when found in EPS (D'Abzac et al., 2010). In this study, heating did not extract significantly more DNA from *D. geothermalis* biofilms than did shaking, a considerably more mild method, suggesting that cell lysis was not promoted by heating. Then again, the use of nucleic acids as a marker for cell lysis might not be ideal and should be used with caution, as they are by now considered an integral part of the EPS matrix, in some cases also associated with structural functions (Flemming & Wingender, 2010). Heating, however, notably reduced both total cell count and culturability, indicating that cells were indeed harmed during treatment. Wingender et al. (2001) recommend using the intracellular enzyme glucose-6-phosphate dehydrogenase as a marker for cell lysis by measuring its activity in the EPS solution. This method should be used in future experiments in order to shed more light on the tolerance of *D. geothermalis* to cell lysis during EPS isolation, provided that the enzyme is not damaged by heat treatment (McSwain et al., 2005).

Shaking. Of all five methods tested, shaking can be considered the least deleterious, as the separation of cells and EPS is pursued by the application of shear forces (Frølund et al., 1996). In agreement with this, cell lysis was not found to be promoted. Out of all methods tested, shaking extracted the least amount of EPS from *D. geothermalis* biofilms. This is not surprising, as mild EPS extraction procedures are generally less efficient than harsh (chemical) methods (D'Abzac et al., 2010).

Cation-exchange resin. EPS, if charged, may be stabilised by cross-linking via multivalent ions (Flemming & Wingender, 2010). This stabilising effect can be diminished by the addition of CER which scavenge divalent cations such as Ca^{2+} or Mg^{2+} under release of the monovalent cation Na^{+} (D'Abzac et al., 2010; McSwain et al., 2005). CER are commonly used for EPS isolation, for example from sludges, where they effectively aid in the disruption of flocs (D'Abzac et al., 2010; Frølund et al., 1995; Frølund et al., 1996; Liu & Fang, 2002; McSwain et al., 2005). In agreement with previous observations (D'Abzac et al., 2010), EPS extraction using CER Dowex® (in combination with shaking) did not seem cause extensive cell lysis, as total cell counts and plate counts remained stable during treatment. However, compared to other methods, the extraction efficacy of proteins and polysaccharides by CER was

low and EPS yield was similar to the EPS yield obtained by shaking only. This suggests that a) shaking is sufficient to destabilise the EPS matrix of *D. geothermalis*, b) cations are not involved in stabilisation of *D. geothermalis* EPS, c) *D. geothermalis* EPS do not contain negatively-charged polymers that permit bridging by multivalent ions, and/or d) polymers are positively-charged and interlinked by negatively charged molecules. Kolari (2003) found evidence for negatively-charged polysaccharides in the EPS of *D. geothermalis* strain E50051 based on observations made from Alcian blue staining and the successful inhibition of intercellular adhesion using uronic acid-containing carbohydrates. The present study, however, could not confirm these observations; the presence of uronic acids in *D. geothermalis* DSM 11300 biofilms was ambiguous. Dowex®-extracted EPS from TSA-grown biofilms contained the highest concentrations of DNA amongst all methods tested. Since abnormal cell lysis by CER-mediated extraction is unlikely, this could represent evidence for eDNA in *D. geothermalis* EPS. DNA molecules are negatively charged due to phosphate groups located along the DNA backbone (Englander et al., 2004). Cation bridging of eDNA that is released upon CER treatment could explain the high DNA concentrations measured.

EDTA. Similarly to CER, EDTA treatment facilitates EPS extraction by scavenging cations which stabilise negatively-charged EPS by cross-linking. Amongst all extractions, isolation of *D. geothermalis* EPS by EDTA resulted in the highest protein yields by far. This, however, is likely to be an overestimation. EDTA is notorious for interfering with colorimetric protein quantification assays: With respect to the Lowry assay, which was applied here to measure the protein concentration in the EPS, EDTA can reduce the phosphomolybdate contained in the Folin-Ciocalteu's phenol reagent (Neurath, 1966; Peterson, 1979) and form complexes with EPS constituents (D'Abzac et al., 2010; Liu & Fang, 2002). Both mechanisms may potentially alter the sample absorbance during photometric analysis, causing under- or overestimation of the protein concentration (D'Abzac et al., 2010). Hence, removal of EDTA prior protein analysis is paramount (Douterelo et al., 2014; Peterson, 1979). In this study, dialysis was performed to remove EDTA from EPS extracts, but the extent of removal of EDTA was not determined. When quantifying extracellular proteins isolated from drinking water biofilms, Michalowski (2012) reported that dialysis did not suffice to prevent interference of the modified Lowry assay by EDTA, possibly because EDTA was retained in the EPS extract due to complex formation with EPS constituents (Liu & Fang, 2002). Although mean protein concentrations in EDTA-extracted EPS were higher compared to other extraction methods tested, they were still significantly lower (17.3 μg cm^{-2} for R2A, 450 μg cm^{-2} for TSA) than the protein concentrations of untreated biofilms (84.0 μg cm^{-2} for R2A, 564.3 μg cm^{-2} for TSA), which means that the values are not implausible *per se*, but could still be an overestimation. Treatment with EDTA did not extract more polysaccharides than treatment with Dowex® or shaking, supporting the notion that the EPS of *D. geothermalis* are not stabilised by

negatively-charged polysaccharides (e.g. uronic acids) interlinked by divalent cations. Whilst others (Liu & Fang, 2002; Michalowski, 2012) have reported reduction of culturability and promotion of cell lysis by EDTA, no significant evidence for the damaging of cells and the abnormal release of intracellular DNA was found in this study. This is not surprising, considering that the cell envelope of the closely-related *D. radiodurans* is highly resistant to chemicals and has been reported to withstand washing with, for example, 1 M sodium hydroxide, 1 M EDTA, or 1% sodium dodecyl sulphate (Kolari, 2003; Thompson et al., 1982). Nevertheless, as EDTA restricts the biochemical characterisation of extracted EPS, its use is not recommended.

Formaldehyde/sodium hydroxide. The combined use of formaldehyde and sodium hydroxide represents a very efficient EPS extraction method and has been reported to yield the highest amounts of EPS when used on sludge (D'Abzac et al., 2010; McSwain et al., 2005). Also in this study, formaldehyde/sodium hydroxide treatment resulted in highest yields with respect to proteins (neglecting the possibly overestimated values obtained from extraction by EDTA) and polysaccharides. This might be explained by an increased pH value caused by the addition of sodium hydroxide. In alkaline solution, acidic EPS groups dissociate and negatively-charged polymers show increased repulsion, what promotes destabilisation of the matrix. Also, the solubility of EPS may be increased, thus allowing for more EPS to be extracted (Wingender et al., 1999). The method was the only EPS isolation procedure tested, which yielded evidence for the presence of uronic acids in the EPS of *D. geothermalis*. However, their concentrations were below the limit of quantification, suggesting that uronic acids – if present at all – contribute insignificantly to the total amount of EPS produced by the organism. Formaldehyde/sodium hydroxide treatment extracted comparatively high amounts of DNA. Whether this is a sign for promoted cell lysis remains equivocal, as it could be explained by the highly efficient nature of this extraction procedure. Although the method reportedly disrupts cells (McSwain et al., 2005), deinococci appear to be resistant to up to 1 M of sodium hydroxide (Kolari, 2003; Thompson et al., 1982). Furthermore, the addition of formaldehyde should counter-act cell lysis, since it reacts with and thus cross-links cell components such as proteins or the cell membrane (Liu & Fang, 2002; Sutherland et al., 2008). This action of formaldehyde is reflected by total cell counts which did not decrease in the course of EPS extraction. The loss of culturability can be explained by the fact that formaldehyde chemically altered vital cell components, thus arresting their functionality. To further understand the effects of this EPS isolation procedure on lysis of *D. geothermalis* cells, the use of different cell lysis assays might be necessary. Glucose-6-phosphate dehydrogenase cannot be used as a marker for cell lysis under these conditions, as the high pH value would disrupt the enzyme (McSwain et al., 2005). Taking into account the fact that the addition of formaldehyde could impede biochemical analysis of the extracted EPS, as the molecule reacts with amino groups of

proteins and amino sugars of polysaccharides (D'Abzac et al., 2010; McSwain et al., 2005), the method is not recommended for the isolation of EPS with unknown composition, such as the EPS of *D. geothermalis*.

4.2.2 EPS characterisation

This study identified proteins and polysaccharides as abundant fractions of the EPS of *D. geothermalis* DSM 11300 isolated using the CER Dowex®, though their concentration and ratio was shown to differ with the mode of growth of the organism and the nutrients provided. Biofilms grown on TSA produced by far the highest amounts of EPS per cm^2 of biofilm area. On TSA, the protein fraction dominated with a concentration of 37.6 μg cm^{-2}, followed by the polysaccharide fraction with 9.5 μg cm^{-2}. When grown on R2A, biofilms produced significantly less EPS (3.3 μg cm^{-2} and 4.5 μg cm^{-2}, respectively) and polysaccharides were the dominant fraction. Thus, significantly different protein/polysaccharide ratios of 3.9 (for TSA) and 0.7 (for R2A) were estimated.

There are two obvious reasons that might explain why TSA-grown biofilms showed higher EPS yields than R2A-grown biofilms: Firstly, TSA offers significantly higher concentrations of nutrients than R2A, what possibly could have led to the formation of thicker biofilms containing more cells per cm^2 biofilm area than R2A-grown biofilms. For strain *D. geothermalis* E50051, Kolari (2003) has shown that certain nutrients such as glucose or starch promote biofilm formation, leading to up to 56% thicker biofilms compared to a control. Unfortunately, due to the high degree of cell aggregation, determination of the total cell count of TSA-grown biofilms was impossible and, as a result, the amount of cells per cm^2 biofilm area could not be compared to R2A-grown biofilms. Secondly, the properties of the nutrient medium could have influenced the amount and quality of EPS to be produced per cell. Changes in pH, temperature, or salt concentration have been shown to affect EPS production in unsaturated biofilms of *Pseudomonas putida* (Lin et al., 2014). Also in *D. geothermalis* E50051, the ionic strength of the nutrient medium was shown to influence biofilm formation (Kolari, 2003). Saarimaa et al. (2006) identified glycosylated proteins with a distinct molecular mass of 10 kDa in the EPS of biofilms of *D. geothermalis* E50051 grown on modified R2A medium. When the starch contained in the medium was replaced with glucose, this protein seized to be produced. Whilst increased cell densities in TSA-grown biofilms cannot be ruled out, the second explanation seems conceivable, as TSA caused production of EPS with a significantly higher protein/polysaccharide ratio compared to the EPS from R2A-grown biofilms. This indicates that indeed more EPS, particularly proteins, were produced on TSA. This is supported by ESEM observations as well as lectin studies. ESEM micrographs depicted an amorphous EPS matrix covering the superficial layer of biofilm cells. Due to the immobile nature of the cells (Ferreira et al., 1997), it is likely that this EPS film had been pro-

duced by precisely this uppermost layer of cells. In the case of TSA-grown biofilms, the EPS film appeared to be thicker. TSA-grown biofilm cells must have produced more and possibly different EPS than R2A-grown cells in order to form the amorphous matrix observed. Lectin staining did not necessarily demonstrate differences in the amount of EPS produced by both biofilm types. However, it clearly showed that both types indeed formed exopolysaccharides of different spatial distribution and arrangement. This further supports the assumption that, depending on the nutrient medium used, quantitatively and qualitatively different EPS were produced by *D. geothermalis*.

Proteins. The EPS of *D. geothermalis* biofilms contain proteins of yet unknown nature and function. Cultivation on TSA stimulated the production of proteinaceous EPS compared to cultivation on R2A. This is likely due to the excess of the complex and amino acid-rich nutrient peptone which is the main constituent of TSA. The EPS of TSA-grown biofilms depicted a relatively high protein/polysaccharide ratio of 3.9. Upward shifts of the protein/polysaccharide ratio can be an indication for cell lysis during EPS isolation (McSwain et al., 2005), in which case the EPS extract is contaminated by intracellular and membrane-associated proteins. Although the cell envelope of deinococci is protein-rich (Farci et al., 2014), it seems improbable that the relatively mild EPS extraction procedure using CER Dowex® might have disrupted it, since it withstands far more aggressive treatments (Thompson et al., 1982). However, it is worth considering, that surface-associated cell appendages may have been sheared off during EPS extraction. These structures generally may include pili or flagella, but, although present extracellularly, they are per definition not part of the EPS (Flemming & Wingender, 2010). When in sessile form, strain *D. geothermalis* E50051 forms protein-containing, thread-like cell appendages which have been characterised as type IV pili (Saarimaa et al., 2006). Evidence for type IV pili in biofilms of *D. geothermalis* DSM 11300 has only been obtained by lectin staining. If the organism truly expressed pili under the conditions tested, these structures may have contributed to the total amount of protein found in the EPS. Treatment with proteinase K eliminated extracellular material from TSA-grown biofilm cells, after which cell aggregation was diminished. This suggests that extracellular proteins are involved in intercellular cohesion.

Polysaccharides. Polysaccharides were found to be the main constituent of the EPS of R2A-grown biofilms (4.5 μg cm^{-2}), and represented the second largest fraction of EPS in TSA-grown biofilms (9.5 μg cm^{-2}). Previous works detected carbohydrates in the EPS of sessile cultures of *D. geothermalis* – either by periodic acid Schiff staining (Ferreira et al., 1997; Saarimaa et al., 2006) or by lectin assays (Kolari, 2003; Peltola et al., 2008; Saarimaa et al., 2006) – but these methods offer only limited information about the composition of the carbohydrates detected. This study attempted to identify the monomers of the exopolysaccharides of *D. geothermalis* by TLC after degradation of the polymers by acidic hydrolysis (0.1 M hydro-

chloric acid, 100 °C). A range of monosaccharides including galactose, glucose, mannose, xylose, fucose, and rhamnose were chosen as references. Evidence for the presence of galactose, glucose, and mannose was obtained from a lectin study on *D. geothermalis* E50051 (Peltola et al., 2008). The sugars glucose, mannose, galactose, fucose, and rhamnose were of further interest as they have been identified as the main constituents of exopolysaccharides of intertidal biofilms, which form in habitats that are subjected to periodic desiccation and re-wetting (Passarelli et al., 2015). Mannose was suspected to be found in the EPS of *D. geothermalis*, as it was the only carbohydrate tested which succeeded to interfere with cell aggregation in TSA-grown biofilms.

During TLC, the hydrolysed EPS from R2A- and TSA-grown biofilms each yielded a single spot, suggesting that either the polysaccharide fraction is composed of a single carbohydrate monomer, or that hydrolysis of the polysaccharide(s) was incomplete. Furthermore, inappropriate experimental conditions (choice of solvent, TLC plate, and derivatisation agent) might have impeded analysis by preventing the separation and identification of the hydrolysate. In terms of R_f values, the sample spots did not match any of the spots of the reference sugars. The extracellular appendages of *D. geothermalis* E50051 consist of proteins some of which are glycosylated (Saarimaa et al., 2006). Attached proteins could have altered the polarity of the carbohydrates which thus may have migrated less far on the TLC plate.

Staining of *D. geothermalis* biofilms with the fluorescently-labelled lectins ACA, HAA, and DBA indicated the presence of galactosides in the EPS of *D. geothermalis*. This was confirmed by a lectin study on *D. geothermalis* E50051, although the authors suspected the EPS to consist of more than one type of polysaccharide (Peltola et al., 2008). Lectin staining of both R2A- and TSA-grown biofilms showed that indeed multiple types of polysaccharides of different spatial arrangement exist within the matrix. However, it must be considered that the carbohydrate specificity of lectins is somewhat limited. Unspecific binding to other biofilm constituents may occur, and often the range of carbohydrate targets is broader than indicated by the manufacturer (Kolari, 2003; Neu et al., 2001).

eDNA. The EPS of *D. geothermalis* were found to contain relatively small amounts of eDNA. Although it cannot be ruled out that its presence was due to the release of DNA from lysed cells, eDNA might represent a structural component of the EPS of *D. geothermalis*. A number of studies have shown that eDNA has a functional role in biofilms of other bacteria (Flemming & Wingender, 2010). In *P. aeruginosa*, for example, it is required for the initial establishment of a biofilm (Whitchurch et al., 2002). In *Myxococcus xanthus* or the aquatic bacterial strain F8, the negatively-charged eDNA interacts with other EPS components to confer structural integrity to the biofilm matrix (Böckelmann et al., 2006; Hu et al., 2012).

4.2.3 EPS of planktonic cells

Planktonic cultures of *D. geothermalis* seemed to contain EPS in concentrations similar to those of biofilms cultivated under comparable nutrient conditions (R2B, R2A). Generally speaking, the production of EPS is not restricted to the biofilm mode of life (Evans et al., 1994). With respect to *D. geothermalis*, however, it has been reported that the extracellular features prominent in sessile cells (thread-like EPS, capsular EPS) are not expressed in planktonic cells (Ferreira et al., 1997; Liedert et al., 2010; Saarimaa et al., 2006).

The observed high EPS concentrations in planktonic cultures could be due to high growth rates during cultivation, enabled by the supply of yeast extract and glucose at a temperature of 45 °C (Bornot et al., 2014a; Bornot et al., 2014b; Ferreira et al., 1997). In *S. epidermis*, the extent of EPS production has been associated with the growth rate of the organism: Slow-growing biofilms of *S. epidermis* exceeded slow-growing planktonic cells of the same species in terms of EPS yield under the same cultivation conditions. When the cultivation conditions were modified to obtain high growth rates, however, biofilms and planktonic cells produced similar amounts of EPS (Evans et al., 1994). In the present study, *D. geothermalis* might have behaved in a similar way.

The employed EPS isolation procedure comprised multiple steps along which the true amount of EPS could have been falsified. Centrifugation and washing steps inevitably lead to a loss of loosely-bound EPS, resulting in an underestimation of the EPS concentration (Zhang et al., 1999). Cell lysis as it may occur in stationary-phase planktonic cultures (Nyström, 2004), on the other hand, would cause a contamination by intracellular material, resulting in an overestimation of the amount of EPS produced (D'Abzac et al., 2010). The same is true for soluble microbial products which are released or excreted into the liquid medium (Laspidou & Rittmann, 2002). Most importantly, it is possible that nutrient medium constituents were carried over during EPS isolation and, thus, may have contaminated the EPS extracts.

4.3 Tolerance of *D. geothermalis* to desiccation and to space and Mars stress

In this study, biofilms and planktonic cells of *D. geothermalis* DSM 11300 were air-dried and exposed to a range of space-relevant stressors, including desiccation, UV irradiation, vacuum, artificial Martian atmosphere, and extreme temperatures. These stressors were applied either individually or in combination. The survival of the organism following exposure was determined by plate counts on R2A agar as well as by culture-independent techniques in order to measure the cells' membrane integrity and ATP levels, and to detect the presence of 16S rRNA as a marker for active protein biosynthesis. The resilient nature of *D. geothermalis* (Makarova et al., 2007) was confirmed, as it survived desiccation as well as stressors simulating aspects of

space and Martian environments. Biofilms were found to be even more stress-tolerant than planktonic cells.

4.3.1 Differential tolerance of biofilms and planktonic cells

This study demonstrated that biofilms of *D. geothermalis* were more tolerant to the various stressors applied to simulate aspects of space or Martian conditions than were planktonic cells. This was particularly obvious when the organism was exposed to long-term desiccation and to UV radiation, either in vacuum or in artificial Martian atmosphere.

Differential stress tolerance of biofilms and planktonic cells has been observed in a number of microorganisms. For example, biofilms of *P. putida* were more tolerant against air-drying, desiccation, and osmotic stress than their planktonic counterparts (Lin et al. 2014; van de Mortel et al. 2004; van de Mortel and Halverson 2004). Similarly, cell aggregates of *P. syringae* on periodically dry leaf surfaces showed better survival compared to single cells of the same organism (Monier and Lindow 2003). When in biofilm form, the cyanobacterium *Chroococcidiopsis* sp. accumulated less cellular damage during exposure to polychromatic UV radiation under simulated space and Mars conditions than in planktonic form (Baqué et al. 2013b).

It is unclear which mechanisms caused *D. geothermalis* to be more stress-tolerant in biofilms than in its planktonic state. So far, differences in stress tolerance between biofilms and planktonic cells have been attributed to the physiological state and metabolic activity, to the degree of aggregation, and – probably most importantly – to the amount, nature, and arrangement of EPS produced by the organism (Baqué et al. 2013b; Lin et al. 2014; Monier and Lindow 2003; van de Mortel et al. 2004).

The physiological state of a microbial cell may influence its tolerance towards environmental stress, in that stationary-phase cells have often been characterised as less susceptible to stress than exponentially growing cells (Nyström, 2004; Vriezen et al., 2006). For example, the survival of *S. meliloti* upon desiccation was enhanced when the cells were dried during stationary phase (Vriezen et al. 2006). In stationary phase, nutrient depletion and starvation trigger stress responses which may protect the cells against others stressors such as desiccation (Morgan et al. 2006), a process called 'stasis-induced cross protection' (Nyström, 2004). Liedert et al. (2012) reported that the enzymes catalase and superoxide dismutase, both of which are directed against oxidative stress, are abundant in stationary-phase cells of *D. geothermalis*, whilst the expression of the reactive radical-generating enzyme lysine 2,3-aminomutase was downregulated. In this study, the exposed planktonic cells were in stationary phase what might have contributed to an increased resistance against desiccation- and UV radiation-induced oxidative stress.

The distribution of physiological states within a biofilm is more heterogeneous than in planktonic cultures, as chemical gradients (e.g. nutrients, oxygen) lead to the establishment of zones with less microbial activity and phenotypes distinct from planktonic cells (Ackermann, 2015; Stewart & Franklin, 2008). In contrast to planktonic cells of *D. geothermalis*, a substantial proportion of biofilm cells (approximately 25%) was void of 16S rRNA, indicating reduced metabolic activity. High levels of membrane integrity suggest that these cells were in fact viable but metabolically inactive. Low metabolic activity might aid in overcoming oxidative stress, considering that pathways which typically generate ROS within the cell are attenuated (Cabiscol et al., 2010; Ghosal et al., 2005). Additionally, starvation-induced stress responses generally occurring in nutrient-deprived zones within biofilms (Nguyen et al., 2011) confer protection against other stressors, including desiccation (Morgan et al., 2006).

The protective function of EPS is widely acknowledged (Flemming and Wingender 2010). A multitude of studies has demonstrated a relationship between EPS and an increased tolerance towards stressors such as desiccation, metal ions, ROS, sodium chloride, and UV radiation (Tab. 4.1). This study demonstrated the presence of EPS in biofilms and – contrary to previous observations (Ferreira et al., 1997; Saarimaa et al., 2006) – also in planktonic cultures of *D. geothermalis*. In both lifestyles, the organism seemed to produce similar quantities of EPS, although the procedure employed for isolating EPS from liquid cultures may have caused an overestimation of the true EPS concentrations due to the contamination of the EPS extract with nutrient medium constituents or soluble microbial products (Laspidou & Rittmann, 2002).

While it appears as though biofilms and planktonic cells of *D. geothermalis* produce EPS of similar quantity and composition, planktonic cells were still more susceptible to desiccation than biofilm cells. This suggests that in *D. geothermalis* the mere presence of EPS does not result in an increased tolerance to desiccation. It rather seems that the increased tolerance of biofilms is due to the spatial arrangement and distribution of the EPS within the matrix as it is formed only in biofilms. ESEM observations of *D. geothermalis* indicated the presence of a surficial EPS layer that covered the uppermost layer of biofilm cells but was not visible on planktonic cells. Such an EPS layer could for instance function as a water barrier by forming a skin on the surface of the biofilm in the event of desiccation (Sutherland, 2001). Peltola et al. (2008) reported the presence of a dense carbohydrate core within *D. geothermalis* biofilms which in this case may act as storage for water. In accordance with this, lectin staining showed an extensive polysaccharide network present within R2A-grown biofilms. The EPS of *D. geothermalis* contain substantial amounts of proteins, some of which might be hygroscopic as well (Flemming & Wingender, 2010). In planktonic samples, the EPS are likely distributed homogeneously throughout the culture. Although they may surround each individual cell, the

Table 4.1. List of selected studies in which the production or addition of EPS has been correlated with an increased microbial tolerance to different environmental stressors.

Stressor	Result of the study	Reference
Desiccation	▪ EPS synthesis significantly increased the desiccation tolerance of *Methanosarcina barkeri* under both aerobic and anaerobic conditions	Anderson et al. (2012)
	▪ Water limitation stimulated the production of EPS (alginate) in *P. putida*.	Chang et al. (2007)
	▪ The EPS of *N. commune* inhibited the fusion of membrane phospholipids during desiccation.	Hill et al. (1997)
	▪ The addition of EPS derived from *N. commune* increased the survival of the algae *Chlorella* sp. and the cyanobacterium *Chroococcidiopsis* sp. during desiccation.	Knowles and Castenholz (2008)
	▪ Mucoid (i.e. EPS-producing) strains of *E. coli*, *A. calcoaceticus*, and *E. stewartii* were more desiccation-tolerant (up to 30% higher survival rates) than non-mucoid mutants of the same species.	Ophir and Gutnick (1994)
	▪ Soil *Pseudomonas* spp. produced hygroscopic EPS in response to desiccation.	Roberson and Firestone (1992)
	▪ In contrast to EPS-depleted cells, EPS-embedded cells of *N. commune* were highly desiccation-tolerant and maintained photosynthesis in desiccated state.	Tamaru et al. (2005)
	▪ Biofilms of *P. putida* were more desiccation-tolerant than planktonic cells grown in liquid medium, possibly due to EPS production.	van de Mortel et al. (2004)
Metal ions	▪ Biofilms of *Pseudomonas fluorescens* reacted differently to metal (copper) stress than planktonic cells. Metal stress stimulated EPS production in biofilm cells.	Booth et al. (2011)
	▪ Biofilms of *P. aeruginosa* were 2-600 times more tolerant to heavy metals (copper, lead, zinc) than planktonic cells, presumably due to sorption of the metal ions by the EPS matrix.	Teitzel and Parsek (2003)
ROS	▪ EPS-engulfed cells of *P. syringae* were more resistant to ROS than EPS-lacking mutants or washed cells deprived of EPS.	Kiraly et al. (1997)
	▪ EPS-producing cells of *P. aeruginosa* were more tolerant to toxic mineral surfaces enriched in ROS than EPS-lacking mutants.	Xu et al. (2012)
Salt	▪ *Nostoc* sp. responded to high levels of sodium chloride by increasing the amount of capsular polysaccharides in its EPS. The salt-sensitive *Anabaena* sp. became more salt-tolerant when exogenous EPS extracted from *Nostoc* sp. were added.	Yoshimura et al. (2012)
UV radiation	▪ *P. aeruginosa* was more tolerant to polychromatic UV radiation when embedded in the polysaccharide alginate.	Elasri and Miller (1999)

limited thickness of the EPS layer would insufficiently protect against stressors such as desiccation (Lin et al., 2014; van de Mortel et al., 2004).

4.3.2 Effects of desiccation

In biofilms, *D. geothermalis* remained viable for a desiccation period of at least two months, since a significant fraction of cells had maintained culturability, membrane integrity, ATP levels, and the presence of 16S rRNA. Planktonic cells, on the other hand, showed significantly lower levels of viability after a two-month desiccation period. Still, the residual culturability and membrane integrity of dried planktonic cells was higher compared to a dead control (cells inactivated by isopropyl alcohol treatment), confirming the exceptional desiccation tolerance of the organism. Although deinococci generally are referred to as desiccation-resistant (Mattimore & Battista, 1996), little experimental data is available regarding the actual desiccation resistance of the species *D. geothermalis*. In a previous study by Daly et al. (2004), a desiccation period of > 30 days led to a reduction in the culturability of *D. geothermalis* grown in liquid culture to 10%. This observation is in agreement with this study, in which a dry period of 28 days reduced the culturability of planktonically grown cells to 6.5%. The culturability of biofilms, however, was only reduced to 36.1% compared to wet samples, which demonstrates their superior desiccation tolerance.

This study identified proteins and polysaccharides as the main constituents of the EPS of *D. geothermalis*, both of which might be hygroscopic and store water (Flemming & Wingender, 2010). These EPS have the potential to slow down the drying rate and could therefore help biofilm cells to adapt to dry conditions (Or et al., 2007). The biofilms used in this study formed at the solid-air interface and, thus, were water-unsaturated. In natural unsaturated biofilms as they occur, for example, on soil particles or plant leaves, microorganisms face periodic dryness or reduced water availability compared to biofilms growing in fluids (Chang & Halverson, 2003). Microorganisms can modify their EPS in order to cope with these conditions. The desiccation-sensitive bacterium *P. putida* adapts to periodic desiccation events by increasing the thickness of the EPS layer at the biofilm-air interface (Auerbach et al., 2000; Chang & Halverson, 2003; Chang et al., 2007; van de Mortel et al., 2004). Upon water loss, such a surficial EPS layer may form a skin that retards the dehydration of the biofilm due to evaporation (Peltola et al., 2008; Sutherland, 2001). ESEM provided evidence for the presence of a surficial EPS layer in *D. geothermalis* biofilms that superimposed the uppermost layer of biofilm cells. In contrast to planktonic cells which were cultivated in liquid nutrient medium, the growth of *D. geothermalis* in unsaturated biofilms could prepare the organism to sudden dehydration (van de Mortel et al., 2004). ESEM further showed that the cells in both biofilms and planktonic samples were tightly packed. Due to this, the intercellular space and, thus, the surface area of the cell clusters were minimised. A reduced surface area helps to re-

duce or slow down water loss (Lin et al., 2014; Monier & Lindow, 2003). By providing a habitat that dries more slowly than the immediate environment, the cells could be provided with sufficient time to initiate adequate counter-measures such as the upregulation of genes expressing oxidative stress-combating enzymes (Bauermeister et al., 2011; Liedert et al., 2010; Liedert et al., 2012).

Membranes, proteins, and nucleic acids represent the main target of desiccation-induced damage (Billi & Potts, 2002). When their hydration shell is gradually lost upon dehydration, membranes become susceptible to ROS-induced damage and leakage, proteins may denature and lose their activity, and nucleic acids are subjected to chemical alterations which impair their function and could lead to cell death (Fredrickson et al., 2008; García, 2011; Potts, 1994; Prestrelski et al., 1993). Some organisms such as cyanobacteria accumulate the disaccharides trehalose and sucrose in order to increase their desiccation tolerance (Hershkovitz et al., 1991). These sugars can stabilise drying biomolecules by replacing the water molecules in their hydration shell. Additionally, they can induce a glass-like state in the dehydrated cytoplasm, in which biomolecules are stabilised and harmful chemical reaction rates are decelerated (Billi & Potts, 2002; Buitink & Leprince, 2004; Crowe, 2014). *D. geothermalis* possesses a trehalose synthase gene (Filipkowski et al., 2012), and members of the genus *Deinococcus* indeed seem to be able to produce trehalose (Gerber et al., 2015). The sugar could play a role in the desiccation tolerance of deinococci, as it does in other bacteria (Leslie et al., 1995).

In contrast to planktonic cells, membrane integrity was maintained at high levels in desiccated biofilms of *D. geothermalis*, suggesting that biofilm cells are able to conserve membrane integrity and membrane fluidity more efficiently. This could be due to the EPS matrix. Hill et al. (1997) have demonstrated that the EPS of *N. commune* facilitate the preservation of membrane integrity by inhibiting the fusion of phosphatidylcholine membrane vesicles. Desiccation did not affect the presence of nucleic acids (DNA and rRNA) in both biofilms and planktonic cells, as shown by successful staining of the cells using the DNA-staining dyes DAPI and Syto® 9, and a 16S rRNA-targeted FISH probe. Deinococci are able to survive high rates of DNA fragmentation and can quickly repair extensive DNA upon rehydration (Cox & Battista, 2005; Daly, 2012). The ubiquitous presence of rRNA in dried and killed cells suggests that ribosomes are well protected against desiccation in *D. geothermalis.* In dried state, the highly viscous cytoplasm exhibits low molecular mobility. The rate of potentially detrimental chemical reactions occurring with nucleic acids and other biomolecules may therefore be slowed down in dehydrated cells (Bauermeister et al., 2011; Kranner & Birtić, 2005).

Different results were obtained between cultivation-dependent and -independent viability markers. Upon desiccation of biofilms, the proportion of culturable cells decreased, whereas total cell counts and membrane-intact cell counts were less affected. Along with high

levels of ATP and 16S rRNA-containing cells, this implied that a significant proportion of the non-culturable population was still viable. Declining amounts of culturable cells accompanied by stable total cell counts have been reported to be an indicator for cultures becoming VBNC (Oliver, 2005). Induction of the VBNC state is considered a response to environmental stressors, including extreme temperatures, starvation, oxidative stress, but also desiccation (Li et al., 2014; Oliver, 2005; Trevors, 2011; Vriezen et al., 2012). For example, desiccation has been shown to induce a VBNC state in *E. coli* and *Alcaligenes eutrophus* (Pedersen & Jacobsen, 1993), and in *S. meliloti* (Vriezen et al., 2006; Vriezen et al., 2012). To the author's knowledge, the occurrence of a VBNC state in deinococci has not been reported, yet. However, this study presented evidence that *D. geothermalis* became VBNC in response to desiccation. VBNC cells exhibit reduced metabolic activity compared to actively growing cells (Li et al., 2014). Induction of the VBNC state might aid *D. geothermalis* in overcoming periods of oxidative stress, for example by arresting ROS-generating metabolic pathways (Cabiscol et al., 2010; Ghosal et al., 2005; Kranner & Birtić, 2005).

4.3.3 Effects of space vacuum and Martian atmosphere

Desiccated samples of *D. geothermalis* were exposed to high vacuum (3.5×10^{-2} Pa) and to medium vacuum in artificial Mars gas (6.5×10^{2} Pa; > 95% carbon dioxide) in order to simulate the effects of space vacuum and Martian atmosphere on cellular viability. The stressors did not affect the viability of both biofilms and planktonic cells, indicating that *D. geothermalis* is very tolerant to low pressures in desiccated state.

Also other *Deinococcus* spp. have been shown to endure exposure to vacuum. Experimental evidence has been obtained for *D. radiodurans*, *Deinococcus aerius*, and *Deinococcus aetherius* to survive exposure to vacuum for one year with a 10- to 100-fold reduction in culturability (Kawaguchi et al., 2013). Bauermeister et al. (2011) reported that the survival of desiccated cells of *D. radiodurans* was superior when the cells were stored in an evacuated exsiccator compared to storage at ambient air. Exposure to vacuum results in the rapid dehydration of microbial cells and can lead to the destruction of the cell membrane, as the water outflow exceeds the hydraulic permeability of the membrane (Gervais & Marechal, 1994). For already desiccated cells, however, storage in vacuum has been reported to be beneficial for survival compared to storage in air (Bozoğlu et al., 1987; Yang & Sandine, 1979). In this study, exposure to medium or high vacuum seemed to affect neither the colony-forming ability nor the membrane integrity of *D. geothermalis*, suggesting that the water content of the air-dried cells was already low enough to prevent membrane damage induced by excessive water outflow. The highly desiccation-tolerant spores of *B. subtilis* were shown to have a water content of approximately 40.3% (Moeller et al., 2009). This study did not attempt to measure the water content of desiccated cells of *D. geothermalis*. However, air-dried cells of the closely-

related *D. radiodurans* were shown to contain even less water (26.9%) than *Bacillus* spores (Bauermeister et al., 2011). The low amount of residual water in deinococcal cells might be trapped in the glass-like cytoplasm and/or be tightly bound to intracellular biomolecules such as DNA or proteins (Bauermeister et al., 2011; García, 2011), and could therefore resist evaporation.

Exposure to artificial Mars atmosphere did not significantly affect the viability of *D. geothermalis*. The gas mixture mainly contained carbon dioxide (95.5%), as well as low amounts of nitrogen (2.7%) and argon (1.6%). Similar to vacuum, the survival of cells stored in nitrogen has been reported to be superior to those stored in oxygen-containing air (Bozoğlu et al. 1987; Yang and Sandine 1979). The oxygen content of the Mars gas mixture was much lower (0.13%) than the concentration of oxygen in ambient air (20.9%). Although reported to be strictly aerobic (Ferreira et al., 1997), *D. geothermalis* has been shown to thrive under low (1%) oxygen conditions (Liedert et al., 2012). Whilst it seems unlikely that active growth has occurred in desiccated cells of *D. geothermalis*, this suggests that the oxygen-poor Mars gas did not impair cell viability. Furthermore, storage in Mars gas may have been beneficial for survival as exogenous sources of oxidative stress (i.e. atmospheric oxygen) were limited (França et al., 2007; Fredrickson et al., 2008; Kranner & Birtić, 2005).

The results strongly suggest that *D. geothermalis* is able to survive both space vacuum as well as the atmospheric conditions of Mars for a certain period of time.

4.3.4 Effects of extreme temperatures

Dried biofilms and planktonic cells of *D. geothermalis* were subjected to 1-h periods of extreme temperatures (-25 °C; +60 °C), and to temperatures oscillating 48 times between -10 °C and +45 °C. Neither culturability nor cultivation-independent viability markers were significantly affected by these treatments.

In general, the viability of dried cells is affected by temperature. Within the range of 4-37 °C, microbial survival rates have been shown to decrease with increasing temperature (e.g. Castro et al., 1995; Champagne et al., 1996; Higl et al., 2007; Morgan et al., 2006; Teixeira et al., 1995; Wang et al., 2004). Low temperatures favour survival by reducing the extent of cellular damage (e.g. membrane lipid oxidation) caused by ROS, as ROS-generating processes are attenuated (Cabiscol et al., 2010; Castro et al., 1995; Ghosal et al., 2005).

The results presented here are in line with previous observations made on other deinococci. Kawaguchi et al. (2013) reported that three different *Deinococcus* spp. (*D. radiodurans*, *D. aerius*, *D. aetherius*) tolerated the repeated exposure to 5,840 temperature cycles in the range of -60 °C to +60 °C (at 10^{-1} Pa) with culture-based survival rates of 0.5-3%, indicating that deinococci in general are very temperature-tolerant. In a study published by Bauermeister et al. (2012), the tolerance of *D. radiodurans* to high temperatures was shown to be increased

in desiccated cells. The organism sustained high levels of membrane integrity after a 10-min exposure to 80 °C when in desiccated state, but was much more sensitive to temperatures of 40 °C or higher when fully hydrated (Bauermeister et al., 2012). Low molecular motion in the dehydrated cytoplasm might protect biomolecules such as proteins from denaturation by heat (Setlow, 2006). Stress responses such as the expression of heat shock proteins or mechanisms induced during stationary phase might contribute to an increased tolerance to adverse temperatures (García, 2011; Makarova et al., 2007; Morgan et al., 2006).

D. geothermalis is a moderately thermophilic organism with an optimum temperature for growth of 45-50 °C, 15-20 °C higher than the growth optimum of *D. radiodurans*, (Battista, 1997) and a G+C content of 65.9 mol% (Ferreira et al., 1997). It is therefore not surprising that *D. geothermalis* tolerated temperatures of 60 °C well. The results suggest that *D. geothermalis* is fit to endure the extreme temperatures encountered during the BOSS experiment in LEO, and possibly also the temperatures prevailing in open space and on the Martian surface.

4.3.5 Effects of UV radiation

Desiccated samples of biofilms and planktonic cells of *D. geothermalis* were exposed to monochromatic UV radiation (254 nm; 0-10 kJ m^{-2}) in air, and to polychromatic UV radiation (200-400 nm; up to 2.2×10^2 kJ m^{-2}) in either vacuum or artificial Mars gas.

D. geothermalis endured a 10 kJ m^{-2} fluence of monochromatic UV radiation with virtually no effect on membrane integrity and a 1- to 2-log reduction in culturability for planktonic cells and biofilms, respectively. A previous study has reported the organism to be more susceptible to monochromatic UV, as a fluence of 4 kJ m^{-2} caused a 4-log reduction in culturability (Makarova et al., 2007). Regarding the closely-related *D. radiodurans* R1, a UV fluence of 500 J m^{-2} has been shown to have no effect on its colony-forming ability (Moseley & Mattingly, 1971). Fluences of 550-600 J m^{-2} rendered all but 37% of the tested population non-culturable, and fluences of 900-1,000 J m^{-2} severely impacted survival (Battista, 1997; Moseley & Mattingly, 1971; Tian et al., 2007). This study produced evidence that *D. geothermalis* endures monochromatic UV fluences 10 times higher than those reported previously for *D. radiodurans*, supporting the fact that *D. geothermalis* is indeed extremely UV radiation-resistant (Makarova et al., 2007).

Whilst all of the studies mentioned above examined the effect of UV radiation on fully-hydrated cells, the present work employed desiccated cells and biofilms. The results suggest that desiccated deinococci and deinococcal biofilms are more UV radiation-tolerant than hydrated, single cells. This concurs with previous observations made on *D. radiodurans*, which was shown to be 2- to 4-times more tolerant to mono- and polychromatic UV radiation in dried state than in hydrated state (Bauermeister et al., 2011). Dehydration of *Deinococcus* cells seems to increase their tolerance to cellular damage induced by heat but also by radiation

(Bauermeister et al., 2012; Bauermeister et al., 2011). In deinococcal cells, DNA, which is a sensitive target of UV radiation-induced damage (Gao & Garcia-Pichel, 2011), is organised in a highly condensed toroid (i.e. ring-like) structure that restricts the diffusion of DNA fragments and, thus, facilitates DNA repair (Englander et al., 2004). Stabilisation of the toroid occurs by interactions of the phosphate groups of the DNA backbone with manganese ions (Cox & Battista, 2005), a process which is amplified under dehydrating conditions (Englander et al., 2004).

When *D. geothermalis* was desiccated and exposed to monochromatic UV radiation in ambient air at nominal pressure, the planktonic cells sustained culturability better than biofilms. However, when *D. geothermalis* was irradiated with polychromatic UV under the dehydrating conditions of high or medium vacuum, this effect was not observed. Instead, biofilms were superior in surviving UV radiation under these conditions. It can be assumed that biofilms were able to retain some residual water during air drying due to their EPS matrix (Peltola et al., 2008). Membrane-deposited planktonic cells lacked an organised EPS matrix. Insufficiently protected against desiccation, they might have been dehydrated to a higher degree than biofilm cells. Although the stored water may aid biofilms during dehydration, it could promote cellular damage during UV irradiation: When exposed to highly energetic radiation such as UV, water molecules can dissociate, leading to the formation of harmful ROS (e.g. $OH^{\cdot}$ radicals) which damage biomolecules (He & Hader, 2002; Heck et al., 2003; Ito et al., 1980; Voeikov, 2001). Exposure the vacuum may have caused the residual water trapped in biofilms to evaporate. Thus, cellular damage due to UV radiation-generated ROS might have been minimised. In biofilms of *N. commune*, EPS (glycan) have been reported to be a source of significant amounts of superoxide radicals upon irradiation with UV-A and UV-B (Shirkey et al., 2000). Similar processes are imaginable to occur in *D. geothermalis* biofilms, although no experimental evidence was obtained to support this idea.

The exposure to UV radiation rendered *D. geothermalis* nonculturable, but did not affect membrane integrity and the presence of DNA, 16S rRNA, and ATP. The fact that these cultivation-independent viability markers were maintained at high levels suggests that a large part of the population remained viable during irradiation. However, the viability markers membrane integrity, ATP, and 16S rRNA have their limitations in detecting radiation-induced cellular damage (discussed below in more detail; Hammes et al., 2011; McKillip et al., 1998; Schuerger et al., 2008; Villarino et al., 2000; Zhang et al., 2015). Therefore, the amount of viable cells in irradiated samples could have been overestimated. Nevertheless, the fact that a residual part of the irradiated population remained culturable demonstrated the extreme radiation tolerance of the organism (Makarova et al., 2007).

The observed discrepancy between plate counts and the high number of seemingly viable cells indicates that *D. geothermalis* entered a VBNC state as a response to radiation

stress. The same was observed in desiccation-stressed cells (see 4.4.2). Since all UV-irradiated cells had been desiccated before, the data allows no conclusion on whether UV radiation alone would cause the same effect on the viability of hydrated cells of *D. geothermalis*. Still, irradiation of desiccated cells further increased the gap between cultivation-dependent and -independent viability. Desiccation and UV radiation cause similar cellular damage, and deinococci make use of the same strategies to cope with both stressors (Mattimore & Battista, 1996). The VBNC state might represent such a strategy, as it can result in an increased chemical and physical resistance of the cell (Li et al., 2014). Induction of the VBNC state following UV irradiation has been shown before for *E. coli* and *P. aeruginosa* (Said et al., 2010; Zhang et al., 2015). Both organisms were able to resuscitate from the VBNC state (Zhang et al., 2015). The ability of VBNC *D. geothermalis* to resuscitate remains yet to be proven.

This study showed that high fluences of UV radiation significantly reduced the culturability of *D. geothermalis*. Under genuine space or Mars conditions, extraterrestrial UV radiation, therefore, would represent a major threat to the survival of the organism. Thus, shielding from UV seems to be necessary in order to sustain viability of *D. geothermalis* under these conditions. UV radiation did neither significantly affect membrane integrity, nor the presence of ATP or 16S rRNA. This could indicate that (i) a part of the population became VBNC upon irradiation, or that (ii) the selected cultivation-independent viability markers failed to detect loss of viability.

4.4 Evaluation of cultivation-independent viability markers

The use of cultivation-independent viability markers is necessary in order to distinguish VBNC cells from dead cells. These markers target aspects of microbial viability other than the cell's ability to form colonies (Hammes et al., 2011; Oliver, 2005). In this study, the viability markers membrane integrity, ATP, and 16S rRNA were considered. Additionally, total cell counts using the DNA-staining dye DAPI were performed and compared to plate counts. Following the exposure to different stressors, particularly desiccation and UV radiation, these markers indicated that *D. geothermalis* maintained a level of viability which would have been underestimated if survival was assessed by using plate counts alone. Stable total cell counts and high levels of cultivation-independent viability indicated that *D. geothermalis* became VBNC upon desiccation and, possibly, UV radiation (Oliver, 2005). Since no universal method exists that permits the clear identification of VBNC cells, it is necessary to combine a range of markers. Depending on the organism and the type of stress the organism is exposed to, these markers need to be evaluated for their suitability (Hammes et al., 2011).

4.4.1 Membrane integrity

Integrity of the cell membrane is a basic prerequisite for microbial viability. The membrane represents a physical barrier between the cell's interior and exterior, and keeps the cytoplasm in equilibrium with the environment. Structural damage to the membrane is usually irreversible and therefore considered to be a sign of cell death (Hammes et al., 2011).

In this study, Live/Dead® staining was used to differentiate between membrane-intact and membrane-damaged cells. The method is a widely-used tool in the detection of VBNC cells which utilises two different fluorescent DNA stains, the membrane-permeable Syto® 9 and propidium iodide (Li et al., 2014). Propidium iodide is a positively-charged molecule that usually does not penetrate intact membranes due to repulsion from the negative net surface charge of a bacterial cell. However, the method has its limitations. Valid results require a careful optimisation of the ratio in which the two dyes are administered (Li et al., 2014). In this study, such an optimisation of the staining protocol showed that a 100-fold dilution of propidium iodide with respect to the manufacturer's instructions was necessary to avoid false-negative results in *D. geothermalis* (data not shown). Furthermore, propidium iodide has been shown to penetrate also intact cell membranes of the soil bacteria *Sphingomonas* sp. and *Mycobacterium frederikbergense*, both of which remained culturable after staining (Shi et al., 2007). This questions the validity of propidium iodide as a dead cell marker.

When *D. geothermalis* was treated with heat or isopropyl alcohol in order to produce dead controls, membrane integrity was significantly reduced by one to two or by over two orders of magnitude, respectively. This shows that the marker is sensitive in detecting cellular damage in *D. geothermalis*. When exposed to desiccation, biofilm cells of *D. geothermalis* sustained membrane integrity over a period of at least 2 months. Desiccation can cause extensive membrane damage (Potts, 1994), which is why membrane integrity can be considered a suitable marker for the detection of viability in organisms exposed to dehydration. Hence, the maintenance of membrane integrity in desiccated cells of *D. geothermalis* suggests that these cells remained viable.

During exposure of *D. geothermalis* to mono- or polychromatic UV radiation, membrane integrity remained high in both biofilms and planktonic cells, indicating that the organism remained viable during UV exposure. However, whilst membrane-damaged cells as such can usually be considered dead, the reverse assumption that membrane-intact cells are alive is not necessarily true (Hammes et al., 2011). Hammes et al. (2011) stated that UV-C radiation, as it is used in disinfection, can potentially cause cell death without immediate detectable damage to the cell membrane. These cells may exhibit an intact membrane, but lethal injuries to, for example, DNA would eventually result in cell death. The use of membrane integrity should, thus, be accompanied by further cultivation-independent viability markers when detecting radiation-stressed VBNC cells (Berney et al., 2007). Zhang et al. (2015) has

demonstrated that UV radiation induces a VBNC state in *E. coli*. Assessment of the membrane integrity using Live/Dead® staining showed that the radiation-sensitive bacterium remained intact up to fluences of 3 kJ m^{-2} (Zhang et al., 2015). The authors evaluated the integrity of the genome in order to describe the viability of radiation-stressed cells more accurately, since nucleic acids represent the prime target of radiation-induced damage (Horneck et al., 2010).

4.4.2 ATP

Microorganisms require energy in the form of ATP to sustain their vital functions. Since the intracellular concentration of ATP often corresponds to a cell's metabolic activity, ATP can be used as a target molecule for detecting microbial survival (Hammes et al., 2011).

Non-stressed cells of *D. geothermalis* were found to contain ATP in the order of 10^{-20}-10^{-19} mol $cell^{-1}$. These values are well in the (broad) range of intracellular ATP concentrations typically observed in other bacteria cultured in the laboratory or isolated from the environment: Depending on the species and the environmental conditions, ATP levels ranging from 10^{-21}-10^{-17} mol $cell^{-1}$ have been described (summarised by Shama & Malik, 2013). It is difficult to compare the ATP levels of specific microorganisms amongst studies, mainly because the intracellular ATP pool of a microbial cell is influenced by a range of factors, including cell division, growth cycle, pH, temperature, nutrients, and oxygen availability (Stanley, 1989). A single study has yet reported data regarding the intracellular ATP concentration of a *Deinococcus* sp. Isolated from a table in a clean room facility used for the assembly of spacecraft, the organism was found to contain 2.2×10^{16} mol ATP ml^{-1} (Venkateswaran et al., 2003). However, since the aforementioned study did not attempt to measure ATP on a single cell level but referred to bulk samples instead, the results are virtually impossible to compare to the present work (Siebel et al., 2008).

When *D. geothermalis* was exposed to desiccation for 2 months, the ATP concentrations in biofilms remained stable. Planktonic cells, on the other hand, experienced a reduction of ATP by two orders of magnitude during this desiccation period. This suggests that ATP is degraded or consumed in planktonic cells desiccated for an extended period of time, whilst biofilm cells are able to sustain their initial ATP levels, either due to conservation or regeneration of their ATP pool. ATP does not seem to be susceptible to short-term desiccation. This study showed that free ATP can be dehydrated without immediate quantitative loss of the substance. In *N. commune*, intracellular ATP was shown to be stable for 30 min and 2 h during short-term desiccation in air at -99.5 MPa (Angeloni & Potts, 1986; Potts & Bowman, 1985). The rehydration period to which desiccated cells of *D. geothermalis* were subjected prior to analysis could have influenced the ATP content of the cells. Usually, a 30-min rehydration period was applied. A control experiment, in which the rehydration period was decreased to 0 min, verified that ATP levels in rehydrating cells of *D. geothermalis* did not in-

crease during a 30-min rehydration period. However, cells may alter their intracellular ATP pool instantaneously. Cells of *N. commune* dried for 2 years have been shown to generate new ATP immediately after rewetting (Potts & Morrison, 1986).

During exposure to space and Mars stress including irradiation with UV, ATP levels of *D. geothermalis* remained within the same order of magnitude compared to a non-exposed control. ATP has been shown to persist under irradiation with Mars-like UV for extended periods of time (Schuerger et al., 2008). Therefore, its suitability as a marker for the detection of viability in UV radiation-stressed cells is questionable.

Environmental stress seems to have a significant impact on the microbial ATP pool, and ATP levels may increase or decrease, depending on the type of stress the organism is exposed to. For example, pressure stress has been shown to cause a 90%-decrease in ATP concentrations in both *L. monocytogenes* and *Salmonella typhimurium* (Tholozan et al., 2000). ATP concentrations of *L. monocytogenes* increased, however, when the organism was exposed to cold temperatures and to carbon dioxide (Li et al., 2003). The authors suggested that *L. monocytogenes* responded to stress by accumulating energy in order to boost repair mechanisms in a later resuscitation event. ATP levels of *D. geothermalis* did not increase upon exposure to either desiccation or simulated space and Mars conditions, suggesting that these stressors did not trigger a similar response in *D. geothermalis*, or that the organism does not accumulate ATP as a stress response *per se*.

Though ATP is susceptible to biological degradation and abiotic removal due to sorption processes (Cowan & Casanueva, 2007), is a relatively stable molecule when degradation processes are absent (Hammes et al., 2010), for example on the surfaces of clean room facilities (Venkateswaran et al., 2003). In this particular environment, levels of extracellular ATP have been shown to be up to two to three orders of magnitude higher than the concentrations of intracellular ATP (Venkateswaran et al., 2003). The present work confirmed the stability of the molecule by showing that free desiccated ATP persisted for weeks under ambient temperature and relative humidity. The persistence of ATP has serious implications for ATP quantifications that do not distinguish between intracellular and extracellular ATP (Venkateswaran et al., 2003). As a consequence for this study, free ATP originating either from extraneous sources or from *D. geothermalis* cells which lysed during the stress experiments could have led to overestimations of the intracellular ATP pool of *D. geothermalis*. Future studies employing ATP as a viability marker should consider the removal of free ATP prior quantification, in order to differentiate between extracellular and intracellular ATP levels. The removal of extracellular ATP could be achieved by separation of the cells from the ATP-containing matrix (Hammes et al., 2010), or by enzymatic degradation of the free ATP (Hammes et al., 2010; Venkateswaran et al., 2003).

This study used ATP as one out of three cultivation-independent viability markers for the detection of VBNC cells. ATP has been used before to differentiate between live and VBNC cells, but its significance is limited if it is used alone (Hammes et al., 2011). In some cases, VBNC cells have been found to maintain their intracellular ATP pool, whilst other studies showed that ATP levels decline in VBNC cells. For *Campylobacter jejuni*, for example, it has been reported that intracellular ATP concentrations rapidly fell after cell death, but were conserved in VBNC cells (Beumer et al., 1992). Another study, however, reported a significant decrease in ATP in VBNC cells of the same species (Federighi et al., 1998). In a different case, Lindbäck et al. (2010) have observed high levels of ATP in *L. monocytogenes* cells which had been VBNC for one year. This confirms that the VBNC state cannot be determined using a single parameter (Hammes et al., 2011).

Biofilm cells of *D. geothermalis* maintained their ATP pool during desiccation and space and Mars stress, but suffered a significant loss of ATP when inactivated by isopropyl alcohol treatment. This shows that the suitability of ATP as a marker for viability in *D. geothermalis* seems to depend on the type of stress the organism is exposed to.

4.4.3 16S rRNA

16S rRNA represents a common target molecule used for FISH, because of its genetic stability and high copy number (Moter & Göbel, 2000). In contrast to DNA, RNA is considered to be relatively unstable (Cenciarini-Borde et al., 2009). The presence of RNA would therefore represent an indication for microbial activity in terms of protein synthesis, which could be interpreted as viability (Hammes et al., 2011).

16S rRNA could be detected in *D. geothermalis* irrespective of the stress the organism was subjected to. Levels of 16S rRNA remained stable during desiccation for 2 months, suggesting that ribosomes are well-protected from dehydration in *D. geothermalis.* Also space and Mars stressors including UV radiation did not influence the presence of 16S rRNA. In compliance with this, several studies have observed that UV irradiation did not affect intracellular rRNA levels in other microorganisms such as *E. coli* (McKillip et al., 1998; Villarino et al., 2000; Zhang et al., 2015), *P. aeruginosa* (Zhang et al., 2015), and *S. aureus* (McKillip et al., 1998). Similarly, rRNA in *E. coli* and *S. aureus* was also found to be unaffected by heat treatment for 20 min at 80 °C (McKillip et al., 1998). A study by Cenciarini et al. (2008) showed that 16S rRNA was one of the most persistent types of RNA in heat-killed (65 °C, 30 min) cells of *E. coli* and *P. aeruginosa*. McKillip et al. (1998) argued that rRNA seems to be an unsuitable target molecule for detecting bacterial viability after exposure to moderate heat or UV radiation. Zhang et al. (2015), however, suggested that high rRNA levels in radiation-stressed cells can be attributed to the cells being VBNC.

In this study, dead controls produced by inactivating *D. geothermalis* using either heat or isopropyl alcohol exhibited unchanged levels of 16S rRNA compared to non-treated cells. This strongly suggests that the detection of 16S rRNA in stressed cells of *D. geothermalis* has little significance for interpreting their viability status.

4.5 Conclusions

In the course of the present study, a model biofilm of *D. geothermalis* was developed that was suitable for the exposure to desiccation and to simulated space and Martian conditions. Water-unsaturated biofilms were grown at the solid-air interface on a porous, mobile support placed on solid nutrient medium (R2A) by incubation at 45 °C for 2 days. Membranes made from CME or cellophane were used as a substratum for biofilm formation.

Depending on the type of nutrient medium used (R2A or TSA), *D. geothermalis* formed biofilms that differed significantly in their appearance, morphology, and adhesion properties. With increasing concentrations of peptone added to the medium, biofilm cells exhibited an increasing tendency to form tenacious aggregates which were resistant to numerous physical and chemical means of dispersion. Treatment with the proteolytic enzyme proteinase K relieved aggregation by disrupting an amorphous matrix engulfing the cell aggregates, suggesting that proteins are involved in the cell-to-cell adhesion of *D. geothermalis*, possibly in the form of adhesion threads that have been described to mediate adhesion in *D. geothermalis* E50051 and are composed of glycosylated proteins (Peltola et al., 2008; Raulio et al., 2006; Saarimaa et al., 2006).

After their extraction using the CER Dowex®, the EPS of *D. geothermalis* were found to contain proteins and polysaccharides as their dominant fractions. Evidence obtained from the use of fluorescently-labelled lectins and potentially adhesin-inhibiting carbohydrates suggested that the polysaccharide fraction may contain mannosides and/or galactosides. Contrary to previous assumptions (Kolari, 2003), the concentrations of uronic acids in the EPS were insignificant, suggesting that negatively charged polysaccharides are not responsible for the pronounced adhesion properties of the organism. Still, the exopolysaccharides may be polycationic or non-charged. The EPS contained minor quantities of eDNA, which might represent a structural component of the matrix.

D. geothermalis remained viable during desiccation and storage in ambient air for a period of at least 2 months, during which the culturability of biofilms and planktonic cells decreased from 19.8% and 28.4% to 5.6% and 0.8%, respectively. Cultivation-independent viability parameters (membrane integrity, ATP, 16S rRNA) were maintained in biofilms to a significantly higher extent than in planktonic cells, indicating that *D. geothermalis* is more desiccation-tolerant in the biofilm mode of life. In desiccated state, the organism seemed to be resistant to the exposure to extreme temperatures, space-like vacuum, and artificial Mars at-

mosphere. UV radiation, either monochromatic (254 nm) or polychromatic (200-400 nm), significantly decreased the culturability of the organism. Also when exposed to simulated space or Martian environments, a trend was observable that the survival of *D. geothermalis* is facilitated in biofilms compared to planktonic cells.

The distinct composition, distribution, and arrangement of the EPS matrix could have contributed to an increased stress tolerance of *D. geothermalis* in biofilms. EPS could, for example, promote the retention of water by forming a skin at the biofilm-air interface which decreases water loss by evaporation, or by storing water in the biofilm core (Peltola et al., 2008; Sutherland, 2001). Upon exposure to desiccation and UV irradiation, *D. geothermalis* exhibited significantly reduced culturability whilst sustaining cultivation-independent viability and total cell counts. Altogether, this indicates that a part of the population entered a VBNC state as a response to desiccation and radiation stress (Li et al., 2014; Oliver, 2005). To the author's knowledge, this represents the first evidence for the ability of *D. geothermalis* to become VBNC. VBNC cells may show increased chemical and physical resistance (Li et al., 2014). In *D. geothermalis*, induction of the VBNC state could have contributed to an elevated stress tolerance.

The present work gave new insights in the extreme robustness of *D. geothermalis* and demonstrated its suitability as a model organism in astrobiology. The results suggest that biofilms of *D. geothermalis* have the potential to endure an 18-months residence in LEO, as intended in the course of the space mission BOSS. Although highly unlikely, such a small time frame may once have sufficed for meteorite exchanges between Earth and Mars to occur (Gladman, 1997; Mileikowsky et al., 2000b). Thus, the outcome of BOSS will broaden our understanding regarding the feasibility of a lithopanspermia scenario within our solar system. Certain aspects of space or Mars such as galactic cosmic rays (Mileikowsky et al., 2000a) or the high reactivity of Martian soil (Martín-Torres et al., 2015; Yen, 2000) are difficult to simulate on Earth and could not be addressed in the present study. However, it seems as though *D. geothermalis* would only survive in space or on Mars when shielded from UV and other forms of deleterious extraterrestrial radiation, suggesting that the organism would have to exist within the rock or the Martian subsurface in order to persist for extended periods of time (Böttger et al., 2012; de la Vega et al., 2007; Mileikowsky et al., 2000b; Westall et al., 2013; Yen, 2000).

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6. APPENDIX

6.1 Publikationsliste

6.1.1 Fachartikel

Frösler, J., Panitz, C., Wingender, J., Flemming, H.-C., Rettberg, P. (2015): Survival of *Deinococcus geothermalis* under desiccation and simulated space and Martian conditions. *In Vorbereitung*.

Frösler, J., Leadbeater, B.S.C. (2009): Role of the Cytoskeleton in Choanoflagellate Lorica Assembly. *J Eukaryot Microbiol*, *56*(2), 167-173.

6.1.2 Poster

Frösler, J., Wingender, J., Panitz, C., Rabbow, E., Rettberg, P., Flemming, H.-C. (2014): Survival of *Deinococcus geothermalis* under simulated space and Martian conditions. EANA 2014, 12.-16. Oktober, Edinburgh, Großbritannien.

Frösler, J., Wingender, J., Panitz, C., Rabbow, E., Rettberg, P., Flemming, H.-C. (2014): The desiccation and UV tolerance of biofilms and planktonic cells of *Deinococcus geothermalis*. Wasser 2014, 26. Mai 2014, Haltern am See, Deutschland.

Frösler, J., Wingender, J., Flemming, H.-C. (2014): The choice of growth medium affects *Deinococcus geothermalis* biofilm morphology and EPS composition. Biofilms VI, 11. Mai 2014, Wien, Österreich.

Frösler, J., Flemming, H.-C., Wingender, J. (2013): Influence of extracellular polymeric substances on the tolerance of *Deinococcus geothermalis* to space and Mars environments. EANA 2013, 21. Juli 2013, Stettin, Polen.

Meeßen, J., de Vera, J.-P., **Frösler, J.**, Rettberg, P. (2013): BIOMEX and BOSS: A Tool for Studies on Habitability of Planets, Panspermia and Space Exploration. HGF-Evaluierung: Review for the Programme Space. 18. April 2013, Köln, Deutschland.

Frösler, J., Marko, A., Flemming, H.-C., Wingender, J. (2012): The influence of extracellular polymeric substances on the survival of desiccated biofilms of *Deinococcus geothermalis*. Biofilms V, 10. Dezember 2012, Paris, Frankreich.

Frösler, J., Marko, A., Flemming, H.-C., Wingender, J. (2012): Influence of extracellular polymeric substances on the desiccation tolerance of *Deinococcus geothermalis* biofilms. EANA 2012, 15. Oktober 2012, Stockholm, Schweden.

Tewes, M., **Frösler, J.**, Petry-Hansen, H., Flemming, H.-C., Wingender, J. (2011): Association of Hygienically Relevant Bacteria With Freshwater Plankton. Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie, 3.-6. April 2011, Karlsruhe, Deutschland.

6.1.3 Vorträge

Frösler, J., Wingender, J., Flemming, H.-C., Panitz, C., Rettberg, P. (2015): Das Überleben von *Deinococcus geothermalis* unter Weltraum- und Mars-ähnlichen Bedingungen - Toleranz gegenüber Austrocknung, Vakuum, Mars-Atmosphäre und Temperaturextremen. DLR Symposium „Forschung unter Weltraumbedingungen“, 28.-30. Oktober 2015, Bonn, Deutschland.

Frösler, J., Flemming, H.-C., Wingender, J. (2013): The influence of extracellular polymeric substances on the tolerance of *Deinococcus geothermalis* to the environmental conditions of space and Mars. EANA 2013, 21. Juli 2013, Stettin, Polen.

Frösler, J. (2013): *Deinococcus geothermalis* – a model organism for the astrobiology experiment BOSS. Astrobiology Workshop, 10. Juni 2013, Düsseldorf, Deutschland.

Frösler, J. (2012): Biofilms in Space: The Experiment BOSS on the EXPOSE R-2 Mission. IWG EXPOSE project meeting, 23.-25. Januar 2012, Köln, Deutschland.

Frösler, J. (2012): Biofilme im Weltraum: Das BOSS-Projekt. Neujahrskolloquium der Fakultät für Chemie der Universität Duisburg-Essen, 11. Januar 2012, Essen, Deutschland.

6.2 Lebenslauf

Dieser Abschnitt ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

6.3 Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„Differential tolerance of biofilms and planktonic cells of *Deinococcus geothermalis* to desiccation and to simulated space and Mars conditions"

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Einzelne Teile dieser Arbeit wurden im November 2015 bei der wissenschaftlichen Fachzeitschrift „Astrobiology" zur Veröffentlichung unter dem Titel „Survival of *Deinococcus geothermalis* in biofilms under desiccation and simulated space and Martian conditions" eingereicht. Ich selbst bin Hauptautor und Verfasser des Artikels.

Duisburg, im Dezember 2015

(Jan Frösler)